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# Nuclear and mitochondrial DNA polymorphisms in three mitotic parthenogenetic *Meloidogyne* spp.

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**Abstract**

In order to expand our understanding of the genetics of root-knot nematodes, 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 43–65% of the total diversity. Many individuals contained more than one mtDNA size variant. *M. incognita* harboured the most heteroplasmic individuals and was 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*. Both TR and AFLP data showed that *M. arenaria* is the most heterogeneous species. The comparison of the genetic distances based on AFLPs and mtDNA size variants revealed a significant correlation for the six *M. arenaria* populations, whereas no consistent correlation was observed for the populations of the other two species.

## 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, information about the genome of the individuals should at least be partly revealed. Quantification of genetic variability has a pivotal role in contemporary phytonematology, but is considered as one of the least understood aspects of nematode population biology (Caswell and Roberts, 1987). This especially holds true for obligatory parthenogenetic root-knot nematodes that include *Meloidogyne incognita, M. arenaria* and *M. javanica* (Triantaphyllou, 1981). In these species parthenogenesis is mitotic 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 (Noe, 1992). 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 (Taylor and Sasser, 1978). 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) (Hyman, 1988). Population genetic studies can be conducted by studying polymorphisms within either DNA type. 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 substantially higher rate (e.g. Blouin et al., 1992; Denver et al., 2000). The mtDNA data are, therefore, more likely to display useful variation at population and individual levels (Avise, 1994).

To date, several polymerase chain reaction (PCR)-based approaches using genomic DNA have been applied to investigate inter- and intrapopulation genetic diversity in *Meloidogyne* species. Random amplified polymorphic DNAs (RAPDs) have been used as a tool to address variation (Welsh and McClelland, 1990; Williams et al., 1990; Cenis, 1993; Castagnone-Sereno et al., 1994; Lynch and Milligan, 1994; Guirao et al., 1995). The use of restriction fragment length polymorphisms (RFLPs) is more reproducible and sensitive, thus enabling more precise estimates of genetic variations (Garate et al., 1991; Carpenter et al., 1992; Fargette et al., 1994; Guirao et al., 1995). 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 (Zabeau and Vos, 1993) generates virtually unlimited numbers of DNA fragments from nanogram quantities of genomic DNA and the stringent reaction conditions guarantee reproducibility and quantification (van Eck et al., 1995; Folkertsma et al., 1996). The discriminatory power of this approach was demonstrated by Semblat et al. (1998) and Tzortzakakis et al. (1999) 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 (Avise, 1994). Hyman and Slater (1990) were the first to study mtDNA sequence variations in *Romanomermis culcivorax*, a parasitic nematode of mosquitoes. Tandemly repeated (TR) sequences have been characterised in the mtDNA genome of root-knot nematodes (Okimoto et al., 1991) and were explored for studying polymorphisms in *M. incognita* populations and individuals (Hyman and Whipple, 1996; Whipple et al., 1998). Such TR sequences in mtDNA have been characterised in over 150 species ranging from nematodes, fishes, amphibians, birds to mammals (Lunt et al., 1998).

In this study, the genetic variation in 16 geographically unrelated *M. incognita, M. javanica* and *M. arenaria* populations was assessed using AFLP markers in nuclear DNA and TR 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*, five of *M. javanica*, and six of *M. arenaria* were propagated on *Lycopersicum esculentum* cv. Moneymaker under glasshouse conditions (Table 1). The geographically unrelated populations were taken from our laboratory collection or obtained from the Plant Protection Service, Wageningen, the Netherlands. The species identification of each isolate was done prior to the experiments, by testing (i) 15 young egg-laying female per population for their isozyme phenotypes of esterase and malate dehydrogenase (Esbenshade and Triantaphyllou, 1990) and (ii) the intergenic spacer region between the cytochrome oxidase II gene and the 16S rRNA gene in the mtDNA of 20 single juveniles per population (Powers and Harris, 1993).

### *AFLP procedure*

Egg masses were harvested from infected tomato roots and treated with 0.5% NaOCl (Hussey and Barker, 1973). After hatching at 22 °C, the mixture of second stage juveniles (J2s) and eggs was homogenised in proteinase K buffer (20 mM Tris – pH 8, 100 mM EDTA, 0.5% SDS, 2 mg/ml proteinase K) and incubated at 50 °C for 4 h. 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 µl TE buffer pH 7.5 (Sambrook et al., 1989).

The AFLP procedure was performed as described by Zabeau and Vos (1993). The digestion of genomic DNA with *Eco* RI and *Mse* I was followed by the

*Table 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 | *Lycopersicon esculentum* | — |
| | Mi-2 | C 3055 | China | Bonsai | 1990 |
| | Mi-3 | C 4756 | Hungary | *Lycopersicon esculentum* | 1991 |
| | Mi-4 | C 9956 | NL | *Rhodochiton* sp. | 1997 |
| | Mi-5 | D 385-C | China | *Ligustrum* sp. | 1997 |
| *M. javanica* | Mj-1 | L1 | NL | *Lycopersicon esculentum* | — |
| | 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. | *Lycopersicon esculentum* | 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 | *Lycopersicon esculentum* | 1997 |
| | Ma-5 | C 6460 | Columbia | *Livinstonia rotundofolia* | 1993 |
| | Ma-6 | C 9891 | NL | *Philodendron* sp. | 1997 |

*Table 2*. Sequences of adapters and primers used in this study

| | | |
|---|---|---|
| *Eco* RI adapter | | 5′-CTC GTA GAC TGC GTA CC-3′<br>3′-CTG ACG CAT GGT TAA-5′ |
| *Mse* I adapter | | 5′-GAC GAT GAG TCC TGA G-3′<br>3′-CTA CTC AGG ACT CAT-5′ |
| *Eco* RI + 0primer | EOO | 5′-GAC TGC GTA CCA ATT C-3′ |
| *Eco* RI + 2primer | E + GA | 5′- GAC TGC GTA CCA ATT CGA-3′ |
| *Mse* I + 0primer | MOO | 5′-GAT GAG TCC TGA GTA A-3′ |
| *Mse* I + 2primer | 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′ |
| *Mse* I + 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′ |

ligation of an *Eco* RI and *Mse* I adapters (Table 2). The ligation mix was used in a non-selective amplification (preamplification) using primers that annealed to the *Eco* RI (E + 0) and *Mse* I adapter sequences (M + 0) (Table 2). The PCR reaction was performed in a PE-9600 thermal cycler (Perkin Elmer, Norwalk, USA) using the following profile: 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 56 °C and 60 s extension at 72 °C. Verification of the PCR products was done in 1% agarose gel in TAE buffer stained with

ethidium bromide (Sambrook et al., 1989). In a typical reaction, the DNA fragments appeared on gel as a smear from 50 to 500 bp. The preamplification product was diluted 10× 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 10 μCi/μl $\gamma$33P-ATP according to the recommendations of the manufacturer (ICN Biomedicals, Zoetermeer, the Netherlands). In each reaction 5 ng of labelled and 30 ng of unlabelled selective primers (M + 2 and M + 3, Table 2) were used. The PCR proceeded according to a touch-down profile of 24 cycles: 12 cycles of 30 s denaturation at 94 °C, 30 s annealing at 65 °C, 60 s 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 12 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 min at 95 °C and loaded on a 5% polyacrylamide gel (Sequagel-5, BioZym, Georgia, USA) in 1 × TBE electrophoresis buffer (Sambrook et al., 1989). 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 analysed by a computer package 'Cross Checker' that provides a semi-automatic analysis, developed for quantitative analysis of DNA fingerprinting patterns (download URL: *http://www.dpw.wau.nl/pv/pub/CrossCheck/*). In each reaction the presence/absence of bands was scored.

### *63-bp TR 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 μl of sterile water. The primers TR-F and TR-R (Table 2) and amplification conditions used have been according to Hyman and Whipple (1996). The forward primer specifically anneals to a non-coding region 15-bp upstream of the 63-bp repeating unit array. The reverse primer binds 5-bp downstream of the TR within the mitochondrial tRNAmet gene (Hyman and Whipple, 1996). PCR products were fractionated on Tris–Acetate–EDTA buffered (Maniatis et al., 1982) 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 pairwise 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 (Nei and Li, 1979). The dendrogram of the 16 *Meloidogyne* populations was constructed from the genetic distance matrix by using the UPGMA algorithm (Sneath and Sokal, 1973). One thousand bootstrap replicates were performed to test the support of the grouping (Felsenstein, 1985). The binary tables were analysed with a Treecon software package (version 1.3b) for construction and drawing of dendrograms and trees (Van de Peer and De Wachter, 1993). Principal coordinate analysis was performed to access interspecies relationship based on the Nei and Li (1979) coefficient using the NtSYS-pc software (Rohlf, 1989).

*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 in some cases visual estimation. Gene diversity was calculated from the mtDNA size class frequencies by $K$-indices (Birky et al., 1989), where $K = 1 - (\Sigma x_i^2)$, where $x_i$ is the frequency estimate of mtDNA size class $i$ within individuals. $K$b is the diversity within an individual, $K$c within a population and $K$d is a measure for the total diversity within a species. For each species, diversity measures were calculated at three hierarchical levels: (i) within individuals (Ci = average$K$b/$K$d), (ii) among individuals within a population [Cip = (average$K$c − average$K$b)/$K$d] and (iii) among populations within a species [Cps = ($K$d-average$K$c)/$K$d]

(Rand and Harrison, 1989). By definition, Ci + Cip + Cps = 1.0 (Arnason and Rand, 1992). To test the differences in allele frequency distributions among populations the *G*-test was employed (Sokal and Rohlf, 1981).

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 (GD based on AFLP and VNTR markers) and one fixed parameter (the populations), we investigated a possible correlation between the AFLP and TR data by calculating the 'product–moment correlation coefficient' (PMCC). The PMCC between the intraspecies GD values of AFLP and TRs was computed by PMCC $= \Sigma y_1 y_2 / \sqrt{\Sigma y_1^2 \Sigma y_2^2}$ (Sokal and Rohlf, 1981) using the SAS/STAT software package (SAS Institute, Inc., 1987).

## Results

### *AFLP*

The presence/absence of 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 (e.g. Figure 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 compared to proportions in *M. javanica* (52%) and *M. arenaria* (61%). There were 168, 64 and 69 species-specific bands for *M. incognita*, *M. javanica* and *M. arenaria*, respectively.

Based on GD (Table 3) and UPGMA analysis, the 16 *Meloidogyne* populations were grouped into distinct clusters, which corresponded with their species identities. It is clearly shown that *M. javanica* and *M. arenaria* are more related to each other than to *M. incognita*. 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

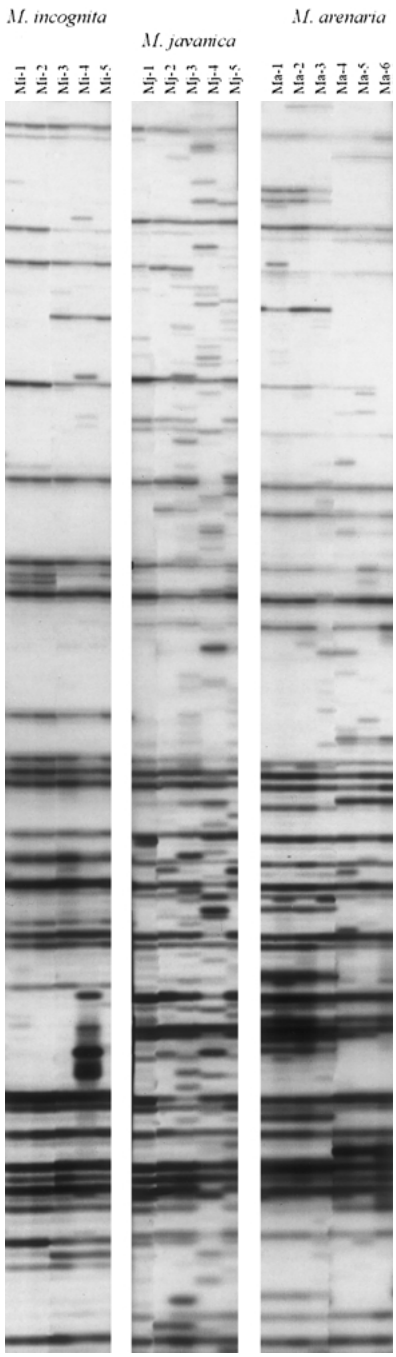


*Figure 1.* AFLP fingerprints generated with primer combination E + GA/M + AC. Subsequent lanes contain amplification products from 16 *Meloidogyne* populations, five *M. incognita*, five *M. javanica* and six *M. arenaria*. Population codes are given in Table 1.

*Table 3*. Genetic distance matrix of 16 *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 | — |

and 0.40, respectively. The branches in the similarity dendrogram were evaluated by 1,000 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 2). The bootstrap support within *M. incognita* and *M. arenaria* was higher than within *M. javanica.* These findings were supported by principle coordinate analyses (Figure 3).

*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

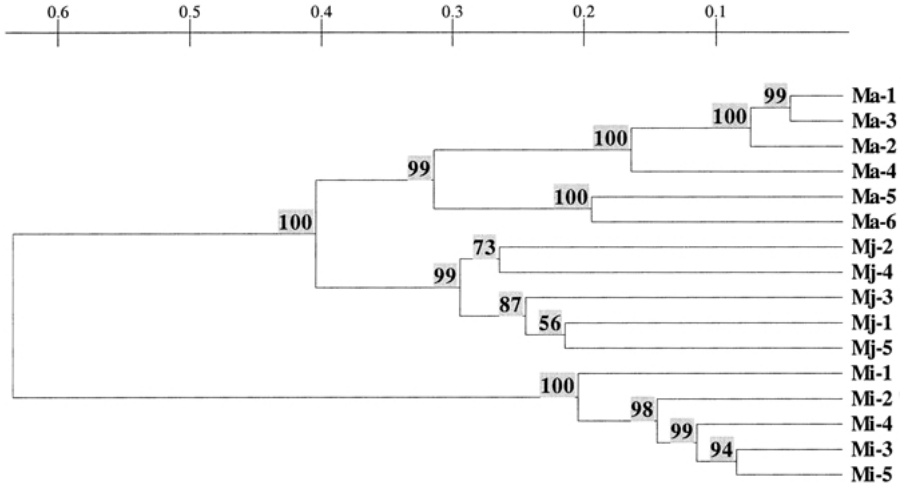


*Figure 2*. Dendrogram of 16 *Meloidogyne* populations belonging to *M. incognita*, *M. javanica* and *M. arenaria*, based on AFLP data. Population codes are given in Table 1.

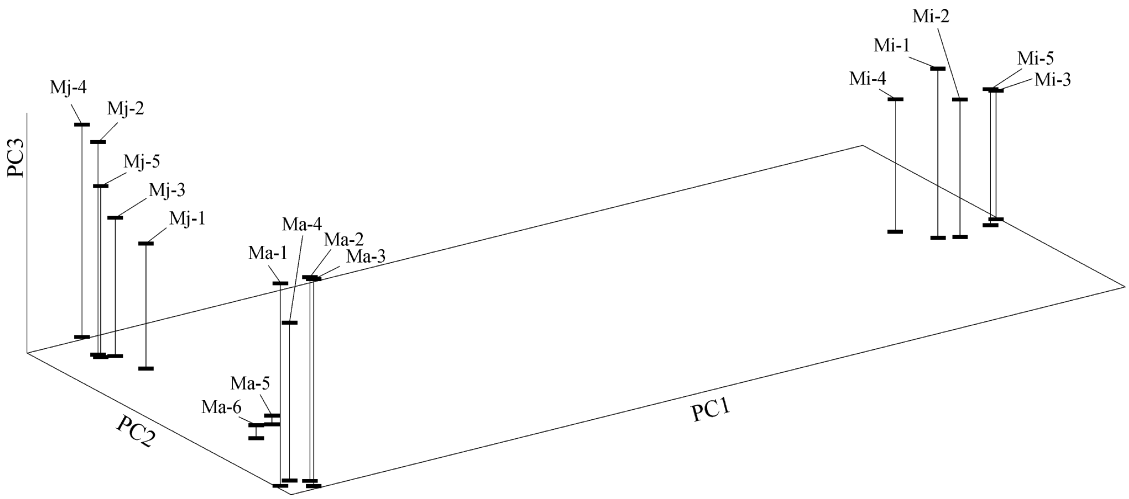


*Figure 3*. Relationships among 16 *Meloidogyne* populations belonging to *M. incognita, M. javanica* and *M. arenaria* obtained by principle coordinate analysis using AFLP data. The three principal coordinate accounted for 58.5% of the total variation. Population codes are given in Table 1.

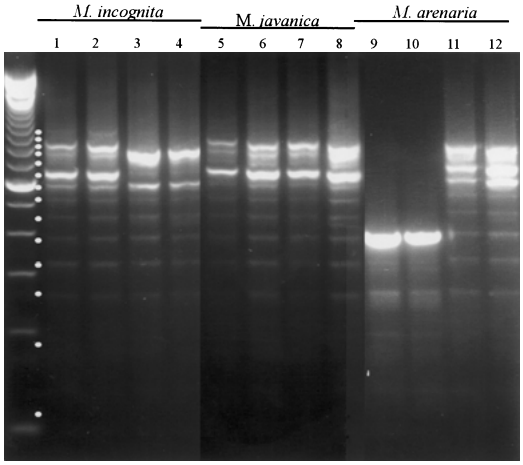


*Figure 4*. 63-bp TR profiles generated from *M. incognita*, *M. javanica* and *M. arenaria* individuals. Each lane represents single second stage juvenile. Lane 1 and 2 individuals from Mi-1, Lane 3 and 4 individuals from Mi-2; Lane 5 and 6 individuals from Mj-1; Lane 7 and 8 individuals from Mj-2; Lane 9 and 10 individuals from Ma-1; Lane 11 and 12 individuals from Ma-2. The positions of the 2–15 63-bp repeating units are indicated by white dots. The 100-bp marker is on the left. Population codes are given in Table 1.

intensities reflected the copy number of the mtDNA size variants. Similar banding patterns were obtained from DNA prepared from individual J2s, indicating that heteroplasmic nematodes containing multiple mtDNA size variants contributed to the genetic structure of the nematode populations (Figure 4).

In this study, the various types of TRs and their frequencies were studied in 10–50 J2s per population. All populations contained substantial numbers of heteroplasmic individuals ranging from 17% to 100% (Table 4). *M. incognita* had on average the highest number of heteroplasmic individuals. The observed number of 63-bp repeats varied from 7 to 20. In general heteroplasmic nematodes have a higher number of repeat 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 6). The Ci values range from 65% for *M. incognita* to 43% and 48% for *M. javanica* and *M. arenaria*, respectively (Table 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 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).

### *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). The number of polymorphic TRs between the *M. incognita* populations was 20%, whereas this figure was 40% for *M. javanica* and 50% for *M. arenaria*. 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 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 'PMCC' 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 5).

*Table 4*. Hierarchical analysis of 63-bp repeating units of mtDNA in five *M. incognita*, five *M. javanica* and six *M. arenaria* populations

| *Meloidogyne* species | Population | $n^1$ | $F(h)^2$ | Kb range | | Kb[3] | Kc[4] | Kd[5] | Ci[6] | Cip[7] | Cps[8] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Min | Max | | | | | | |
| *M. incognita* | Mi-1 | 50 | 20 | 0.00 | 0.65 | 0.52 | 0.59 | | | | |
| | Mi-2 | 50 | 34 | 0.00 | 0.54 | 0.24 | 0.31 | | | | |
| | Mi-3 | 12 | 8 | 0.00 | 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 | 0.00 | 0.50 | 0.17 | 0.29 | | | | |
| | Mj-2 | 12 | 67 | 0.00 | 0.73 | 0.17 | 0.22 | | | | |
| | Mj-3 | 12 | 0 | 0.18 | 0.72 | 0.45 | 0.72 | | | | |
| | Mj-4 | 10 | 10 | 0.00 | 0.58 | 0.43 | 0.73 | | | | |
| | Mj-5 | 14 | 36 | 0.00 | 0.73 | 0.28 | 0.68 | | | | |
| | | | | | | 0.30 | 0.53 | 0.69 | 0.43 | 0.33 | 0.24 |
| *M.arenaria* | Ma-1 | 16 | 50 | 0.00 | 0.63 | 0.27 | 0.34 | | | | |
| | Ma-2 | 10 | 50 | 0.00 | 0.50 | 0.17 | 0.24 | | | | |
| | Ma-3 | 12 | 83 | 0.00 | 0.32 | 0.05 | 0.06 | | | | |
| | Ma-4 | 16 | 50 | 0.00 | 0.66 | 0.25 | 0.35 | | | | |
| | Ma-5 | 10 | 0 | 0.35 | 0.72 | 0.55 | 0.78 | | | | |
| | Ma-6 | 12 | 17 | 0.00 | 0.77 | 0.52 | 0.68 | | | | |
| | | | | | | 0.30 | 0.41 | 0.62 | 0.48 | 0.17 | 0.35 |

[1]number of individuals sampled; [2]frequency of homoplasmic individuals; [3]mtDNA diversity within individual; [4]mtDNA diversity within population; [5]mtDNA diversity within a species; [6]% total genetic diversity within individual; [7]% total genetic diversity among individuals within populations; [8]% total genetic diversity among population within species (lineages).

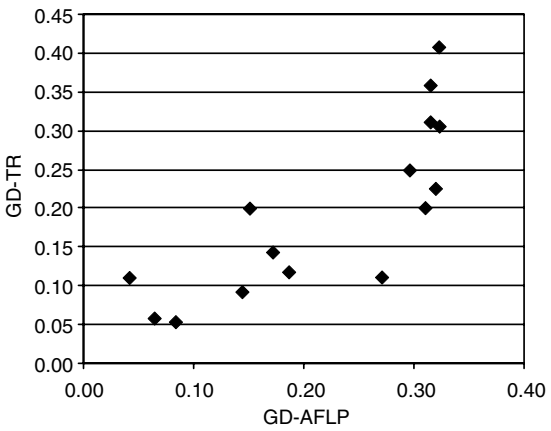


*Figure 5*. Comparison of GD based on AFLP and TR data from six *M. arenaria* populations.

## 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, these populations were also analysed with the AFLP technique. To our knowledge, this is the first study in which both variations in nuclear and mitochondrial DNA have been investigated in plant-parasitic nematode populations.

AFLP fingerprints of 16 *Meloidogyne* populations, representing the three major mitotic parthenogenetic *Meloiodygne* species, displayed common and differential (e.g. species specific) bands within and between species. The three *Meloidogyne* species were consistently grouped in three separate clusters. 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*, which is in agreement with the AFLP analysis of Semblat et al. (1998) and Van der Beek et al. (1998). Baum et al. (1994) and Tastet et al. (2000) found a similar genetic relationship based on RAPDs and protein analysis. However, evidence from mtDNA (Xue et al., 1992; Hugall et al., 1994; Guirao et al., 1995) suggests that *M. arenaria* does not have a closer relationship to *M. javanica* than it does to *M. incognita*. Others have placed *M. arenaria* as the most distinct species (Powers and Sandall, 1988). This

latter finding is also supported by the observation that *M. arenaria* is the only species that exists in triploid form with a somatic chromosome number larger than 50 (Lapp and Trintaphyllou, 1972).

There was no correlation between the AFLP data and the geographical origins of the populations (Figure 2). Several studies (Blok et al., 1997; Semblat et al., 1998; Tzortzakakis et al., 1999) have lead to similar conclusions, while others (Baum et al., 1994; Tastet et al., 2000) 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; 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 (Figure 2).

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 (Hyman, 1988), and (ii) occurrence of polymorphic forms. Differences in the geographic distribution of mtDNA haplotypes have been used to estimate genetic differentiation within and among populations of various organisms (Avise, 1994). Intra- and interindividual variation in repeat numbers in mtDNA has been found in *M. incognita* (Whipple et al., 1998) and *Romanomermis* spp. (Azevedo and Hyman, 1992; Hyman and Slater, 1990). 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). A similar result has been obtained in a previous study on *M. incognita* (Whipple et al., 1998). The results for root-knot nematodes 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 (Arnason and Rand, 1992). Similar high heteroplasmy levels have also been reported for species of crickets (Rand and Harrison, 1986; 1989), frogs (Monnerot et al., 1984) and *Drosophila* (Hale and Singh, 1986). As compared to *M. incognita* and *M. javanica*, *M. arenaria* shows strongest differentiation among populations with the lowest genetic diversity within populations (Figure 6).

Mutation to different 63-bp repeat copies is the primary mechanism by which size variation is

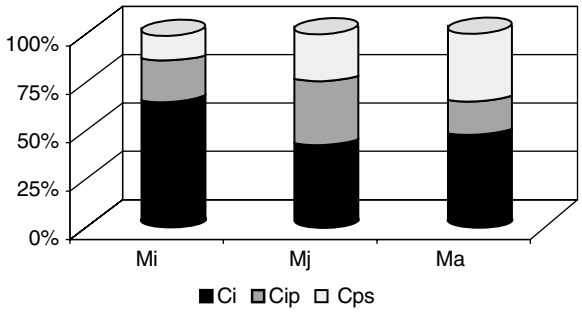


*Figure 6.* Genetic diversity based on 63-bp TR on different hierarchical levels. Cps – % genetic diversity among population within species, Cip – % genetic diversity among individuals within populations and Ci – % genetic diversity within individuals.

generated in obligatory mitotic parthenogenetic root-knot nematodes, because a paternal contribution is excluded (Whipple et al., 1998). The diversity of the banding patterns produced by amplifying the 63-bp TRs, 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 (Lunt and Hyman, 1997; Lunt et al., 1998).

Both AFLP and TR data show that the variation among populations is the lowest in *M. incognita* and the highest in *M. arenaria*. Also the comparison of the GD among *M. arenaria* populations based on AFLP and binary coded TR data revealed a significant positive correlation (see Figure 5). A similarity dendrogram constructed from the binary coded TR data showed that all *M. incognita* populations were more related to each other than to *M. arenaria* or *M. incognita*. However, the bootstrap values are low, and the six *M. arenaria* and six *M. javanica* populations are not clustered in separate groups and were completely intermingled (data not shown). These data support the view that care should be taken to use variations in TRs as robust measures for genetic relationships, because of the high levels of homoplasy (Lunt et al., 1998). On the other hand, it is also shown here that there are in several cases significant correlations between both data sets, indicating that in specific situations the advantages of TRs may be exploited to study genetic structures of populations. The observation that even among closely related root-knot nematodes distinct patterns of variation in TRs

are found, suggests that more plant-parasitic nematode species have to be studied to obtain a definitive view about the possibilities of this sensitive technique in plant nematology.

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* (Folkertsma et al., 1996) which 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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