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Protein Variability in *Meloidogyne* spp. (Nematoda: Meloidogynidae) Revealed by Two-Dimensional Gel Electrophoresis and Mass Spectrometry

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Total protein variation as revealed by two-dimensional electrophoresis (2D-E) was studied in 18 isolates from populations of *Meloidogyne arenaria* (six isolates), *Meloidogyne incognita* (10 isolates), and *Meloidogyne javanica* (one isolate) plus an unclassified isolate. Gels (80 × 60 × 0.75 mm) were silver-stained and digitized in order to compare their protein patterns. Optical density and position of protein patterns were measured using statistical cluster analysis and computer-assisted image analysis software. Only those protein stains or positions that were clearly defined (i.e., without background) were considered. The number of positions in gels ranged from 86 to 203. Each of these positions had 95 clearly expressed proteins that were present in at least two replicates for each isolate. Spot position was considered a taxonomical character with two different states: presence (1) and absence (0). Accordingly, genetic distance was estimated among isolates and species, and a phylogenetic tree was constructed following the cladistic approach based on maximum parsimony analysis. Isolates of *M. arenaria*–*M. javanica*–*Meloidogyne* sp. and of *M. incognita* formed two separate monophyletic groups. Both groups were clearly defined on the basis of two sets of protein positions that can be considered as diagnostic characters. An attempt to identify these proteins by mass spectrometry was made. Group diagnostic proteins for *M. incognita* and *M. arenaria* (and for other proteins common to all isolates) were distinguished by protonated mass signals in the MALDI fingerprinting spectrum.

Keywords: *Meloidogyne* • nematodes • proteomics • 2D electrophoresis • phylogeny • diagnosis

1. Introduction

Meloidogyne spp. (root-knot nematodes) are an important group of plant-parasitic nematodes.[1] Of the roughly 80 species currently described, three of them, *M. arenaria*, *M. incognita*, and *M. javanica*, are responsible for the greatest amount of nematode damage to the world's major crops.[2] Their taxonomy has principally been defined on the basis of the perineal morphology of adult females.[3] The comparison of esterase isoenzymatic patterns[4−7] has been the most reliable and useful method for species diagnosis, although other enzymatic systems can be applied.[8,9]

Protein electrophoretic patterns are phenotypic characters that reflect genetic differences among species or populations better than do morphological characters.[10] For this reason, since the 1960s[11] they have become a valuable tool for taxonomy, systematic, and evolutionary studies.[12−15] While two-dimensional protein electrophoresis[16] was originally used for sytematic purposes,[17,18] it has also proven to be a valuable technique for analyzing protein constituents of tissues and organs. It is a particularly useful tool for studying phylogenetics[19] and closely related species.

Two-dimensional electrophoresis has been mainly applied to cyst nematode genera such as *Heterodera* [22−27] and *Globodera*,[28−31] although nematologists have employed 2-D electrophoresis to make taxonomical diagnoses[20] and phylogenetic inferences[21] in plant and soil nematodes in the Dorylaimida. Surprising little is known, however, regarding the amount of variation in protein expression among population/species of *Meloidogyne* spp.[32−36] especially considering their agroeconomical impact.

Two-dimensional gel databases have proven useful as bioinformatic tools, but their role is changing as zoologists move toward protein expression rather than position variability in two-dimensional gel maps. Thus, the purpose of this study was to identify the proteins carrying phylogenetic information that could be used as stable taxonomical markers.

2. Materials and Methods

Eighteen isolates of *Meloidogyne* belonging to three different species were used for comparison: *M. incognita* (Mi9705, Mi9683, Mi9639, Mi9681, Mi9714, Mi9727, Mi9660, M3, M4 and M5), *M. arenaria* (Ma9647, Ma9713, Ma9688, Ma9730, Ma9742, Ma9671), and *M. javanica* (Mj9712). In addition, an unidentified isolate (Msp) was also included in the study. Isolates were extracted from females in their reproductive stage that were

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collected off of field crops in the Extremadura Region (Spain). The susceptible tomato cv, Rio Fuego, was used in order to increase the number of isogenic isolates. The cv was inoculated with one egg mass per isolate. This multiplication process was conducted in a growth chamber at 25 °C day/16 °C night temperature, with a photoperiod of 16 light and 70% relative humidity. Plants were grown singly in 20 cm-*d* pots in sterilized fine sand and fertilized once a week with Gambor's solution.

After 3 months of culture, females were extracted by macerating tomato roots in a water solution of Celluclast (35%) plus Pectinex (15%) (Novo Nordisk Ferment Ltd, Denmark). Roots were then crushed with a hand whisk and the macerate filtered through 150 μm sieves to recover adult females. The females were separated from the plant debris with a Pasteur pipet and washed several times in 0.9% saline solution.

For each isolate, protein was extracted from two samples of approximately 30 clean, white, mature females in a 1.5 mL of Ependorff tube. Females were crushed with a Teflon pestle in 200 μL of extraction buffer (Tris-HCl, 50 mM, pH 8, 5% (v/v) β-mercaptoethanol, 3% SDS, 0.1 mM EDTA), and all proteins were precipitated using cold acetone.[37] Protein concentration was estimated following the method of Lowry et al.[38] Each sample of protein extracts was aliquoted to about 150 μg of protein and frozen at −80 °C for up to, but not exceeding, 2 months. Prior to use, extracts were centrifugated for 5 min (13000*g* at 4 °C), and the supernatant protein fraction was dried and stored at −80 °C.

Preceding electrophoresis, this fraction was dissolved in 20 μL of lysis buffer (urea 9.5 M, 5% 2-mercaptoethanol, 2% Triton X-100, 1.6% Ampholytes pH range 5−7, 0.4% Ampholytes pH range 3.5−10). Essentially, electrophoresis was carried out according to O'Farrell,[16] but certain adaptations[39] were made in order to utilize the Mighty Small II apparatus (Hoefer, Amersham Pharmacia Biotech). Isoelectric focusing was performed in capillary tubes (75 mm long and 1.9 mm inner diameter). Tubes were filled according to the manufacturer's instructions with an acrylamide gel solution composed of 9 M urea, acrylamide/bisacrylamide (T 4%, C 0.5%), 3.3% Triton X-100, 2.66% ampholytes (pH range 5−7), 0.7% ampholytes (pH range 3.5−10), 0.12% TEMED, and 0.26% PSA.

After polymerization, 12 μL of protein samples were applied to the top of capillary tubes with a Hamilton syringe and covered with 10 μL of overlay buffer (urea 8 M, 1.5% ampholytes pH range 5−7, 0.4% ampholytes pH range 3.5−10). A pre-run focusing was performed according to the following schedule: 18 min, 170 V; 18 min, 350 V; 18 min, 520 V; 18 min, 700 V. Following that, isoelectric focusing was performed for 3 h 30 min at 700 V and for 1 h at 1000 V. After extraction from the tubes, gels were equilibrated for 10 min in the following buffer solution: Tris-HCl 0.0625 M pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol. Second-dimension electrophoresis (SDS−PAGE) was performed with 25 mA/gel in a stacking zone, followed by 35 mA/gel in a separation zone. Electrophoresis was performed simultaneously on two gels. Gels were developed following the silver stain procedure.[40] Gels were kept overnight in 30% methanol and then dried between two sheets of cellophane. Molecular weight and pH estimation were done with Biorad calibration.

Analysis of Protein Patterns. The Image Quant program (Computer Densitometer, Molecular Dynamic) was used to record the optical density and coordinates of each spot. Protein patterns were compared (a) by cluster analysis (NTSYS program, UPGMA method) of coordinates and optical density of spots and (b) through the use of computer-assisted image analysis software (Image Master Elite 3.01, Amersham Pharmacia Biotech). The first procedure was especially useful in cases where position assignment was uncertain, and it ensured that only homologous proteins were matched among the 18 different isolates analyzed. A gel with the largest number of identifiable positions was chosen as a master (Ma9647). This permitted the comparison of species/populations using a presence (1) and absence (0) matrix. Genetic distance[41] and parsimony analyses were made using the PAUP program.[42] Mass spectrometry was used on the synapomorphies (proteins carrying phylogenetic information) in an attempt to establish their identity.

In-Gel Digestion of Proteins. Protein spots were excised manually and incubated for several minutes in a solution containing 50 mM sodium thiosulfate and 15 mM potassium ferricyanide to remove Ag ions. The digestion protocol used was that of Schevchenko et al.[40] with some minor variations: the gel plug was washed with 25 mM ammonium bicarbonate and acetonitrile prior to reduction with 10 mM dithiothreitol in 25 mM ammonium bicarbonate and alkylation with 100 mM iodoacetamide in 50 mM ammonium bicarbonate.

The gel piece was then rinsed with 50 mM ammonium bicarbonate and acetonitrile and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, Madison WI) at a final concentration of 16 ng/μL in 25 mM ammonium bicarbonate was added to the dry gel piece and the digestion proceeded at 37 °C for 12 h. Peptides were eluted with acetonitrile, 25 mM ammonium bicarbonate, and 10% formic acid (v/v) for a final extraction volume of 100 μL. The digestion solution was vacuum-dried and redissolved in 10 μL of 33% aqueous acetonitrile with 0.1% trifluoroacetic acid.

MALDI Peptide Mass Fingerprinting and Database Searching. A 0.5 μL aliquot of the final digestion solution was manually deposited onto the stainless steel MALDI probe and allowed to dry at room temperature. Then, 0.5 μL of matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid) was added and again allowed to dry at room temperature. Mass fingerprints were measured automatically on a Bruker Reflex III MALDI-TOF mass spectrometer (Bruker-Franzen Analytic GmbH, Bremen, Germany) equipped with the SCOUT source in positive-ion reflector mode using delayed extraction and AutoXecute acquisition software. Ion acceleration voltage was 25 kV.

The equipment was first externally calibrated employing protonated mass signals from a peptide mixture covering the m/z 1000−4000 range. Thereafter, every spectrum was internally calibrated using selected signals arising from trypsin autoproteolysis to reach a typical mass measurement accuracy of ±30 ppm. MS BioTools was used to convert and send the tryptic peptide mass data to the NCBInr database, and Mascot software (Matrix Science, London, UK) was utilized to search the database for protein matches and identification.

Initially, no restrictions were placed on the species of origin of the protein and a protein molecular mass of 1−200 kDa was allowed. Up to one missed tryptic cleavage was considered, and a mass accuracy of 40 ppm was used for the tryptic-mass search. A minimum of four tryptic peptides were required for identification.

3. Results

Position Identification. Isolates of *Meloidogyne* species were highly variable. The number of protein positions ranged from

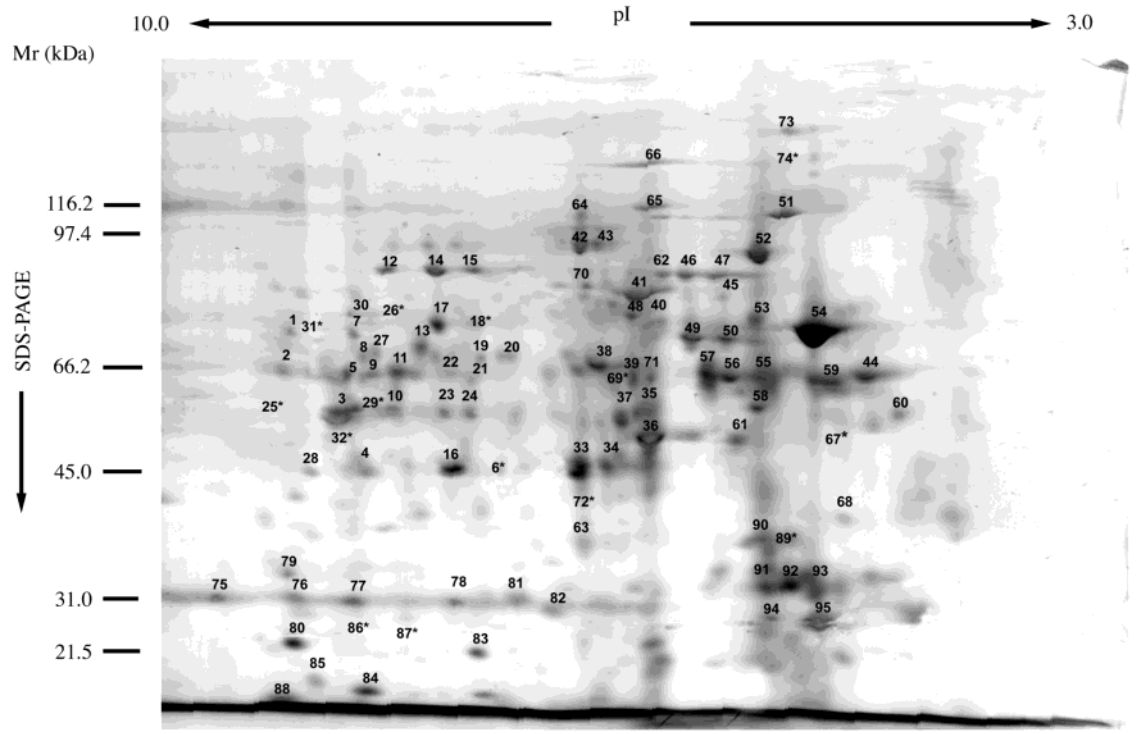

Figure 1. Distribution of protein positions used for similarity and phylogenetic analysis. Positions with an asterisk are not presented on the referenced master gel (Ma9747). Their presence in isolates is shown in Table 1.

Table 1. Matrix of Proteins Position (1 = Present; 0 = Absent) in Figure 1 on Which the Phylogenetic Analysis Is Based

	1 2 3 4 5 6 7 8 9 0 0 0 0 0 0 0 0 0
Ma9647	11111011111111111011111100110100111111111111111111111111111111111101011010111111111111010111111
Ma9713	00101011001111111000000111110100110111101111111111111111111101111110100101111011111011011011111
Ma9688	11111101011111111010110101110100111111101111111111011111011011101101011010111111111111110111111
Ma9730	00111111101111111011111111111010011111111111111111111111111111101110110100101011111111101111101111
Ma9742	00111110111111111010010111111010011111111111111111111111111111111101101011010111111111111100101111
Ma9671	00111001011111101101001011111010011011110111111111111111111111111111101011010111111111111111101111
Mi9705	01111111011111111011000010011111010111111111111111011111111110111010111110101011111111100111111111
Mi9683	01111111101111010110000000011111011111110111111111011111111100111111111111011101111111111001111111
Mi9639	00111111101111111011100010011111010111111111111101101111111111001101011111101011111111111001111111
Mi9681	00111111101111010110000100111111011011101111110110011111010101101101111100011101111111101111111
Mi9714	01111111101111111011000010011111111111111111111111101111111110111111111111111111111111111001111111
Mi9727	00111111101111010110000100111111010011110111111111011111111000110101110100011111111111001111111
Mi9660	11111111111111111011000010011111101101110011111111100111111111111?1011110?10101011111111001111111
M4	11111111111111111011100010011111101111110111111111101111111111111111111111001111111111111001111111
M5	00111111111111111011000010011111101111110111111111101111111111101101111110010111111011111001111111
M3	11111111111111111011000010011111101111110111111111101111111111011101111110111111111111111011111111
Mj9712	01111011011111011010000001?110000110111101111110111111111111110110100101101111111111111111011111111
Msp	11111011101111111000011111111010010011100110111111111111111111111101101000010111111101110010111111

86 to 203 positions in *M. arenaria* (average is 148 positions); from 73 to 171 positions in *M. incognita* (average is 118 positions); and from 122 to 134 positions in *M. javanica*—*Meloidogyne* sp. (average is 128 positions). A master gel was constructed with a total of 2321 positions. It was at times difficult to assess whether differences in positions were due to either real differences between proteins or to small deformations in the gels. Using the UPGMA method, these 2321 spots were clustered taking into account similarity of coordinates (x and y) and optical density (data not shown). Differences in positions due to small variations in gel size were avoided using this approach. Final similarities among spots were determined using Image Analysis Software. Only protein spots without background were considered. Ninety-five clearly expressed proteins or positions were present in two replicates of each isolate. Of the 95 main protein spots, 37 could be considered monomorphic (present in all isolates). The distribution of spot positions in the master gel is shown in Figure 1, while the respective species are shown in Table 1. Taxonomical character is considered a protein position with two states—protein the presence (1) or protein absence (0) (Table 1).

Phylogenetic Analysis. Table 2 shows total position differences (above the diagonal) and genetic distance among isolates (below the diagonal). Figure 2a illustrates the UPGMA tree, which classified the entire set of isolates according to overall genetic distance. Clustering the 18 isolates divided the set into two distinct groups. One group consisted of *M. incognita* isolates and the second of *M. arenaria* plus *M. javanica* and *Meloidogyne* sp. isolates. The average distance for both groups was $D = 0.27$. Maximum inner distance averages were also

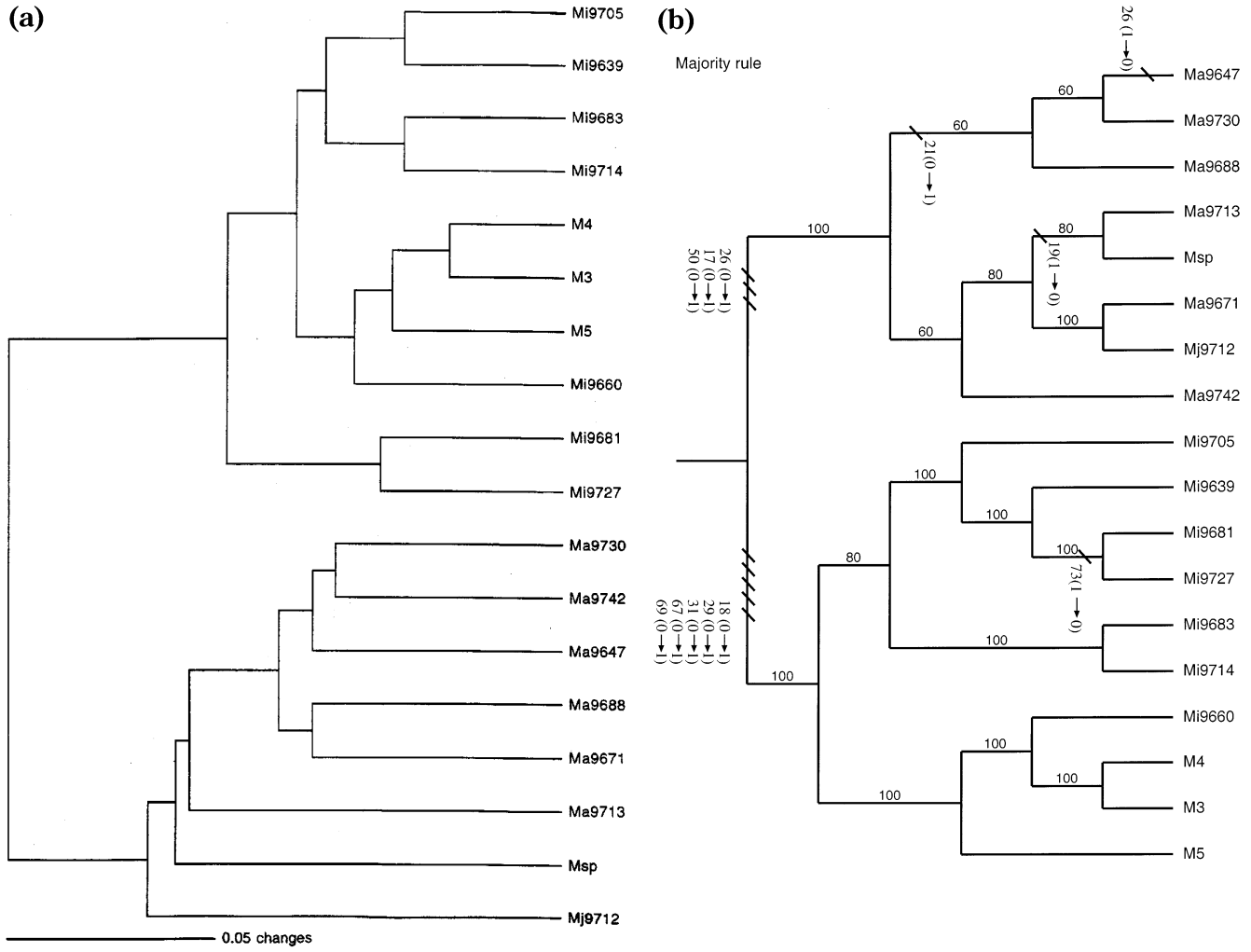

Figure 2. (a) Tree of similarity among isolates obtained by UPGMA according to genetic distance (Table 2); (b) Topology consensus of the five most parsimonius trees (cladograms), representing the phylogenetic relationship of isolates. Numbers on the branches express in percentage the level of significance. Also included are the protein positions carrying the most consistent phylogenetic information (synapomorphies) and how they change.

approximately the same [$D = 0.14$ (*M. arenaria* group) and $D = 0.132$ (*M. incognita* group)]. The relationships established by these genetic values appear to confirm that protein positions are good markers for the systematization of *Meloidogyne* species/populations. The cladistic approach based on parsimony analysis reveals an evolutionary genetic relationship by allowing us to detect those proteins (synapomorphies) which carry phylogenetic information. A heuristic search was performed with a simple addition sequence (TBR, branch-swapping, mulpars options), and characters were reweighted according to their maximum rescaled consistency index value. Five trees were obtained whose consensus (50% majority rule) is represented in Figure 2b. When characters were unweighted (i.e., all proteins were considered to have equal analytical relevance) the topology was the same but consistency (CI = 0.427) was lower than that for weighted characters (i.e., not all proteins were given equal analytical relevance) (CI = 0.909). Bootstrapping, using 100 data sets, separated the isolates into two monophyletic groups: (1) *M. incognita* and (2) *M. arenaria* plus *M. javanica* and *Meloidogyne* sp. Characters with CI = 1.00 were considered to be unambiguous synapomorphies (excluding terminal taxa or autopomorphies). These characters are expressed by either a gain (change from 0 to 1) or a loss (change 1 to 0) of state character (Figure 2b). Most of the 95 proteins were homoplasious, due to either a state reversal or parallelism (convergence) in different branches or groups of isolates. The synapomorphies that defined monophyly were 17, 26, and 50 for the *M. arenaria* group and 18, 29, 31, 67, and 69 for the *M. incognita* group. These proteins, therefore, offer genuine diagnostic value for species identification.

The database search using MALDI peptide mass fingerprinting (Table 3) failed to identify the proteins. This was most likely due to the lack of records in the database for the studied species. However, as can be seen in Table 3a, the spectra suggested that position differences were produced by different proteins and not by different alleles or by post-translational protein modifications. Common positions for three species, such as 3, 5, 36, and 54, in contrast, share the same tryptic peptides masses (Table 3b).

4. Discussion

Understanding population genetics and evolution within species requires recognition of variation within and between populations. Several molecular techniques have been applied to *Meloidogyne* spp. identification and genetics such as RFLPs,[43,44] RAPDs, [45−47] Multiplex PCR,[48−50] AFLPs,[32,33] and satellite DNA.[51]

Table 2. Total Position Differences (Above Diagonal) and Genetic Distance of Li and Nei, 1979 (Below Diagonal)

	1 Ma9647	2 Ma9713	3 Ma9688	4 Ma9730	5 Ma9742	6 Ma9671	7 Mi9705	8 Mi9683	9 Mi9639	10 Mi9681	11 Mi9714	12 Mi9727	13 Mi9660	14 M4	15 M5	16 M3	17 Mj9712	18 Msp
1	–	21	12	11	11	13	21	23	22	28	20	28	22	16	21	19	18	16
2	0.233	–	19	14	16	12	26	24	27	31	23	25	29	27	28	30	18	17
3	0.133	0.211	–	13	13	11	21	25	26	26	24	28	23	24	25	21	19	20
4	0.122	0.156	0.144	–	10	14	24	26	21	29	23	25	30	25	24	26	22	17
5	0.122	0.178	0.144	0.111	–	10	18	22	19	25	19	23	22	19	16	20	16	15
6	0.144	0.133	0.122	0.156	0.111	–	24	24	27	25	25	25	25	25	24	26	12	17
7	0.233	0.289	0.233	0.267	0.200	0.267	–	10	7	17	9	11	10	11	10	12	21	27
8	0.256	0.267	0.278	0.289	0.244	0.267	0.111	–	13	15	7	11	13	11	14	14	21	29
9	0.244	0.300	0.289	0.233	0.211	0.300	0.078	0.144	–	12	10	8	15	12	9	15	22	28
10	0.311	0.344	0.289	0.322	0.278	0.278	0.189	0.167	0.133	–	18	8	13	20	15	17	20	32
11	0.222	0.256	0.267	0.256	0.211	0.278	0.100	0.078	0.111	0.200	–	14	13	8	11	9	24	28
12	0.311	0.278	0.311	0.278	0.256	0.278	0.122	0.122	0.089	0.089	0.156	–	15	18	13	19	22	26
13	0.250	0.330	0.261	0.341	0.250	0.284	0.114	0.148	0.170	0.148	0.148	0.170	–	8	10	9	23	23
14	0.178	0.300	0.267	0.278	0.211	0.278	0.122	0.122	0.133	0.222	0.089	0.200	0.091	–	7	5	24	24
15	0.233	0.311	0.278	0.267	0.178	0.267	0.111	0.156	0.100	0.167	0.122	0.144	0.114	0.078	–	8	23	25
16	0.211	0.333	0.233	0.289	0.222	0.289	0.133	0.156	0.167	0.189	0.100	0.211	0.102	0.056	0.089	–	23	25
17	0.202	0.202	0.213	0.247	0.180	0.135	0.236	0.236	0.247	0.225	0.270	0.247	0.264	0.270	0.258	0.258	–	21
18	0.178	0.189	0.222	0.189	0.167	0.189	0.300	0.322	0.311	0.356	0.311	0.289	0.261	0.267	0.278	0.278	0.236	–

Table 3. Protonated Mass Signals Observed in the MALDI Fingerprinting Spectra

(a) Species Diagnosis Bands 17, 18, 50, 29, 31, 67, and 69

17	18	29	31	50	67	69
1254.75	865.43	990.60	1011.46	1119.64	1263.64	1020.49
1335.70	1068.55	1030.42	1267.68	1607.90	1313.63	1215.59
1370.77	1438.71	1109.54	1370.70	1890.04	1340.62	1405.66
1463.78	1522.78	1872.97	1787.75	2509.67	1350.66	2150.10
2343.03	1584.74				1787.77	
2413.39	1618.75				2163.15	

(b) Common Species Bands 3, 5, 36, and 54

3	5	36	54
807.42	807.43		807.42
	897.35	897.31	897.37
1060.40	1060.48	1060.42	
1132.45	1132.50	1132.49	1132.47
	1283.55		1283.59
1349.79			1349.73
1393.60	1393.67		1393.62
	1450.73	1450.70	
	1521.75		
1947.90		1947.82	1947.89
2082.99	2082.91	2082.88	2082.85
	1649.82		
			2435.26
1821.72	1821.,76		1821.,69
	1911.88		
	2095.01		2095.09
	2584.23		

Even species with parthenogenetic reproduction show a very high variability.[9] This likely arose from a primitive sexual condition of reproduction[52] which was subsequently fixed as an adaptation to a wide range of hosts and soil environments.[45,53]

According to Hugall et al.,[54] the agreement between patterns of variation in mtDNA and esterases indicates discrete genetic entities within *M. arenaria*, *M. incognita*, and *M. javanica* that do not correspond precisely to classical taxonomic boundaries. We support the view of these authors that it is necessary to define groups of populations (isolates) based on their genetic background. Characterization of inter- and intraspecific genetic differences is easily detected by two-dimensional electrophoresis. Additionally, the application of principles and methods of Phylogenetic Systematics allows us to recognize the value of synapomorphic proteins to define monophyletics groups. Aquadro and Avise,[17] have shown that genetic distances obtained from two-dimensional electrophoresis are highly concordant with taxonomical levels in vertebrate species (rodents) while Miyazaki et al.[55,56] and Tsubokawa and Miyazaki[57] considered two-dimensional electrophoresis to be a valuable systematic tool for estimating and evaluating genetic distance in invertebrates (Crustacea and Mollusca).

The number of spots revealed that protein quantities differed among the three species. So much variability has allowed quantitative comparison as has been done in other studies.[26,31] Although the utility of this approach was confirmed in this study, the large degree of variability made it necessary to specifically select the proteins used for analysis. We chose proteins that were easily detectable (expressed proteins), and that had clearly confirmable shared positions. Despite using slightly different techniques, the genetic distance and isolate values we obtained were quite similar to those obtained for other species and isolates (such as *M. chitwoodi* and *M. fallax*),[32,36] in which the number of spots considered was higher.

Inner genetic distances detected in groups of *M. incognita* and *M. arenaria*−*M. javanica* also fell within the range of variation for *M. chitwoodi* and *M. fallax*. These results confirm the efficiency of the methodology we applied to the construction of the master gel. Only well-stained proteins were considered. Therefore, the removal of poorly expressed proteins did not significantly change the results of the analysis.

Theoretically, proteins with identical sequences should appear in the same position within two compared patterns. Quantitative and qualitative protein variation due to mutations also influence the amount of variance among two-dimensional gels. Hence, an unambiguous identification of positions is needed for a later characterization. Although two-dimensional electrophoresis is used as a routine method of identification, the number of *loci* detected also provide an efficient method for screening genetic changes. The level of genetic variation, as well as the number of polypeptides resolved by two-dimensional electrophoresis is clearly dependent on the procedure used.[37,58] The level of estimated heterozygosity (allelic variants) is considerably lower than when one-dimensional electrophoresis is used.[59] In addition, two-dimensional electrophoresis allows identification (sequencing) of specific proteins and, therefore, of the coding gene.

One advantage of two-dimensional electrophoresis analysis for phylogenetic inference is that data can be analyzed by phenetic methods (genetic distance) or by cladistic methods (ancestor descendants succession) to construct parsimony based phylogenetic trees.[19] The cladistic approach consistently shows the anagenetic changes in the same tree and how these changes manifest during taxa evolution. Due to the lack of protein records in the databases, no single protein is proposed as a species indicator. In addition, while there may be a great deal of data on an organism such as *Caenorhabditis elegans*, there may be no match due to the strong phylogenetic divergence that exists between plant-parasite nematodes and the Rhabditids. Nevertheless, two-dimensional electrophoresis does permit us to make a rapid selection among hundreds of potential candidates.

According to the protonated mass values listed in Table 3 (different for different positions), these proteins did not have the characteristic of proteins encoded by alleles at the same *locus*.[60] The protein patterns obtained by two-dimensional electrophoresis can be extremely complicated. For this reason, mass protonated spectrometry allows an easy recognition of protein when different laboratories may be involved. The results of the two-dimensional electrophoresis analysis presented here support the close association of some *M. javanica* isolates to *M. arenaria*,[9] while other *M. javanica* isolates could be associated to *M. incognita*,[54] as has been suggested in other studies.

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