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Genetic variation in tropical *Meloidogyne* spp. as shown by RAPDs

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Summary – Random Amplified Polymorphic DNA (RAPD) fingerprinting was used to assess within and between species variability in tropical *Meloidogyne* spp. Reproducibility of the technique was assessed. *M. arenaria* showed the most within species variability; species were distinguished with only a few primers though some primers group some of the *M. arenaria* lines more closely with *M. javanica* than with other *M. arenaris* lines. Three virulent lines of *M. arenaria* were differentiated from both avirulent of this species and the other lines of *M. javanica, M. incognita, M. mayaguensis* and *M. hapla*. Primers contributed more to variability in the data than genotypes. Comparison of RAPD with RFLP (restriction fragment length polymorphism) data showed similar levels of interspecific difference between species variation but greater within species variation with the RAPDs.

Summary – ***Variabilité chez les* Meloidogyne *tropicaux telle que le montre une étude par RAPD*** – La technique des RAPD a été utilisée pour évaluer la variabilité intra- et inter- spécifique chez des lignées de *Meloidogyne* tropicaux. La reproductibilité de la technique a été évaluée; pour chaque couple « amorce - ADN testé », l'ADN des différentes lignées de *Meloidogyne* testées n'est pas en cause pour expliquer les différences de reproductibilité; ce sont les amorces qui en sont responsables, certaines se révélant meilleures que d'autres en terme de reproductibilité des résultats et donc de fiabilité. *M. arenaria* montre la variabilité intraspécifique la plus importante; quelques amorces seulement suffisent à distinguer les espèces bien que les données produites par certaines d'entre elles révèlent plus de similitude entre certaines lignées de *M. arenaria* et de *M. javanica* qu'au sein même du groupe de lignées de *M. arenaria*. Trois lignées virulentes de *M. arenaria* peuvent être distinguées tant des autres lignées, non virulentes, de *M. arenaria* que des autres lignées de *M. javanica, M. incognita, M. mayaguensis* et *M. hapla*. La comparaison des données obtenues sur le même jeu de lignées par RFLP et RAPD révèle des valeurs équivalentes en terme de différence interspécifique mais la technique des RAPD met en évidence une variation plus importante au niveau intraspécifique.

Key-words : Genetic diversity, Random Amplified Polymorphic DNA, *Meloidogyne,* nematodes.

In tropical regions, several parthenogenetic *Meloidogyne* spp. with wide host ranges (Jepson, 1983) are major crop pests. These species are relatively consistant in their isozyme phenotypes and can be readily identified by their esterase banding patterns (Esbenshade & Triantaphyllou, 1985). The presence of populations of *Meloidogyne* spp. in West Africa which overcome resistance in cultivars as diverse as tomato cv. Rossol, sweet potato cv. CDH and soyabean cv. Forrest has been reported (Prot, 1984). Many of these populations have a distinct esterase phenotype (Fargette & Braaksma, 1990) which is characteristic of *M. mayaguensis,* a species described from Mexico (Rammah & Hirschmann, 1988). This implies that these populations are biologically and genetically distinct from *M. incognita, M. javanica* and *M. arenaria,* the other major tropical root-knot nematode species. The resistance-breaking populations from West Africa were confirmed as *M. mayaguensis* by RFLP studies using clones established from single egg masses (Fargette *et al.,* 1995). These RFLP studies readily discriminated between the species and grouped the resistance-breaking lines with *M. mayaguensis.* This technique also revealed polymorphisms within species with most variation being found between the *M. arenaria* populations. In contrast, the populations of *M. incognita, M. javanica* and *M. mayaguensis* were more homogeneous. The RFLP technique, however, requires relatively large amounts of high quality DNA. In contrast PCR based methods require much less DNA and this paper reports a study of the use of randomly amplified polymorphic DNA (RAPDs) to investigate the relationships between populations of tropical *Meloidogyne* spp. The efficacy of this method for examining inter- and intra-specific variation is discussed.

Materials and methods

NEMATODE POPULATIONS

A total of 26 clonal lines were studied. These were five lines of *M. mayaguensis,* nine lines of *M. incognita,* seven lines of *M. arenaria,* four lines of *M. javanica* and one

line of *M. hapla*. The source of these lines and their virulence on the tomato cv. Rossol, which contains the major (Mi) gene for resistance to *Meloidogyne* spp., are given in Table 1. The lines were established from single egg masses from different field populations. Each clonal line was established by inoculating the egg mass from the single female onto a susceptible tomato plant (cv. Moneymaker).

The lines were maintained, in glasshouse cultures at 25 °C, on susceptible tomato plants (cv. Moneymaker) in pots containning *c* 1000 g soil. Six to 7 weeks after inoculation juveniles were extracted from the roots by placing the washed roots in a mist unit at 25-27 °C. The juveniles were collected twice a week and checked for the presence of saprophagous nematodes. Only samples that contained less then 3 % saprophagous nematodes were kept. The juveniles were concentrated by centrifugation and after freezing in liquid nitrogen were stored at - 80 °C.

Table 1. *Single egg mass lines of* Meloidogyne *spp., their codes, country of origin and resistance status.*

Species	Line Number	Country	Virulence to Mi
M. javanica	22	Burkina Faso	–
	23	Burkina Faso	*
	24	Spain	–
	25	Portugal	–
M. arenaria	10	Ivory Coast	–
	26	Portugal	–
	28	French West Indies	*
	29	French West Indies	**
	31	French West Indies	+
	32	French West Indies	+
	34	French West Indies	+
M. incognita	9	Ivory Coast	–
	11	USA (Race 3)	–
	12	USA (Race 4)	–
	15	Thailand (Race 1)	–
	16	Senegal	+
	17	Burkina Faso	–
	18	Chad	+
	19	French West Indies	+
	27	USA	+
M. mayaguensis	3	Ivory Coast	+
	5	Ivory Coast	+
	7	Ivory Coast	+
	13	Puerto Rico	+
	30	Burkina Faso	+
	33	The Netherlands	+

+ : High multiplication levels on cv. Rossol; – : No reproduction on cv. Rossol; * : The first generation on cv. Rossol gave low reproduction and there after failed; ** : The first generation on cv. Rossol gave high reproduction initially and there after declined.

To assess virulence the lines were cultured on the resistant tomato cv. Rossol in the same way as on the susceptible cv. Moneymaker and inoculated with a piece of root from the susceptible host that had galls and well-formed egg masses. After 6 to 8 weeks galls and egg masses that were present were re-inoculated onto cv. Rossol and the process repeated. Lines that were able to produce galls and egg masses and sustain multiplication consistently were classified as virulent.

DNA extraction

A packed volume of 50 µl of juveniles from each egg mass line was ground in liquid nitrogen until a fine powder was obtained. Extraction buffer (7 M urea, 0.35 M NaCl, 2 % SDS, 10 mM EDTA, 0.1 M Tris buffer, pH 7.5) was added and the mixture left at room temperature with gentle agitation for 15 min. The DNA was extracted twice with an equal volume of phenol-chloroform (1:1), followed by one chloroform-isoamyl alcohol (24:1) extraction. The DNA was precipitated by adding 2.5 volumes of absolute ethanol followed by centrifugation and was resolublised in TE (10 mM Tris, 1 mM EDTA, pH 8). The quantity of DNA was estimated as described by Fargette *et al.* (1995).

PCR reactions

Single random primers ten nucleotides long were used as described earlier (Williams *et al.,* 1990; Welsh & McClelland, 1990) in the random amplified polymorphic DNA-PCR technique. Seven of the oligonucleotides were obtained from Operon Technologies (OPG kit; Alameda, CA, USA) and two SC10-30 (5′-CCGAAGCCCT-3′) and SC 10-87 (5′-CGTGGGGGGC-3′) from R. Waugh (SCRI).

Amplification reactions included 10 ng of DNA in 50 µl of 10mM Tris-HCl (Ph 8.3), 1.5mM $MgCl_2$, 50mM KCl, 200 µm dATP, dCTP, dGTP, and dTTP and one unit of Boehringer Taq polymerase. Amplifications where preceded by a 2 min denaturation at 94 °C followed by 45 cycles of 15 at 94 °C, a 1.5 min ramp to the annealing phase at 38 °C for 1 min, a 1 min ramp to extension at 72 °C for 2 min using a Perkin-Elmer 480 thermal cycler. The products from these PCR reactions were separated by electrophoresis in TBE buffered 1.5 % agarose gels (Sambrook *et al.,* 1989) and the products visualized with ethidium bromide and UV illumination. Reproductibility was assessed by repeating the amplifications on three separate occasions, taking

considerable care to maintain as consistent reaction conditions as possible.

The results were assessed in two ways. Firstly all bands from the three runs were scored (presence = 1, absence = 0) in order to assess the variation between reactions. Secondly the amplifications products from the three separate runs were compared and only reaction products which were present in all the amplifications were recorded (single scores).

Statistical analysis

To assess the repeatability of the RAPD assays the band data were considered on a primer by genotype basis. Each band within a primer by genotype combination was considered " consistent " if all three amplifications gave the same expression, either all present or all absent. Bands which were expressed on one or two runs only were considered " inconsistent ". The proportion of consistent bands in each cell of the primer by genotype table was then analysed using a generalised linear model assuming a logit link function. The aim was to estimate overall repeatability and investigate whether the level of reproducibility varied with primer or genotype.

To assess inter-and intra-species variation among the *Meloidogyne* lines the " single score " data was used to produce a similarity matrix using the formula of Nei and Li (1979) which assesses similarity on the basis of the number of shared bands. The similarity matrix produced was used to produce a dendrogram by average linkage clustering and was also subjected to a principal coordinate (PCO) analysis. Scatter diagrams of the first two PCO dimensions were used to obtain a visual assessment of the inter-and intra-specific variation. The between and within species covariance matrices based on the first three PCO axes scores, were derived. The diagonal elements of these matrices measure the variation on each PCO axis. The covariance matrices for the RAPD scores were compared with the covariance matrices from a similar analysis (Fargette *et al.,* 1995) from an RFLP study on the same clonal lines.

Results

Virulence

The results of the tests for virulence are given in Table 1. Three lines of *M. arenaria,* four *M. incognita,* all the *M. mayaguensis* and the *M. hapla* population reproduced well on the resistant cv. Rossol and it was established that it is possible to maintain these lines on this genotype. Other populations were avirulent and unable to reproduce at all. However two of the lines (123 and 128) did produce a few females after the initial inoculation from the susceptible but these were unable to produce a second viable generation. A third line (129) reproduced well in the first generation but then declined in subsequent generations. Several attempts to culture these three lines in cv. Rossoll all ultimately failed and they are thus classified as avirulent. Within the *M. incognita* lines and some of the *M. arenaria* lines there is a clear distinction between virulence and avirulence. However within the *M. arenaria* group there are two populations which showed some reproduction but were not sufficiently virulent to maintain a viable population.

Repeatability

For any primer the amplification products for a particular line were generally very similar. As an example differences between some of the *M. incognita* lines are shown in Fig. 1. The Louisiana Race 3 (In 3) and North Carolina Race 4 (In 12) are clearly differentiated from the other seven *M. incognita* lines and these bands are consistently amplified in all three replicates. Some variation between replicates can also be seen, particularly in the first track of the In 11 group. Although the proportion of consistent bands was high (95 %) when averaged over all primers and genotypes, not one of the genotypes or primers was completely consistent. Table 2 shows one method for quantifying the contribution of primer and template to the variation in the PCR reactions. The residual contribution takes into account the uncontrolable variation in the reaction preparation and amplification. This approach showed that there was much more

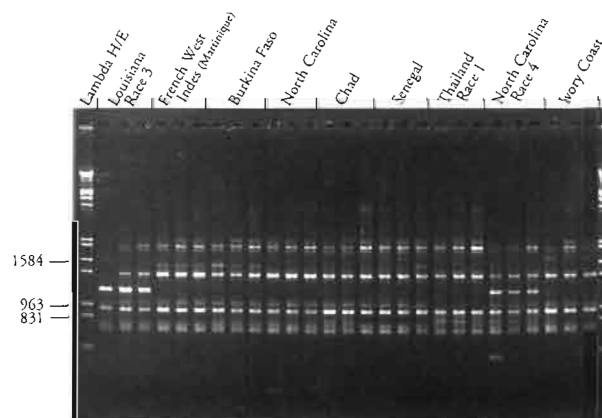

Fig. 1. *RAPD patterns of nine* Meloidogyne incognita *populations using primer SC10-87.*

Table 2. *Analysis of deviance.*

	Degrees of freedom.	Mean deviance
Template DNA (Lines)	25	1.69
Primes	8	13.48
Residual	200	1.39

variation among the primers than among the genotypes (template DNA) with the latter accounting for only a little more variation than the residual. OPG6 produced the highest proportion of consistent bands, 99 %, OPG2 the lowest, 81 % (Fig. 2). Figure 2 also shows that the percentage of consistent bands was not related to the number of amplification products. As the whole experiment was not replicated there is no test for the interaction between lines and primers. The relatively small residual deviance (when compared to that for primers) suggests that if interactions are significant their effects are small compared to the main effects.

The " single score " data was used to generate a similarity matrix from which a between- and within-species similarity matrix (Table 3) and a dendrogram was then produced (Fig. 3). The *M. incognita, M. mayaguensis* and *M. hapla* groups are distinct from each other (similarities of 51.3 % or less; Table 3) and show limited variability within groups (average within group similarity of 95.7-95.9 %) relative to that within the *M. arenaria* lines (average within group similarity of 76.5 %). The *M. arenaria* and *M. javanica* (72.3 % similarity) groups are less distinct from each other with a subgroup of *M. arenaria* (lines 10, 26, 28 and 29) being more similar to the *M. javanica* group than to the other *M. arenaria* lines. The *M. javanica* group itself showed a high within group similarity (94.7 %).

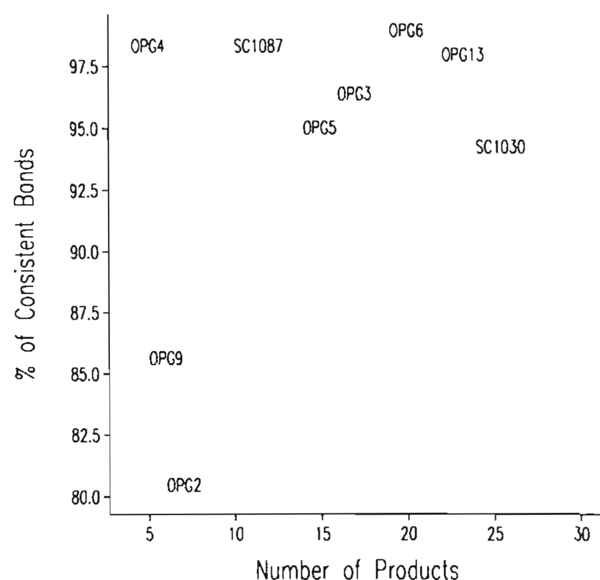

Fig. 2. *Plot of percentage consistent bands against the number of amplification products produced by nine RAPD primers.*

Table 3. *Between and within* Meloidogyne *species similarites produced from " single score " data. The numbers in brackets from* Guirao et al. *(1955).*

M. javanica	94.7				
M. arenaria	72.3 (55-62)	76.5			
M. incognita	51.3 (36-41)	44.8 (27-33)	95.7		
M. mayaguensis	6.9	8.2	17.9	95.9	
M. hapla	13.4 (10-11)	19.8 (7-10)	17.4 (11-13)	20.5	100

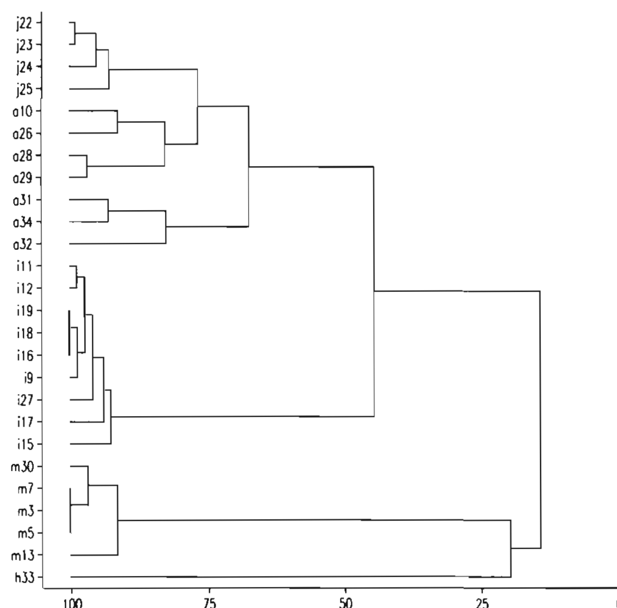

Fig. 3. *Dendrogram of 26 lines of* Meloidogyne *spp.*

Figure 4 shows a bandmap (Powell *et al.*, 1991) of the data arranged so that both the most similar lines and most similar bands are grouped together making group specific bands easy to identify. A summary of the source of bands that are monomorphic within a species and which are thus species specific is given in Table 4. No primer yielded separate bands that were specific to all the species. Four primers (OPG3, 6, 13 and SC10-30) produced bands that were specific to *M. incognita, M. hapla* and *M. mayaguensis*. Of these SC10-30 produced three bands specific to *M. javanica*. OPG5 was the only primer to produce a band specific to *M. arenaria* whilst SC10-87 produced a band specific to the one *M. hapla* line. The remaining primers (OPG 2, 4 and 9) each produced fewer bands which were polymorphic but contributed to differentiating lines within species. Within the *M. arenaria* group there are three lines (ln31, ln32 and ln34; Table 1) from the French West Indies which are virulent on the Mi resistance gene; the isolates could be differentiated from all other lines by three bands produced by OPG13. Within the *M. arenaria* group thre were a further four bands from SC10-87, SC10-30, OPG6 and OPG13 that differentiated these

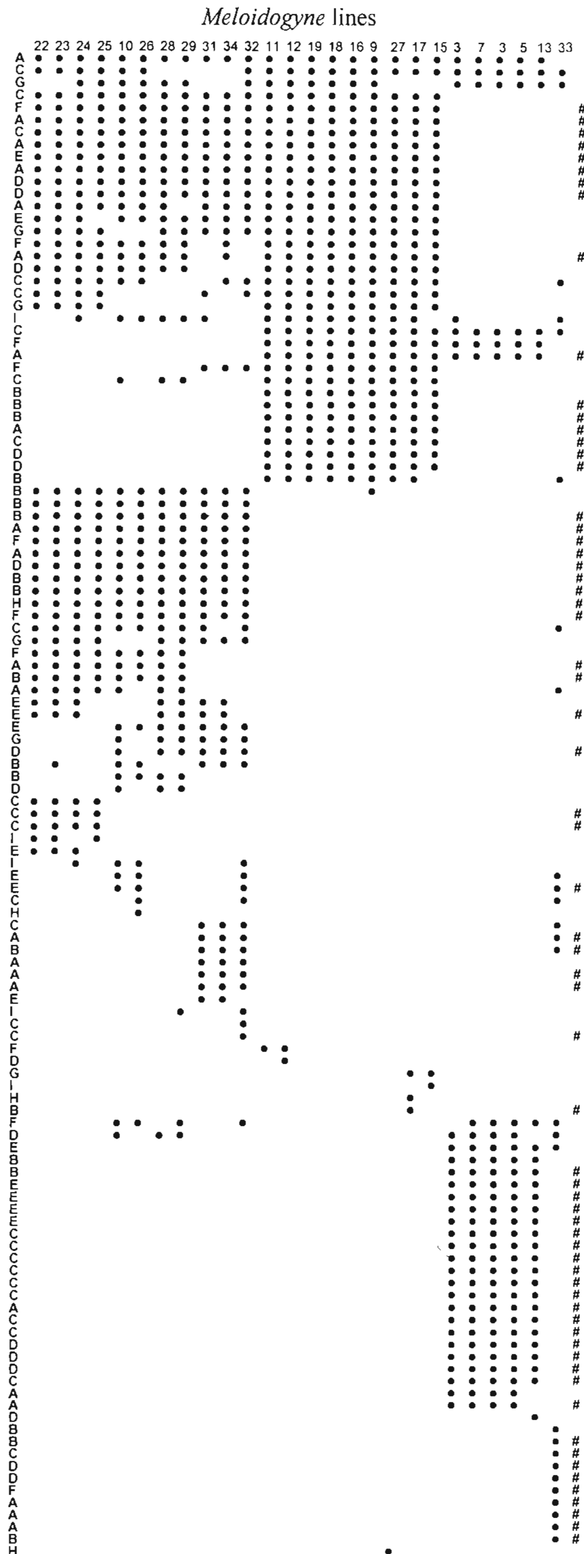

Fig. 4. *Bandmap of Single score data (● indicates the presence of a band; # indicates identity with the band above).*

virulent lines from the other avirulent *M. arenaria* lines. Primers OPG3, SC10-87, OPG13 and OPG6 produced bands which grouped the *M. javanica* lines with the four avirulent *M. arenaria* lines.

Comparison of RAPDs and RFLPs

Data from an RFLP analysis of the same lines (Fargette *et al.*, 1995) was compared with the RAPD data. The RFLP data had been generated by probing five different endonuclease restriction enzyme digests with each of four probes derived from a *Meloidogyne* total DNA library. The total within species variation, based on the first three PCO axes scores from the analysis of the RAPD and RFLP data are shown in Table 5 and highlight that while the between species variances are similar, greater within species variation is revealed by the RAPD assays.

Discussion

Results of examining the scores of the replicated data show that the main differences are between the primers in their reliability rather than the template DNA. These observations on reproducibility between primers and between reactions have been commented on before (Black, 1993) but not quantified. The analyses of the scores of the individual runs showed that there were marked differences between the repeatability of results from the primers and to a much lesser extent from the template DNA with no strong evidence for marked primer X line interactions. This would suggest that factors other than the quantity and quality of the template DNA are differentially affecting the performance of the different primers.

This measure of similarity was devised in relation to the interpretation of RFLP data but is frequently used with binary data generated from RAPDs. Methods of analysing RAPD data to provide estimates of several population – genetic parameters have been devised (Lynch & Milligan, 1994) and used by Guirao *et al.* (1995) in the study of *Meloidogyne* spp. Clark and Lanigan (1993) addressed the problem of using RAPDs to assess nucleotide divergence. They conclude that with certain assumptions the Nei and Li (1979) fomula is an appropriate starting point. In this study our aim was to estimate the relative similarities of the clonal lines of *Meloidogyne* spp. revealed by RAPDs and to compare this with similar estimates from RFLP studies.

RAPs have been shown to have value in discriminating species of *Meloidogyne* (Cenis, 1993; Castagnone-Sereno *et al.*, 1995) and our results confirm this but also show that this technique has value in revealing intraspecific variation. In comparing species similarities using RAPDs, bands which comigrate are assumed to be homologous and depending on the degree of species genetic divergence this assumption may be invalid hence linking species in the dendrogram may be inappropriate. In an RFLP analyses of the same populations (Fargette

Table 4. *RAPD primers used and the number species specific amplification products that they produced.*

Primer	Code in Fig. 4	Bands	Bands specific for				
			M. javanica	*M. arenaria*	*M. incognita*	*M. hapla*	*M. mayaguensis*
OPG13	A	22		3*	1	3	1
OPG6	B	19			3	3	2
SC10-30	C	24	3		1	1	9
OPG3	D	16			1	2	3
OPG5	E	14		1			4
SC10-87	F	10				1	
OPG2	G	6					
OPG4	H	4					
OPG9	I	5					
Total		122					

* Unique to three *M. arenaria* populations with virulence to the Mi gene

Table 5. *Total between lines and within species variances derived from the PCO analysies of RFLP and RAPD assays of 25* Meloidogyne *egg mass lines.*

Species	RFLP variance × 10^2	RAPD variance × 10^2
Between species	58.51	67.29
M. javanica and *M. arenaria*	5.99	31.99
M. javanica	0.29	2.64
M. arenaria	6.08	20.56
M. mayaguensis	0.63	3.03
M. incognita	0.73	2.40

et al., 1995), where cross-hybridisation between species does permit assessment of species relationships, similar relationships between species to those in the RAPD study were found. It thus seems likely that the assumptions that the RAPD bands are identical can be made here. The RAPD data, however, revealed more intraspecific variation than RFLPs which is partly explained by the greater proportion of monomorphic bands observed within groups using the populations in this study were produced. Intraspecific variation was greatest within *M. arenaria.* In contrast, the levels of variability were lower within *M. javanica* and *M. incognita,* despite the lines representing a wide geographic spread. Guirao *et al.* (1995) also observed the same relative relationships between species in their study though the values of the similarities they observed were lower than obtained here (Table 3). The relative lack of genetic diversity in these species suggests that they may have been dispersed recently from a single source rather than representing indigenous populations. The grouping of lines within these species does not reflect their geographic provenance or indicate how they might have been spead.

Carpenter *et al.* (1992), using RFLPs, also reported the relatively high levels of variation in the *M. arenaria* group even with populations which were geographically closely related. Garate *et al.* (1991), Xue *et al.* (1993), Baum *et al.* (1994) and Guirao *et al.* (1995) observed that *M. arenaria* and *M. javanica* were closely related and some authors have suggested that these species could possibly be considered as one group. Both these results are consistent with these RAPD and RFLP studies (Fargette *et al.,* 1995) which indicate that *M. arenaria* is a heterogeneous mixture of clonal lines and *M. javanica* a homogeneous group of related lines. Studies of mitochondrial DNA, however, have not shown that *M. arenaria* is more closely related to *M. javanica* than it is to *M. incognita* but they have shown that lines of *M. arenaria* can show as much or more intraspecific variation than in any interspecific comparisons (Powers & Sandall, 1988; Hyman & Powers, 1991; Hugall *et al.,* 1994) and thus indicate that these two groups have diverged.

Additionally the RAPD analysis showed groupings of lines within *M. arenaria* relating to virulence. The three virulent lines, which were also grouped by the RFLP analysis, were from the same geographic area (although the two other lines from the French West Indies are avirulent) and further investigations on a broader range of *M. arenaria* populations is required to determine whether the amplification products unique to these po-

pulations can be used to discriminate virulent populations.

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