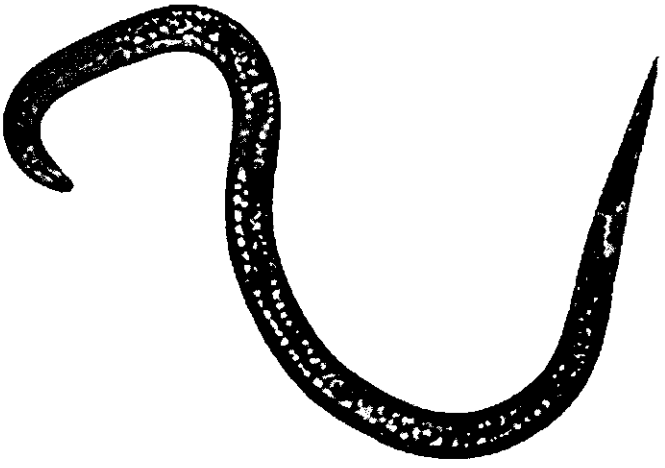
Population and molecular genetics of root-knot nematodes



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Population and molecular genetics of root-knot nematodes

Makedonka Dautova

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Bibliographic Abstract: This thesis describes studies of root-knot nematodes *Meloidogyne* spp. - an economically important pest in agriculture - using population and molecular genetics. Variability in virulence to *Mi* bearing tomato genotypes is shown for *Meloidogyne* spp. isolates and their impact of implementation of proper management system is discussed. Genetic polymorphisms in *Meloidogyne* spp. were assessed using molecular techniques that reveal nuclear and mitochondrial DNA divergence on different hierarchical levels. cDNA library constructed from the pre-parasitic second stage juveniles of *Meloidogyne incognita* was screened by single pass 5'-end sequencing to investigate genes expressed at the onset of parasitism. One expressed sequence tag homologous to an endoxylanase was used to identify a novel cell wall degrading enzyme - xylanase. Several lines of evidence support the endogenous origin of the nematode xylanase. The amino acid sequence revealed a high similarity with bacterial xylanases indicating that nematode xylanase may have been acquired from bacteria by horizontal gene transfer.

CONTENTS

Propositions **Wageningen, 22 June 2001**

1. Root-knot nematodes have acquired xylanase genes from bacteria by horizontal gene transfer as an evolutionary adaptation to parasitise monocotyledons.

This thesis
Hurlbert JC and Preston JF (2001) Functional characterization of a novel xylanase from a corn strain of *Erwinia chrysanthemi*. *J Bacteriol* 138: 2093-2100

2. Mitotic parthenogenetic and amphimictic plant-parasitic nematodes generate a similar level of genetic diversity.

This thesis

3. In contrast to vertebrates saliva of sedentary plant-parasitic nematodes is essential not only for digestion but also for production of the food.

Bird AF (1962) The inducement of giant-cells by *Meloidogyne javanica*. *Nematologica* 8: 1

4. The time consuming morphological diagnosis of certain plant parasitic nematodes can efficiently be replaced by DNA-based identification methods.

5. Scientific data alone cannot address a public's concern over biotechnology.

6. I understand the world as a field for cultural contest among the nations.

Goce Delčev (1872-1903)

7. If you carry within you something unsaid, something that pains and burns, bury it within the depths of silence - the silence will say it for you.

Aco Šopov (1923-1982)

8. De gretigheid waarmee Nederlanders hun talenkennis etaleren maakt het voor buitenlandse studenten volstrekt zinloos een cursus Nederlands te volgen.

***PhD thesis* of Makedonka Dautova**
Population and molecular genetics of root-knot nematodes

Chapter 1

GENERAL INTRODUCTION

Nematodes constitute the largest and most ubiquitous phylum of the animal kingdom (78). Often they comprise 80 to 90% of all the animals of the soil fauna. They can be present in numbers ranging from 1.8 to 120 millions per square meter of soil (54). This observation inspired Cobb (25) to make the following remark:

> *"even if all matter in universe, except nematodes were swept away, the world will still be recognisable dimly by its mountains, hills, valleys, lakes, and oceans represented by a film of nematodes".*

Most nematodes are microbivores, fungivores, predators or omnivores surviving in various terestrial, aquatic or marine environments (5). Only a small fraction of the nematodes species has the ability to parasitise animals and plants.

Plant parasitic nematodes

During the course of evolution plant-parasitic nematodes have adapted to parasitise completely unrelated organisms. They are frequently obligatory parasites that have evolved various modes of actions varying from simple feeding strategies to highly complex relationships with the host plant. The vast majority of plant-parasitic nematodes are soil-dwelling and feed from the plant roots. Parasitism is established when the nematode pierces the cell wall by the hollow stylet – possessed only by plant parasitic nematodes – that has a narrow lumen through which salivary secretions are injected into punctured cells (90).

Plant parasitic nematodes show great diversity in parasitic behaviour. Migratory nematodes bluntly remove the cytoplasm from plant cells, frequently causing cell death, and then move on to another cell to repeat the feeding process. Other nematodes are more subtle and feed from a single cell or a group of cells for prolonged periods of time. The cell and tissue modifications that are induced by root parasitic nematodes have recently been reviewed by Cohn and Spiegel (26) and Sijmons et al. (77). Root parasitic nematodes belonging to the order *Dorylamida* cause serious root damage when they occur in large populations. In addition to the physical damage to plant tissues they have economical importance as a vectors of several soil born viruses. Nematodes in the order *Tylenchida* seriously affect plant growth by inducing profound alternations in the structure of the cell from which they feed as well as in the anatomy and function of the roots (90). The worst effect of plant-parasitic nematodes is debilitation of the plant without producing any specific and visible symptoms leading to underestimation of the economic effects by the growers (4).

Distribution and economical importance of root-knot nematodes

Root-knot nematodes (RKN, *Meloidogyne* spp.) (39) are responsible for billions of dollars in annual crop losses. The majority of the plant species that account for the world's food supply are susceptible to root-knot nematode infection. They are widely distributed and have established a complex and long-lasting parasitic relationship with more than 2.000 plant species (74) including monocotyledons, dicotyledons, herbaceous and woody plants. Decline of RKN infected host plants is usually further increased by secondary infections because the nematode's invasion often coincides with other soil-borne pathogenic bacteria and fungi (68). Frequently there is more than one nematode species involved (63) causing more economic damage to food crops than any other group of plant-parasitic nematodes among the *Tylenchida* (49, 73). RKN are considered as important pathogens on tomato (*Lycopersicon esculentum* Mill) (18, 58) especially in tropical, subtropical and warm temperate areas of the world where soil temperature is high, seasons are long and several nema-

tode generations are completed annually, resulting in high population densities. *M. incognita, M. javanica, M. arenaria* are found in tropical and subtropical regions and in glasshouses while *M. hapla* (and *M. chitwoodi)* prevail in temperate climate zones.

The estimated yield loss caused by RKN is over 10% world-wide, although the damage inflicted in certain regions of developing countries may be as much as 25% to 50% (80, 75). The four common species, *M. incognita, M. javanica, M. arenaria* and *M. hapla*, are responsible for nearly 90% of the losses caused by RKNs. For instance, Barker and Olthof (7) have shown that *M. incognita* suppresses yield of tomato cultivars by 20-30% in mountain cultivations and up to 85% in coastal plains (3). Some other species, like *M. chitwoodi* (38), are also of economic importance, but have a more restricted geographical distribution (50, 30).

The history of RKN

For a long time the symptoms caused by *Meloidogyne* spp. have been attributed to other pathogens. Barkeley (6) noticed for the first time that nematodes were present in galls of cucumber roots. Since then the overall history of the genus *Meloidogyne* can be divided into three periods. Period 1855-1878, during which a correlation was observed between root galls and nematode incidence, followed by a relatively long confusing period (1879-1948) during which root-knot nematodes were included in the same genus as cyst nematodes (*Heterodera*; 76). In the final 'revival'-period root-knot nematodes were placed into a separate genus (24) and were hold accountable for large economic losses. According to the revision of Maggenty et al. (62, 60) the taxonomic position of root-knot nematodes is as follows below:

Phylum *Nematoda*
Class *Secernentea*
Subclass *Diplogasteria*
Order *Tylenchida*
Suborder *Tylenchina*
Superfamily *Tylenchoidae*
Family *Heteroderidae*
Subfamily *Meloidogyninae*
Genus *Meloidogyne* Göldi, 1892

At present, there are more than 80 nominal root knot nematode species described. The available evidence strongly indicates that the four so-called common species *Meloidogyne javanica* (81), *Meloidogyne arenaria* (66), *Meloidogyne incognita* (55), *Meloidogyne hapla* (24) account for 95% of all populations all over the world.

Biology of RKN

Root knot nematodes are obligatory endoparasites, which have evolved a very specialised and complex feeding relationship with their host plant. The juvenile can move within the egg but is not very active (Figure 1.1A). Hatched second stage juveniles (body width and length is approximately 290 and 912 mm respectively), are the infective stage of the species (Figure 1.1B). They migrate in the soil and prior to penetration probe the root epidermal cells at the zone of elongation by continuous head rubbings and occasional stylet protrusion. As vigorous stylet thrusting does not occur at this stage, the involvement of cell wall degrading enzymes has been suggested (11, 12). The release of secretions, that may include cell wall degrading enzymes, through the stylet prior to penetration has been observed *in vitro* (91). To complete their life cycles, J2s penetrate roots of susceptible hosts behind the root cap (Figure 1.1C) (42) and migrate intercellularly to the developing protoxylem in the vascular cylinder (91). A successful host-parasite relationship requires that these nematodes modify several plant root cells to obtain nourishment necessary for their development and reproduction (42, 51). So, initial feeding activities by J2 induce localized modification of host cells to form an elaborate feeding site consisting of several multinucleate cells, so-called giant-cells (Figure 1.1D)(41, 52).

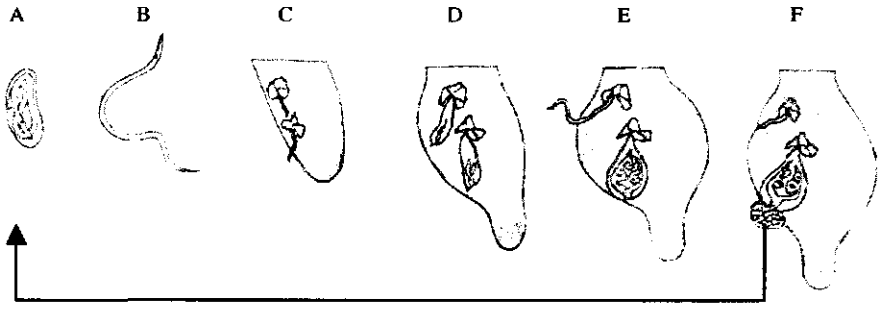

Figure 1.1- Life cycle of a root-knot nematode, *Meloidogyne* spp. Depending of environmental conditions, the cycle is completed within one or two months.

Giant-cells are larger than normal cells with multiple nuclei, thickened walls with extensive ingrowths and dense cytoplasm with increased numbers of organelles (9, 51). They are metabolically highly active and serve as a metabolic sink for host photosynthates that are consumed by the nematode (15, 65). The morphological changes involved in the formation and development of giant-cells have been thoroughly studied. The multinucleate state arises from repeated karyokinesis uncoupled from

cytokinesis, but the DNA content of the nuclei increases nonsystematically on a per-nucleus basis (41, 53, 87). Stylet secretions, originating from the nematode's oesophageal glands are presumed to be responsible for protein synthesis, nuclear division, cell growth and differentiation in giant cells (44, 48). Other components in the stylet secretions may form feeding tubes, which are structures that facilitate withdrawal of nutrients by the feeding parasite (47). Induction and maintenance of giant-cells are possibly separate phenomena mediated by different development stages of the parasite (17). The nematodes are completely dependent on these giant cells for their development and reproduction (41).

After feeding for about 10-12 days in roots of susceptible plants, the J2 ceases feeding and moults three times over a 48-hour period. The females continue to feed and grow considerably larger than the males. They are swollen (median length 440 – 1300mm, median width 330 – 700 mm) (29) and remain in the root. After the fourth moult the males are still slender (body length 700 – 2000 mm), leave the root (Figure 1.1E) and fertilise the females in the case of amphimictic species. However, parthenogenesis is often encountered in root-knot nematodes, thus fertilisation is not necessary for egg development and does not occur even if sperm is present in the spermotheca (80). The females lay their eggs in a gelatinous matrix that normally protrudes out of the host tissue (Figure 1.1F) (78). After completion of its life cycle, the female nematode dies and the giant-cells degenerate (10). The eggs hatch freely in water, however, the rate of hatch is higher in the presence of host root exudates (78).

Population genetics

Genetic changes in nematode population are partially driven by the environment including the use of resistant varieties and pesticides. Studying such genetic changes at the population level is crucial for understanding and predicting the behaviour of nematodes in the field. Theoretical population geneticists have mainly been concerned with sexually reproducing species, often supposing that these species are outbreeders with much opportunity for recombination between different genomes. However, in asexually reproducing species many generations may pass in which only a very limited number of recombinations will occur. The majority of root-knot nematodes species, *Meloidogyne* spp., are obligatory mitotic parthenogenetic and therefore, the offspring is expected to be identical to the parent. However, many years ago root knot nematode populations of the same species were shown to differ in their ability to reproduce on a variety of crops (69, 37).

To reveal the origin of these variations in virulence nematologists have to enter deeply into the secrets of root-knot nematode genetics (nuclear and mitochondrial). A wide range of molecular tools (mainly based on the polymerase chain reac-

tion) has been adapted to investigate intra- and interpopulation genetic diversity and phylogenetic affinities in *Meloidogyne* species and populations:

- Since 1990 RAPD (Random Amplified Polymorphic DNA) has been used as a genetic marker (88, 85). RAPD markers are generated by the amplification of random DNA segments in the target genome with single oligonucleotide primers of arbitrary nucleotide sequences. Because of the vast number of primers that can be generated the number of DNA markers is enormous (89). Since the introduction of the RAPD technique it has been used to establish relative degrees of polymorphism between individuals, populations and species of the genus *Meloidogyne*, however, the detected intraspecific polymorphisms remained rather low (22, 23, 40, 61).
- Restriction Fragment Length Polymorphism (RFLP) are the second type of genetic markers that have been used to assess genetic variation in root knot nematodes (19, 33, 35, 40, 93). RFLPs are the result of genomic DNA restricted with specific endonucleases generating a unique set of DNA fragments typical for the nucleotide sequence of the genome. Nucleotide substitutions, insertions or deletions in the endonuclease recognition sites of species, population and isolates modify the RFLPs in a DNA fingerprint that can be used as a set of diagnostic characters. RFLPs frequently arise from stretches of repetitive DNA. Due to the extensive variation in repetitive DNA RFLPs are valuable to assess diversity both between and within plant-parasitic nematode species (1).
- Amplified Fragment Length Polymorphism (AFLP) technique is a new PCR-based approach, used for detecting and evaluating genetic variation, that has a considerable potential for analysing nematode genomes (34, 71, 84, 94). AFLP is based on selectively amplifying a sub-population of restriction fragments from a genomic DNA. Polymorphisms are detected as differences in lengths of amplified fragments. Prior to PCR amplification genomic DNA is digested by two restriction enzymes. Subsequently, oligonucleotide adapters are ligated to the resulting restriction fragments to generate template DNA for PCR. In contrast to the RFLP procedure, the AFLP technique generates virtually unlimited numbers of DNA fragments from nanogram quantities of genomic DNA. In comparison to the RAPD approach, the AFLP technique uses stringent reaction conditions, which guarantees a better reproducibility. Furthermore, this technique is quantitative and AFLPs can therefore be used as co-dominant markers (82).

Molecular genetics

The rapid development of molecular tools in the last decades enabled scientists to reveal directly the secrets hidden in the genomes of organisms. Complete characterization of a certain gene of interest starts with knowing the nucleic acid sequence,

however, true value is only achieved when the biochemical basis for its regulation is understood and a functional relation can be found to other biochemical pathways and processes. As such molecular nematologists aim at unraveling the genetic code of plant parasites in order to understand the underlying causes of pathogenicity. Conventional procedures require relatively large amounts of starting material for RNA preparation (72). Frequently, it is difficult to apply these methods to the minute plant-parasitic nematodes, because it takes a huge effort to obtain sufficient quantities of mRNA. The introduction of the PCR largely enabled nematologists to overcome this limitation (64).

To date, various methods have been applied to prepare and screen cDNA libraries of plant-parasitic nematodes (28, 46, 56, 57, 70), but only a limited number of genes were identified and all procedures required prior knowledge of candidate genes or technically advanced pre-selections in mRNA pools. To address this problem, it has proven to be successful to sequence at random only small portions of cDNAs from a library, called expressed sequence tags (EST) (2). Although the ESTs are short (200-500 bp) DNA sequences generated from the 3' and 5' ends, they contain enough information to indicate the gene's probable function and its relationship with other genes (2).

Good quality cDNA-libraries constructed from different life stages of the nematode may generate sequence tags expressed from genes at various time points in the parasitic cycle. The parasitic cycle involves various distinct stages, plant penetration and intercellular migration, and feeding site initiation and maintenance. Therefore, cDNA libraries covering these main stages may provide insight in the molecular fundaments of plant parasitism by RKN.

Oesophageal glands - source of pathogenicity factors

Root-knot nematodes, as all other plant parasitic nematodes, have adapted to plant parasitism by evolving i) protrusible stylet, ii) muscular metacorpus containing a triradiate pump chamber and iii) three large oesophageal glands. The oesophageal glands enlarged as plant parasites evolved from free-living nematodes to the *Rabditida*, indicating a change in their primary function (43). Each oesophageal gland is a single large cell, which is entirely specialised to its secretory function (31). The gland cells are packed with membrane-bounded granules in which secretory proteins are stored and released from into the lumen of the oesophagus. This process is under the control of the nervous system of the nematode (32). Each of the two subventral glands has a short cytoplasmic extension that terminates in an ampulla at the base of the pump chamber in the metacorpus. The dorsal gland has a long cytoplasmatic extension that terminates in an ampulla near the stylet knobs (44). The secretory granules formed in the nuclear region of the gland cells migrate forward

through the extensions in order to accumulate near the release valves in the ampullae (45).

Distinct morphological changes of the oesophageal glands occur during parasitism. At the onset of parasitism during host penetration, the subventral oesophageal glands of *Meloidogyne* J2 initially increase in length but not volume (Figure 1.2A)(14). Following penetration the secretory granules accumulated in the subventral glands in parasitic J2 start to decrease in number (12, 91).

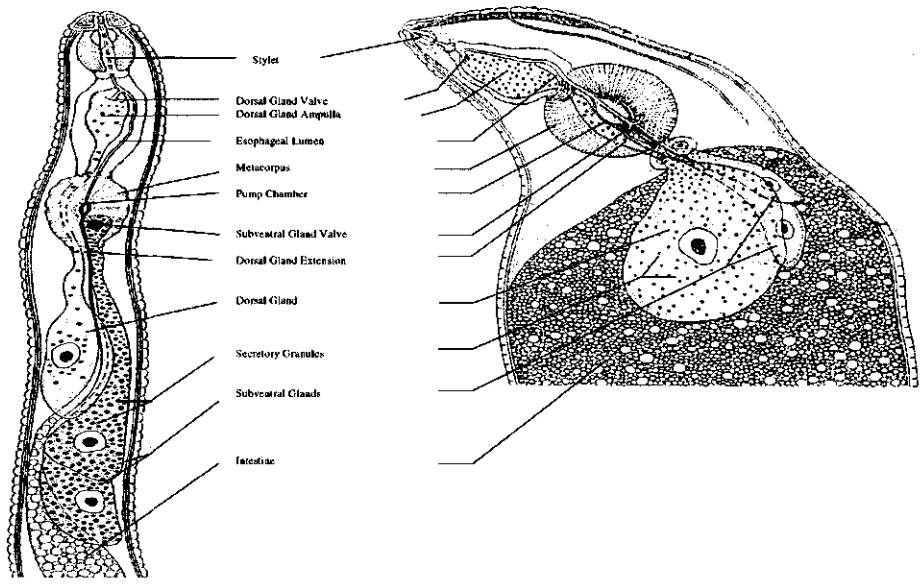

Figure 1.2 - The esophagus of a phytoparasitic second stage juvenile(A) and of an adult female (B) root-knot nematode, *Meloidogyne* spp. (48).

Following feeding site initiation the dorsal gland increases in size whereas the subventral glands decrease again (13, 14). The dorsal gland in preparasitic J2 contains few secretory granules. However, soon after juveniles penetrate the roots, secretory granules accumulate in the dorsal gland cell lobe and ampulla (16). In adult females, the dorsal gland predominates (Figure 1.2B) whereas the subventral glands are greatly reduced in size (13).

The changes in oesophageal gland and secretory granule morphology during parasitism by *Meloidogyne* species indicate a different role for the two types of oesophageal glands and their secretions at successive stages of the nematode's life cycle. Based on the developmental changes in the gland cells subventral gland se-

cretions are presumably involved in initial phases of parasitism – plant penetration and intercellular migration. The dorsal gland secretions may be involved in feeding cell initiation and maintenance as well as facilitating food extraction from the feeding site cytoplasm.

Plant cell wall - the first barrier for plant-parasitic nematodes

The architecture and function of plants depend on the structure of the cell walls, which is conceived as a highly organized network composed of polysaccharides, proteins and aromatic compounds. Polysaccharides are long carbohydrate molecules covalently linked at various positions and with side chain decorations of various natures and length. The cell wall carbohydrates represent a vast spectrum of polyhydroxyl aldexydes (aldoses). In aldoses, including the hexose glucose and the pentose arabinose, the C-1 is the only carbon that binds to two oxygen atoms (anomeric). The hydroxyl group of the anomeric carbon can either be in a or b positions, and is always accompanied by D or L designation which refers to the position of the hydroxyl group on the asymmetric carbon most distant from the C-1 (21). All monosaccharides in the cell wall polymers are derived from a-D-Glucose. The homopolymer of b-1,4- linked D-glucose is the cellulose, which is the most abundant polysaccharide found in the plant biomass. The complete degradation of the cellulose involves 4 classes of enzymes (Figure 1.3).

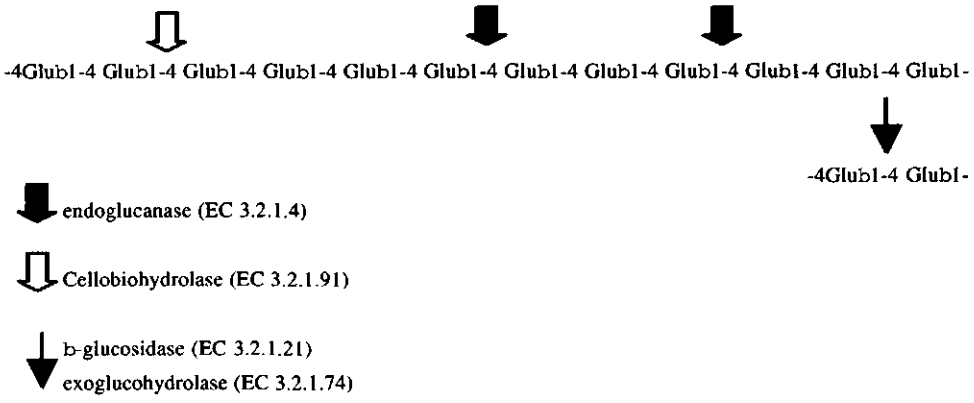

Figure 1.3 - Cellulose composition and enzymes involved in cellulose degradation.

When the C-6 primary alcohol of the a-D-Glucose is oxidized to a carboxylic acid group a-D-Glucuronic acid is formed. Enzymatic removal of the carboxyl group

from the a-D-Glucuronic acid forms the pentapyranose a-D-Xylose, a sugar in which all of the carbons are part of the heterocyclic ring. The b-1,4- linked polymers of D-xylose form a polysaccharide named xylan. Next to cellulose, xylans are one of the most abundant biopolymers synthesized in the biosphere. Structure of xylans may vary from linear poly-D-xylose to highly branched hetero-polysaccharide (*e.g.* arabinoxylan or glucuronoarabinoxylan). Hardwood xylan is typically o-acetyl-4-o-methylglucuronoxylan with approximately 10% of xylose units a-1,2-linked to a 4-o-methyl-glucurononic acid side-chain, and 70% of xylose residues acetylated at the C-2 or C-3 positions. Softwood xylans are commonly arabinoxylans in which 10% of the xylose units are substituted with a-2,3-linked arabinofuranose residues (86). Xylan, as a non-cellulosic polysaccharide, is synthesized in the Golgi apparatus, packaged in secretory vesicles, and exported to the cell surface, where they are interlinked with cellulose microfibrils (20). There is no evidence to confirm covalent linkage between cellulose and any other component of the plant cell wall, including xylans. Xylans are also believed to interact via hydrogen bonds to cellulose microfibrils with a strength that could be inversely proportional to the degree of side-chain substitution (83). The two aromatic side-groups and 4-*O*-methyl glycuronic acid of xylan enables a covalent cross-linking to other cell wall components such as pectin or lignin.

Degradation of the heterogenous and variable polysaccharide xylan (linear or highly branched hetero-polysaccharide) requires a whole suite of enzymes (8, 27) (Figure 1.4).

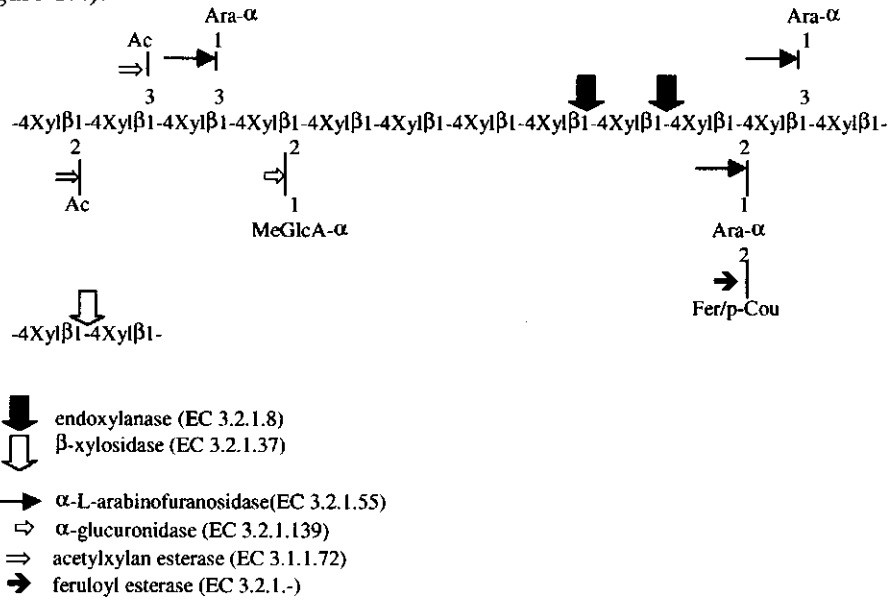

Figure 1.4 - Xylan composition and enzymes involved in xylan degradation.

Between these enzymes a considerable degree of synergy exists (67). For example, many xylanases will not cleave glycosidic bonds between xylose units that are substituted. Thus, side chains must be cleaved before the xylan backbone can be completely hydrolysed (59). Xylans are structurally more complex than cellulose, and therefore require more enzymes to achieve efficient hydrolysis. However, they do not form tightly packed structures such as is the case with cellulose, and are more accessible to hydrolytic enzymes. Consequently, the specific activity of xylanases is generally 2-3 orders of magnitude greater than that cellulases have for their appropriate substrates (36).

To invade the plant, pathogens and parasites have to degrade cell walls. In order break down the complex network, as the plant cell wall is, pathogens require a diverse set of enzymes, including cellulases, xylanases, pectinases and proteases. Although, the biochemistry and mode of action of most hydrolytic enzymes have been studied extensively for bacteria and fungi (36), it is a new area of research for plant-nematode interactions.

For a long time nematologists assumed that only mechanical force is involved in the migration of the nematodes through the plant root (92). Present findings indicate that a combination of mechanical force and enzymatic softening (see also 70, 79) of plant cell walls takes place. To date, only b-1,4-endoglucagenases have been identified in root-knot nematodes (70). It is expected that other types of enzymes are also involved in the migration through plant tissues of this nematodes species.

Outline of this thesis

The overall objective of this thesis is to study root-knot nematodes *Meloidogyne* spp. - an economically important pest in agriculture - using population and molecular genetics.

In Chapter 2 observations on the distribution of *Meloiodgyne* populations in Macedonian vegetable and tobacco fields are presented. Variability in virulence to *Mi* bearing tomato genotypes is shown for 73 *Meloidogyne* isolates. The impact of the occurrence of extensive variations in virulence is discussed for the implementation of proper management system.

In the third chapter the genetic variation in 16 *Meloidogyne* populations was assessed using molecular techniques that reveal nuclear and mitochondrial DNA divergence on different hierarchical levels. The AFLP technique was used to assess the phylogenetic relationships based on polymorphisms of total DNA. Polymorphisms generated by differences of mtDNA in the tandem array of 63-bp repeats were quantified down to the level of an individual nematode.

Chapter 4 describes the construction of a cDNA library from the pre-parasitic second stage juveniles of *Meloidogyne incognita* to investigate gene expression at the onset of parasitism. The results show that a good quality cDNA library and single-pass cDNA sequencing of randomly chosen primary transcripts is a powerful method to identify candidates for parasitism related genes in plant parasitic nematodes. The efficiency of the method is illustrated with the production of expressed sequence tags for virtually all parasitism-related genes cloned to date.

In chapter 5, one expressed sequence tag homologous to an endoxylanase of various bacterial origins was used to identify a xylanase in *M. incognita* (*Mi-xyl1*). Transcription of this gene is shown to be localized in the subventral oesophageal glands of second stage juveniles. A recombinant *Mi-xyl1* protein exhibited hydrolytic activity on both xylan and carboxymethylcellulose.

Finally, in chapter 6 the state of the art in population and molecular genetics is discussed with respect to root-knot nematodes in order to summarise and illustrate the impact of the results describe in this thesis.

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ABSTRACT

The occurrence of *Meloidogyne* in several areas of Macedonia was surveyed. Seventy-three isolates from 9 locations (fields and glasshouses) were identified and their behaviour on two susceptible and two *Mi* resistant tomato cultivars compared. *M. incognita* (47,9%) and *M. javanica* (35,6%) are the predominant species followed by *M. arenaria* (13,7%), and, sporadically, *M. hapla (2,7%)* was found. Mixtures of species were present at nearly all locations. Virulent isolates were found in *M. incognita* (11%) and *M. javanica* (46%) as well as in *M. arenaria* (50%). *M. hapla* isolates were compatible with all tomato genotypes tested. The impact of the occurrence of virulence for *Mi* carrying tomato genotypes is discussed.

INTRODUCTION

The widely distributed polyphagous root knot nematodes (*Meloidogyne* spp.) are among the most damaging plant parasitic nematodes in many economically important crops. Damage by the different species, its populations (intraspecific variation) and subsequent control are important issues. A means of control that is environmentally satisfactory is the growing of resistant cultivars but this is generally hampered because resistance is often not universal i.e. not directed to all *Meloidogyne* species present in an area or by the occurrence of virulent field populations. Resistance against root knot nematodes has been described for various crops, among them tomato *(Lycopersicum esculentum)* (e.g. 11, 29).

Resistance in tomato to root knot nematodes was found about 50 years ago in an accession of *L. peruvianum* and introgressed in *L. esculentum* using embryo rescue (34). All currently available root-knot resistant tomato cultivars are derived from this source (21). A major gene (*Mi*) located on chromosome 6 (12) controls resistance. The *Mi* gene confers resistance to *M. incognita, M. javanica* and *M. arenaria* but not to *M. hapla* (1, 14).

There are two major limiting factors in the use of *Mi*: (i) at high soil temperature (28-30°C) the resistance does not function (9) and (ii) the occurrence of resistance breaking root-knot nematode populations. *M. incognita, M. arenaria* and *M. javanica* include virulent field populations and isolates selected on *Mi*-bearing cultivars after several generations (e.g. 22, 24, 28, 31). Variation in virulence in *M. incognita* populations towards the *Mi* gene has been studied by many authors (3, 5, 7, 16, 19). There are also several reports on virulent *M. arenaria* and *M. javanica* populations (23, 27, 33, 37). Currently seven additional independent dominant *Meloidogyne* resistance genes, designated *Mi-2* to *Mi-8,* have been identified from *L. peruvianum* accessions and one from *L. chilense.* These genes are not yet available in commercial cultivars. They display resistance with different properties from *Mi*

and some are resistant to *M. hapla* or confer resistance at 32 °C. (see: 38). Because *L. esculentum* and *L. peruvianum* do not normally cross, the behaviour of these resistance genes in a tomato background is as yet hardly known.

In spite of the economic importance of the tomato crop in Macedonia, knowledge on the distribution of *Meloidogyne* species is scarce and even completely absent if intraspecific variations in virulence are considered. These omissions seriously impede implementation of proper management systems for *Meloidogyne*. As a first step towards such a system we report in this chapter on the distribution of *Meloidogyne* populations (a)virulent on *Mi*-bearing tomato genotypes in Macedonian vegetable and tobacco fields.

MATERIALS AND METHODS

The susceptible tomato, L. esculentum Mill., cvs Moneymaker and Vivia - F 172, and the *Mi* resistant genotypes cv. Carmello GC 204 and cv. Manthos GC 785 were used. S&G Sandoz Seeds, The Netherlands, kindly provided us with the seeds, except for cv. Moneymaker. To exclude nullifying the *Mi* gene at high temperature (9) experiments were done at 20-25 °C in a glasshouse. Two weeks old seedlings were transplanted into 20cm plastic pots filled with sterilised sand and allowed to establish for two weeks before inoculation with second stage juveniles. Pots were fertilised at regular intervals and watered with tap water as required. To avoid contamination pots were kept separate on saucers.

In 1996 and 1997 populations of *Meloidogyne* from nine areas (Table 2.1), seven cropped with vegetables (mainly tomatoes) in glasshouses and two tobacco fields were collected from infested roots and rhizosphere soil.

Only in the Gevgelija glasshouse was the *Mi* resistant tomato cv. Suzo grown. The seventy-three isolates (lines) were set up from single egg masses and propagated and maintained for about seven months on cv. Moneymaker. To obtain enough second stage juveniles (J_2) for the virulence tests eggs were harvested approximately 12 weeks after inoculation by dissolving egg masses in 0.5% NaOCl-solution (15). J_2s were hatched in water and stored at 4 °C until required. The average egg mass contained 200 J_2s. Prior to inoculation nematodes were allowed to pass through a cotton filter and viable juveniles were collected after one day. In virulence tests 400 J_2s (~ 2 egg masses (P_i)) in suspension were pipetted with an automatic syringe onto the soil surface around the stem base of the test plant seedlings followed by light watering.

Seven weeks later plants were harvested individually, their roots washed free from sand stained with Phloxine B solution (8) and the egg masses (P_f) and galls counted. Multiplication rates (P_f/P_i) were assessed. Host status was divided into three categories as follows: $P_f/P_i \geq 1.0$, suitable host (SH); $0.1 < P_f/P_i < 1.0$,

poor host (PH) and P_f/P_i £ 0.1, non-host (NH). Each nematode population was tested in four replicates on the susceptible and resistant genotypes. For each isolate, after square root transformation of the data (data not shown), pair-wise differences between the means were analysed for significance and Least Significant Difference (LSD) at P = 0.05 with ANOVA using Genstat (25).

Table 2.1 - Origin, preceding crop and species designation of populations and isolates used.

Location	Preceding crop	Number of populations	Number of isolates	Species identified	Code*
Bogdanci	tomato, cv. Priska	1	7	*M. incognita*	MiB
Hamzali	cucumber	3	12	*M. incognita*	MiSH
		1	2	*M. javanica*	MjSH
	tomato, cv. Balka	1	3	*M. arenaria*	MaSH
Bansko	cucumber	1	4	*M. incognita*	MiSB
Ilovica	tobacco	2	5	*M. incognita*	MiI
Prosenikovo	cucumber	2	7	*M. incognita*	MiP
		1	2	*M. javanica*	MjP
		1	1	*M. arenaria*	MaP
Kocani	cucumber	1	3	*M. javanica*	MjK
	tomato, cv. Balka	2	8	*M. javanica*	MjK
		1	1	*M. arenaria*	MaK
		1	1	*M. hapla*	MhK
Gevgelija	tomato, cv. Suzo	1	7	*M. javanica*	MjG
Kumanovo	tomato, cv. Vivia	1	4	*M. javanica*	MjKu
		1	1	*M. hapla*	MhKu
Stuka	tobacco	1	5	*M. arenaria*	MaS

* isolates are coded as indicated followed by numbers for field population and isolate.

Species composition of the isolates was determined in two ways. (i) Using the morphological criteria of perineal pattern of adult females and morphometrics of males and J_2s (17). At least 15 perineal patterns and 25 males and juveniles of each isolate were examined. (ii) By amplifying the intergenic spacer region between the cytochrome oxidase II gene and the 16S rRNA gene in the mitochondrial genome of single juveniles (26). In this approach ten individual J_2s were handpicked, homogenised in a 15 µl drop of sterile water, frozen for future analysis or immediately processed in a PCR procedure in a final volume of 25 µl as described. Follow-

ing DNA amplification the products were separated on agarose gel, stained with ethidium bromide and visualised on a UV box. *M. arenaria* is characterised by a unique 1.1 kb fragment. To allow discrimination among species with identically sized amplification products standard restriction digestions (Dra I or Hinf I) of 5 µl of the amplified products were conducted for 2-4 hours at 37 °C and evaluated on 1.5 % agarose gels. Digestion with Dra I was carried out in case of the presence of a 0.52 kb amplification product. A four-banded pattern (0.44, 0.29, 0.23 and 0.08 kb fragments) separated *M. hapla* from *M. chitwoodi, M. marylandi, M. nataliei M. naasi* and *M. fallax.* In case of the presence of a 1.7 kb fragment a Hinf I digestion distinguished *M. javanica* from *M. incognita* on basis of an undigested band and two-banded patterns (0.4 and 1.3 kb fragments) respectively (39). Results were obtained with about 90% of the individual J_2s tested.

RESULTS

There were hardly any discrepancies between the time consuming morphological identifications and the rather straightforward and rapid identifications based on amplification of intergenic spacer region of the mtDNA (Figure 2.1). Contradictory results between the morphological and DNA-based identifications were obtained with three isolates (4.1%). The perineal patterns pointed at *M. javanica* and the DNA based identifications at *M. incognita.* The outcomes of the DNA analyses were followed.

Seventy-three isolate originating from 22 *Meloidogyne* populations were collected in 11 areas previously cropped with cucumber, tobacco or tomato. Two glasshouses turned out to be free from *Meloidogyne* infestations. Mostly mixtures of species were found and *M. javanica* (Treub) Chitw. was present in nearly all tomato crops. Thirty-five isolates were identified as *M. incognita* (Kofoid and White) Chitw., 26 as *M. javanica*, 10 as *M. arenaria* (Neal) Chitw. and 2 as *M. hapla* Chitw.

Virulence characteristics of the isolates were determined on the susceptible cvs Moneymaker and Vivia and the *Mi* resistant cvs Carmello and Manthos (Table 2.2, Figure 2.2). All *M. incognita* isolates reproduced on both susceptible cultivars. It was noticed that often the numbers of egg masses on cv. Moneymaker were higher than the numbers of galls. On cv. Vivia the opposite was true. Four out of the 35 *M. incognita* isolates (11 %) (two MiB, MiS and MiI) also produced egg masses on the resistant cultivars. One of the two MiB isolates, being the most virulent, reproduced equally well on cv. Moneymaker and both resistant cultivars.

As also shown in Table 2.2 the *M. javanica* isolates generally reproduced well on both susceptible cultivars although numbers of egg masses on cv. Vivia tended to be less than on cv. Moneymaker.

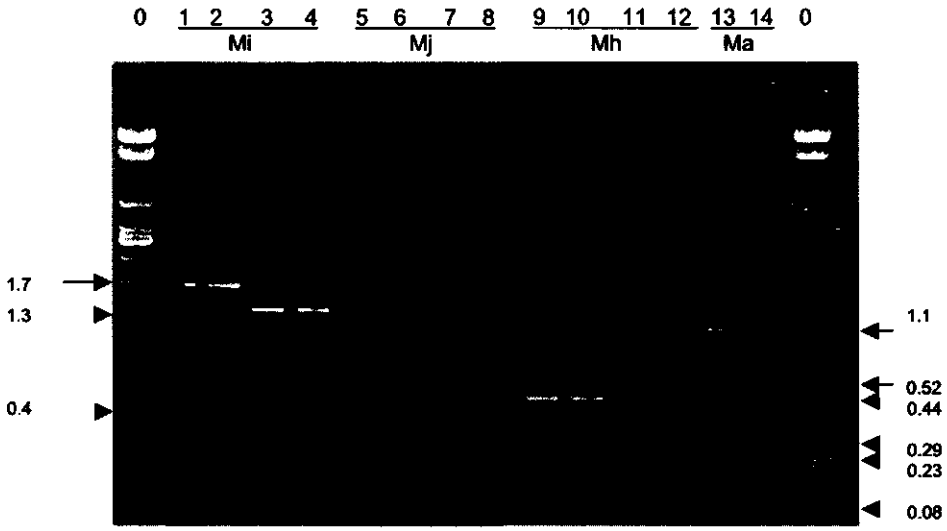

Figure 2.1 - PCR amplification of mtDNA of individual J_2s. Primers and conditions are as described by Powers and Harris (26). The 1.7 kb product is characteristic for *M. incognita* and *M. javanica* (lanes 1, 2 and 5, 6 respectively). After a Hinf I digestion of the 1.7 kb fragment *Meloidogyne incognita* (lane 3, 4) is identified by a two banded pattern and the *M. javanica* (lane 7, 8) fragments remains undigested. The 0.52 kb product (lanes 9, 10) is characteristic for a number of *Meloidogyne* species. After a Dra I digestion four unique fragments identify *M. hapla* (lanes 11, 12). *M. arenaria* gives a 1.1 kb product (lanes 13, 14). The first and last lanes are standard markers.

Twelve out of the 26 isolates (46 %) were virulent at least on one of the *Mi*-bearing cultivars. Some of the isolates (MjG, MjK and MjSH) produced slightly more egg masses on cv. Carmello than on cv. Manthos, while for some other isolates from the same locations (MjG, MjK and MjKu) the opposite was true.

The highest number of virulent isolates (50 %) was found with *M. arenaria*. All isolates reproduced on the two susceptible cultivars. Isolate MaK and two MaS isolates were also fully compatible with the both resistant cultivars and MaSH and one of the MaS lines reproduced better on cv. Manthos than on cv. Carmello.

Both *M. hapla* isolates were fully compatible with all four tomato genotypes.

Table 2.2 - Means of the square root numbers of egg masses of 73 *Meloidogyne* isolates on roots of four *Lycopersicum* genotypes differing in presence or absence of the M*i* gene (means are of four replicates; virulent populations are shaded).

Isolates	Egg masses / Root system			
	Money-maker	Vivia	Carmello	Manthos
MiB12.4	8,25 *a	4,47 **b	0,00 c	1,41 c
MiB12.5	10,72 *a	6,40 **b	1,41 c	0,00 d
MiB12.6	7,35 *a	11,83 *b	0,00 c	0,00 c
MiB12.7	3,46 *a	9,64 **b	2,45 **a	2,65 a
MiB12.8	8,83 *a	9,43 **a	0,00 c	0,00 c
MiB12.9	9,49 *a	8,43 a	2,00 c	5,10 d
MiB12.10	5,66 *a	7,28 **b	1,00 c	0,00 d
MiSH17.4	6,93 *a	0,00	0,00 c	0,00 c
MiSH17.5	7,87 *a	4,47 **b	0,00 *c	0,00 *c
MiSH17.7	7,35 a	6,56 a	0,00 c	0,00 c
MiSH17.9	10,15 a	12,04 *b	0,00 c	1,00 d
MiSH17.10	16,09 *a	10,10 *b	1,00 c	0,00 d
MiSH17.12	6,00 a	18,28 *b	0,00 c	1,41 d
MiSH25.1	6,78 *a	8,77 b	0,00 c	0,00 c
MiSH25.2	11,96 a	10,91 *a	1,00 c	0,00 d
MiSH25.3	8,19 **a	13,27 **b	0,00 **c	1,41 *d
MiSH25.8	13,49 *a	9,80 *b	2,00 c	0,00 d
MiSH26.2	9,90 a	9,43 a	0,00 c	0,00 c
MiSH26.3	13,67 a	10,30 **b	0,00 **c	1,00 d
MjSH15.3	8,72 a	2,65 *b	0,00 c	1,00 d
MjSH15.10	8,49 a	3,74 b	2,24 c	1,00 d
MaSH25.4	15,52 a	14,63 *a	1,41 c	1,00 c
MaSH25.5	7,07 a	7,35 a	1,41 c	1,41 c
MaSH25.10	14,18 a	11,18 **b	1,41 c	2,00 c
MiSB19.3	9,38 *a	8,06 a	1,00 c	1,00 c
MiSB19.6	16,22 a	9,95 b	1,00 *c	0,00 d
MiSB19.7	7,21 a	4,90 *b	1,00 c	0,00 *d
MiSB19.9	9,59 a	8,37 *a	0,00 c	0,00 c
Mil29.3	3,87 a	2,83 a	0,00 c	0,00 c
Mil29.6	3,16 a	3,16 a	0,00 c	0,00 c
Mil29.10	11,09 a	8,25 b	1,00 c	2,00 *d
Mil30.4	7,21 a	7,35 a	0,00 c	0,00 c
Mil30.7	6,93 a	7,68 a	1,41 c	0,00 d
MiP32.5	7,62 a	5,10 *b	0,00 c	0,00 c
MiP32.9	4,00 a	2,00 *b	0,00 c	0,00 c
MiP32.11	4,58 a	2,45 b	0,00 c	0,00 c
MiP33.1	6,71 a	5,83 a	0,00 c	0,00 c

Isolates	Egg masses / Root system			
	Money-maker	Vivia	Carmello	Manthos
MiP33.3	9,33 a	8,25 a	0,00 c	0,00 c
MiP33.6	5,74 a	4,00 *b	0,00 c	0,00 c
MiP33.8	9,06 a	8,72 a	1,00 c	1,00 c
MjP31.1	9,33 a	5,00 **b	0,00 c	1,00 d
MjP31.2	10,39 *a	8,89 **b	0,00 *c	0,00 c
MaP33.5	8,12 *a	7,07 **a	0,00 c	0,00 c
MjK21.1	8,66 a	3,61 b	2,65 bc	2,00 c
MjK21.4	3,61 *a	5,29 *b	1,00 *c	2,45 d
MjK21.8	8,89 a	6,00 *b	2,45 c	1,41 d
MjK2.2	8,25 a	7,00 b	0,00 c	0,00 c
MjK2.4	9,59 a	5,83 b	0,00 c	0,00 c
MjK2.6	7,35 a	4,80 b	0,00 c	1,00 d
MjK2.7	9,49 a	3,61 **b	0,00 c	0,00 c
MjK2.10	11,05 *a	6,86 *b	0,00 c	0,00 c
MjK3.3	8,25 *a	8,60 a	1,00 c	3,46 d
MjK3.12	7,87 a	4,58 b	0,00 c	1,00 d
MjK3.13	14,35 a	10,10 b	1,00 c	0,00 d
MaK22.1	11,58 a	12,17 a	2,83 *c	4,58 *d
MhK2.8	8,66 a	6,71 a	1,73 c	3,61 a
MjG4.3	9,22 a	6,48 b	1,41 c	3,16 d
MjG4.4	3,16 a	3,46 a	1,00 c	0,00 d
MjG4.5	9,27 a	12,53 a	2,24 c	8,89 a
MjG4.6	11,40 a	5,20 *b	3,00 c	2,65 c
MjG4.10	7,48 *a	2,83 **b	1,41 *c	0,00 d
MjG4.11	9,00 a	4,69 b	1,73 c	1,41 c
MjG4.13	8,49 a	4,58 b	0,00 c	1,41 d
MjKu7.3	9,59 *a	4,90 *b	1,41 c	2,00 c
MjKu7.4	8,37 a	7,21 a	0,00 c	0,00 c
MjKu7.7	10,20 *a	7,35 **b	1,00 c	2,24 d
MjKu7.9	8,72 *a	5,57 *b	0,00 *c	1,73 d
MhKu7.6	9,90 *a	5,29 **b	2,00 *c	2,65 **b
MaS27.2	8,49 a	8,00 a	0,00 *c	0,00 *c
MaS27.5	13,67 a	12,49 a	0,00 c	1,41 d
MaS27.6	11,58 a	11,09 a	2,24 c	3,87 d
MaS27.9	8,83 a	7,94 *a	2,65 *c	2,00 c
MaS27.11	7,28 a	6,16 a	0,00 c	2,00 d

* numbers of galls less that numbers of egg masses

** numbers of galls more that numbers of egg masses

Isolate-genotype combinations sharing a common letter do not differ significantly at P = 0.05.

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ABSTRACT

In order to expand our understanding of the unpredictable behaviour of root-knot nematodes, the genetic variation in nuclear DNA and mitochondrial DNA in *Meloidogyne incognita*, *M. arenaria* and *M. javanica* was investigated. Despite the obligate mitotic parthenogenetic mode of reproduction, a large number of AFLP polymorphisms were observed among all 16 populations studied. Both UPGMA and principle coordinate analyses revealed three distinct groups that corresponded with the respective species identities of the 16 populations. *M. incognita* was genetically most distinct. Amplification of 63 bp tandem repeats (TR) in mtDNA from single individuals enabled the calculation of diversity measures at three hierarchical levels: within individuals, among individuals of a single population and among populations. For all three species, the highest diversity was observed within individuals explaining 43 to 65% of the total diversity. Many individuals contained more than one mtDNA size variant. *M. incognita* harboured the most heteroplasmic individuals and was also the most homogenous at the population level. Only 13% of the total diversity was observed among populations, while this figure was 35% for *M. arenaria*. No consistent relation was observed between intraspecific distances based on nuclear DNA and mtDNA. The discordance between the two data sets derived from the two unlinked genomes and the possible causes are discussed.

INTRODUCTION

Knowledge of the genetic structure at the population level is a prerequisite for understanding and predicting the behaviour of pathogens in the field. In order to study how genetic principles apply to a population of a pathogen the genetic information enclosed in the genome of the individuals should at least be partly revealed. Quantification of genetic variability has a pivotal role in contemporary phytonematology too, but is considered as one of the least understood aspects of nematode population biology (10). This especially holds true for obligatory parthenogenetic species such as the three most common species of root knot nematodes *Meloidogyne incognita, M. arenaria* and *M. javanica* (49). In these species parthenogenesis is of the mitotic type and the somatic (2n) number of chromosomes is maintained during maturation of the oocytes. Sperm for fertilisation of the oocyte is not necessary for egg development. The progenies of a single female should, therefore, be identical but genetic differences appear within and among populations. These genetic differences within species and within populations of a single species may account for the variable results of various nematode control strategies such as the growth of resistant crops (34). Since there is no definite species concept that includes parthenogenetic organisms, root knot nematode species are subjective entities based on morphology and,

to some extent, on host response (48). For practical reasons each of these species consists of a large number of field populations that share characteristics of taxonomic value.

As with all animals, the nematode genome consists of nuclear and mitochondrial DNA (mtDNA)(25). Population genetic studies can be conducted by studying polymorphisms at either DNA level. The evolution of the nuclear and mtDNA are unlinked, and discordance between the two DNA types can be expected since the mitochondrial genome mutates at a substantial higher gear (*e.g.* 7, 13). The mtDNA data are therefore more likely to display useful variation at population and individual levels (2).

To date, several PCR (Polymerase Chain Reaction)-based approaches using genomic DNA have been applied to investigate inter- and intrapopulation genetic diversity in *Meloidogyne* species. RAPDs (Random Amplified Polymorphic DNA) have been broadly used as a tool to address variation (11, 12, 20, 29, 55, 59). The use of RFLPs (Restriction Fragment Length Polymorphism) is more reproducible and sensitive thus enabling more precise estimates of genetic variations (9, 15, 19, 20). The technique, however, requires substantial amounts of high quality DNA, which is a major drawback when plant parasitic nematodes are the subject of the analysis. In contrast to the former approaches, the AFLP technique (59) generates virtually unlimited numbers of DNA fragments from nanogram quantities of genomic DNA and the stringent reaction conditions guarantee reproducibility and quantification (17, 18, 53). The discriminatory power of this approach was among others demonstrated by Semblat et al. (43, 50) who have explored the AFLP procedure in a genetic analysis of root knot nematodes.

Since the late 1970s similar attempts have been made to assess interpopulation and individual genetic variations based on polymorphisms within mtDNA of vertebrates and arthropods (2). Hyman and Slater (25) were the first to study mtDNA sequence variations in *Romanomermis culcivorax*, a parasitic nematode of mosquitoes. Tandemly repeated sequences have been characterised in the mtDNA genome of root knot nematodes (36) and were explored for studying polymorphisms in *M. incognita* populations and individuals (26, 56). Polymorphisms within the repetitive regions in mtDNA is limited to only a few organisms such as the white sturgeon *Acipenser transmontanus* (8) and the Atlantic cod *Gadus morrhua* (1).

In this chapter the genetic variation in 16 geographically unrelated *M. incognita, M. javanica* and *M. arenaria* populations was assessed using AFLP markers in nuclear DNA and tandem repeats in mtDNA. The AFLP technique was used to assess the genetic diversity among populations, while the tandem array of 63-bp repeats allowed an estimate of the diversity at three hierarchical levels: within individuals, among individuals within populations and among populations.

MATERIALS AND METHODS

Nematodes. Five isolates of *M. incognita,* 5 of *M. javanica,* and 6 of *M. arenaria* were propagated on *Lycopersicum esculentum* cv Moneymaker under glasshouse conditions (Table 3.1). The geographically unrelated populations were either taken from our laboratory collection or obtained from the Plant Protection Service, Wageningen, The Netherlands. The species identification of each isolate was done by testing their isozyme phenotypes of esterase and malate dehydrogenase (14) and the intergenic spacer region between the cytochrome oxidase II gene and the 16S rRNA gene in the mtDNA of single juveniles (36).

Table 3.1 - Origin of *Meloidogyne* populations used in this study

Meloidogyne spp.	Isolate	Code	Origin	Host of origin	Year of sampling
M. incognita	Mi - 1	L48	NL	tomato	-
	Mi - 2	C 3055	China	bonsai	1990
	Mi - 3	C 4756	Hungary	tomato	1991
	Mi - 4	C 9956	NL	*Rhodochiton sp.*	1997
	Mi - 5	D 385 - C	China	*Ligustrum sp.*	1997
M. javanica	Mj - 1	L1	NL	tomato	-
	Mj - 2	C 3059	China	bonsai	1990
	Mj - 3	C 2539	Costa Rica	*Chrysanthemum sp.*	1990
	Mj - 4	C 8032	NL	Celosia sp.	1995
	Mj - 5	13	U.S.A.	tomato	1997
M. arenaria	Ma - 1	L1	NL	unknown	1994
	Ma - 2	C 8526	U.S.A.	*Hosta lancifolia*	1996
	Ma - 3	C 7277	NL	*Hosta sp.*	1994
	Ma - 4	14	Belgium	tomato	1997
	Ma - 5	C 6460	Columbia	*Livinstonia rotundofolia*	1993
	Ma - 6	C 9891	NL	*Philodendron sp.*	1997

AFLP procedure. Egg masses were harvested from infected tomato roots and treated with 0.5 % NaOCl (23). After hatching at 22°C the mixture of second stage juveniles (J2s) and eggs was homogenised in proteinase K buffer (20 mM Tris - pH8, 100 mM EDTA, 0.5% SDS, 2 mg/ml proteinase K) and incubated at 50°C for 4 hours. A single phenol and chlorophorm:isoamyl extraction was followed by adding 10 M ammonium acetate and absolute ethanol to precipitate the DNA. The DNA pellet was washed twice with 70% ethanol, air dried and resuspended in 20 ml TE buffer pH7.5 (41).

The AFLP procedure was performed as described by Zabeau and Vos (59). The digestion of genomic DNA with *Eco* RI and *Mse* I was followed by the ligation of an Eco RI-adapter and an Mse I-adapter (Table 3.2). The ligation mix was used in a nonselective amplification (preamplification) using primers that annealed to the *Eco* RI (E+0) and *Mse* I adapter sequences (M+0) (Table 3.2). The PCR reaction was performed in a PE-9600 thermal cycler (Perkin Elmer, Norwalk, USA) using the following profile: 30 cycles of 30" denaturation at 94°C, 30" annealing at 56°C and 60" extension at 72°C. Verification of the PCR products was done in 1% agarose gel in TAE buffer stained with 0.5 ml/ml ethidium bromide (41). In a typical reaction the DNA fragments appeared on gel as a smear from 50 bp up to 500 bp. The preamplification product was diluted 10x with ddH_2O to prepare it as the secondary template for the selective amplification.

For selective amplification of restriction fragments, only one primer (E+GA) was labelled using 10mCi/ml g33P-ATP according to the recommendations of the manufacturer. In each reaction 5ng of labelled and 30 ng of unlabeled selective

Table 3.2 - Sequences of adapters and primers used in this study

EcoRI adapter		5' -CTC GTA GAC TGC GTA CC 3' -CTG ACG CAT GGT TAA- 5'
MseI adapter		5' -GAC GAT GAG TCC TGA G 3' -TAC TCA GGA CTC AT- 5'
EcoRI + 0primer	EOO	5' -GAC TGC GTA CCA ATT C- 3'
EcoRI + 2primer	E+GA	5' -GAC TGC GTA CCA ATT CGA- 3'
MseI + 0primer	MOO	5' -GAT GAG TCC TGA GTA A- 3'
MseI + 2primers	M+AC	5' -GAT GAG TCC TGA GTA AAC- 3'
	M+AG	5' -GAT GAG TCC TGA GTA AAG- 3'
	M+AT	5' -GAT GAG TCC TGA GTA AAT- 3'
	M+CC	5' -GAT GAG TCC TGA GTA ACC- 3'
	M+CG	5' -GAT GAG TCC TGA GTA ACG- 3'
	M+CT	5' -GAT GAG TCC TGA GTA ACT- 3'
	M+GA	5' -GAT GAG TCC TGA GTA AGA- 3'
	M+GC	5' -GAT GAG TCC TGA GTA AGC- 3'
	M+GT	5' -GAT GAG TCC TGA GTA AGT- 3'
	M+TA	5' -GAT GAG TCC TGA GTA ATA- 3'
	M+TG	5' -GAT GAG TCC TGA GTA ATG- 3'
	M+TT	5' -GAT GAG TCC TGA GTA ATT- 3'
MseI + 3primers	M+AAA	5' -GAT GAG TCC TGA GTA AAA A- 3'
	M+AAC	5' -GAT GAG TCC TGA GTA AAA C- 3'
	M+AAG	5' -GAT GAG TCC TGA GTA AAA G- 3'
	M+AAT	5' -GAT GAG TCC TGA GTA AAA T- 3'
	M+ACC	5' -GAT GAG TCC TGA GTA AAC C- 3'
TR-F		5'- CTA TTT TAA AGT TAT CGA CTG-3'
TR-R		5'-CCT AAA GAC TTT TTA TCC TAA C-3'

primers (M + 2 and M+ 3, Table 3.2) was used. The PCR proceeded according to a touch-down profile of 24 cycles: 12 cycles of 30" denaturation at 94°C, 30" annealing at 65°C, 60" extension at 72°C; the annealing temperature was reduced each cycle by 0.7°C for the next 12 cycles, and was continued at 56°C for the remaining 24 cycles. A total of twelve M+2 and five M+3 primers were used in a combination with the labelled E+GA primer. The PCR products were mixed with an equal volume of formamide-loading buffer, denaturated for 5' at 95°C and loaded on a 5% polyacrylamide gel (Sequagel-5, BioZym, Georgia, USA) in 1xTBE electrophoresis buffer (41). Following transfer on Whatmann 3MM paper (Model 583, Bio-Rad), gels were dried, and used to expose X-Ray films (Konica, Tokyo, Japan) for 3 and 6 days at room temperature. The autoradiograms of the DNA fingerprints were scanned and cross-checked with a computer package providing a semi-automatic analysis, developed for quantitative analysis of DNA fingerprinting patterns. In each reaction the presence/absence of bands was scored.

63-bp TR (tandem repeats) procedure. Template DNAs were prepared from the following sources: i) pooled genomic DNA from preparasitic second stage juveniles and ii) genomic DNA from individual J2s. The DNA from these individual J2s was obtained by maceration of the larvae in 10 ml of sterile water. The primers TR-F and TR-R (Table 3.2) and amplification conditions used have been according to Hyman and Whipple (26). The forward primer specifically anneals to a non-coding region fifteen bp upstream of the 63 bp repeating unit array. The reverse primer binds five bp downstream of the TR within the mitochondrial tRNAmet gene. PCR products were fractionated on Tris-Acetate-EDTA buffered (30) 2% agarose gels (MethaPhor, FMC, USA). The gels were stained with gelstar (BioProduct, Rockland, ME, USA) and photographed with Polaroid negative film under UV light. The bands were scored for intensity and presence/absence.

Data analysis.

AFLP - The presence/absence of bands was scored for all populations. Experiments were repeated at least once, and only DNA fragments consistently present or absent were taken into account and treated as binary characters. Calculation of the genetic distances (GD) between populations was based on pair-wise comparisons and calculated according the following equation: GDxy=1-[2Nxy/(Nx+Ny)], where Nx is the number of fragments in genotype x, Ny is the number of fragments in genotype y and Nxy is the number of fragments shared by genotypes x and y (33). The dendrogram of the 16 *Meloidogyne* populations was constructed from the genetic distance matrix by using the UPGMA algorithm (44). Thousand bootstrap replicates were performed to test the support of the branches (16). The binary tables were analysed with a Treecon software package (version 1.3b) for construction and drawing of dendrograms and trees (51). Principal coordinate analysis was performed to ac-

cess interspecies relationship based on the Nei and Li (33) coefficient using the Nt-SYS-pc software (40).

63-bp TR - To estimate the relative frequencies of mtDNA length variants within and among individual nematodes, the relative intensities of bands reflecting heteroplasmic variations were assessed by a combination of ImageBioMax densitometry software and visual estimation. Characterisation of the variation within and among samples was done by K-indices (5). Gene diversity was calculated from the mtDNA size class frequencies by K-indices, where $K = 1-(Sx_i^2)$, where x_i is the frequency estimate of mtDNA size class i within individuals. Kb is the diversity within an individual, Kc within a population and Kd is a measure for the total diversity. For each species, diversity measures were calculated at three hierarchical levels: i) within individuals (Ci=averageKb/Kd)), ii) among individuals within a population (Cip=(averageKc-averageKb)/Kd) and iii) among populations within a species (Cps=(Kd-averageKc)/Kd) (39). By definition, Ci+Cip+Cps = 1.0 (1). To test the differences in allele frequency distributions among populations the G-test was employed (46).

The 63-bp repeating units were also scored as presence/absence between populations and treated in the same way as the AFLP fragments using the software package Treecon (1.3b).

AFLP and TR Correlation – Taking into account that we deal with two variables (genetic distances based on AFLP and VNTR markers) and one fixed parameter (the examined populations) we have performed a statistical analysis. To investigate a possible correlation between the AFLP and TR data the 'product-moment correlation coefficient' (PMCC) was calculated. The PMCC between the intraspecies GD values of AFLP and TRs, was computed by $PMCC=Sy_1y_2/ \ddot{O}Sy^2{}_1Sy^2{}_2$ (47) using the SAS/STAT software package (42).

RESULTS

AFLP. Presence/absence polymorphisms in AFLP fingerprints were scored for five *M. incognita*, five *M. javanica* and six *M. arenaria* populations. The number of fragments per primer combination ranged from 29 to 72 with sizes varying from 50 to 500 bp (Figure 3.1). The total number of fragments ranged from 295 to 338, 258 to 323 and 319 to 454 for the *M. incognita*, *M. javanica* and *M. arenaria* populations, respectively. The proportion of polymorphic DNA fragments between *M. incognita* populations (32%) was low as compared to proportions in *M. javanica* (52%) and *M. arenaria* (61%). There were 168, 64 and 69 bands species specific for *M. incognita*, *M. javanica* and *M. arenaria*, respectively.

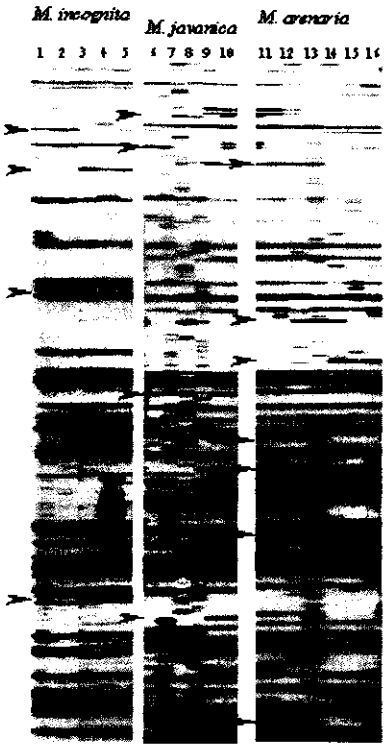

Figure 3.1 - AFLP fingerprints generated with primer combination E+GA / M+AC. Subsequent lanes contain amplification products from 16 *Meloidogyne* populations, five *M. incognita*, five *M. javanica* and six *M. arenaria*. Polymorphic bands for *Meloidogyne* spp. are designated with arrows. Population codes are given in Table 3.1.

Based on genetic distances (Table 3.3) and UPGMA analysis the 16 *Meloidogyne* populations were grouped into three clusters, which corresponded with their respective species identities. The average GD among populations within a species was 0.15 for *M. incognita*, 0.27 for *M. javanica* and 0.22 for *M. arenaria*. The GD values between species were much higher. Between *M. incognita* and *M. javanica* populations the GD value was 0.64. Between *M. incognita*/M. *arenaria* and *M. javanica/M. arenaria* the GD values were 0.62 and 0.40, respectively. The branches in the similarity dendrogram were evaluated by 1000 bootstrap replicates. The *M. incognita* populations were assigned to one cluster with 100% bootstrap support, whereas *M. javanica* and *M. arenaria* populations were grouped in separate clusters with a bootstrap value of 99% (Figure 3.2). These findings were supported by principle coordinate analyses (Figure 3.3).

Table 3.3 - Genetic distance matrix of 16 screened *Meloidogyne* populations belonging to *M. incognita, M. javanica* and *M. arenaria,* generated by the formula of Nei and Li (1979), after binary transformation of the AFLP fragments (below the diagonal) and the TR fragments (above the diagonal).

	Mi-1	Mi-2	Mi-3	Mi-4	Mi-5	Mj-1	Mj-2	Mj-3	Mj-4	Mj-5	Ma-1	Ma-2	Ma-3	Ma-4	Ma-5	Ma-6
Mi-1	-	0,03	0,03	0,03	0,09	0,36	0,07	0,11	0,20	0,07	0,20	0,25	0,30	0,11	0,00	0,12
Mi-2	0,18	-	0,07	0,00	0,13	0,33	0,04	0,08	0,17	0,03	0,17	0,22	0,27	0,08	0,03	0,15
Mi-3	0,20	0,13	-	0,07	0,06	0,39	0,10	0,14	0,23	0,03	0,23	0,28	0,33	0,14	0,03	0,09
Mi-4	0,21	0,15	0,10	-	0,13	0,33	0,04	0,08	0,17	0,03	0,17	0,22	0,27	0,08	0,03	0,15
Mi-5	0,22	0,15	0,08	0,11	-	0,44	0,16	0,20	0,29	0,09	0,29	0,33	0,38	0,20	0,09	0,08
Mj-1	0,60	0,59	0,65	0,54	0,64	-	0,30	0,26	0,18	0,36	0,18	0,13	0,07	0,26	0,36	0,46
Mj-2	0,66	0,66	0,68	0,59	0,68	0,27	-	0,04	0,13	0,07	0,13	0,18	0,24	0,04	0,07	0,19
Mj-3	0,64	0,62	0,66	0,58	0,67	0,22	0,28	-	0,09	0,11	0,09	0,14	0,20	0,00	0,11	0,23
Mj-4	0,67	0,69	0,72	0,63	0,72	0,34	0,26	0,29	-	0,20	0,10	0,16	0,22	0,09	0,20	0,31
Mj-5	0,64	0,64	0,68	0,57	0,68	0,21	0,28	0,26	0,26	-	0,20	0,25	0,30	0,11	0,07	0,12
Ma-1	0,62	0,61	0,68	0,62	0,67	0,35	0,44	0,39	0,44	0,39	-	0,05	0,11	0,09	0,20	0,31
Ma-2	0,59	0,59	0,65	0,57	0,63	0,35	0,45	0,39	0,45	0,37	0,08	-	0,06	0,14	0,25	0,36
Ma-3	0,60	0,60	0,66	0,59	0,65	0,35	0,46	0,40	0,47	0,40	0,04	0,07	-	0,20	0,30	0,41
Ma-4	0,64	0,63	0,68	0,61	0,68	0,37	0,46	0,41	0,48	0,41	0,15	0,17	0,15	-	0,11	0,23
Ma-5	0,59	0,58	0,62	0,54	0,62	0,34	0,43	0,36	0,42	0,36	0,31	0,30	0,32	0,27	-	0,12
Ma-6	0,62	0,58	0,63	0,57	0,63	0,29	0,42	0,35	0,45	0,38	0,32	0,32	0,32	0,32	0,19	-

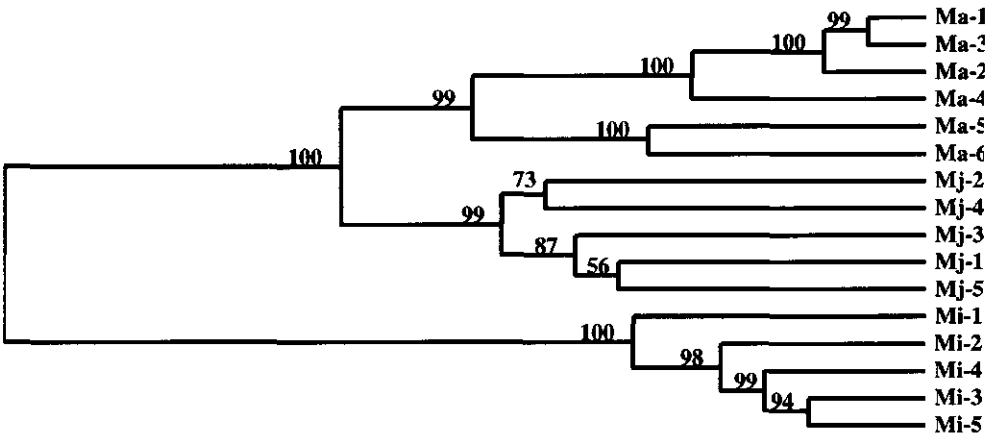

Figure 3.2 - Dendrogram of 16 *Meloidogyne* populations belonging to *M. incognita, M. javanica* and *M. arenaria*, based on AFLP data. Population codes are given in Table 3.1.

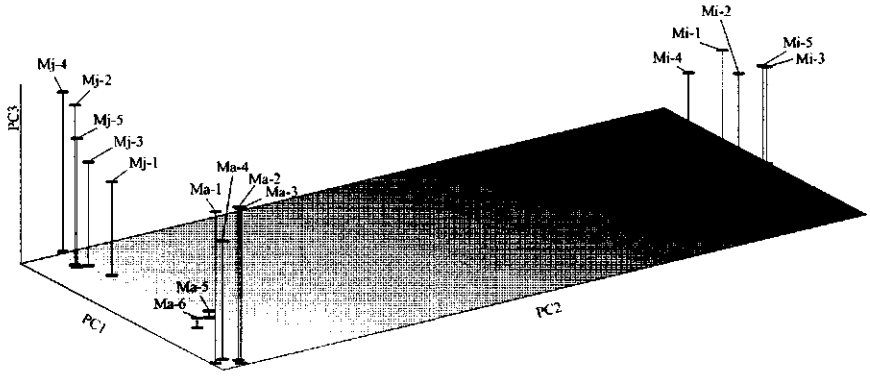

Figure 3.3 - Relationships among 16 *Meloidogyne* populations belonging to *M. incognita, M. javanica* and *M. arenaria* obtained by principle coordinate analysis using AFLP data. Population codes are given in Table 3.1.

63-bp TR. Successful amplifications of 63-bp TRs from pooled individuals were achieved for all populations. When electrophoretically resolved, banding patterns appeared in which each successive band was 63-bp larger than its immediately adjacent band. This indicated that the multiple PCR products resulted from the presence of mtDNA template molecules having different numbers of the 63-bp repeating unit. The band intensities refelected the copy number of the mtDNA size variants. Similar banding patterns were obtained from DNA prepared from individual J_2s, indicating that heteroplasmic nematodes containing multiple mtDNA size variants help comprise the genetic structure of the nematode populations (Figure 3.4).

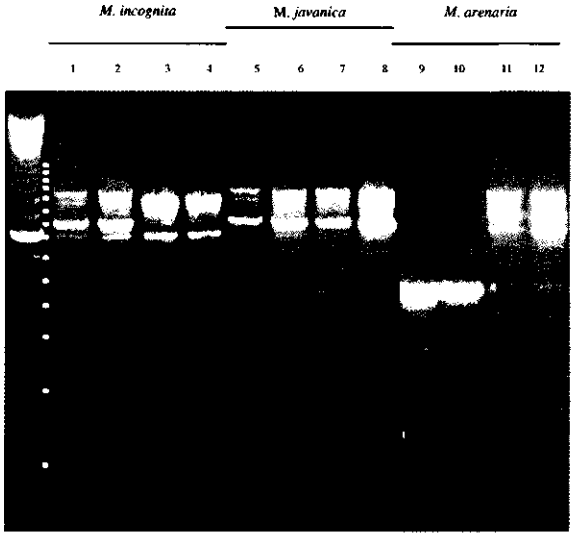

Figure 3.4 - 63-bp TR profiles generated within *M. incognita*, *M. javanica* and *M. arenaria* individuals. Each line represents single second stage juvenile.

- Lane 1,2 individuals from Mi-1,
- Lane 3,4 individuals from Mi-2,
- Lane 5,6 individuals from Mj-1,
- Lane 7,8 individuals from Mj-2,
- Lane 9,10 individuals from Ma-1,
- Lane 11,12 individuals from Ma-2.

The positions of the 2nd till the 15th 63-bp repeating unit are indicated by white dots. The 100-bp marker is on the left. Population codes are given in Table 3.1.

In this study the various types of TRs and their frequencies were studied in ten to fifty J_2s per population. All populations contained substantial numbers of heteroplasmic individuals ranging from 17 to 100% (Table 3.4). *M. incognita* had on average the highest number of heteroplasmic individuals. The number of 63 bp repeats varied from 7 to 20. In general heteroplasmic nematodes have a higher number of repeats numbers than homoplasmic individuals. The genetic diversity was evaluated at three levels: i) within individuals (Ci), ii) among individuals (Cip), and iii) between populations (Cps). For all three species, the highest diversity is observed within individuals (Figure 3.6). The Ci values range from 65% for *M. incognita* to 43 and 48% for *M. javanica* and *M. arenaria*, respectively (Table 3.4). At the level of the population *M. incognita* was the most homogenous (Cps value of 13 %) followed by *M. javanica* (24 %) and *M. arenaria* (35 %) (Table 3.4). A G-test for the frequency distributions of the various mtDNA size variants within the three species revealed significant differences between the three data sets (G=259,5756, d.f.=36; G=106,4144, d.f.=36 (Tv=67,985) and G=117,9254, d.f.=40, (Tv=73,402), P<0.001 , for *M. incognita, M. javanica* and *M. arenaria*, respectively).

Table 3.4 - Hierarchical analysis of 63-bp repeating units of mtDNA in five *M. incognita*, five *M. javanica* and six *M. arenaria* populations.

Meloidogyne				Kb range							
species	Population	n	F(h)	min	max	Kb	Kc	Kd	Ci	Cip	Cps
M. incognita	Mi - 1	50	20	0	0,65	0,52	0,59				
	Mi - 2	50	34	0	0,54	0,24	0,31				
	Mi - 3	12	8	0	0,75	0,43	0,73				
	Mi - 4	10	0	0,32	0,69	0,53	0,64				
	Mi - 5	12	0	0,18	0,77	0,60	0,81				
						0,46	0,62	0,71	0,65	0,22	0,13
M. javanica	Mj - 1	12	67	0	0,50	0,17	0,29				
	Mj - 2	12	67	0	0,73	0,17	0,22				
	Mj - 3	12	0	0,18	0,72	0,45	0,72				
	Mj - 4	10	10	0	0,58	0,43	0,73				
	Mj - 5	14	36	0	0,73	0,28	0,68				
						0,30	0,53	0,69	0,43	0,33	0,24
M. arenaria	Ma - 1	16	50	0	0,63	0,27	0,34				
	Ma - 2	10	50	0	0,50	0,17	0,24				
	Ma - 3	12	83	0	0,32	0,05	0,06				
	Ma - 4	16	50	0	0,66	0,25	0,35				
	Ma - 5	10	0	0,35	0,72	0,55	0,78				
	Ma - 6	12	17	0	0,77	0,52	0,68				
						0,30	0,41	0,62	0,48	0,17	0,35

n – number of individuals sampled
F(h) – frequency of homoplasmic individuals
Ci – % total genetic diversity within individual
Cip – % total genetic diversity among individuals within populations
Cps – % total genetic diversity among population within species (lineages)
Kb–mtDNA diversity within individual
Kc–mtDNA diversity within population
Kd- mtDNA diversity within species

Comparison of AFLP and TR data. In order to investigate the degree to which nuclear and mitochondrial variations in DNA are associated, the AFLP and TR data were compared in different ways. One way was to compare the Cps values with the number of AFLP polymorphisms among populations from a single species. The Cps value was the lowest for *M. incognita* (13%) and the highest for *M. arenaria* (35%), which is in agreement with the level of AFLP polymorphisms within *M. incognita* (32%) and *M. arenaria* (61%).

Another way was to transform the TRs to binary data. Similar to the AFLP data, the 63-bp repeating units were treated as presence/absence data by comparison of all 16 populations and the binary table was used for calculating GDs (Table 3.3). The number of polymorphic TRs between the *M. incognita* populations was 20%, whereas this figure was 40% and 50% for *M. javanica* and *M. arenaria*, respectively. The average GD between populations of a single species were 0.06, 0.17 and 0.20 for *M. incognita*, *M. javanica* and *M. arenaria*, respectively. UPGMA analyses showed that there is no consistent relationship between the two types of data when analysing the whole range of GD values. In contrast to the AFLP data (Figure 3.2), the similarity dendrogram based on the GD values of the TR data of the 16 *Meloidogyne* populations showed low bootstrap values for most clusters (data not shown). In addition, the *M. arenaria* and *M. javanica* populations were not clustered in separate groups and were completely mixed. Only the five *M. incognita* populations occurred as a distinct group.

Statistical tests for the correlation between the two types of GD data are also not consistent. Using the 'product-moment correlation coefficient' we found no significant correlation between the GD as determined with AFLP and the GD as determined with TR between *M. incognita* (PMCC=-0.226973, d.f.=8) and *M. javanica* (PMCC=-0.514644, d.f.=8) populations, whereas a significant correlation (PMCC =0.000328, d.f.=13) exists between the two data sets for the *M. arenaria* populations (Figure 3.5).

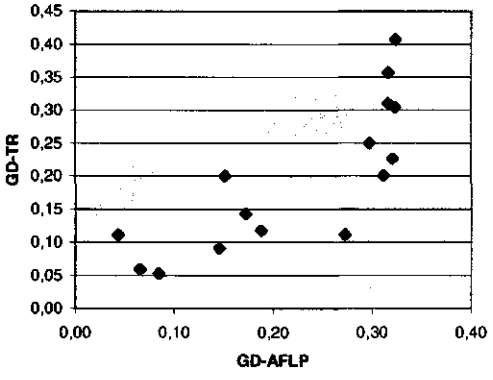

Figure 3.5 - Comparison of genetic distances based on AFLPs and TRs data from six *M. arenaria* populations.

DISCUSSION

The small size of nematodes, an obstacle for analysing single genotypes, has been overcome by using a PCR-based approach to study size variations in mtDNA molecules. To obtain an overall view of the genetic architecture of the 16 *Meloidogyne* populations, we analysed these populations also with the AFLP technique. To our knowledge, this is the first study in which both variations in nuclear and mitochondrial DNA are investigated in plant parasitic nematode populations.

AFLP fingerprints of 16 *Meloidogyne* populations, representing the 3 major mitotic parthenogenetic *Meloiodygne* species, displayed both common and differential (e.g. species specific) bands within and between species. In our experiments the three *Meloidogyne* species were consistently grouped in three separate clustered. Further analysis of the AFLP fingerprints showed that at the species level *M. javanica* and *M. arenaria* are more related to each other than to *M. incognita,* what is in compliance with the AFLP analysis of Semblat et al. (43) and Van der Beek et al. (52). Baum et al. (4) and Tastet et al. (47) have found a similar genetic relationship based on RAPDs and protein analysis. However, evidence from mtDNA (20, 22, 58) suggests that *M. arenaria* is not closer related to *M. javanica* than it is to *M. incognita*. Others have placed *M. arenaria* even as the most distinct species (37). This latter finding is also supported by the observation that *M. arenaria* is the only one that exists in triploid form with a somatic chromosome number larger than 50 (28).

The dendrogram (Figure 3.2) shows no correlation between the AFLP data and the geographical origins of the populations. Several studies (6, 43, 50) have lead to similar conclusions, while others (4, 47) revealed consistent clusters due to common geographical origin. In our study, clustering geographically widely separated populations together (*e.g.*, Mi-3 from Hungary and Mi-5 from China; Mj-1 from the Netherlands and Mj-5 from USA; Ma-5 from Columbia and Ma-6 from the Netherlands), suggests common origins. Clustering Ma-1 and Ma-3 both from the Netherlands were exceptions.

In addition to the results obtained with AFLP the structures of the 16 populations were investigated using the mtDNA of individual nematodes. Generally, mtDNA is used for population genetic studies because of two main peculiarities, i) high cellular copy number (24), ii) occurrence of polymorphic forms. Differences in the geographic distribution of mitochondrial DNA haplotypes have been used to estimate genetic differentiation within and among populations of various organisms (2). Intra- and interindividual variation in repeat numbers in mtDNA has been found in *Meloidogyne incognita* (56) and *Romanomermis spp.* (3, 25). At present no population genetics have been done on *M. javanica* and *M. arenaria* using 63-bp TRs.

In all three *Meloidogyne* species included in our investigation the highest diversity in TRs was found within individuals (Ci). These results commensurate the diversity in mtDNA size variants in *Gadus morhua* (atlantic cod) for which it was

concluded that the generation of variation by length mutation overcomes the loss of variation due to random drift during cell division (1). Similar high heteroplasmy levels have been also reported for species of crickets (38, 39), frogs (31) and *Drosophila* (21).

As compared to *M. incognita* and *M. javanica, M. arenaria* shows strongest differentiation among populations with the lowest genetic diversity within populations (Figure 3.6). These findings provide further evidence that *M. arenaria* is the most heterogenous of the three major mitotic parthenogenetic *Meloidogyne* species.

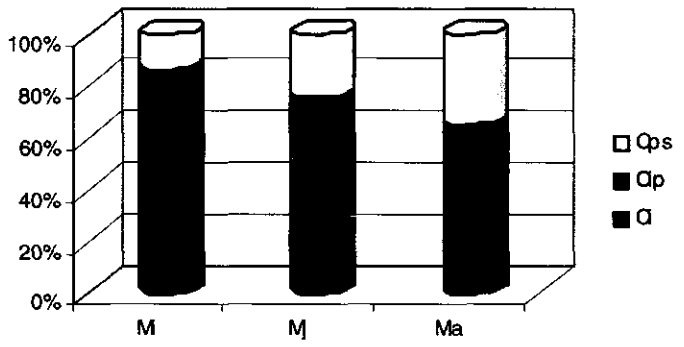

Figure 3.6 – Genetic diversity based on 63-bp tandem repeats on different hierarchical levels.

Cps - % genetic diversity among population within species,
Cip - % genetic diversity among individuals within populations,
Ci - % genetic diversity within individual.

Mutation to different 63-bp repeat copies is the primary mechanism by which size variation is generated in obligatory mitotic parthenogenetic root-knot nematodes, because a paternal contribution is excluded (56). The diversity of the banding patterns produced by amplifying the 63 bp tandem repeats, together with the observation that these patterns are stable (*e.g.*, absence of the 8th 63-bp in all Mj-4 individuals), suggest that this is a sensitive assay for substructuring of the *Meloidogyne* populations. One explanation for the lower incidence of heteroplasmy in some isolates may derive from the higher frequency of the single 63 bp repeat, because most mechanisms proposed for mutations require multiple 63-bp repeats. Several types of genetic rearrangements have been reported (27, 32, 45, 54).

With regard to the level of diversity among populations within a single species, both AFLP (% polymorphism) and TR (Cps values) data show that *M. incognita* has the lowest diversity and *M. arenaria* the highest. However, analyses of the relation between the genetic distances based on 63-bp TRs and AFLPs shows that the degree of divergence within species is not always correlated. For the six *M. are-*

naria populations a positive correlation was found, whereas no correlation was observed for the other two species. Also similarity dendrograms based on the two types of genetic distances were not congruent. Only the five *M. incognita* populations clustered as expected, while the other two species were intermingled. These inconsistencies between the two data sets are most likely the result of the forces that drive the variation in the mitochondrial and nuclear genome. It is thought that the rate of mutation to different 63 bp repeats copies is much higher than the rate of mutation in the nuclear genome. Also selection and drift are relatively strong. For example, not all repeat copies will be replicated at the same speed and extremely high copy numbers are probably not tolerated in the mitochondrial genome, setting a limit to the number of potential polymorphisms. In contrast to variation in nuclear DNA, drift is also operating on the TRs during vegetative germ line cell divisions, which would ultimately result in 100% homoplasmic individuals when not countered by mutations. Altogether, these processes account for a highly dynamic system maintaining variation at the level of mtDNA. Together with the relatively small number of potential polymorphisms in 63 bp repeat copies, which favours convergence and parallelisms masking ancestral relationships, this is probably the most likely explanation for the observed disparities.

This study shows that the species *M. incognita*, *M. javanica* and *M. arenaria* display a wide range of variations within individuals, among individuals within populations, among populations within a single species and among species. AFLP analyses at the latter two hierarchical levels indicated that variations in the number of 63 bp repeats are not appropriate to study long term evolutionary processes. The rate at which these mitochondrial repeats evolve is probably too fast to retain the footprints of ancestral relationships, not only between species but also between populations of a single species. Nevertheless, mtDNA remains appealing because of the ability to study variation within a single genotype and the high evolution rate may also be an advantage when studying more short term evolutionary processes.

AFLP analyses showed that despite the mitotic parthenogenetic mode of reproduction the three species have a level of variation that is not very different from the potato cyst nematode species *Globodera rostochiensis* and *G. pallida* (17, 18) that have an amphimictic mode of reproduction. The intraspecific distances were even somewhat larger for the *Meloidogyne* species than for the *Globodera* species. These data challenge the prevailing view that mitotic parthenogenetic plant parasitic nematodes are less variable than amphimictic nematodes. The variation observed in this study at various hierarchical levels probably reflects the ability of *Meloidogyne* species to parasitise a wide range of hosts and to adapt to various environments. One of the challenges in the future will be to unravel the mechanism by which mitotic parthenogenetic organisms generate variation and at the same time maintain the integrity of the species.

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Chapter 4

Single pass cDNA sequencing – a powerful tool to analyze gene expression in preparasitic juveniles of the southern root-knot nematode *Meloidogyne incognita*

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ABSTRACT

Expressed sequence tags (ESTs) have been widely used to assist in gene discovery in various organisms (*e.g.*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Mus musculus*, and *Homo sapiens*). In this paper we describe an EST project, which aims to investigate gene expression in *Meloidogyne incognita* at the onset of parasitism. Approximately 1000 5'-end sequence tags were produced from a cDNA library made of freshly hatched preparasitic second stage juveniles (J2). The ESTs were identified in the primary transformants of the cDNA library, and assigned to nine different functional groups, including (candidate) parasitism genes. A large fraction of the ESTs (45%) did not have a putative homologue in public databases. Sixty five percent of the ESTs that could be clustered into a functional group had putative homologues in other nematode species. ESTs were found for virtually all parasitism related genes that have been cloned from *M. incognita* to date. In addition, several novel genes were tagged, including a xylanase and a chitinase gene. The efficiency of EST projects, which produce sequence data for thousands of genes in months time without any difficult pre-selections of mRNA pools, makes random sequencing cDNA libraries a superior method to identify candidates for parasitism related genes in plant-parasitic nematodes.

The sequences in this chapter are retrievable from Genbank with the accession numbers BE191640 to BE191741, BE217592 to BE217720, BE225324 to BE225598, BE238852 to BE239221, and BE240829 to BE240865.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are polyphagous endoparasites, responsible for billions of dollars in annual crop losses. The majority of the plant species that account for the world's food supply are susceptible to root-knot nematode infection. The hallmark of the complex nematode-plant interaction is the feeding cell structure that is induced by the nematode in the host plant. The feeding cell structure – the giant cell – facilitates a permanent flow of plant nutrients from the vascular tissue to the feeding nematode. Inadequate feeding cells result in poor development and reduced fecundity of the nematodes. Knowledge of nematode genes that are involved in host penetration, migration and feeding may help to design resistance strategies for pest control (61).

The only published estimate of the haploid genome size of *Meloidogyne* is 51 Mb (38), however, further estimates of the physical properties of the *G. rostochiensis* genome using AFLP analysis indicate that the size and the average G+C composition of plant parasitic nematodes are similar to the estimates obtained for *Caenorhabditis elegans* (9.7×10^7 bp) (49). Hence, it is reasonable to assume that

the gene number of plant parasitic nematodes is also similar to that predicted for *C. elegans* (»19,000).

To date various approaches have been applied to investigate gene expression in plant parasitic nematodes in order to identify candidate parasitism genes. In summary, these approaches include screening of cDNA libraries either with monoclonal antibodies specific for nematode secretions (14, 15, 27) or with homologous plaque hybridization (29), PCR based cloning using degenerate primers (53), RNA fingerprinting (17), and differential screening of cDNA libraries (31, 48). Although these methods have proven to be successful for a limited number of genes, all require prior knowledge of candidate genes or technically advanced pre-selections in mRNA pools.

Random sequencing of cDNA libraries of various developmental stages has been applied to animal parasitic nematodes such as the filarial nematodes *Brugia malayi* and *Onchocerca volvulus* in order to identify expressed sequence tags (ESTs) of nematode genes (http://helios.bto.ed.ac.uk/mbx/fgn/net/librarylist.html and http://math.smith.edu/~sawlab/fgn/net/librarylist.html). In the *Brugia* genome project, a combination of expressed sequence tag sequencing from multiple cDNA libraries representing the complete filarial nematode lifecycle, and comparative analysis of the sequence dataset has proven to be very effective in gene discovery. With the advent of high throughput sequencing facilities, the affordable prices of a single sequence run make similar EST projects feasible for plant parasitic nematodes too. Hence, approximately 1000 ESTs were recently produced from the potato cyst nematodes, *Globodera pallida* and *G. rostochiensis,* and proved to be efficient tools for identifying novel parasitism genes (39, 40).

This chapter describes an EST project to investigate gene expression in *Meloidogyne incognita* at the onset of parasitism. We have chosen to start with pre-parasitic second stage juveniles to cover the initial phases of the parasitic cycle, plant penetration and intercellular migration. Single-pass sequences were obtained from the 5' end of the cDNA inserts of the primary transformants of the cDNA library, and grouped into 9 functional classes, including (candidate) parasitism genes (16, 43).

MATERIAL AND METHODS

Nematodes. *Meloidogyne incognita,* line 48, was propagated in greenhouse cultures on tomato cultivar Moneymaker at 20-25 °C. Eggs were harvested approximately 12 weeks after inoculation and isolated using 0.5% NaOCl-solution (26). Second stage juveniles (J_2) were collected from eggs in water on a cotton wool filter, purified using 70% sucrose, and stored at –80 °C until further processing.

RNA Isolation. Total RNA was extracted from 100 000 frozen pre-parasitic J_2 using Trizol Reagent (Life Technologies, Gaithersburg, MD, USA). Following a chloro-

form extraction, the RNA was precipitated in isopropyl alcohol. The pellet was subsequently washed in 75% ethanol, and the remaining RNA was dissolved in an appropriate volume of sterile dimethyl pyrocarbonate-treated water. Analysis of the total RNA on denaturing agarose gel resulted in a smear from 50 to 3000 bp with two distinct bands of ribosomal RNA.

cDNA synthesis. The cDNA for the library was prepared using the Smart cDNA library construction system (Clontech, Palo Alto, CA, USA) with a few modifications. Briefly, 3 ml containing 50ng total RNA was transcribed into single strand cDNA using a Smart oligonucleotide, a modified oligo $d(T)_{30}$ anchor primer, and Superscript II reverse transcriptase (Life Technologies). The single strand cDNA was amplified in 23 cycles (long-distance) of PCR according to the manufacture's protocol (Clontech). The amplified cDNA was digested with *Sfi* I restriction enzyme, and fractionated in a Chroma-Spin-400 column (Clontech). Only the size fractions including cDNA ranging from 700 to 1500 bp were pooled, and subsequently ligated into the vector plasmid pMAK1.

Construction of library into plasmid pMAK I. The plasmid pMAK1 was derived from the plasmid pcDNA II (Invitrogen, San Diego, CA, USA). The *Eco* RI – *Bam* HI element in the multiple cloning site of pcDNA II was replaced by a fragment, which includes two *Sfi* I restriction sites that allow for directionally cloning (Figure 4.1). Briefly, two oligonucleotides 5'-AATTCGCTAGGCCATTATGGCCGCTAGGCCGCCTCGGCCGCTAG-3' and 5'-GATCCTAGCGGCCGAGGCGGCCTAGCGGCCATAATGGCCTAGCGA-3' were annealed to construct a fragment that would produce two different overhangs (underlined) upon digestion with *Sfi* I restriction enzyme. Following propagation of the plasmid in *E. coli*, pMAK1 was digested with *Sfi* I, dephosphorylated using alkaline phosphatase (Life Technologies), and purified from Sea Plaque agarose gel using Glass MAX DNA Isolation Matrix System (Life Technologies). To construct a library the fractionated cDNA was directionally ligated in the *Sfi* IA restriction site at the 5' end (ATTAT) and *Sfi* IB restriction site at the 3' end (GCCTC) of pMAK I. The ligation mix was introduced into *E. coli* TOPO10 cells (Invitrogen) using electroporation, which resulted in 2.2×10^6 primary transformants on Luria-Bertani (LB) medium including ampicillin.

5'End cDNA sequencing. Approximately one thousand colonies directly following ligation and transformation were randomly picked from the plates for single pass sequencing at the 5' end of the library inserts (Incyte Pharmaceuticals, Palo Alto, CA, USA). Either the T7 promoter primer (5'-TAATACGACTCACTATAGGG-3') or the Universe M13 forward primer (5'-GTAAAACGACGGCCAG-3') was used for sequencing using the dye terminator chemistry. The vector sequences and bases called with Phred value £ 20 were automatically trimmed off (19).

Sequence analysis / EST characterisation. Batches of EST sequences were analysed using Basic Local Alignment Search Tool (BLAST) (3) of the 'BLAST client' (blastcl3) server at the National Centre of Biotechnology Information (NCBI). Ini-

tially, each sequence was translated in six reading frames, and all reading frames were compared with published sequences in all non-redundant databases (nr) of GenBank using the BLASTX algorithm (with BLOSUM62 substitution matrix and the standard genetic code). Sequences that produced no significant or poor homology were also compared with nucleotide databases (nr) using the BLASTN. To evaluate the redundancy of each EST sequence to all other isolated sequences we analysed our local *M. incognita* EST database with a 'Stand-alone BLAST' search engine (Blastall) downloaded from NCBI.

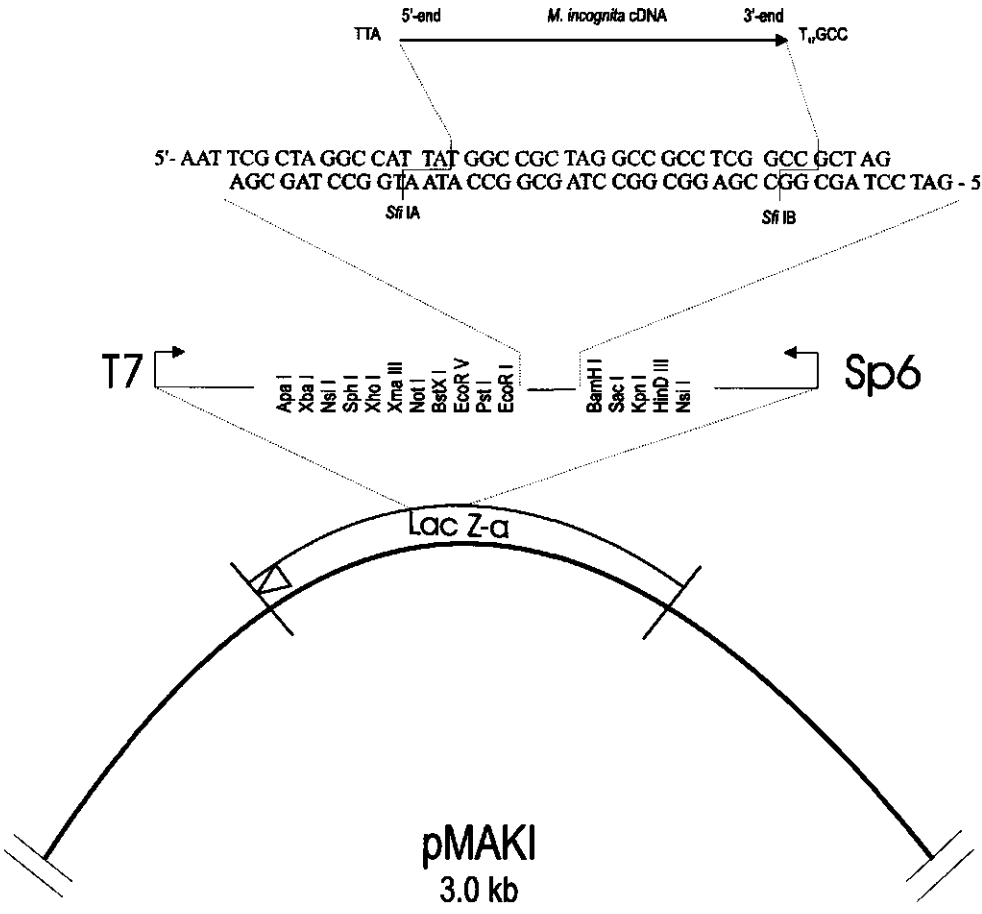

Figure 4.1 - Construction of the cDNA library in the *Sfi* I restriction sites of the multiple cloning site of plasmid pMAK I.

RESULTS

The average insert size of the cDNA library that is used in this paper was 1.1 kb (range of 300 bp to 3.0 kb). The average length of the 1096 single read sequences was 650 bp. Only 2.5% of the library plasmids did not contain an insert and 14% of the sequences failed to meet our quality criteria (Phred value >20). For the evaluation of redundancy in our local EST database two nucleotide sequences were considered to be similar or highly homologous if the bit score S' in the BlastN algorithm was larger than 200 (~E-value < 1 * e^{-50}). According to this stringent threshold the percentage of clones appearing only once or without highly conserved homologues in the data set was 78.0 %.

In total single pass cDNA sequencing of randomly chosen colonies resulted in partial nucleotide sequences of 914 primary transcripts of *M. incognita* that were used to search protein databases for putative homologues. Similarities were determined using BLASTX algorithms, and ESTs were subsequently annotated and grouped by putative function (Table 4.1). The grouping is based on the top 'hit'. A similarity was considered as a 'hit' when the obtained BLAST score had an E-value less than $1x10^{-5}$, which means that 37.4% of predicted proteins had significant similarity to known sequences deposited in various databases. Out of these, approximately 7.8% of the predicted proteins – so called undefined homologues – have significant similarity to sequences with poor definitions only. For 28.7% of the predicted protein the searches resulted in less significant similarities (10^{-5}£ E-value £10^{-1}). A substantial number of ESTs resulted in BLASTX E-values higher than $1x10^{-1}$, and those are considered to encode novel translation products (33.9%), unique to this data set. A total of 398 ESTs resulted in a hit (E-value£ $1x10^{-1}$) with deposited sequences from nematode origins, which is 65.8% of all found similarities. All sequences with BLAST-score probabilities of $1x10^{-1}$ and less were categorized in 9 groups (Table 4.1) leaving 45% of the ESTs ungrouped. ESTs encoding proteins involved in the categories 'Metabolic processes' (12.6%), 'Gene expression' (9.8%), and 'Structure and muscle' (7.3%) are most abundantly represented in the cDNA library. These three categories contain many house keeping genes that have conserved homologues in many unrelated organisms and for which high expression levels, and therefore many ESTs, can be expected.

These three categories are the main sources of redundancy of the cDNA library. Although our clustering method may be considered as arbitrary, as the same EST may be assigned to more than one group, the catalogue still reveals relatively reliable proportions of the genes expressed in pre-parasitic second stage juveniles of *M. incognita*.

The power of single pass cDNA sequencing is well illustrated by EST *MD0774* (Figure 4.2), which tags a novel chitinase gene. A segment of 623 bp was found in the EST that matched chitinases of various nematodal and arthropod origins (Figure

4.2). This chitinase fragment contains the conserved glutamic acid of class II chitinases (glycosyl hydrolase family 18), which acts as proton donor in the active site of the enzyme.

DISCUSSION

Single pass cDNA sequencing. The success of an EST project largely depends on the quality of the cDNA library that is used for random sequencing, which is determined by the average insert size, the percentage of full-length clones, the redundancy in the library, and the number of plasmids that carry no insert. The average transcript size in *C. elegans* is predicted to be 1.33 kb. If the same holds true for *M. incognita* then the insert size distribution in the cDNA library is similar to the mRNA size distribution of the nematode. For reasons of large differences in cloning efficiencies most of the small transcripts have been excluded from the library (see Material and methods). The percentage of the ESTs with significant or no similarity with the known sequences deposited in various databases are in accordance with previous reports on other nematode EST projects (5).

ESTs with $1x10^{-5}$£E-value £ $1x10^{-1}$ were considered to have less significant homologies (28.7%), and require further sequence analysis using more sophisticated algorithms (*e.g.*, PSI-BLAST). This percentage includes 3.8% of the predicted proteins that align to some extent with undefined homologues and a subset of 11.4% that have some sequence similarity with other database accessions that have poor descriptions.

Sequencing the 5' end of nematode mRNA is usually more informative as compared to the 3' end, because with the shorter 5' untranslated regions the sequences usually include a larger part of the coding region. In addition, the signal sequences that target proteins to be secreted into the plant are generally also located at the 5'end, which is in our view a crucial feature of many candidate parasitism genes (43). In conventional methods reverse-transcriptase frequently terminates before transcribing the complete mRNA sequence rendering many clones in a cDNA library incomplete. This is particularly true with long mRNAs primed with oligo-dT or if the mRNA molecule contains abundant secondary structures. To improve the percentage of full-length clones in cDNA libraries oligonucleotide primers based on a spliced-leader (SL) sequence have been used to amplify full-length cDNA only from the first-strand synthesis products (60). The number of mRNA species preceded by a spliced-leader (SL) sequence is estimated to be 70 percent for *C. elegans* (8) and more than 80 percent for *Ascaris lumbricoides* (37). In our laboratories cDNA-AFLP analysis showed that approximately 60 percent of the transcripts in the potato cyst nematode *Globodera rostochiensis* have a spliced leader SL1 (L. Qin, Pers. Comm.). Messenger RNAs carrying a SL sequence have been isolated from *M. in-*

Table 4.1 - A subdivision of the conceptual translations of 914 EST sequences of *M. incognita* preparasitic J2. The subdivision is based on the most significant homologues according to the BLASTX E-values. The total 'number of ESTs' is broken down into two fractions – the number of ESTs ('high') that have E-values < $1x10^{-5}$, and the number of ESTs ('low') that resulted in E-values between $1x10^{-5}$ and $1x10^{-1}$. Percentages between brackets indicate the relative numbers of ESTs as a ratio of the total number of 914 ESTs.

	Category	Description Nand examples	Number of ESTs	Similarity	
				High	Low
1	Structural and muscle	Cytoskeletal and muscle proteins (e. g. myosin, actin, calponin)	67 (7.35%)	52 (5.7%)	15 (1.6%)
2	Enzymes and Metabolic	Proteins involved in diverse metabolic processes (e. g. GAPDH, GPD, fatty CoA ligase, catalase)	115 (12.6%)	86 (9.4%)	29 (3.2%)
3	Gene expression and protein synthesis	Proteins involved in transcription and Translation (e.g. transcription factor, translation elongation factor, ribosomal proteins)	90 (9.8%)	54 (5.9%)	36 (3.9%)
4	Cell cycle	Proteins involved in cell division and DNA replication (e.g. cyclin, mitogen inducible gene, DNA topoisomerase, centrin)	11 (1.2%)	3 (0.3%)	8 (0.9%)
5	Transport	Membrane transporters and lipid transport proteins (e. g. clathrin heavy chain, axonal transport protein, transportin)	13 (1.4%)	10 (1.1%)	3 (0.3%)
6	Neuron function	Proteins involved in neuronfunction (e. g. neurofilament protein, synaptic vesicle protein, FMRF neuropeptide)	11 (1.2%)	9 (1%)	2 (0.2%)
7	Protein domains	Proteins defined by specific domains and repeats (e. g. C2 domain, Ca2+ binding protein, lipoprotein-binding prot.)	59 (6.5%)	35 (3.8%)	24 (2.6%)
8	Candidate parasitism Genes	Nematode-host interaction specific (e. g. cellulases, chitinase, xylanase, SEC2, CBP 1, Ov T1, Ov 20, cysteine proteinase)	28 (3.1%)	22 (2.4%)	6 (0.7%)
9	Undefined homologues	C. elegans, B. malayi, H. sapiens ESTs (e. g. yk8c7.5, proteins in chrom. III)	106 (11.6%)	71 (7.8%)	35 (3.8%)
10	Unknown	No matching	414 (45.2%)	310 (33.9%)[1]	104 (11.4%)[2]

[1] ESTs with E-values > $1x10^{-1}$ in BLASTX. These ESTs are unique to this data set.

[2] These ESTs have E-values of $1x10^{-5} \leq E \leq 1x10^{-1}$ in BLASTX, however, the aligning sequences have poor definition lines.

cognita too, however, it is unclear if this counts for the majority of the transcripts. Some of the parasitism related genes that have been cloned to date are not preceded by a SL sequence (48). Therefore, we have used the SMART-oligonucleotide system to essentially obtain the same effect as libraries made of SL amplified cDNA, a higher percentage of full-length clones, without any possible bias (4, 12).

Parasitism related genes. Our main focus is aimed at cDNAs encoding proteins that may be involved in the plant-nematode interaction. For many ESTs that are either poorly characterized (10^{-5} £ E-value £ 10^{-1}) or produce open reading frames without significant matches in databases (E-value > 10^{-1}) it is more difficult to determine whether the corresponding gene is involved in parasitism. These genes require additional information regarding their function. For others the function of putative homologues may be indicative for a role in plant parasitism.

Products of two genes, *msp-1* (*Meloidogyne secretory protein 1*; 18) and a *gp-sec2* homologue (*G. pallida secretory protein 2*; 42), are secreted by plant-parasitic nematodes at relatively high levels. We found three ESTs (*MD0906*, *MD0676*, and *MD0432*) similar to the *msp-1* gene of *M. incognita*. Homologues of *msp1* have previously been described as venom allergen antigens in other nematodes (25, 50). Despite its presence in parasitic stages of *M. incognita* a function in plant-parasitism is not evident (18).

ESTs (*MD0814*, *MD0596*, *MD0421*, *MD0351*, *MD0254*, and *MD0298*) showing significant homology with gp-*sec2* in *G. pallida* (42) also appeared at a relatively high abundance in the dataset. Homologues of this transcript are characterized in several other animal-parasitic nematodes (56, 57). Moreover, a high similarity of these ESTs with an ABA-1 allergen of *Ascaris lumbricoides* (35) and *Ov-20* in *O. volvulus* (56) suggests retinol- and fatty acid-binding activities for this gene. Retinol deficiency in animals results in an impaired immune response to parasitic nematodes (10) and similarly vitamin A deficiency contributes to the pathogenesis of animal-parasitic nematodes (47).

Several ESTs showed homology with genes encoding cell wall degrading enzymes produced in the oesophageal glands of nematodes. A large gene family encoding b-1,4-endoglucanases appears to be present in *M. incognita* (M. Rosso, Pers. Comm.). This observation is confirmed by four distinct ESTs (*MD0118*, *MD0139*, *MD0340*, and *MD0369*) with homology (59 to 98% identity) to *eng-1* (Genbank accession number AF100549) previously cloned from *M. incognita* (48). Furthermore, *MD0915* identifies a novel gene encoding a b-1,4-endoxylanase (unpublished), which is another type of cell wall degrading enzyme. *MD0790* is homologous to the *cbp-1* from *M. incognita*. *Cbp-1* is characterized by a cellulose binding-domain and despite the absence of any enzyme activity it is expected to play a role in parasitism of root knot nematodes (17).

Chitinases (EC 3.2.1.14) (Figure 4.2) have been characterised in bacteria, fungi and animal-parasitic nematodes such as *B. malayi* (21), *Acanthocheilonema viteae*

(2), and *O. volvulus* (23). Studies showing a reaction of murine antibody with the cuticle of post-infective L3 of *O. volvulus* (23) implies that it is secreted via the hypodermis. Wu et al. (62) concluded that chitinases expressed in infective stages of filarial nematodes may play a role in moulting during post-infective development. In plant parasitic nematodes chitin is present in the eggshell only (6), therefore it is anticipated that chitinase in *M. incognita* is involved in hatching of the juveniles. However, derivatives of chitin may a function as signal molecules in plants-microbe interactions (33).

Hydroxyl-3-methylglutaryl CoA reductase activity (HMGR, *MD0756*) has been localized in oesophageal glands of *M. incognita* and is believed to be the key enzyme for sterol synthesis (7). It is suggested that HMGR activity in giant cells is related to the high rate of sterol biosynthesis required to sustain the active demand of sterols for nematodes (13, 51). HMGR secreted by the nematode may regulate the de-alkylation of phytosterol into sterols to satisfy the extensive feeding requirements of the developing nematode (7).

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AE003477        CYYTNWSQYRVKIGKFVPEDIPADLCTHIIFAFGWLKKNKLS-SYESNDETKDNV-PGLY 184
AF250997        CYYTNWAQYREGEGKFLPENIPNGLCTHILYAFAKVDELGDSKAFEWNDEDSEWS-KGMY 85
Q11174          CYFTNWAQYRQGRAKFVPEDYTPGLCTHILFAFGWMNADYTVRAYDPADLPNDWAGEGMY 116
Mi_MD0774       CYFTNWAIYRSGRAKFAPEDYAPGLCTHIFYAFAYFNESFEAYAIDPNDLPNDSDPLGQY 74
                **:***: **   .** **: . .*****::**. ..      : :  *  .:    * *

AE003477        ERMMTLKKANPKLKILLALGGWSFG--TQKFKDMSSTRYTRQTFVYSAIPFLRKRGFDGL 242
AF250997        SGVTKLKETNPELKILLSYGGYNFG--SAIFTEIAKSAQKTERFIKSAIEFLRKNNFDGF 143
Q11174          RRVNKLKVTDTQLKTLLSFGGWSFG--TALFQGMAASSASRKVFIDSAITFVRTWGFDGI 174
Mi_MD0774       ARVVALKKYDPNLKFVMSFGGWTFSTTTTLFQNMTSSKQNRGKFIKSSIAFIKKHGFDGI 134
                  :  **  :.:** ::: **:.*.  :  *  :: :  .   *: *:* *::. .***:

AE003477        DMDWEYPKGSDDKKNFVLLLKELREAFEAEAQELKKPRLLLSAAVPVGPDNIRGGYDVPA 302
AF250997        DFDWEYPLG--VAKEHAKLVEAMKSAFVEEAKTSGKQRLLLTAAVSAGKETIDGSYDVES 201
Q11174          DIDWEYPSGATDMANYVALVKELKAACESEAGSTGKDRLLVTAAVAAGPATIDAGYDIPN 234
Mi_MD0774       DLDIEYPDS---KENFNLLLQEFHLASCNEK--NVKTKLIITAAVAAGIDIVKNSYDIAT 189
                *:* *** .     :.  *:: :: *   *     * :*:::***..*   :  .**:

AE003477        IASYLDFINLMAYDFHGKWERETGHNAPLYAPSTDSEW--- 340
AF250997        LGKNFDLLFLMSYDLHGSWEKNVDLHGKLRPTKGEVSGI-- 240
Q11174          LAPNFDFILLMSYDFFGAWASLVGFNSPLYATTELPAEW-- 273
Mi_MD0774       MAKYVDFINLMTYDFHITSENKTGYNSPLRSKGLFEYYRCW 230
                :.  .*:: **:**:.      .. :. * .
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Figure 4.2 - Amino acid sequences of chitinases of various origins aligned using the algorithm Clustal W, version 1.8 (55). Only fragments of accession with significant homology to MD0774 of *M. incognita* are shown. The putative proton donor – a glutamic acid (E) – in the active site of the chitinases is in bolded font. Two chitinases of nematode origin (*C. elegans*, swiss-prot accession Q11174, *Wuchereria bancrofti*, genbank accession AF250997) and one chitinase sequence of arthropod origin (*Drosophila melanogaster*, genbank accession AE003477) are included in the alignment. Asterisk, identical or conserved resiudes in all sequences of the alignment; colon, conserved substitutions; single dots, semiconserved substitutions.

Diverse antioxidant proteins as peroxiredoxin (*MD0522* and *MD0716*), catalase (*MD0897*, *MD0884*, and *MD0672*), glutathione peroxidase (*MD0641*, *MD0352*, and *MD0334*), and thioredoxin peroxidase (*MD0127* and *MD0137*) were tagged by several ESTs. Antioxidant enzymes have been identified in many helminths (36, 9), and were shown to be one of the major surface-associated molecules that may shield the parasites by inactivating toxic products produced by host phagocytes. Recently, thioredoxin peroxidases (TPx) are described as a new class of anti-oxidant enzymes (32) and it is strongly suggested that this is probably the major H_2O_2-metabolizing system in filarial nematodes that enhance defence against the host immune response or limit damage from host inflammatory cells. A similar protective function could be envisioned for plant-parasitic nematodes to counteract the active oxidants released by host-plants. TPx genes have been found in vertebrates, fungi, plants, bacteria (45) and also were cloned and characterized from the nematodes *Dirofilaria immitis* (28), *O. volvulus* (11, 32), *B. malayi* (22) and *G. rostochiensis* (46).

MD0736 identifies a cysteine proteinase with homology to a *Haemonchus contortus* proteinase (52). Cysteine proteinase has been intensively studied in animal parasitic nematodes as *Ostertagia ostertagi* (41) and *Ancylostoma caninum* (24). They are also abundantly expressed in the intestine of *C. elegans* (44). Similarly, two cysteine proteinase genes (*hgcp-I* and *hgcp-II*) have been cloned from the plant parasitic nematode *Heterodera glycines* (59). Specific protease inhibitors expressed as transgenes in hairy roots of host plants resulted in a reduced fecundity of feeding soybean cyst nematodes (58).

'Pioneering' sequences. The most challenging ESTs (33.9 %) are the ones for which no putative homologues were found in the public databases. Based on our experience with the potato cyst nematode *Globodera rostochiensis* many genes potentially related to parasitism are pioneering sequences. A number of analytical steps may help to assign a function to pioneering gene sequences.

First, it is most likely that the majority of parasitism related genes encode secreted proteins (61). Single pass 5' end sequences may include the N-terminus of the encoded proteins, which allows for prediction of a signal peptide for secretion. The latest release of Signal-P at http://www.cbs.dtu.dk/services/SignalP-2.0/ has improved capabilities to discriminate between signal peptides and uncleaved signal anchors. Combined with computer software that searches for transmembrane domains a good overall prediction for a protein to be secreted is possible. A small number of secretory proteins lack a typical hydrophobic signal peptide for translocation via the classical secretory pathway. In these cases secreted proteins require the interaction with a helper-protein - usually ATP-binding-cassette (ABC)-transport proteins (30). Examples of this mechanism of secretion have been found in both prokaryotic and eukaryotic organisms. Several secreted proteins of *O. volvulus* infective larvae, reported initially as a host protective antigen, lack a classical N-terminal signal peptide (1).

Secondly, large scale *in situ* hybridisation procedures would provide valuable data on unique EST sequences. A large scale *in situ* hybridisation procedure was developed for *C. elegans* by Tabara et al. (54). Similar high-throughput methods to obtain spatial expression patterns are being developed in our laboratories at the moment.

Thirdly, a novel high-throughput RNA fingerprinting based strategy named cDNA-AFLP has been recently applied to obtain temporal expression data for unknown genes of the plant parasitic nematode *G. rostochiensis* (43). The biology of the potato cyst nematode especially during hatching lends itself perfectly to a differential display procedure like cDNA-AFLP. Despite more experimental difficulties a similar succession of distinct phases in the transition from preparasitic to parasitic juveniles of *M. incognita* should be amenable to analysis with cDNA-AFLP too.

Alternatively, introducing double-strand RNA to disrupt gene activity may be an ideal contribute to assess the function of genes in plant parasitic nematodes. This strategy has been shown to be useful for RNA interference in *C. elegans* (20) and the effects have persisted well into the next generation. No RNA interference mutants of plant parasitic nematodes have been reported yet, which may be the consequence of their more complex mode of reproduction and obligatory parasitic nature.

In conclusion, this small pilot EST sequencing project has produced EST tags for virtually all parasitism related genes that have been cloned from *M. incognita* at present. The efficiency of EST projects, which produce sequence data for thousands of genes in months time without any difficult pre-selections of mRNA pools, makes random sequencing of cDNA libraries a superior method to identify candidates for parasitism related genes. This method has a high potential for cost-effective analyses of nematode-plant interactions that mainly for economical reasons have had little attention of molecular nematologists so far. For each of the candidates with interesting homologies in this study further expression analysis or biological tests will have to demonstrate their role in parasitism. At the time this chapter was prepared an EST sequencing project dealing with various parasitic nematode species was initiated by the St. Louis Genome Sequencing Centre and Hinxton Sanger Centre (34). This promising initiative will reveal the presence of thousands of interesting genes in nematodes for which nematologists will have to provide a biological understanding to assess their relative importance in the plant-nematode interaction.

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ABSTRACT

To invade plants parasitic nematodes have to degrade the plant cell walls – a protective armour against intruders. For complete hydrolysis of a highly organised network as the plant cell wall parasites require an array of enzymes. So far, all previously identified cell wall degrading enzymes in *M. incognita* are endoglucagenases. In this study we report the first hemicellulolytic enzyme in a plant-parasitic nematode. Single-pass 5'end sequencing of approximately 1,000 clones from a cDNA library constructed of preparasitic *M. incognita* J_2 has revealed a partial sequence with a homology to xylanases of various bacterial origins. This expressed sequence tag was used to obtain a full-length transcript of 1220 nt encoding an open reading frame (*Mi-xyl1*) of 37.6 kDa. Hydrophobic cluster analysis classified the putative xylanase as a type 5 glycosyl hydrolase. Whole mount *in situ* hybridization showed specific labelling of the *Mi-xyl1* in the subventral oesophageal glands of second stage juveniles. DNA blot hybridisation indicated the presence of two homologues in *M. incognita* whereas no hybridisation was found with genomic DNA fragments of *C. elegans* and cyst nematodes. Recombinant Mi-XYL1 protein exhibited hydrolytic activity on xylan and carboxymethyl cellulose. Conclusively, root knot nematodes (*Meloidogyne* spp.) make use of a suite of cell wall degrading enzymes with overlapping activities to facilitate plant invasion.

INTRODUCTION

The plant cell wall is a highly organized composite of different polysaccharides, proteins and phenolic compounds. Models of the cell wall architecture describe several independent but interacting structural networks. The primary structural network is made of scaffolding cellulose interlocked by heterogenous polymeric glycans and xylans. This (hemi)cellulose framework lies embedded in a matrix of pectic polysaccharides, which is sometimes reinforced by phenolic compounds. Growing cells only maintain a primary cell wall, however, when growth seizes the cell wall is locked in place by structural proteins and the elaboration of secondary cell wall layers. The secondary cell wall, mainly made of cellulose microfibrils coated with polymeric xylans, allows the cell to build specialized structures suited to the cell's functions.

The primary function of plant cell walls is to dictate size and shape of the protoplasts during cell growth and differentiation. However, in addition to these structural functions cell wall components may be involved in cell-to-cell communications or metabolite transport. The cell wall may become impregnated with structural proteins and lignins or deposited with callose to armour against invading or-

ganisms. Moreover, fragments of cell wall polysaccharides may elicit specific resistance responses to invading pathogens and parasites.

Of the hemicelluloses known xylan is the most abundant compound. Particularly in monocots xylans form a substantial part of the cell walls (13). The main chain of this polysaccharide consist of b-1,4-linked D-xylopyranoside residues that can be partly modified by acetylation, depending on the plant species from which the xylan originates. The polymeric backbone can also be branched as a result of substitution with L-aranbinofuranose, 4-*O*-methyl-D-glucuronic acid and acetic acid (48). The L-arabinofurosyl residues can be esterified with furelic or *p*-coumaric acid, which have been shown to cross link lignins in the secondary cell wall. In order to degrade the heterogenous xylans completely a complex set of enzymes is required (6, 15). Endoxylanases (EC 3.2.1.8) are capable to hydrolyse the xylan backbone at non-modified residues only.

In order to make use of the plant nutrients as a food source plant pathogens have evolved ways to overcome the fortifications of cell walls by secreting cell wall degrading enzymes. Xylanases involved in cell wall breakdown are found to contribute to the pathogenicity of bacteria (10, 11, 30). Similarly, xylan degrading enzymes are important factors for the colonization of plant tissue by plant pathogenic fungi. For instance, endoxylanases have been identified in the corn pathogen *Cochliobolus carbonum* and the rice pathogen *Magnaporthe grisea*. It is temping to suggest that the endoxylanase activity in pathogens is correlated to the ability to attack grasses.

Root-knot nematodes, *Meloidogyne* spp., are obligatory plant parasites that feed from thousands of different plant species both monocotydelons and dicotydelons. They use a hollow, protrusible stylet to penetrate plant cell walls, inject oesophageal gland secretions and withdraw nutrients from the cytoplasm. Before feeding commences the infective nematode migrates intercellularly through several cell layers in the plant root. At an appropriate site in the vascular cylinder the nematode establishes a giant-cell from which it feeds for weeks to follow. It has only recently been discovered that nematodes use cell wall degrading enzymes in addition to the physical impact of their stylets to penetrate cell walls in the initial stages of the parasitic cycle. Nematodes were the first animals for which it has been shown that they produce cellulases independent from endosymbionts.

There is a large variety in the molecular composition and organization of plant cell walls components among plant species. Root knot nematodes, parasites of monocots and dicots, have to deal with this variety of complex substrates. This would implicate that these nematodes are equipped with a large suite of genes encoding cell wall degrading enzymes. So far, a gene family encoding b-1,4-endoglucanases have been identified in root knot nematodes (18, 41). In addition, expressed sequence tags made from cDNA libraries of root knot nematodes have been found for pectate lyases and endopolygalacturonases, however, the true nature of these

transcripts still needs to be confirmed. Over a hundred hemicellulolytic enzymes have been purified and characterized from both fungi and bacteria (44) but to date no hemicellulolytic enzyme has been isolated from plant parasitic nematodes. In this paper we present evidence for a hemicellulolytic xylanase gene in the root knot nematode species *M. incognita*. The nematode b-1,4-endo/exoxylanase gene belongs to the glycosyl hydrolase family 5 as do the b-1,4-endoglucanases in this species, however, no significant similarity exits between the two types of cell wall degrading enzymes. b-1,4-endo/exoxylanases catalyse the hydrolysis of 1,4-beta-D-xylosidic linkages in xylans, and as such it may be involved in the unlocking the microfibrils of the secondary cell walls during migration of the nematode in plant tissue.

MATERIAL AND METHODS

Nematodes. *Meloidogyne incognita*, line 48, was propagated in greenhouse cultures on tomato cultivar Moneymaker at 20-25 ºC. Eggs were harvested approximately 12 weeks after inoculation and isolated using 0.5% NaOCl-solution (28). Second stage juveniles (J_2) were collected from eggs in water on a cotton wool filter, purified using 70% sucrose, and stored at –80ºC until further processing.

Cloning of Mi-xyl1. A partial sequence of the Mi-xyl1 gene was generated by random single-pass 5'-end sequencing of approximately 1000 clones from a cDNA library constructed by Dautova et al. (17). The mRNA that served as template for this cDNA library was isolated from pre- parasitic second stage juveniles of *Meloidogyne incognita*. The clone that yielded EST MD0915 was further sequenced at the 3'-end. The open reading frame contained on the cDNA clone was incomplete, therefore, vector specific (pMAK15f) and internal (MI2r) primers (Table 5.1) were used to amplify the missing 5' end of the Mi-xyl1 cDNA from a plasmid prep of the cDNA library using Pwo-DNA Polymerase (Roche Molecular Diagnostics, Indianapolis).

Nucleotide sequence analysis. Sequencing reactions were performed using the ThermoSequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham-Pharmacia, Buckinghamshire) with T7 promoter primer and pMAK16r primer (Table 5.1). The sequencing reactions were analysed on an Automated Laser Fluorescent DNA sequencer (Amersham-Pharmacia) using ALFwin SA2.10 software. Nucleotide sequence data were analysed using the Lasergene Biocomputing Software for Windows (DNASTAR, Madison). The DNA sequences were analysed using the Basic Local Alignment Search Tool algorithm (BLAST) via the world-wide-web-interface at the National Centre for Biotechnology Information. The prediction of signal peptides was done at Signal-P server using the world-wide-web interface at http://www.cbs.dtu.dk/services/SignalP-2.0/.

***In situ* hybridisation.** Single strained cDNA synthesised with linear PCR (45) was used to obtain digoxigenin-11-dUTP (Roche Molecular Diagnostics) labelled sense and anti-sense probes. The probes were amplified from the 625 to 1095 nucleotide region of the MI-xyl1 cDNA using the MI1f and MI1r primers (Table 5.1) in two separate reaction. The probes were checked on 1% agarose gel and purified through Mini Quick Spin DNA Columns (Roche Molecular Diagnostics). Freshly hatched nematodes were fixed overnight in 2% paraformaldehyde (in M9 buffer; 20) and cut into 3 to 4 pieces. The permeabilisation and hybridisation (at 50 ℃) were performed as described previously (20). Alkaline phosphatase activity was detected with X-phosphate and 4-nitro blue tetrazolium cloride (Roche Molecular Diagnostics). The juveniles were examined using differential interference contrast microscopy (Leica, Deerfield).

Table 5.1 – Oligonucleotide sequences of the primers used in this study

Primer	Location	DNA Sequences of the oligoprimers
MI-1f	Svg (in situ)	5' – TTATGGCACCCGAATGTGC – 3'
MI-1r	Svg (in situ)	5' – CTGTGATGCACACTTAACG – 3'
MI-2r	Internal (5' end)	5' – GAGCACATTCGGGTGCCA – 3'
MI-2f	N-terminal (full length)	5' – GGTACTATATTCATTTAATTC – 3'
MI-3r	C-terminal (full length)	5' – CGACTAGAAATCCTAATATC – 3'
MI-3f	N-terminal (expression)	5'– GTCGACGAATTCGATAATATAGXAAAAATAAATTCTG – 3'
MI-4r	C-terminal (expression)	5'– CAAGCTTGGATCCCCTAAAATTTATAGAATATTGTTG – 3'
MI-4f	N- terminal (southern)	5' – GGTGGTTCTAGTGCTTGG – 3'
MI-5r	Internal (southern)	5' – AATTCATCAACAGCAGATTG – 3'
PMAK1-6r	PMAKI (3'-end, vector)	5' – GCTATGCATCAAGCTTGGTAC – 3'
PMAK1-5f	PMAK1(5'-end, vector)	5' – ATATCTGCAGAATTCGCTAG – 3'
PcDNAII	Vector specific	5' – GTAAAACGACGGCCAG – 3'
PcDNAII	Vector specific	5' – TAATACGACTCACTATAGGG – 3'

Heterologous expression in *Escherichia coli*. The open reading frame (117 to 1045 bp) of Mi-xyl1 was amplified with Pwo-DNA Polymerase using oligonucleotide primers MI3f and MI4r (Table 5.1) in order to introduce *Eco* RI and *Bam* HI restriction sites at the 5' and 3' ends respectively. Cloning into pMAL-c2 and pMAL-p2 expression vectors was done according the manufacturer's instructions (New England Biolabs, Beverly) using the Eco *RI* and Bam *HI* restriction sites. In the case of pMAL-p2, the Eco *RI* site in the oligonucleotide primer was introduced directly behind the predicted signal-peptide. The recombinant plasmids were transformed into *E. coli* strain TB1. Promoter P*tac* driven expression of the recombinant protein was induced with 0.3 mM isopropylthio-b-D-galactoside at 37℃ for 4h.

Western blot analysis. The fusion proteins were transferred to semi-dry western blots and immunodetection were performed as described by De Boer et al. (19). The

recombinant protein was detected on blot with an anti-malE monoclonal antibody (New England Biolabs).

DNA blot analysis. The 172 to 483 bp region of Mi-xyl1 was amplified using MI4f and MI5r oligonucleotide primers (Table 5.1) and digoxigenin-11-dUTP in a PCR (Roche Molecular Diagnostics). The probes were checked on 1.5% gel and purified using Rapid Gel Extraction System (Life Technologies, Grand Island). In each hybridisation reaction 10 ng DIG-labelled DNA probe was used per millilitre DIG Easy hybridisation solution (Roche Molecular Diagnostics). Genomic DNA was isolated from second stage juveniles with alkaline/SDS lysis and phenol/chloroform extraction (42). Prior separation on 0.6% agarose gel the genomic DNA was digested with Bam *HI* and Eco *RI* restriction endonucleases. The separated DNA was transferred on to a positively charged Nylon Membrane (Roche Molecular Diagnostics). Pre-hybridization (in DIG Easy Hyb at 38°C for 30 min.), hybridization (in DIG Easy Hyb at 38°C overnight) and stringency washes (in 0.1% SSC and 0.1% SDS at 68°C) were performed as recommended by the manufacturer (Roche Molecular Diagnostics). The immunological detection of the digoxigenin-11-dUTP -labelled DNA probe with CSPD (Roche Molecular Diagnostics) solution preceded exposure of the membrane to X-Ray films (Konica, Tokio, Japan) for 6,12, and 20 minutes at room temperature.

Enzyme Activity assays. A semi-quantitative birchwood xylan and carboxymethylcellulose cup plate assay was used to determine the hydrolase activity of affinity-purified heterologous fusion proteins (34). The effect of the pH on enzyme activity was determined by measuring the production of reducing sugars in the reaction (3). To this purpose birchwood xylan was resuspended to 1% in 0.05 M buffers. The buffers included were in the assays were acetate (pH 3.6-5.4), phosphate (pH 6.0-7.0), Tris/HCl (pH 7.0 – 9.0), and carbonate-bicarbonate (pH 10.0-11.0). Xylan substrate (0.9 ml) was incubated with crude *E. coli* lysates harbouring the *Mi-xyl1* gene (0.1 ml) for 24 hours at 30°C. Aliquots of 0.2 ml were boiled with 0.3 ml dinitrosalicicylic acid (36) to determine the amount of reducing sugars. The sample was diluted five time prior the measurement at 540 nm using Shimadzu UV-160 recording spectrophotometer (Shimadzu, Kyoto, Japan). The monomer D-xylose was used as standard (3). The absorbance was converted to activities expressed as nanomolar reducing sugars released in 1 ml for a time period of 1 second. To follow the activity over a period of 10 days, aliquots of 0.2 ml were removed each second day and activity was measured as described above.

Sequence comparison using hydrophobic cluster analysis (HCA). HCA plots were made by using the program HCA-PLOT V3.0 (Doriane, Le Chesnay, France). In these plots, the amino acid sequence of the proteins is drawn on a duplicated helical net using the standard one-letter code except for P, G, T and S which are represented by stars, diamonds, squares and pointed stars, respectively. Clusters of hydrophobic residues (V, I, L, F, W, M, and Y) are automatically drawn on the bi-di-

mensional helical representation. Analysis of the plots was performed as described in the results (32)

Nucleotide sequence accession number. The nucleotide sequences for the b-1,4-endoxylanase encoding gene of *Meloidogyne incognita* is retrievable from the GenBank database under accession number AF224342.

RESULTS

Cloning and characterization of an endoxylanase from *M. incognita*. Random sequencing of an *M. incognita* cDNA library generated a 740 bp long expressed sequence tag with homology to a b-1,4-endoxylanase in *Aeromonas cavia* (SWISS-PROT accession number U86340). Further extension of the cDNA sequence at the 5'- and 3'-end revealed a full-length transcript of 1220 nt. The largest open reading frame contained on the cDNA clone included 329 amino acids of which the first 21 amino acids are predicted to function as signal peptide for secretion (Figure 5.1).

The SignalP software indicted that the most likely cleavage site of the signal peptide is between Cys_{21} and Asp_{22} rendering a mature protein of 34,9 kDa. The predicted isoelectric point of the mature protein sequence is 8.8. Two consensus sites for N-linked glycosylation (N_{317} to T_{319} and N_{321} to N_{323}) are present close to carboxy terminus of the open reading frame.

Sequence comparison. The encoded open reading frame was compared with protein sequences deposited in the public database using the BLAST algorithm (2). The amino acid sequence showed significant similarity (E-value<$1.e^{-5}$) with b-1,4-endoxylanases of various bacterial origins (*Bacillus subtilis*, SWISS-PROT accession E69892; *Aeromonas caviae*, SWISS-PROT accession, U86340; *Pectobacterium chrysanthemi*, SWISS-PROT accession U41750). The homologous b-1,4-endoxylanases are all members of the glycosyl hydrolase family 5 (GH 5; 27). Because of its sequence similarity with endoxylanases we named our cDNA clone *Meloidogyne incognita* b-1,4-endoxylanases-1 (*Mi-xyl1*).

A b-1,4-endoglucanase produced in *M. incognita* is also found in GH family 5, however, direct comparison with Mi-xyl1 resulted in a sequence identity of 10%. In glycosyl hydrolases structural aspects are more conserved than primary amino acid sequences. Therefore, with have used HCA to compare structural similarities using hydrophobic clusters in cellulases and xylanases of GH family 5 (Figure 5.2). Four out of five hydrophobic clusters conserved in GH family 5 cellulases are also present in the xylanases (including Mi-XYL1) of the same GH family. The conserved clusters III and V facilitate in the identification of two putative active site

glutamic acids in the Mi-xyl1 sequence (E_{158} and E_{258}). An asparagine residue, which is conserved in GH family 5 members directly amino terminal to the putative proton donor site (E_{158}) is replaced by serine (S_{157}) in Mi-XYL1.

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                                                                  M   K   L   F   N   F          6
GGTACTATATTCATTTAATTGAATTAAACAAATTCAAATAAATTCAAAAAAAAG ATG AAA TTA TTT AAT TTT              72

 F   F   L   F   N   L   I   L   F   Y   Y   S   V   K   C   D   N   I   A   K             26
TTC TTT TTA TTT AAT TTA ATT TTA TTT TAT TAT TCT GTA AAA TGC GAT AAT ATA GCA AAA            132

 I   N   S   D   I   T   Y   Q   S   I   D   G   F   G   G   S   S   A   W   L             46
ATA AAT TCT GAT ATT ACT TAT CAA TCA ATA GAT GGT TTT GGT GGT TCT AGT GCT TGG TTG            192

 G   N   I   P   D   K   G   I   G   N   I   F   G   K   L   G   L   S   I   L             66
GGT AAT ATA CCA GAT AAA GGA ATT GGA AAT ATT TTT GGT AAA CTT GGT TTA TCA ATT TTA            252

 R   V   G   I   V   D   L   C   K   N   Q   K   W   G   N   Y   R   C   I   G             86
CGT GTC GGA ATT GTT GAT TTA TGT AAA AAT CAG AAA TGG GGT AAT TAT CGT TGT ATT GGA            312

 Q   E   A   L   T   A   Q   K   A   S   K   Y   G   V   K   I   F   S   S   P            106
CAA GAA GCA TTA ACA GCA CAA AAA GCA TCA AAA TAT GGA GTT AAA ATT TTT TCT TCG CCA            372

 S   T   S   P   I   S   F   K   T   N   N   N   E   V   M   G   E   L   R   E            126
AGT ACT TCG CCA ATA TCA TTT AAA ACA AAT AAT AAT GAA GTT ATG GGA GAA CTT AGA GAA            432

 D   K   Y   N   D   Y   V   E   Y   L   Q   S   A   V   D   E   L   N   K   V            146
GAT AAA TAT AAT GAT TAT GTT GAA TAT TTA CAA TCT GCT GTT GAT GAA TTA AAT AAA GTT            492

 G   V   N   L   Y   A   I   S   L   Q   S   E   P   D   F   S   P   P   Y   C            166
GGA GTT AAT TTA TAT GCA ATA TCA TTA CAA AGT GAA CCC GAT TTT TCA CCA CCA TAT TGT            552

 S   I   K   W   S   P   K   Q   I   A   A   F   L   K   S   Y   S   R   K   I            186
TCA ATT AAA TGG TCA CCA AAA CAA ATA GCT GCT TTT TTA AAA TCG TAT TCT AGA AAA ATT            612

 K   G   P   K   I   M   A   P   E   C   A   H   F   V   P   E   Y   N   D   A            206
AAA GGA CCG AAA ATT ATG GCA CCC GAA TGT GCT CAT TTT GTA CCT GAA TAT AAT GAT GCT            672

 I   L   N   N   P   D   V   A   K   G   V   D   I   I   A   W   H   M   Y   G            226
ATT TTG AAT AAT CCT GAT GTA GCT AAA GGA GTT GAT ATA ATA GCT TGG CAT ATG TAT GGA            732

 M   Q   L   V   S   Q   T   K   A   Q   K   M   G   K   S   S   L   D   D   R            246
ATG CAA TTA GTA TCA CAA ACA AAA GCT CAA AAA ATG GGA AAA TCC AGC TTG GAT GAC AGA            792

 K   N   K   W   K   L   E   K   V   F   M   E   T   A   K   D   I   H   D   C            266
AAA AAC AAA TGG AAA TTG GAA AAA GTT TTT ATG GAA ACA GCA AAA GAT ATA CAT GAT TGC            852

 M   T   I   A   N   Y   N   A   Y   V   Y   F   W   F   K   D   P   K   Y   V            286
ATG ACT ATA GCA AAT TAT AAT GCT TAT GTT TAT TTT TGG TTT AAA GAT CCA AAA TAT GTT            912

 S   I   V   D   N   N   Y   E   I   T   S   R   G   Y   I   L   G   Q   Y   A            306
TCG ATT GTC GAT AAT AAT TAT GAA ATA ACA TCA AGA GGA TAT ATA TTA GGA CAA TAT GCA            972

 K   Y   I   R   P   G   Y   F   R   I   N   A   T   E   N   P   T   T   I   F            326
AAA TAT ATA AGA CCA GGA TAT TTT AGA ATT AAT GCT ACT GAA AAT CCA ACA ACA ATA TTC           1032

 Y   K   F   *                                                                            329
TAT AAA TTT TAG GTATATAAATATACTTTTTCGTGTATATCAGTCGTTAAGTGTGCATCACAGAAGGCTACAGTT           1107

GCAATATCCTGAGATCATTTAATAATTATTTTAATACATAGATAAGAGTTTTAAGATATTAGGATTTCTAGTCGATAAA          1186
ATTTTTGTAACTTTAAAAAAAAAAAAAAAAAAAA                                                        1220
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Figure 5.1- cDNA sequence of *Meloidogyne incognita Mi-xyl-1* gene. Predicted secretion signal sequence is bolded and the putative cleavage site is indicated by vertical arrowhead. The first residue of the mature protein is D. Oligonucleotide primers sequences used in this study are indicated by arrows. The sequence of the deduced encoded protein is showen above the nt sequence and is numbered from the first methionine residue. * indicates the stop codon.

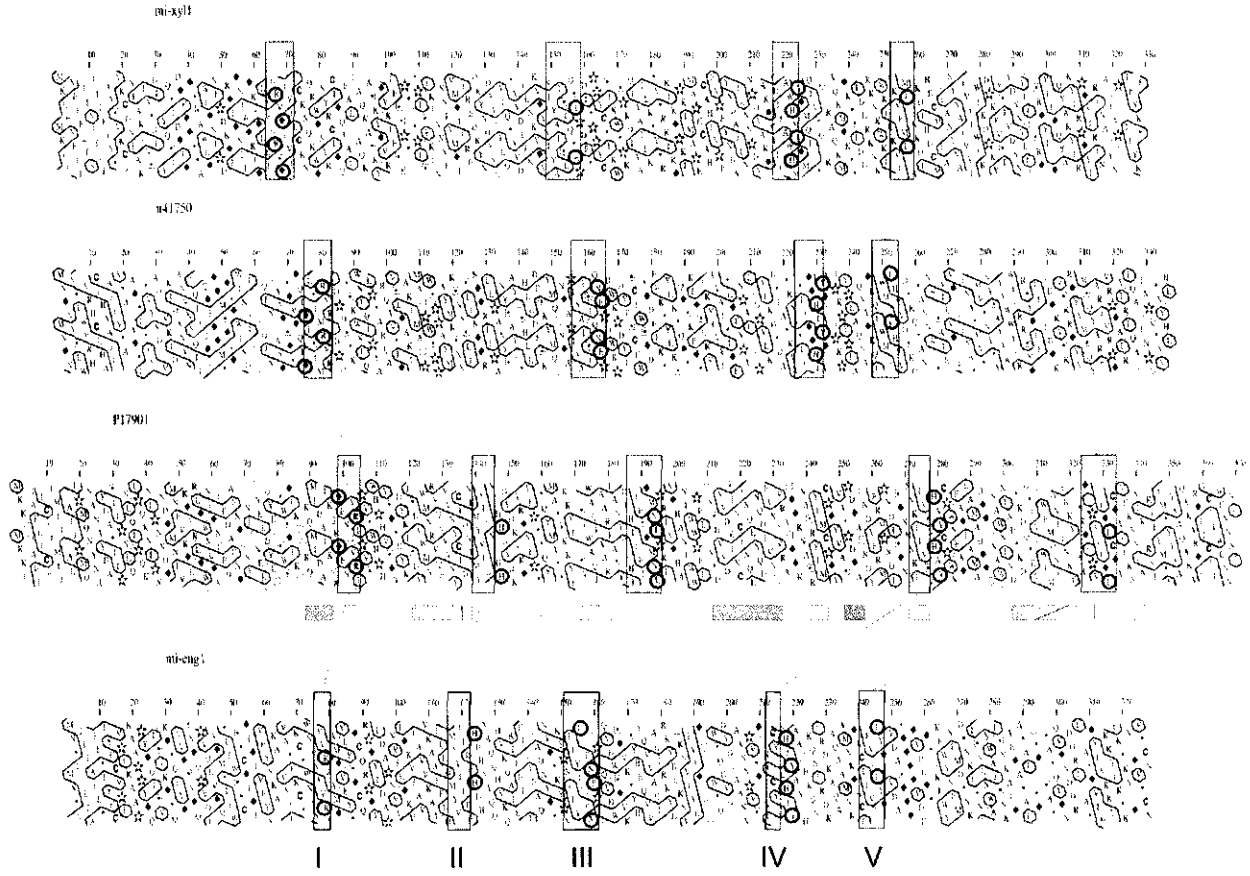

Figure 5.2 - Hydrophobic cluster analysis of Mi-XYL1, xylanase of *Pectobacterium chrysanthemi* (SWISS-PROT accession U41750), endoglucanase of *Clostridium cellulolyticum* (SWISS-PROT accession P17901) and endoglucanase of *M. incognita* (GenBank accession AF100549). The similarity between the ß - strands in xylanases and endoglucanases is shown. The second hydrophobic cluster conserved in the endoglucanesis is lacking in the xylanases. Conserved catalytic residues are circled. ▭ a - helices, ▭ ß - strands.

Heterologous expression of *Mi-xyl1* in *E. coli*. In order to test whether the open reading frame of *Mi-xyl1* encoded b-1,4-endoxylanase activity it was cloned into pMAL-p2 expression vectors and transformed into *E. coli*. The translational fusion with the maltose binding protein *malE* (40 kDa) appeared as single band of approximately 75 kDa (Figure 5.3B) on western blot. *E. coli* lysates tested positive on b-xylan (Figure 5.3C) and carboxymethylcellulose (Figure 5.3D) and as compared to lysates of bacteria containing the empty vector pMAL-p2.

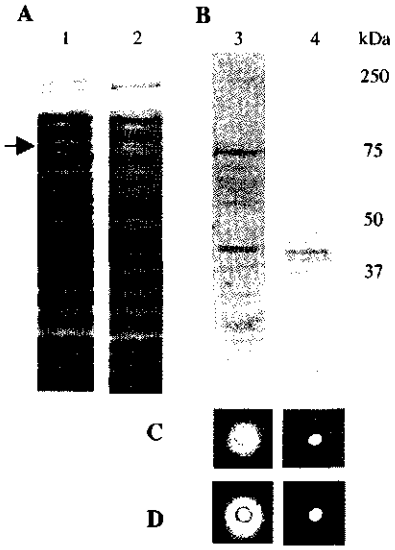

Figure 5.3 - **A.** Comassie Blue stained sodium dodeylsulphate/polyacrilmide gel (SDS-PAGE) of *Escherichia coli* cells (strain TB1) carring expression plasmid (pMAL-p2) containing Mi-XYL1. Line 1: cells induced 4h at 37°C with 0.3mM isopropylthiogalactoside (IPTG), Line 2: uninduced cells. **B.** Detection of recombinant MBP/Mi-XYL1 in *E. coli* lysates on western blot. Line 3: MBP/Mi-XYL1, Line 4: empty vector pMAL-p2. Molecular weight markers are on the *right*. Arrow indicating the location of MPB/Mi-XYL1 is on the *left*. Detection of xylanase activity (halo) on xylan (**C**) and CMC (**D**) substrate.

Enzyme activity. The effect of the pH on the activity of Mi-XYL1 was assessed only on recombinant protein expressed in the periplasm of *E. coli*. The highest amount of reducing sugars, after 24 hours incubation at 30°C with a enzyme: substrate ratio of 1:10, was detected at pH 8.0 (Figure 5.4A). Time incubations conducted at 30°C over a period of 10 days showed a constant increase of released reducing sugars (Figure 5.4B). No activit y was detected in lysates of *E. coli* transformed with empty pMAL-p2 plasmid.

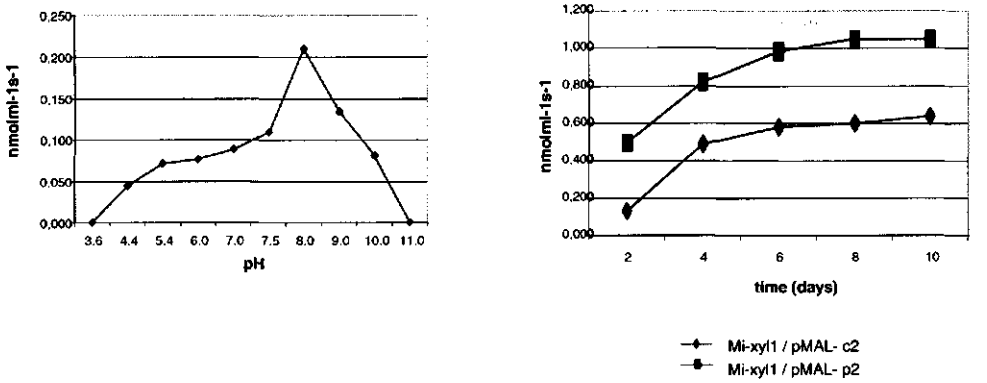

Figure 5.4 - **A.** Effect of pH on activity of *Meloidogyne incognita* xylanase cloned into pMAL-p2. Activity of Mi-XYL1 is expressed in nmol reducing sugars in ml per sec. The optimum pH was determined by using a range of buffers in the standard xylanase assay as described in Material and methods. **B.** Increase of reducing sugars produced from the birchwood xylan after incubation at 30°C in a period of 10 days. Incubations were performed with 0.05M Tris buffer, pH 8.0. Recombinant nematode xylanase cloned into pMAL-p2 and expressed in *E. coli* showes higher activity than the one cloned into the cytoplasmic pMAL-c2.

In situ localization of endoxylanase transcripts. To localize *Mi-xyl1* transcripts in whole mount sections digoxigenin-11-UTP labelled sense and antisense cDNA probes were hybridised with pre-parasitic second stage juveniles of *M. incognita*. The antisense probe showed hybridisation signal specifically within the subventral oesophageal glands (Figure 5.5A). The transcripts were detected within the gland cell lobe but not hybridisation was found in the gland cell extensions and ampullae. Control hybridisations with sense *Mi-xyl1* probe showed no binding to the nematode sections (Figure 5.5B).

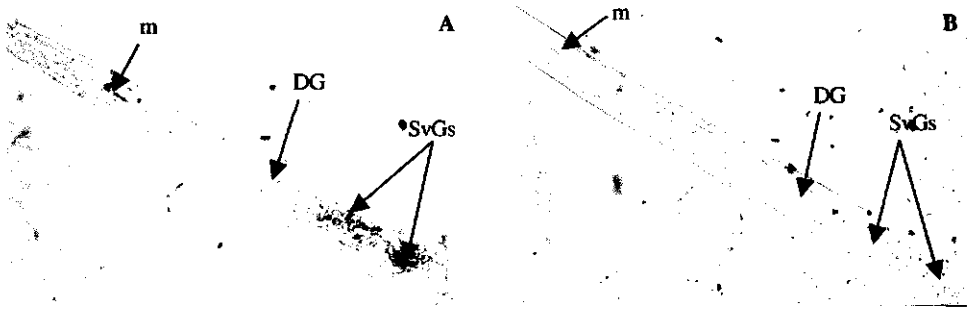

Figure 5.5 - *In situ* localization of Mi-xyl-1 transcripts. Anterior sections of preparasitic J_2 of *M. incognita* hybridized with digoxigenin-labeled cDNA probes. **A.** specific staining of the subventral gland cells (SvGs), no staining observed in dorsal gland cell (DG). m, metacorpal pump chamber. **B.** no staining is observed with the sense probe.

DNA Blot Hybridisation. DNA blot hybridisation using a 311 nt digoxigenin-11-UTP labelled probe of *Mi-xyl1* resulted in two hybridising bands with genomic DNA of *M. incognita* (Figure 5.6). Genomic DNA of seven other nematode species was also analysed for hybridising bands in order to determine if genes homologous to *Mi-xyl-1* were present. One single band was observed in *Eco* RI restricted genomic DNA of *M. javanica (*Figure 5.6, Line 3). No hybridisation signal was observed with genomic DNA from *M. arenaria*, *M. hapla*, *Globodera rostochiensis*, *Globodera pallida* and *Caenorhabditis elegans*.

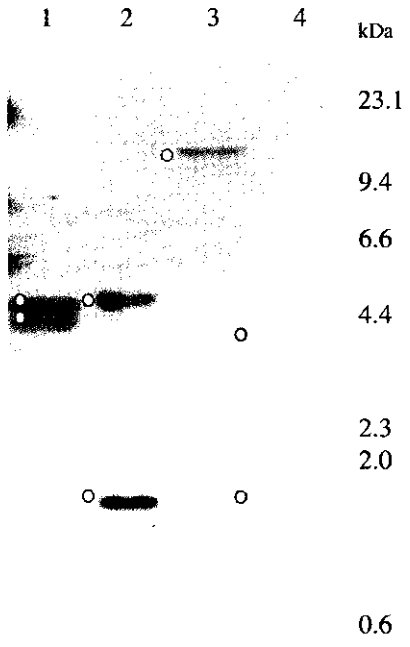

Figure 5.6 - Southern blot hybridization of Mi-xyl-1 fragment in different nematode genomes. Genomic DNA from *M. incognita* (1, 2), *M. javanica* (3, 4) was digested with Eco RI and BamHI respectively and hybridized with dig-labeled *Mi-xyl1* fragment. Two bands per genome were detected for *M. incognita* genomic DNA digested with EcoRI and BamHI (Lane 1,2). One band was observed in EcoRI digested *M. javaniva* genomic DNA (lane 3). BamHI digested *M. javanica* genomic DNA showed 2 bands (lane 4). There was no hybridization observed with genomic DNA of *M. hapla, G. rostichiensis, G. pallida,* and *C. elegans* (data not showen). The sizes of DNA markers are on the *right.* The bands positions are indicated by white dots.

DISCUSSION

Endogenous origin of b-1,4-endoxylanase. Mi-XYL1 only shares significant amino acid sequence identity with endoxylanases of *Pectobacterium chrysanthemi, Bacillus subtilis* and *Aeromonas caviae* (31 to 33%). This observation would suggest that the production of cell wall degrading enzymes in nematodes is depending on endosymbiontic bacteria. Several lines of evidence support an endogenous origin of the *M. incognita* b-1,4-endoxylanase. First, in situ hybridisation shows specific labelling of the *Mi-xyl1* in the subventral oesophageal glands of the nematode. Despite extensive ultrastructural studies no indications to the presence of endosymbiontic bacteria in the oesophageal glands have been found (*e.g.*, 12). Secondly, the coding sequence of *Mi-xyl1* exhibits features that are typical for eukaryotes such as polyadenylation (33), a short 5' untranslated region and a signal peptide with a leucine/phenylalanine-rich hydrophobic core sequence (37).

To date, cellulases and pectinases were found to be expressed in the subventral oesophageal glands of plant parasitic nematodes. In all cases studied so far these cell wall degrading enzymes seem to be more related to their bacterial homologues. Now with the cloning of the first xylanase from the nematode *M. incognita* that only shows similarity to bacterial homologues this trend seems to continue. The lack sequence of identity with eukaryotic homologues raises the question if ancestors of current nematode species have acquired these genes from plant pathogenic bacteria by horizontal gene transfer (43). With the completion of whole genome sequences more evidence is found to support a role for horizontal gene transfer as an evolutionary mechanism. However, it is very difficult to provide conclusive evidence for horizontal gene transfer from one organism to another (18), particularly if the relatedness is based on amino acid sequence analysis only.

Structural characterization. HCA of the catalytic domains have classified all glycosyl hydrolases into 85 different enzyme families (27; http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf.html). Members of each family are considered to have evolved from a common ancestral sequence. Recent analyses of three-dimensional structure of enzymes from different families have showed common folds, which would also suggest a common ancestry (26). Moreover, a shared evolutionary history of some glycosyl hydrolase families deduced from i) the common physical distance between the active site residues, and ii) similarity in the catalytic mechanism (26, 29).

Based on HCA, Mi-XYL1 is classified as a type 5 glycosyl hydrolase and is one of the 6 xylanase members of this family. GH family 5 is characterized by two invariant glutamic acid residues (E_{158} and E_{258} in Mi-XYL1). One glutamic acid corresponds to the experimentally identified nucleophile (47) and, therefore, the other is likely to be the proton donor upstream of the nucleophile. The majority of bacte-

rial and fungal xylanases belong to structural family 10 or 11 (25, 50). Keen et al. (30) have cloned the first xylanase that was positioned between family 5 and 30.

In some bacterial and fungal xylanases a conserved relationship is found between their molecular weight and their pI. Wong et al. (49) have proposed a dichotomy in xylanases variants. One category is made up of low molecular weight proteins that have a basic isoelectric points, whereas the second category should consists of high molecular weight xylanases with acid pI. Among the 6 xylanase members of the GH family 5 there is no strong evidence to support a dichotomy, however, there is a inverse correlation between molecular weight and pI (*i.e.* Mi-XYL1, 34.9 kDa and pI 8.8; Swiss-Prot accessions Q46961, 45.2 kDa and pI 8.5; P70733, 58.5 kDa and pI 6.52; O24852, 62.0 kDa and pI 7.02; Q45397 63.2 and pI 5.35; O66065, 66.9 kDa and pI 4.89).

In addition, the recombinant *Mi-xyl1* activity seems to have an optimum relatively close its pI, which be may the consequence of the fact that a translational fusion with the maltose binding protein is used to assess pH optimum curves. An alternative construct in which the open reading frame only is included would have been superior, however, this has proven to be a difficulty in expression studies.

Functional characterization. *Mi-xyl1* encoded protein possessed detectible activity on xylan and carboxymethyl cellulose. Enzymes with similar cross-activities on xylan as well as on cellulose have been identified in bacteria (1, 22, 24) and fungi (7). The ratio of the two activities may differ considerably. Palindromic sequences that have been found upstream of the cellulases (21, 31, 46) and xylanase genes (8) suggests that both types of enzymes involved in cell wall degradation are regulated by a common mechanism in *Streptomyces* and *Thermomonospora*.

As compared to cellulose, xylans are very heterogeneous polymers. Furthermore, xyanases exhibit a high degree of substrate specificity (40, 49), which suggests that in order to degrade xylans an organism should either have multiple xylan loci or be able to modulate the substrate specificities by post-translational modifications (14, 49). A large multi-family of cellulases have been found in *M. incognita* (41), of which at least one has shown to be inactive on xylan (5). In this paper we present evidence for one xylanase gene and at least one closely related homologue in the genome of *M. incognita*. Moreover, in the Mi-XYL1 protein two consensus sites for N-linked glycosylation are located at the carboxy terminus of the protein, which makes it eligible to functional modifications. These findings indicate that both mechanisms to pertain diversity in substrate specificity are active in the xylanase system of in *M. incognita*. Mi-XYL1 produced in *E. coli* is unable to degrade RBB-xylan, but is capable of degrading birchwood xylan, which may illustrate the substrate specificity of this protein. However, a shift in substrate specificity may also have been introduced in the Mi-XYL1 by the choice of the expression system.

Nematode b-1,4-endoxylanase is a pathogenicity factor. At the initial stages of the parasitic cycle of root knot nematodes the infective juveniles migrate considerable distances intercellularly through root tissue. This process of migration involves a very delicate sequence of steps that is started with the emission of cell wall degrading enzymes and is ended when the nematode separates two aligning cells ahead at the middle lamella in order to move on to the next layer of cells. The plasmodesmata that connect the cytoplasm of the aligning cells are detached, but the cells are left intact. This whole procedure would be impossible if the nematode did not have a suite of cell wall degrading enzymes with overlapping activities at its disposal. Particularly, if one takes into account the large host range of root knot nematodes, including dicots and monocots, with the enormous variety in substrates it represents. To date, this suite of cell wall degrading enzymes appears to be made up of cellulases (41), pectate lyases (EST), endopolygalacturonases (EST), and xylanases (this chapter).

The invasion process of the nematodes seems to have much in common with that of bacteria and biotrophic fungi (4, 35), where cell wall degrading enzymes are important virulence factors (9, 23, 38, 39). In these systems xylolytic activity is associated with virulence on some grass plants (11) due to the relatively high xylan content of the cell walls (16). Similarly, the xylanase genes in *M. incognita* may be an evolutionary adaptation to parasitism on grasses and other monocotyledons.

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Variation in virulence

Plant damage due to root-knot nematode infection is often difficult to control. The difficulties lie in the diverse and unpredictable behaviour of nematode populations in the field. Detailed knowledge on nematode incidence, distribution and virulence is a first step to avoid obstacles that impede implementation of proper management systems.

Parasite populations are always analysed in relation to their host. Mainly two approaches can be used to assess the differences in virulence in populations. First, the single isolate test, which tends to be very labour intensive thus limiting the number of populations that can be tested. The technique, however, yields very accurate results within the constraints of the restricted sample sizes. The bulk sample test makes the second option, in which the number of infections on a resistant plant relative to a (susceptible) control gives an estimate of the relative frequency of the virulent phenotypes in populations.

Differential survival of individuals in a *Meloidogyne* population on *Mi* resistant tomato cultivar may result from:

i) genetic variation because of a certain percentage of virulent genotypes (*e.g.* 56),

ii) overcoming resistance by mutations or genetic rearrangements (*e.g.* 42) being the result of small effective mutations at an unusual high frequency (56),

iii) resistance, which is not effective against all *Meloidogyne* species (*e.g. M. hapla;* 23),

iv) resistance, which is not effective at temperatures above 28°C (*e.g.* 16).

The results presented in Chapter 2 on the occurrence of *Meloidogyne* spp. in Macedonia and their behaviour on susceptible and *Mi* resistant tomato cultivars are of invaluable importance to the growers. Often the apparent populations investigated consisted of mixtures of species (Table 2.1) that had to be separated into *Meloidogyne* isolates derived from single egg masses for further analyses. The various *Meloidogyne* isolates differed in their ability to overcome the resistance gene (Table 2.2). The multiplication rate of the virulent isolates on both resistant cultivars also varied considerably (Figure 2.2).

In the areas where virulence for the *Mi* gene was not found (Table 2.2) a successful control of root knot nematodes could be achieved with *Mi*-gene harbouring cultivars (18). This situation may not hold for long, because after repeated selection by *Mi*-carrying cultivars virulent populations may arise from avirulent ones (11).

In the fields like Bogdanci, Gevgelija, Kocani and Stuka (Table 2.2), where significantly virulent populations are present, a combination of control strategies is necessary. A successful integrated nematode management may include a variety of

environmentally satisfactory approaches such as steam sterilisation, biological control and trap crops, as an alternative to the use of traditional nematicides. Regarding the number of life-cycles of RKN that can be completed during a growth period of a susceptible crop extended fallow periods may be an alternative too (17).

Furthermore, due to the lack of sexual reproduction in the mitotic obligatory parthenogenic *M. incognita*, *M. javanica*, and *M. arenaria*, the inheritance of avirulence and virulence in these major species cannot be tested directly by recombination. One approach is to isolate (a)virulence genes from *Meloidogyne* by differential molecular analyses of near isogenic (a)virulent lines but frequently their diversity in overcoming the *Mi* resistance gene hinders the situation (12). Attempts to detect (a)virulence characters by AFLP analysis of (a)virulent lines failed, as the clustering of the populations was not associated with their (a)virulence against the tomato *Mi* resistance genes (50, 57).

An alternative procedure to address this problem is to make use of transposable elements found in many organisms. Their use in reverse genetics as molecular tools for gene tagging (36, 49) have inspired Leroy et al. (32) to assess the presence of mariner-like elements (MLEs) in different *Meloidogyne* genomes. In four *Meloidogyne* species they detected MLEs and these may become valuable tools for gene (virulence character) tagging in these species.

Assessment of polymorphisms in nuclear and mitochondrial DNA of *Meloidogyne* spp.

Genetic changes driven by the environment, including the use of resistant varieties and pesticides, influence the virulence behaviour of the mitotic parthenogenetic species of *Meloidogyne*. At present, only a few investigations have been conducted to study these genetic changes at different hierarchical levels. RAPD and RFLP, two commonly used techniques, fail to provide us with consistent patterns at low template DNA concentrations (Chapter 1). Therefore, we have applied the AFLP protocol being the most reliable procedure to distinguish genetic polymorphisms in *Meloidogyne* spp.

The AFLP patterns have generated 738 informative markers for genetic analysis of root knot nematodes (Chapter 3). Using 17 primer combinations (Table 3.2) we have characterised five-*M. incognita,* five *M. javaniva* and six *M. arenaria* populations. Based on genetic distance matrixes and UPGMA analysis the populations were clustered in three distinct groups that corresponded with the respective species identities. Populations of a single species from the same state were not clustered together, excluding any correlation between genomic similarity and geographical origin of the populations. On the intraspecific level, *M. arenaria* showed the

highest variability, which is in compliance with Semblat et al (51). In contrast to this latter paper *M. incognita* was the least polymorphic instead of *M. javanica.*

In order to obtain information on lower hierarchical levels we have investigated size variations due to a variable number of tandem repeats in the mtDNA molecules of the same populations. The availability of complete or nearly complete mtDNA sequences for several helminth species provides us with a rich source of genetic markers for studying genetic variability in helminth groups (7, 19).

The metazoan mitochondrion contains at least one relatively large non-coding control region that harbours the promoters for the initiation of transcription (52). The non-coding control region is the most variable portion of the genome both in terms of length and nucleotide sequence. This region has been used for many intra-specific studies in different organisms (*e.g.* 21).

In Chapter 3 we have assessed the genetic polymorphisms in the 63-bp tandem repeats region of mtDNA of *Meloidogyne* spp., which is located in the non-coding region of the *Meloidogyne* mtDNA genome (38). In all three *Meloidogyne* species the highest degree of diversity was detected within individuals (Ci). The highest degree of heterogeneity (Cps) was revealed among populations within *M. arenaria* (Table 3.4).

The correlation between AFLPs- and TR-based polymorphisms was not significant in all cases. With regard to the diversity among populations within a single species, with both approaches *M. incognita* showed lowest genetic diversity whereas *M. arenaria* showed the highest diversity (Table 3.3, Table 3.4). The dendrograms produced from both methods were not congruent. Based on the AFLP patterns, all populations from a single species cluster together in a separate group (Figure 3.2). In contrast, in the dendrogram based on the TR marker *M. incognita* populations clustered together, whereas the populations of *M. javanica* and *M. arenaria* failed to cluster in two separate groups (Chapter 3). The only positive correlation between outcomes of the two approaches was found among the *M. arenaria* populations (Figure 3.5).

The discordance between patterns of mtDNA variation and those seen for nuclear gene markers are expected as mtDNA represents only a single locus unlinked to the nuclear genome. The fact that mtDNA evolves at a faster rate than nuclear genes makes mtDNA suitable for discriminating closely related organisms (3). Indeed, most of the understanding of the population genetics of many eukaryotic species owes much to studies using mtDNA. Furthermore, mutational events and gene rearrangements in the mitochondrial genome are normally uncomplicated and taxon-specific, so that the mtDNA sequences are often regarded as valuable for the study of inter- and intra-specific variation (22). Consequently, sequences generated from the mitochondrial genome provide excellent molecular markers for defining population groups, for tracing the genetic history of an individual or a particular group of related individuals (8).

Conclusively, despite the mitotic parthenogenetic mode of reproduction *Meloidogyne* spp. display a wide range of variations on all hierarchical levels studied in Chapter 3. The AFLP fingerprints gave highly informative marker profile, revealing reliable genetic relationships on inter- and intraspecific level. These results were in compliance with other studies. However, analyses of the relation between the genetic distances based on 63-bp TRs and AFLPs show that the degree of divergence within species is not always correlated between both methods. The main reason for such a discrepancy is the high gear at which the mitochondrial repeats evolve, which is probably too fast to retain the footprints of ancestral relationships. Nevertheless, mtDNA remains appealing offering possibility to study variation within a single genotype and a high evolution rate may also be an advantage when studying more short term evolutionary processes, such as the accumulation of mutations during a limited number of generations.

Expressed sequence tags – an efficient approach for investigating gene expression

Conventional procedures in molecular biology require relatively large amounts of starting material for RNA isolation (45). Frequently, obtaining sufficient quantities of mRNA from microscopic organisms like nematodes is difficult. The introduction of the PCR has largely overcome this limitation (34). Small adaptations of this technique combined with an efficient cDNA synthesis protocol can produce good quality cDNA libraries from only 50-ng total RNA (Chapter 4). A proper screening method of the cDNA library is equally important as the construction of the library. Random sequencing of a small portions of a library, yielding expressed sequence tags, is a rapid method for determining the nucleotide sequence of genes expressed in an organism and offers many advantages above other screening methods (1) (Chapter 4). At the time that we started a small pilot project involving ESTs from *M. incognita* there were only few EST from plant-parasitic nematodes available in the public databases. One year later the number has increased rapidly, which illustrates the attractiveness of the method (Table 6.1). The rapid increase in EST entries in the public database offers an enormous quantity of information concerning the genes expressed in plant-parasitic nematodes. The next step for scientists should be to reveal the function of the most challenging group of ESTs – the 'pioneering sequences'. Much effort is currently invested in the development of protocols that are capable of assigning a function of these sequences (see Chapter 4).

Table 6.1 - Cumulative number of public entries of 'expressed sequence tags' in 2000 and 2001 summarised by organism. * Indicates plant-parasitic nematode species.

Nematode species	February 2000	February 2001
Caenorhabditis elegans	101,232	109,215
Brugia malayi	20,941	22,392
Onchocerca volvulus	8,981	14,347
Haemonchus contortus	246	2,749
*Meloidogyne javanica**	22	1,223
*Heterodera glycines**	2	1,421
*Globodera rostochiensis**	0	849
*Meloidogyne incognita**	0	6,626

cDNA libraries constructed from different life stages of the nematodes will yield ESTs of genes expressed at various time points of the parasitic cycle. Random sequencing of the cDNA library from pre-parasitic J2 of root-knot nematode *Meloidogyne incognita* represented the genes expressed at the onset of parasitism (Chapter 4). As expected, ESTs identifying proteins involved in diverse metabolic processes (12.6%), gene expression (transcription and translation, 9.8%) and cytoskeletal and muscle proteins (7.3%) were most abundantly represented in the library (Table 4.1). Only 3.1% of the ESTs were assigned to the 'candidate parasitism genes' category based on similarity with known pathogenicity factors in bacteria and fungi and parasitism related genes in animal-parasites. The ESTs in preparasitic J2 of *M. incongita* that have no apparent homologue in *C. elegans* have a high potential as evolutionary adaptations to parasitism. Genes involved in parasitism are frequently conserved among animal-parasitic nematodes species, which may also proof to be the case for the plant-parasitic species (29, 40). Evidence for this is already found in the finding of cellulase genes in practically all plant-parasitic nematode species that have been investigated at present (14).

The group of 'candidate parasitism genes' included all parasitism genes identified to date. These genes were often isolated following years of hard work and dedication, and were tagged in the cDNA library of *M. incognita* with only a fraction of that effort. In our view this makes EST projects undoubtedly an extremely powerful tool to identify parasitism genes in nematodes.

From gene-structure to gene-function

The current classification of glycosyl hydrolases (GH) and related enzymes include 90 different Enzyme Commission (EC) entries that are classified into 81 GH families (http://afmb.cnrs-mrs.fr/~pedro/CAZY/ghf.html). Forty-seven families are monospecific (containing only one EC number), while 34 families are found to contain at least 2 EC numbers (polyspecific). Relatedness based on sequence similarity and on 3D structures has yielded 10 major clans of related families.

Degradation of a highly organised and complex network as the cell wall is, requires an entire spectrum of enzymes (Chapter 1), including xylanases. On the basis of sequence similarities and function, the enzymes involved in xylan degradation are classified into cellulases family A, F, and G (27) also known as glycosyl hydrolases (GH) family 5, 10, 11 (26). The majority of bacterial and fungal xylanases belong to families 10 and 11. Recently, three xylanases has been classified as GH family 43 (Table 6.2).

GH family 5 contains mainly endogluganases and only 6 xylanases (Table 6.2). *M. incognita* xylanase (Mi-Xyl1, this thesis) is one of the members of GH family 5 that is classified in clan GH-A, characterised by a catalytic domain shaped as a classical eight-fold b/a-barrel structure in which conserved Glu function as catalytic nucleophile and acid/base catalytic residues. Four out of five hydrophobic clusters conserved in GH family 5 cellulases are also present in *Mi-xyl1* (Chapter5, Figure 5.2) . The conserved clusters III and V facilitate in the identification of two putative active site glutamic acids in the Mi-XYL1 sequence (E_{158} and E_{258}). An asparagine residue, which is conserved in GH family 5 members directly amino terminal to the putative proton donor site (E_{158}) is replaced by serine (S_{157}) in Mi-XYL1. A similar substitution (NE ® SE) at the same position is found in a GH family 17 member - a b-1,3-1,4- endoglucanase of *Hordeum vulgare* (Swiss-Prot accession P12257). A functional explanation for this similarity remains a subject of investigation.

Mode of action of the xylanases

Knowledge of the mechanism of xylan-degrading enzymes has been gained from studies on substrate specificity, the role of side chain substitutions on activity, the specificity of bonds cleaved and the end products. Generally, xylanases appear to be specific towards the intersugar linkage (15). For example, the xylans of fungal origin are characterised as non-debranching (41) and debranching (35). It is commonly observed that substitutions in the highly branched polysaccharides interfere with xylanase activity. However, enzymes having more affinity for main chain linkages, near branch points, are also reported (15).

Table 6.2 - Xylanases from various origins classified into glycosyl hydrolase families.

	Protein	EC number	Sequences
GH-5	**Endoglucanase**	EC 3.2.1.4	113
	Xylanase	EC 3.2.1.8	6
	CMC-xylanase	EC 3.2.1.14	1
	beta-mannosidase	EC 3.2.1.25	2
	exo-1,3-glucanase	EC 3.2.1.58	12
	endo-1,6-glucanase	EC 3.2.1.75	1
	beta-mannanase	EC 3.2.1.78	16
	Cellobiohydrolase	EC 3.2.1.91	2
	Endoglycoceramidase	EC 3.2.1.123	2
	Cellodextrinase	EC 3.2.1.-	3
	non-defined	-	52
GH-10	**Endoglucanase**	EC 3.2.1.4	1
	Xylanase	EC 3.2.1.8	117
	endo-1,3beta-xylanase	EC 3.2.1.32	1
	Cellobiohydrolase	EC 3.2.1.91	1
	non-defined	-	20
GH-11	**Xylanase**	EC 3.2.1.8	97
	non-defined	-	12
GH-43	**Xylanase**	EC 3.2.1.8	3
	beta-xylosidase	EC 3.2.1.37	10
	alfa-L-arabinofuranosidase	EC 3.2.1.55	7
	Arabinanase	EC 3.2.1.99	3
	non-defined	-	7

The hydrolysis reaction catalysed by xylanases as well as cellulases proceeds through an acid-base mechanism involving two residues.The first residue acts as a general catalyst and protonates the oxygen of the oxidic bond. The second one acts as a nucleophile, which in the case of retaining enzymes interacts with the oxocarbonium intermediate. In the case of inverting enzymes it promotes the formation of an OH^- ion from a water molecule. The critical distance between two catalytic carboxylic acids (» 5.5Å) is less in retaining enzymes as compared to that in inverting glycosidases (» 10Å). Lawson et al. (31) has also observed that the precise placement of the acid/base catalyst is not critical, since both shortening and length-

ening this carboxyl side chain resulted in approximately the same modest decrease in kcat/Km values. Thus, the positional requirements for proton transfer are less demanding than for carbon-oxygen formation. The Mi-XYL1 protein is classified in GH family 5, which implicates that xylan hydrolysis occurs with retention of anomeric configuration (53).

Biochemical characteristics of xylanases

The available information on the properties of xylanases stems mostly from studies with bacterial and fungal enzymes, although xylanases are produced as well by a plethora of organisms like algae, protozoa, gastropods and arthropods (15). Microbial xylanases are single-subunit proteins with molecular masses in the range of 8-145 kDa (55). Xylanases are usually stable over a wide pH range (3-10) and show optimum pH in the range of 4 and 7. The xylanases from fungi (20, 28) exhibit optimum pH towards acid (pH 2-6). The isoelectric points for endoxylanases from various sources range from 3 to 10. Generally, bacteria are known to produce two xylanases: i) high molecular mass acidic xylanase, ii) low molecular mass basic xylanase.

Mi-XYL1 showed an optimum pH of 8.0, retaining more than 50% activity in a range of pH 7.5 to pH 8.5. This value is higher than that of the xylanases from other plant pathogens (*e.g. Erwinia chrysanthemi*) (9). The apoplast in plants has a pH between 5 and 6.5, which is also significantly different from the pH optimum of the Mi-XYL1 activity. The translational fusion partner of Mi-Xyl1, the maltose binding protein, may influence the optimum activity, which makes a direct comparison less opportune. In addition, the ionic strength of intercellular fluids and interactions with other proteins and carbohydrates may also contribute to this discordance.

Multiplicity of cell wall degrading enzymes

Frequently, within the genomes of plant pathogens an entire complex of cell wall degrading enzymes is found. This often includes more representatives from a single enzyme class. For example at least 15 cellulases, two xylanases and two b-glucosidase genes have been isolated from the anaerobic thermophilic bacterium *Clostridium thermocellum* (25). To degrade the glycosilic bonds of the polysaccharides anaerobic microorganisms secrete a highly active cellulolytic complex, so-called cellulosome (4, 6) in which xylanase activity is also detected (24, 37). Contrary, aerobic microorganisms secrete individually but synergistically acting enzymes for the same purpose.

Endogenously production of the two cellulases in the cyst nematode *G. rostochiensis* and in *H. glycines* (54) indicates existence of a multiple enzyme complex in plant parasitic nematodes too. Homologous xylanases were detected in each of the genomes of *M. incognita* and *M. javanica* (Chapter 5, Figure 5.6), but at present it's not known how these genes relate to each other.

The existence of multiple cellulases and xylanases in a single organism reflects the heterogeneous nature of plant cell walls that these organisms have to deal with. Although cellulose molecules are chemically homogeneous, structurally they are quite diverse. It is possible, therefore, that various isoenzymes hydrolyse b-1,4 bonds that are in different physical environments within the cellulose molecule. This argument can also be applied to xylan hydrolysis in view of the heterogeneity in both the chemical and the physical nature of the polymer.

Isoenzymes may be transcribed from different genes. However, isoenzymes may also arise from post-translational modifications through glycosylation and partial proteolysis. Based on biochemical studies it is difficult to determine, whether the apparent isozymes are the products of multiple genes, the result of the modification of the single enzymes (59) or a combination of both. We have not raised an antibody against *Mi-xyl1*, therefore, it is not clear at this stage if in addition to the two homologous genes further differentiation in specificity by post-translational modification is occurring in the nematode xylanases.

The role of cell wall degrading enzymes in parasitism and pathogenicity

Knock-out mutants or inhibitory antibodies would provide us with more conclusive evidence for the importance of cell wall degrading enzymes in the parasitic cycle of nematodes. However, the state of the art in molecular nematology has not reach to this level yet. Progress in the development of protocols to knock out genes in parasitic nematodes, either by stable transformation or by RNA interference, is still slow due to the complex mode of reproduction and the obligatory parasitic behaviour of the nematodes.

Instead of providing conclusive evidence for nematodes some lessons may be learned from cell wall degrading enzymes in other plant-pathogens. In 1886, DeBary published the first indications that extracellular enzymes may be involved in the infection process of plant pathogenic fungi. Since then numerous reports of cell wall degrading enzymes in fungi have appeared showing their importance in the breakdown of plant cell walls and colonisation of plant tissue (10, 13). In plant-pathogenic bacteria, it is not always so clear-cut whether cell wall degrading enzymes are crucial for pathogenicity and virulence. The outcome of the experiments largely depended on the species of the bacteria and its host (5, 33, 44). In both bacteria and fungi, disruption of the export mechanism can result in a simultaneous loss

of extracellular enzyme activity and a reduction in virulence or loss of pathogenicity (30, 58).

The difficulty in correlating the enzyme production to virulence or pathogenicity in plant pathogens is the existence of multiple isozymes of cell wall degrading enzyme in most of them. Disruption of individual genes (*e.g.* in *C. carbonum*) fails to show that they are crucial for pathogenicity, as the mutants show the same virulence as the wild type (2, 47, 48). In these cases knocking out a single gene may have little influence on the total fitness of the pathogen that can use similar enzymes to compensate for the loss in specificity.

Mi-xyl1 cloned from root knot nematode *Meloidogyne incognita* is probably one of at least two xylanases (Chapter 5). It is not possible yet to determine whether Mi-Xyl1 is crucial for parasitism of plants by *M. incognita*. However, to date a multigene family of at least five endoglucanases (43) and two xylanases (this thesis) have been identified in this species, and it is difficult to envision that maintaining such an array of glycosyl hydrolases would not pay off for the nematode. Therefore, it is reasonably to assume that penetration and subsequent migration of the nematode through the plant root is facilitated by enzymatic weakening of the cell walls. Typical for *Meloidogyne* spp. is that migration proceeds intercellularly during which the plant cell walls of two aligning cells ahead are separated at the middle lamella. Such a subtle process, that leaves the cells intact, gives some insight in the importance of cell wall degrading enzymes.

In contrast to cyst nematodes, root knot nematodes are polyphagous nematodes, including monocots and dicots in their host range. The cell wall composition of monocots and dicots is significantly different. For instance, the xylan content of monocots is much higher as compared to dicots. Our finding of xylanases next to the previously cloned cellulases in *Meloidogyne* spp. suggests an evolutionary adaptation towards monocots in root knot nematodes. In spite of intensive studies, xylanases have not been found in *Globodera rostochiensis* and *Heterodera glycines*, parasites of dicots only, which is in favour of this hypothesis.

Evolutionary relationships between xylanases

To gain more insight in the evolutionary relationships between xylanases of various plant-pathogens we have done a phylogenetic analysis of representatives of the three main GH families that contain xylanases.

Xylanases of GH-5, GH-10 and GH-11 are clustered as monophyletic groups supported with a bootstrap value of 100%. One exception is the xylanase M83379 of GH-5 that shows high homology to endoglucanases. The two sequences from the GH-5 (M75706 and U94826) that represent ORFs with multiple activities (*e.g.*, en-

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Summary

Variability in the behaviour of a nematode population is a reflection of the genetic structure of that population. Revealing the genetic information contained in the nematode's genome contributes to reliable predictions of their behaviour in the field. The genome of the all animals consists of nuclear and mitochondrial DNA. Population genetic studies can be conducted by studying polymorphism at the DNA level of both subsets.

In **Chapter 3** we have studied genetic variation in 16 *M. incognita, M. javanica* and *M. arenaria*, populations. In this chapter, genetic variation in the *Meloidogyne* spp. populations was assessed using amplified fragment length polymorphism (AFLP) markers in nuclear DNA and tandem repeats (TR) in mtDNA in order to reveal genetic divergence at different hierarchical levels. Even though the examined populations belong to obligatory mitotic parthenogenetic species genetic differences were detected within and among populations. The proportion of polymorphic fragments in the nuclear and mtDNA genomes respectively were lower in *M. incognita* (32% and 20% respectively) as compared to portions in *M. javanica* (52% and 40% respectively) and *M. arenaria* (61% and 50% respectively). Genetic distances based on the AFLP patterns have assigned the populations into three clusters commensurate with their respective species identities. The three principle coordinate analysis situated *M. incognita* as the most genetically distinct of the three species. Polymorphisms generated by differences of mtDNA in the tandem array of 63-bp repeats, showed that *M. incognita* individuals were the most heteroplasmic, whereas at the population level *M. incognita* was the most homogenous (13% overall diversity) followed by *M. javanica* (24%) and *M. arenaria* (35%). Comparing the intraspecific genetic distances based on nuclear and mtDNA markers has only revealed a positive correlation between both approaches with regard to the *M. arenaria* populations.

Exploring the nematode's genome with molecular techniques may directly lead us to the genes involved in parasitism of plants. By definition these nematode genes are pivotal for host penetration, intercellular migration and feeding on plants. Their identification may help to design resistant strategies for pest control as they potentially make good targets for bioengineering anti-nematode strategies. In **Chapter 4,** we have presented the analysis of 1,000 random sequences obtained from a cDNA library. These so-called expressed sequence tags (EST) have shown to be a powerful method to identify genes expressed at a certain time point of the nematode life cycle. The parasitic cycle involves various distinct stages, plant penetration and intercellular migration, and feeding site initiation and maintenance. Therefore, cDNA libraries covering these main stages may provide insight in the molecular fundaments of plant parasitism by root knot nematodes.

In chapter 4 a cDNA library of preparasitic J2s of *Meloidogyne incognita* was used, which covered the initial phases of the parasitic cycle – plant penetration and intercellular migration. The ESTs were clustered into 9 functional groups.

'Candidate parasitism genes' (3.1%) included all parasitism genes identified to date as well as novel ones. A more challenging group is the class 'Pioneering sequences' (33.9% of the ESTs). Several analytical steps that may help in assigning a function to these novel sequences are discussed.

One of the expressed sequence tag was categorised into the 'Candidate parasitism genes' group because it showed homology to a cell wall degrading enzyme – a xylanase (**Chapter 5**). The partial sequence of the EST was used to obtain a full-length transcript of 1220 nt encoding an open reading frame (Mi-Xyl1) of 37.6 kDa. Hydrophobic cluster analysis classified the putative xylanase as a family 5 glycosyl hydrolase. Whole mount *in situ* hybridisation showed specific labelling of a *Mi-xyl1* probe in the subventral oesophageal glands of second stage juveniles. DNA blot hybridisation indicated the presence of two homologues in *M. incognita* whereas no hybridisation was found with genomic DNA fragments of *Caenorhabditis elegans* and cyst nematodes. Recombinant *Mi-xyl1* protein, produced in *Escherichia coli*, exhibited hydrolytic activity on xylan and carboxymethylcellulose.

The plant cell wall can be considered as an effective barrier that protects the plant from invasion by pathogens and parasites. It is a highly organised network composed of different polysaccharides, proteins and phenolic compounds. Recently, endoglucagenases were identified in plant-parasitic nematodes. In this thesis evidence is provided for the presence of hemicellulolytic enzymes in plant-parasitic nematodes. This finding suggests that plant parasitic nematodes make use of a suite of cell wall degrading enzymes with overlapping activities to facilitate plant invasion. Root knot nematodes are able to propagate on both monocots and dicots. It is hypothesised that the xylanases genes in these nematode species enable invasion of monocots, which have a significantly higher xylan content in the cell walls.

Based on phylogenetic and hydrophobic cluster analysis the nematode xylanases seem to be closer related to bacterial xylanases than to homologues in other animals, plants and fungi. This observation is commensurate with the findings with pectinases and cellulases in nematodes, which suggests that this type of genes may have been acquired from bacteria by horizontal gene transfer. More lines of evidence are needed to support these hypotheses.

De resultaten uit **hoofdstuk 2** tonen aan dat het telen van tomatenrassen met het *Mi*-resistentiegen voordelig kan zijn mits er sprake is van avirulente populaties. Het is echter eerder al gebleken dat na langdurige selectie virulente populaties kunnen ontstaan uit avirulente populaties. In die situatie zullen de beheersmaatregelen een combinatie moeten zijn van vruchtwisseling, biologische bestrijding en braaklegging. Eventueel kan dit worden uitgebreid met sterilisatie door stoom en de toepassing van nematiciden.

De variatie in de reactie van nematoden populaties op allerlei beheersmaatregelen is een afspiegeling van de genetische structuur van de populaties. Ontsluiting van de genetische informatie die is opgeslagen in het genoom van de nematoden draagt bij aan de betrouwbaarheid van de voorspellingen van het gedrag van populaties in het veld. Het genoom van dieren bestaat uit het nucleaire DNA en het mitochondriale DNA. Populatiegenetica kan worden bedreven door polymorfismen te onderzoeken in beide DNA subsets.

In **hoofdstuk 3** is genetische variatie onderzocht van 16 populaties met *M. incognita*, *M. javanica* en *M. arenaria*. De variatie in het nucleair DNA is bepaald met behulp van zogenaamde *Amplified Fragments Length Polymorphisms* (AFLP), terwijl de variatie in het mitochondriaal DNA is geanalyseerd via de zogenaamde *Tandem Repeats* (TR). Op basis van deze uitkomsten is de genetische divergentie tussen populaties bepaald op verschillende hiërarchische niveaus. De onderzochte soorten zijn obligaat mitotisch parthenogenetisch, maar ondanks deze wijze van reproductie bleken de verschillen in variatie binnen en tussen populaties aanzienlijk. De fractie polymorfe fragmenten in het nucleaire en mitochondriale DNA was het laagst in *M. incognita* (respectievelijk 32% en 20 %) in vergelijking met *M. javanica* (respectievelijk 52% en 40%) en *M. arenaria* (respectievelijk 61% en 50 %). In de dendrogram op basis van de genetische afstand (GD) afgeleid van de AFLP merkers zijn de drie soorten gegroepeerd in drie monofeletische clusters wat in overeenstemming is met de identiteit van de soorten. Volgens een *three principle coordinate* analyse blijkt dat *M. incognita* het minst verwant is van de drie soorten. Uit de TR analyse bleek dat individuen van *M. incognita* het meest heteroplasmisch zijn. Op populatie-niveau was deze soort het meest homogeen (13% totale diversiteit) gevolgd door *M. javanica* (24% totale diversiteit) en *M. arenaria* (35% totale diversiteit). Een vergelijking van de intraspecifieke genetisch afstand zowel op basis van de AFLP als de TR analyse resulteerde alleen in een positieve correlatie voor *M. arenaria* populaties.

Het onderzoeken van het genoom van nematode met behulp van moleculair biologische technieken kan leiden tot de identificatie van genen die betrokken zijn bij het parasitisme. Per definitie kunnen deze genen van belang zijn voor de penetratie van de plant, de intercellulaire migratie in weefsel en het onttrekken van voeding aan planten. De identificatie van deze genen kan de basis vormen voor biotechnologische strategieën die de ontwikkeling van resistentie tot doel hebben. In

hoofdstuk 4 is de analyse gepresenteerd van 1000 *random* sequenties uit een cDNA bibliotheek van *M. incognita*. Deze zogenaamde *Expressed Sequence Tags* (EST) blijken een uiterst doeltreffend middel om genen te identificeren op een bepaald tijdstip in de levenscyclus van de nematode. De parasitaire cyclus van een nematode bestaat uit een aantal afzonderlijke fasen – penetratie, intercellulaire migratie en voedingscel inductie en onderhoud. Analyse van cDNA bibliotheken gemaakt van al deze fasen kan leiden tot inzicht in de moleculaire basis van de plant-nematode interactie.

In hoofdstuk 4 is een cDNA bibliotheek geanalyseerd van preparasitaire nematoden waardoor de gen expressie in de eerste fasen van de parasitaire cyclus is onderzocht. De ESTs zijn gegroepeerd in negen functionele klassen. De categorie '*candidate parasitism genes*' bevatte tevens alle tot dan toe gekloneerde en gekarakteriseerde parasitisme genen uit *M. incognita*. Een interessante groep zijn de '*pioneering sequences*' (33,9 %van de ESTS). Enkele analytische stappen die kunnen helpen bij het toewijzen van een functie aan sequenties in deze laatste groep worden besproken in dit hoofdstuk.

Een van de ESTs, in de categorie '*candidate parasitism genes*', toonde homologie met een celwand afbrekend enzym – een xylanase (**hoofdstuk 5**). De partiële sequentie van de EST is gebruikt om een transcript (1220 nt) met een compleet open leesraam (37,6 kDa) te isoleren. Vanwege de homologie met xylanases is dit open leesraam Mi-Xyl1 genoemd. Volgens de hydrofobe cluster analyse behoort Mi-Xyl1 tot de familie 5 van de glycosyl hydrolases. In situ hybridisatie met een *probe* afgeleid van Mi-Xyl1 toonde een specifieke interactie met de subventrale speekselklieren van preparasitaire juvenielen. Uit analyse van het genomische DNA bleek dat een vermoedelijk twee homologe xylanase genen in het *M. incognita* aanwezig zijn. In *Caenorhabditis elegans* en in cystenaaltjes zijn geen homologe genen gevonden. Recombinant Mi-Xyl1, geproduceerd in *Escherichia coli*, bleek enzymatisch actief op xylan en op carboxymethylcellulose.

De celwanden in planten vormen een barrière tegen de invasie door pathogenen en parasieten. The celwand is een complexe composiet van allerlei polysacchariden, eiwitten en fenolachtige verbindingen. Recent zijn endoglucanases aangetroffen in plantenparsitaire nematoden. In dit proefschrift is voor het eerst bewijs geleverd voor de aanwezigheid van hemicellulolytische enzymen in deze parasieten. Deze vondst suggereert dat nematoden bij de penetratie en intercellulaire migratie kunnen beschikken over een reeks van celwand afbrekende enzymen met deels overlappende activiteiten. Wortelknobbelaatjes kunnen zich vermeerderen moncotielen en dicotielen. Monocotielen hebben een aanzienlijk hoger xylan gehalte in de celwanden, en naar aanleiding van de resultaten in dit proefschrift wordt verondersteld dat xylanase in wortelknobbelaaltjes met name een rol speelt bij het parasiteren van monocotielen.

Samenvatting

Op basis van de hydrofobe cluster en fylogenetische analyse lijkt Mi-Xyl1 meer verwant met bacteriële homologen, dan met die uit andere dieren, planten en schimmels. Deze observatie is in overeenstemming met de situatie van pectinases en cellulases in nematoden. Dit feit suggereert dat nematoden de vooroudergenen van deze celwand afbrekende enzymen hebben verkregen via horizontale gen overdracht vanuit bacteriën. Er is echter meer eenduidig bewijs nodig om deze hypothese te ondersteunen.

СОДРЖИНА НА ДИСЕРТАЦИЈАТА

Популациона и молекуларна генетика на галовите нематоди

Нематодите го претставуваат најголемиот и најбројниот филум во животинското царство. Главно живеат во водата и почвата. Во почвата се присутни во енормен број кој достигнува од 1.8 до 120 милиони по квадратен метар. Многу мал дел од нив се имат приспособено да ги паразитираат растенијата (фитопаразитни). Фитопаразитните нематоди се проучуваат главно поради економските штети кои ги предизвикуваат како резултат на морфолошките и физиолошките абнормалности што ги причинуваат кај растенијата, кои доведуваат до сигнификантно намалување на приносот.

Галовите нематоди, кои припаѓаат на родот *Meloidogyne* (Goeldi, 1892), ја сочинуваат најголемата група од фитопаразитни нематоди и се од огромно економско значење. Нивната широка распространетост и способноста да опстанат во различни климатски услови им овозможило да воспостават комплексни паразитски односи со повеќе од 2000 растителни видови. Годишните штети од галовите нематоди се проценети на 10% од вкупните светски загуби предизвикани од фитопаразитни нематоди. Овие загуби можат да достигнат и до 25% во земјите во развој. До денес се опишани осумдесет видови галови нематоди, но само четири од нив, и тоа *M. incognita, M. javanica, M. arenaria* и *M. hapla*, се одговорни за 90% од вкупните штети предизвикани од галови нематоди. Еколошка и најпогодна мерка за заштита на производството од напад на галовите нематоди е одгледувањето на отпорни сорти. Доста често, појавата на вирулентни популации, кои ја совладуваат отпорноста, ја намалуваат ефикасноста на овој заштитен пристап.

И покрај големото економско значење на доматот како градинарска култура во Македонија, малку се знае за распространетоста на видовите од родот *Meloidogyne* на површините во Македонија, додека пак податоци за интервидовата варијација и потполно недостасуваат. Овие недостатоци блијаат сериозно врз изработката и примената на погодна и ефикасна програма за заштита на културите од *Melodogyne spp.* во Македонија. Како прв чекор кон успешна заштита е детерминирање на присутните *Meloidogyne* spp. во Македонија како и детектирање на вирулентните популации кон резистентните сорти домати. Отпорноста на доматите кон *Meloidogyne* spp. е резултат на поседувањето на *Mi*-генот кој е лоциран на 6-тиот хромозом во доматниот геном.

Во **второто поглавје** од дисертацијата, идентификувани се седумдесет и три *Meloidogyne* изолати кои потекнуваат од девет локации (полски и оранжериски) и споредувано е нивното влијание врз осетливите и отпорните сорти домати. Најзастапени беа видовите *M. incognita* (47,9%) и *M. javanica* (35,6%), а поретко беше застапен видот *M. arenaria* (13,7%) додека *M. hapla* (2,7%) беше де-

тектирана спорадично. Во скоро сите локалитети е утврдено присуство на повеќе од еден вид. Вирулентни изолати се утврдени кај: *M. incognita* (11%), *M. javanica* (46%) и кај *M. arenaria* (50%). Изолатите од *M. hapla* беа компатибилни со сите тестирани генотипови од доматот, што може и да се очекува поради тоа што *Mi*-генот не допринесува за резистентност кон *M. hapla*.

Горенаведените резултати укажуваат на тоа дека одгледувањето на отпорни сорти домати е предност кај локациите каде има присуство на авирулентни популации. Долгорочен селекционен притисок може да доведе до вирулентност кај невирулентните популации, што доведува до проблем при одгледувањето на отпорните сорти. Во тој случај потребно е комбинирање на мерките за заштита како што се: плодоред, угар и билошка контрола или пак употреба на нематоциди. Варијабилноста во однесувањето на поодделните индивидуи во една популација од нематоди е резултат на генетската структура на таа популација. Откривањето на генетските информации кои се инкорпорирани во нематодниот геном може да допринесе да се предвиди нивното однесување во полски услови. Имајќи во предвид дека геномот на сите животни, вклучувајки ги и нематодите, се состои од јадрена и митохондриална ДНК, испитувањата во рамиките на популационата генетика можат да бидат спроведени со проучување на полиморфизмот кај ДНК и во двата геноми.

Во **третото поглавје** проучувана е генетската варијабилност во 16 популации од *M. incognita, M. javanica* и *M. arenaria*. Во оваа поглавје, со помош на AFLP (amplified fragment length polymorphism) маркери во јадрената ДНК и TR (tandem repeats) во мтДНК, беа откриени генетските разлики на различни хиерархиски нивоа. Иако испитуваните видови се карактеризираат со облигаторно митотско-партогенетско размножување беа детектирани генетски варирања во и помеѓу популациите. Пропорционалната застапеност на полиморфните фрагменти во јадрениот и митохондријалниот ДНК геном беше помала во *M. incognita* (32%, 20%) споредено со *M. javanica* (52%, 40%) и *M. arenaria* (61%, 50%). Генетските разлики базирани на AFLP профили ги класифицира популациите во три групи согласно нивните видови идентитети. Понатамошните анализи покажаа дека од трите испитувани видови *M. incognita e* генетски најоддалечен вид. Полиморфизмите што резултираат од TRs т.е. разликите во бројот на последователно повторуваната 63-бп секвенца покажаа дека ларвите на *M. incognita* се со највисока хетероплазмија додека на популациско ниво *M. incognita* беше најхомогена (13%) во однос на *M. javanica* (24%) и *M. arenaria* (35%). Споредувањето на интерспецифичните генетски разлики на јадрената и митохондријалната ДНК покажа позитивна корелација помеѓу двата приода во однос на популациите од *M. arenaria*.

Примената на молекуларни техники за изучување на нематодниот геном води директно кон гените кои се одговорни за паразитирање на растенијата од страна на нематодите. По дефиниција тие гени имаат главна улога при навлегувањето, меѓуклеточното движење и хранење на нематодите од растението. Нив-

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At the end, to all of you

- I was not trying to be patronising, because everything I said is true.

p.s. To Ling, Herman, Marie-José, Matijas, Hans, Erin, Robert, Evert, Willemien, Ula, Aneta, Erwin - good luck with your thesis.

Curriculum vitae

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Makedonka Dautova finished primary, junior and high school education in her hometown of Strumica, Republic of Macedonia. In 1986 she started the academy study at the University of Skopje-Macedonia, obtaining her bachelor's degree BSc in Biology in 1990. One year later she entered the graduate program MSc studies at the same University and three years later she acquired the title of 'Master of Biological Sciences'. In 1995 she came to Wageningen to attend the Integrated Pest Management program at 'International Agriculture Centre', when she contacted Dr. Fred Gommers at the Laboratory of Nematology for starting PhD programme at Wageningen-UR. Due to lack of experience in practical molecular biology in 1996 she went to California Department for Food and Agriculture, Sacramento, CA, USA, where she learned the basics of contemporary molecular techniques. After her return at 1997 she started PhD program at Wageningen-UR with a 'sandwich fellowship' provided by this University. Since that period to 2001 she carried out PhD research on population and molecular genetics of root-knot nematodes in the Laboratory of Nematology and Laboratory of Monoclonal Antibodies under the supervision of Dr. ir. FJ Gommers, Dr. ir. G Smant and Prof. dr. ir. J Bakker. The results obtained in that period are described in this thesis.

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