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An RFLP study of relationships between species, populations and resistance-breaking lines of tropical species of *Meloidogyne*

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Summary – To determine the identity of resistance-breaking *Meloidogyne* spp. showing the esterase phenotype pVI, six pVI lines were compared by RFLP with ten lines of *M. incognita,* seven of *M. arenaria,* four of *M. javanica,* one of *M. hapla* and one of *M. mayaguensis.* A total of three groups were identified in these analyses. Two groups, represented by *M. incognita* and *M. mayaguensis,* displayed low level of intraspecific variation, but were clearly distinct from each other. The second group included the lines exhibiting the pVI esterase phenotype. A third group was more variable; it consisted of the *M. arenaria* and *M. javanica* lines. From the comparison of *Meloidogyne* spp., the resistance-breaking lines, with the pVI esterase phenotype, were shown to belong to the species *M. mayaguensis,* and they were not lines selected from other existing tropical *Meloidogyne* spp. following the use of resistant cultivars.

Résumé – ***Étude par le polymorphisme en longueur des fragments de restriction (RFLP) des relations entre espèces, populations et lignées brisant la résistance appartenant aux* Meloidogyne *tropicaux*** – Afin d'établir l'identité de certaines espèces de *Meloidogyne,* caractérisées par le phénotype estérasique pVI et pouvant se développer sur certains cultivars résistants, six de ces lignées sont comparées par RFLP à dix lignées de *M. incognita,* sept de *M. arenaria,* quatre de *M. javanica,* une de *M. hapla* et une de *M. mayaguensis.* Deux groupes très homogènes sont identifiés : le premier comprend les lignées de *M. incognita,* le deuxième les lignées pVI et la lignée de *M. mayaguensis;* un troisième, plus variable, regroupe les lignées appartenant à *M. arenaria* et à *M. javanica.* Les lignées se développant sur cultivars résistants et dotées du phénotype pVI apparaissent comme une espèce distincte, *M. mayaguensis,* et non comme la conséquence de la sélection, par l'usage de cultivars résistants, de variants parmi les espèces tropicales.

Key words : *Meloidogyne, Meloidogyne mayaguensis,* RFLP, similarities.

Meloidogyne spp. are major crop pests in tropical areas. Although they have wide host ranges, a number of resistant cultivars and non-host crops are used in their integrated control. However, resistance-breaking populations have been identified (Riggs & Winstead, 1959; Triantaphyllou & Sasser, 1960; Netscher, 1976; Bost & Triantaphyllou, 1982), and in West Africa, populations able to overcome the resistance of tomato cv. Rossol, sweet potato cv. CDH and soybean cv. Forrest are widely distributed (Prot, 1984; Fargette & Braaksma, 1990). It is important to assess the relationship of such populations to the avirulent ones and to determine if they represent a distinct and homogenous biological group or are derived from existing species as a result of short-term selection following the use of resistant cultivars. Effective implementation of quarantine measures and integrated control depend, in part, upon the way these resistance-breaking populations arise.

Initial studies on six resistance-breaking lines of *Meloidogyne* originating from the Ivory Coast showed they all had the same, distinct esterase phenotype (pVI; Fargette & Braaksma, 1990). More detailed studies on host ranges, karyotypes, lectin labelling of amphidial exudates and cuticle surface of juveniles, *Pasteuria penetrans* attachment tests and specific monoclonal antibodies (Fargette & Braaksma, 1990; Fargette *et al.,* 1994) were aimed at relating these populations to the well identified tropical species *M. incognita, M. arenaria* and *M. javanica.* Some relationships with *M. incognita* and *M. arenaria* were established but differences were also observed. These pVI lines were most similar to *M. mayaguensis,* a species described from Puerto Rico (Rammah & Hirschmann, 1988). However, the work previously mentioned (Fargette & Braaksma, 1990; Fargette *et al.,* 1994) revealed variation within the species studied. A broader investigation of *Meloidogyne* including the

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lines expressing the pVI esterase phenotype was undertaken to determine the relationships between and within the tropical species of *Meloidogyne*. This study characterizes five species of *Meloidogyne (M. incognita, M. arenaria, M. javanica, M. hapla, M. mayaguensis)* together with the pVI lines using RFLP analysis.

Materials and methods

The nematodes lines

Meloidogyne *single female lines*

Single female lines, each taken from a different field population, were studied. Each line was obtained from a natural population by inoculating the egg-mass from a single female onto a young susceptible tomato plant (cv. Moneymaker). Prior to establishing the clonal lines, the esterase phenotype of the " mother " was obtained to confirm the species, following the procedures of Fargette (1987 *a, b*).

A total of 29 lines were studied. These included six pVI lines from West Africa (Ivory Coast and Burkina Faso), ten lines of *M. incognita,* seven lines of *M. arenaria,* four lines of *M. javanica,* one line of *M. mayaguensis,* and one line of *M. hapla.* Their origins are shown in Table 1. Several of these lines (1, 2, 3, 5, 7, 9, 10, 11, 12, 13, 15) have been used in related studies (Fargette *et al.,* 1994). The population of *M. mayaguensis* from which line 13 was raised is the type population which was used for the description of the species (Rammah & Hirschmann, 1988) and it is the only population assigned with certainty to this species. *M. hapla* was used as a clearly distinct and contrasting species.

Greenhouse cultures

The lines were maintained on susceptible tomato cv. Moneymaker in pots containing *c.* 1000 g of soil, in greenhouses where the temperature ranged between 25 °C and 27 °C. To avoid cross-contamination, pots (two to ten per line, depending on the numbers of nematodes required) were kept on slatted benches and separated from each other with screens.

Extraction and collection of juveniles

Six to 7 weeks after inoculation, juveniles were mass extracted from the roots by washing the tomato plant roots free of soil and placing them in a mist unit at 25-27 °C. The juveniles were collected twice a week and the level of saprophagous nematodes in samples was checked. Most of the samples were free of them and saprophagous nematodes never exceeded 3 % in any of the samples used. Juveniles were concentrated by centrifugation and excess water removed. Samples were quickly frozen in liquid nitrogen before storing them at – 80 °C.

Table 1. *Specific identification and geographical origin of the 29* Meloidogyne *lines.*

M. javanica	22	Burkina Faso, Sahelian climate
	23	Burkina Faso, Sudanese climate
	24	Spain
	25	Portugal
M. arenaria	10	Ivory Coast
	26	Portugal
	28	French West Indies
	29	French West Indies (Ste Anne)
	31	French West Indies
	32	French West Indies
	34	French West Indies
pVI lines	30	Burkina Faso
	1	Ivory Coast
	2	Ivory Coast
	3	Ivory Coast
	5	Ivory Coast
	7	Ivory Coast
M. mayaguensis	13	Puerto Rico
M. incognita	11	Louisiana, race 3
	12	North Carolina, race 4
	15	Thailand, race 1
	9	Ivory Coast
	17	Burkina Faso, Sudanese climate
	20	French Guiana
	16	Senegal
	18	Chad
	19	French West Indies (Martinique)
	27	North Carolina
M. hapla	33	The Netherlands

RFLP's

DNA extraction and calibration of samples

Juveniles collected from several plants were thoroughly ground in liquid nitrogen until a fine powder was obtained; " urea buffer " (7M urea, 0.35M NaCl, 2 % SDS, 10mM EDTA, 0.1M Tris buffer, pH 7.5) was added and the mixture left at room temperature on a shaker for 15 min. The nucleic acids were extracted twice with an equal volume of phenol – chloroform, followed by one chloroform extraction. The nucleic acids were precipitated by adding two and a half volumes of absolute ethanol followed by centrifugation and were then resolubilised in TE (10mM Tris, 1mM EDTA, pH 8).

Aliquots of each sample were subjected to electrophoresis on 1 % agarose TBE gel (Sambrook *et al.,* 1989) in order to check that the DNA was not excessively sheared and to assess the amount of DNA. The quantity of DNA was visually estimated following ethidium bromide staining and samples equilibrated to ensure similar amounts of DNA in each sample.

Digestion

A 200 ng subsample of DNA from each line was RNAsed and simultaneously digested with the restriction endonuclease; five endonucleases were used : BamH I, EcoR I, EcoR V, Hind III, and Bgl II (Pharmacia & Northumbria Biologicals Ltd). The DNA aliquots were digested by adding 1 µl boiled RNAse A (0.67 µg/ml) and 30 units of endonuclease, adjusting the final volume to 30 µl, and leaving for 4 h at 37 °C.

Electrophoresis, Southern blotting and probing

The entire digestion product (30 µl) was loaded onto a 1 % agarose TBE gel and electrophoresis performed at 30 to 40 volts until the migration front had reached 15 cm. The gel was stained with ethidium bromide and the DNA transferred with 20x SSC (Sambrook *et al.,* 1989) onto a Hybond N+ membrane (Amersham) following the manufacturer's instructions. The membranes were annealed with ^{32} P dCTP labelled probes from a *Meloidogyne* genomic library (Random primed DNA labelling kit; Boehringer Mannheim) and washed at high level of stringency : 0.1 × SSC, 0.1 % SDS for the last wash, 65 °C. Exposure of blots to X-ray film took place at – 80 °C for 7 to 10 days.

The probes : construction of a genomic library and production of genomic probes

Line 5 (pVI) was chosen for the construction of a genomic library (nucleic acid extraction described above). About 500 ng DNA were digested with 80 units of BamH I endonuclease and ligated into 50 ng pBlue-Script vector (Stratagene) which had previously been digested with BamH 1 and treated with alkaline phosphatase (Boehringer). Competent *Escherichia coli,* strain DH5 α [BRL] were transformed according to manufacturer's instructions. Plasmid DNA was prepared from selected white colonies (Sambrook *et al.,* 1989) and digested with BamH I endonuclease. Inserts were purified (Prep A Gene kit; Biorad) and their sizes determined following electrophoresis. Four inserts were selected for use as probes for the Southern blots because of the distinct patterns they produced : inserts Melo 1, Melo 2, Melo 19, and Melo 63. Melo 1, 2, and 19 are relatively large inserts (*c.* 3000, 8000, and 4000 base pairs, respectively) while Melo 63 is smaller (1000 base pairs).

DATA ANALYSIS

Twenty different autoradiographs were obtained following the probing of each of the five blots (one blot for each endonuclease) with four probes. The bands produced on the autoradiographs were scored in two ways :

– on a scale from 0 to 4, referring to intensity of bands,

– or on the presence (+)/absence (–); scores 0 and 1 on the previous scale were scored (–) whereas scores 2, 3, 4 were scored (+).

These data were analyzed using the statistical package Genstat 5 (Payne *et al.,* 1987) to produce similarity matrices.

In addition the data were also examined using parsimony with the PAUP programme developed by D. L. Swofford, Illinois Natural History Survey, Champaign, Illinois. They were analyzed as unordered data using the " Hold = 10 Swap = Global Mulpars " functions.

Results

AUTORADIOGRAPHS

Probes Melo 2 and Melo 19 labelled only a limited number of bands; probes Melo 1 and Melo 63 produced more complicated patterns. From differences in numbers and position of bands produced by all four probes it was possible to distinguish the species (Fig. 1A – B, 2A – B).

Polymorphism within species was also observed. Within *M. arenaria* three groups could be identified, the first one comprising lines 31, 32, and 34 (9th, 10th and 11th tracks) and another, lines 10, 27 and 28 (5th, 7th and 8th tracks respectively); line 26 (6th track) showed differences from the others. For example, following restriction with BamH1 and probing with Melo 2 (Fig. 1A) *M. arenaria* line 26 (6th track) lacks one band present in the lines 10, 28, 29 (respectively tracks 5, 7, 8). Also, the first band is larger in line 26, suggesting that in line 26 one restriction site may have been lost as the larger piece of DNA corresponds, in molecular weight, to the addition of the first and last bands of other *M. arenaria* lines. In some instances, where patterns consisted of numerous bands interpretation was more difficult.

The *M. incognita* group of populations, in contrast to *M. arenaria,* showed very little variation. Figure 2B (Hind III/Melo 2) shows one instance of polymorphism : line 20 (22th track) shows one extra band compared to the other lines of *M. incognita.*

The *M. mayaguensis* group was also very homogenous; however, slight variation was observed which distinguished between lines originating from West Africa (tracks 12 to 17) and that from central America (track 18) : for instance in Figs. 1A, B, 2B an extra band can be observed only on track 18.

SIMILARITY ANALYSIS AND DENDROGRAM

A total of 383 distinguishable bands were identified from the 20 autoradiographs (combination of five endonucleases and four probes). These were scored as described above.

The analysis produced similar results for both scoring systems; although the scoring 0 – 4 system produced slightly more discrimination between closely related lines, it did not alter the groupings of lines in the dendrogram produced using a +/–approach.

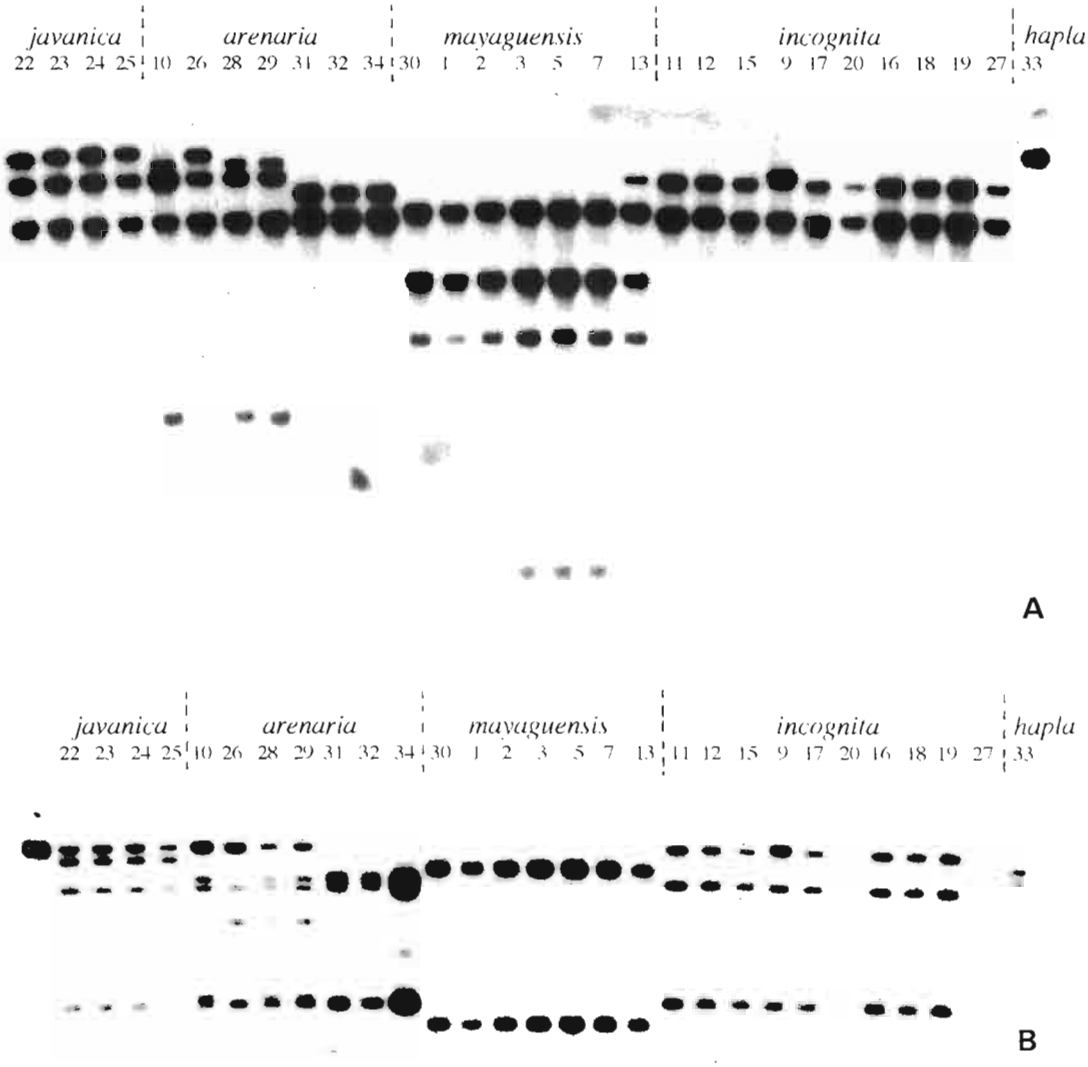

Figure 1. *RFLP's of DNA from 29 lines of* Meloidogyne. *A : DNA was digested with BamH I endonuclease and subsequent blot probed with probe " Melo. 2 ". From left to right :* M. javanica *(lines 22, 23, 24, 25),* M. arenaria *(lines 10, 26, 28, 29, 31, 32, 34),* M. mayaguensis *(lines 30, 1, 2, 3, 5, 7, 13),* M. incognita *(lines 11, 12, 15, 9, 17, 20, 16, 18, 19, 27),* M. hapla *(line 33); B : DNA was digested with EcoR I endonuclease and subsequent blot probed with probe " Melo. 2 ". From left to right : Lambda Marker,* M. javanica *(lines 22, 23, 24, 25),* M. arenaria *(lines 10, 26, 28, 29, 31, 32, 34),* M. mayaguensis *(lines 30, 1, 2, 3, 5, 7, 13),* M. incognita *(lines 11, 12, 15, 9, 17, 20, 16, 18, 19, 27),* M. hapla *(line 33).*

Similarities between lines and species were calculated and the similarity matrix (Table 2) shows that each set of lines identified by their esterase phenotypes as *M. incognita, M. javanica* and pVI / *M. mayaguensis* produced very homogenous groups with average within group similarities of more than 98.5 %. Also each group is clearly distinct from the others with, at most, only 30 to 44 % similarity between groups. The pVI lines and the *M. mayaguensis* from Central America comprised a distinct and very homogenous group.

The *M. arenaria* lines formed a more variable group with an average within group similarity of 84 %. Although the *M. arenaria* group was quite distinct from *M. mayaguensis* and *M. incognita* (35 to 44 % similarity) it was relatively similar to *M. javanica* (73 % similarity). A more detailed examination of variation within *M. arenaria* showed that the populations can be placed in three subgroups (Table 3) : sub-group M.a.1 comprised lines 10, 27 and 28; M.a.2 comprised line 26 and M.a.3 comprised lines 31, 32 and 34. Subgroups M.a.1 and

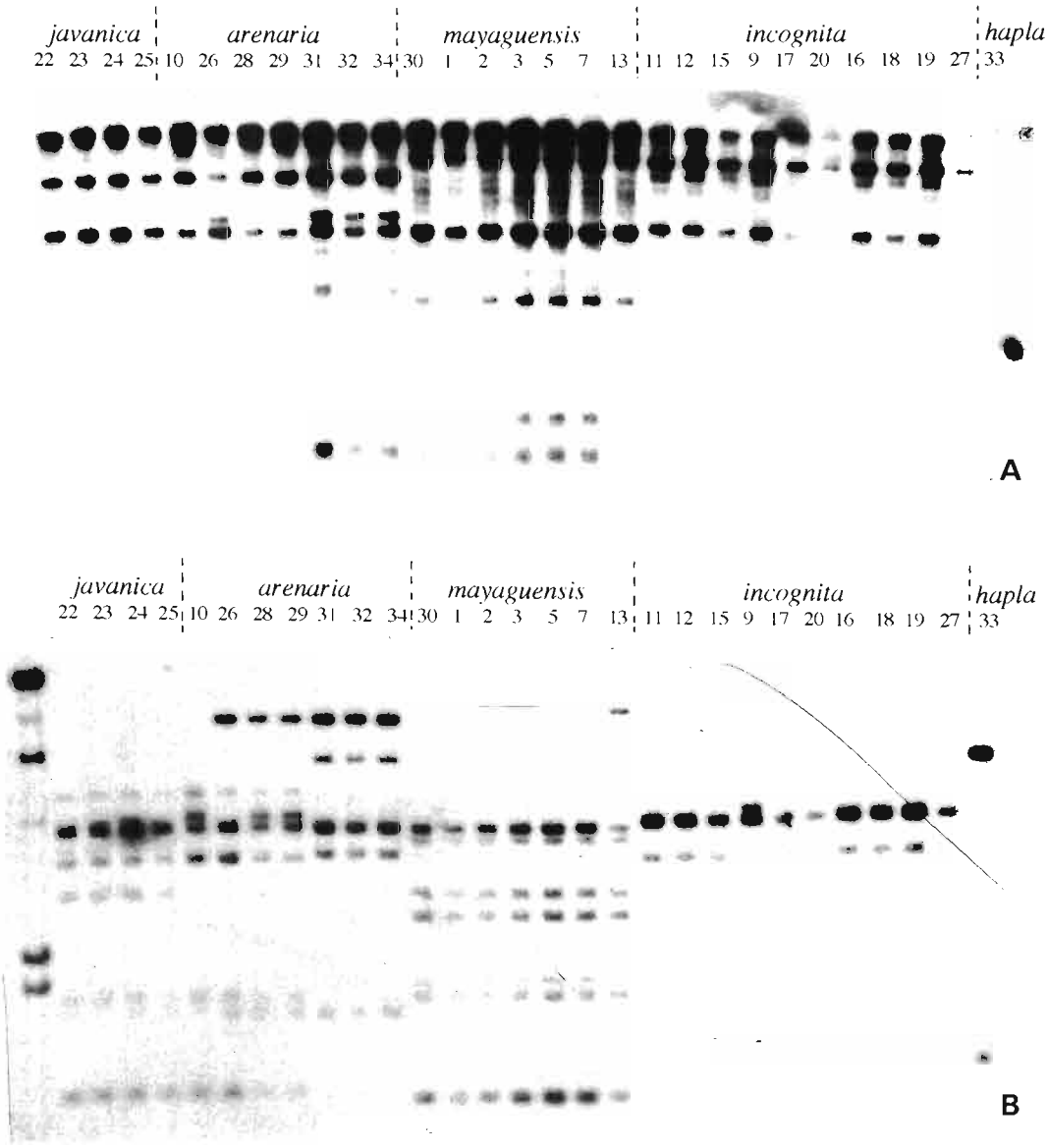

Figure 2. *RFLP's of DNA from 29 lines of* Meloidogyne. *A : DNA was digested with BamH I endonuclease and subsequent blot probed with probe " Melo. 19 ". From left to right :* M. javanica *(lines 22, 23, 24, 25),* M. arenaria *(lines 10, 26, 28, 29, 31, 32, 34),* M. mayaguensis *(lines 30, 1, 2, 3, 5, 7, 13),* M. incognita *(lines 11, 12, 15, 9, 17, 20, 16, 18, 19, 27),* M. hapla *(line 33); B : DNA was digested with Hind III endonuclease and subsequent blot probed with probe " Melo. 2 ". From left to right : Lambda Marker,* M. javanica *(lines 22, 23, 24, 25),* M. arenaria *(lines 10, 26, 28, 29, 31, 32, 34),* M. mayaguensis *(lines 30, 1, 2, 3, 5, 7, 13),* M. incognita *(lines 11, 12, 15, 9, 17, 20, 16, 18, 19, 27),* M. hapla *(line 33).*

M.a.2 were 88.7 % similar and more similar to the *M. javanica* (82.3 and 78.8 % similarity, respectively) than to M.a.3 (76.7 % and 76.8 % similarity). Although more distant, group M.a.3 still shares almost 70 % of similarity with *M. javanica.*

M. hapla is quite separate from the tropical species (less than 15 % similarity).

The data were analyzed by parsimony and the shortest or most parsimonious trees had lengths of 487 and a consistency of 0.7. Ten different trees with the same length were examined and all were found to have similar topologies. There were only slight differences in the arrangement of lines within species and one tree which is representative is shown in Fig. 3; it shows the clear spe-

Table 2. *Between and within species similarity matrix; M.j.* = M. javanica, *M.a.* = M. arenaria, *M.m.* = M. mayaguensis, *M.i.* = M. incognita, *M.h.* = M. hapla.

	M.j.	M.a.	M.m.	M.i.	M.h.
M.j.	99.7				
M.a.	73.5	83.9			
M.m.	35.7	35.2	98.7		
M.i.	43.3	44.4	30.9	99.0	
M.h.	6.3	9.6	11.0	14.5	100

Table 3. *Similarities between and within groups and sub-groups (*M. arenaria *sub-group M.a. 1 = lines 10, 27 and 28;* M. arenaria *sub-group M.a. 2 = line 26;* M. arenaria *sub-group M.a. 3 = lines 31, 32 and 34; M. javanica group = M.j.;* M. incognita *group = M.i.;* M. mayaguensis *group = M.m.;* M. hapla *group = M.h.).*

	M.j.	M.a. 1	M.a. 2	M.a. 3	M.m.	M.i.	M.h.
M.j.	99.8						
M.a. 1	82.3	99.9					
M.a. 2	78.8	88.7	100				
M.a. 3	66.9	76.7	76.8	100			
M.m.	34.8	35.8	36.4	35.0	98.8		
M.i.	44.5	44.8	44.6	45.7	32.2	99.2	
M.h.	9.4	9.9	9.2	10.0	9.8	14.7	100

cies groupings and also visualizes this relationship between the three sub-groups of *M. arenaria* and the *M. javanica* group.

Discussion

Under stringent hybridization, four probes revealed consistent differences between *Meloidogyne* lines in their binding patterns, reflecting differences in restriction sites, in DNA sequences and / or genomic organisation. Conversely, highly consistent binding patterns, such as observed within the pVI lines and *M. mayaguensis* indicate a high degree of similarity. The results therefore confirm the isozyme evidence (Fargette *et al.*, 1994) that the pVI lines are derived from populations of *M. mayaguensis.*

The *M. incognita* lines formed a second, highly homogenous group, even though the populations originated from different parts of the world. Other studies have detected greater variability in *M. incognita* (Curran *et al.*, 1986; Castagnone-Sereno *et al.*, 1991, 1993; Piotte *et al.*, 1992) possibly because their probes were made from a more variable part of the genome such as repetitive sequences. Similarly geographically diverse *M. javanica* lines formed a homogenous group.

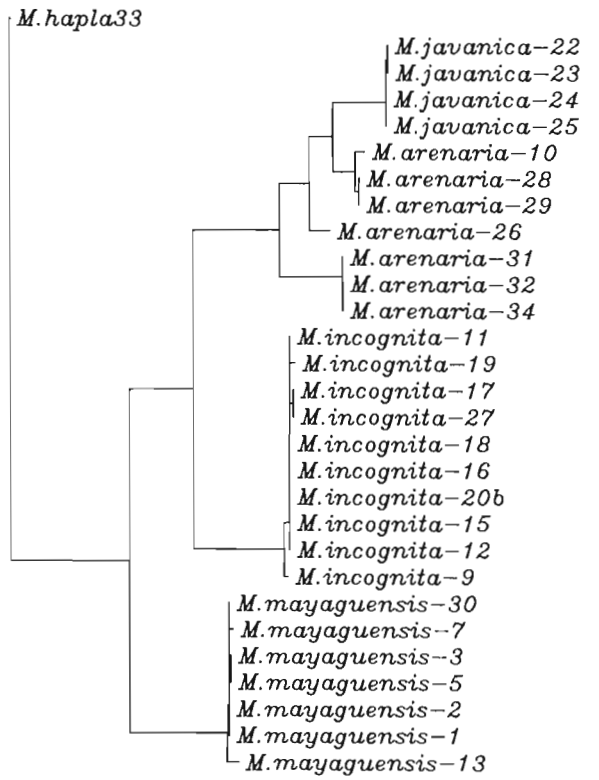

Figure 3. *Dendrogram produced out of similarities between* Meloidogyne *lines and showing the variability and relationships between and within species.*

The *M. arenaria* lines were more variable with three sub-groups apparent. However, there was no apparent geographical basis for these groupings, supporting the observations of Carpenter *et al.* (1992) who observe differences in RFLP patterns between populations of *M. arenaria* all originating from North Carolina. Lines 28 and 29 from the West Indies were more similar to line 10 from the Ivory Coast than to lines 31, 32 and 34 which originated from the West Indies. Also line 26 from Portugal was only slightly different from the group formed from lines 10, 28 and 29.

The similarity analysis (Table 3) indicated that there were smaller differences between *M. javanica* and some of the *M. arenaria* subgroups than there were between the most different *M. arenaria* groups. These observations are in agreement with those of Garate *et al.* (1991) and Xue *et al.* (1992) and indicate that these two species could be regarded as belonging to the same, rather variable group. Analysis of mitochondrial DNA has produced controversial understanding of the relationships between *M. arenaria* and *M. javanica.* Powers and Sandall (1988) and Hyman and Powers (1991) showed that mitochondrial DNA of *M. arenaria* was distinct from that of *M. javanica;* however another study (Hugall *et*

al., 1994) concluded that these species were more similar in their mitochondrial DNA than previously stated and gave an explanation for the discrepancy first observed. Agreement between nuclear and mitochondrial information is confirmed in this study.

The hybridization patterns, especially those produced by the BamH I and EcoR I endonucleases in combination with probes Melo 2 and Melo 19, were particularly useful and could provide diagnostic tools. Probes Melo 1 and Melo 63 produced more complicated patterns than probes Melo 2 and Melo 19 but the reason for these differences is not known. Each probe / enzyme combination samples only a very small fraction of the total genome and therefore the results need interpreting with caution. Even so, where different asessments such as isozyme analysis indicate similar conclusions, i.e. that there is a greater degree of variability in *M. arenaria* than in *M. incognita* or *M. javanica*, confidence in these conclusions is increased.

The work presented here has been carried out on single female lines. Tropical *Meloidogyne* species reproduce clonally by mitotic parthenogenesis (Triantaphyllou, 1985; Rammah & Hirschmann, 1988). Hence each clone is a distinct biological and genetic entity and field populations may consist of a single clone or mixtures of clones of the same or more than one species. These lines were used in this study and in work previously described by Fargette and Braaksma (1990) and Fargette *et al.* (1994) in preference to the original populations to allow genotypic and species pure comparisons to be made. However the lines were obtained from populations of diverse geographical origins with the assumption that the within population variation would be less than the between populations. The results obtained here for *M. incognita, M. javanica* and *M. mayaguensis* are striking in that they reflect high levels of homogeneity within the species, irrespective of their geographical origin. The greater variability observed within *M. arenaria* species is of interest and further study at a local scale using populations collected from the same vicinity would be valuable.

Perhaps the most important conclusion is that *M. mayaguensis* is present in both continents of Africa and America. Another species, *M. enterolobii* (Yang & Eisenback, 1983) from China has been described as having the same esterase phenotype as *M. mayaguensis*. However it is not known whether their DNA are closely related. The virulence displayed by *M. mayaguensis* against several sources of resistance to *M. incognita* (Fargette & Braaksma, 1990) makes it a potential threat against which quarantine measures may be appropriate.

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