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**ARTICLE** *in* JOURNAL OF PROTEOME RESEARCH · JUNE 2005

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# Identification of Proteins Expressing Differences among Isolates of *Meloidogyne spp.* (Nematoda: Meloidogynidae) by Nano-Liquid Chromatography Coupled to Ion-Trap Mass Spectrometry

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**Received February 12, 2005**

***Abstract:*** Total protein variation (up to ninety-five different positions) was revealed by two-dimensional electrophoresis (2-DE) in 18 isolates from populations of *M. arenaria* (6 isolates), *M. incognita* (10), *M. javanica* (1) plus an unclassified isolate in a previously reported study. Isolates of *M. arenaria*, *M. javanica*, *Meloidogyne sp.*, and *M. incognita* formed two separate groups defined on the basis of two sets of protein positions that could be considered as diagnostic characters, but we could not identify these proteins by MALDI-TOF. To identify these marker positions, nano-liquid chromatography as peptides separation method was coupled to an ion-trap mass spectrometer for induced real-time fragmentation of eluted peptides. Group diagnostic proteins for *M. incognita* and *M. arenaria* were in-gel digested and on line analyzed by tandem mass spectrometry (LC−MS/MS). Six proteins out of seven selected spots were unambiguously identified by the analysis of the corresponding MS/MS (MS2) spectrum from parent ions fragmentation: Actin, Enolase, CG3752-PA protein similar to Aldehyde Dehydrogenase, HSP-60 and Translation initiation factor eIF-4A. In *M. incognita* sample, de novo sequencing experiment of doubly charged ion at $m/z = 936.9$ Da in spot 29 identified as enolase, reveals three residue substitutions (K to T, N to T, and D to E) when tentative sequence was compared with that of *Anisakis simplex* and *Onchocerca volvulus* enolase, thus three SNPs (single nucleotide polymorphisms) were also possibly identified.

**Keywords:** *Meloidogyne* • nematodes • proteomics • 2D electrophoresis • diagnosis • nano-liquid chromatography • tandem mass spectrometry • protein identification

## 1. Introduction

*Meloidogyne spp.* (root-knot nematodes) are a highly polyphagous group of plant-sedentary endoparasites nematodes.[1] Although 80 species have been described so far, three of them alone, *M. arenaria*, *M. incognita*, and *M. javanica*, are responsible for the greatest amount of nematode damage to the world's major crops.[2] Those proteins that are fully characterized are primarily related to plant-nematode interactions.[3−6] Little research have been devoted to be used in as species diagnosis.[7] However, advances in the practice and application of proteomics will allow us to confront many of the problems related to phylogeny and its methodology[8,9] exploring specific proteins with evolutionary value.

*Meloidogyne* is into one of the world's major pathogens. Molecular characterization and diagnosis of *M. incognita*, *M. arenaria* and *M. javanica* as well as the development of new techniques for their control, can benefit from proteomics approach. Characterization of inter and intraspecific differences is easily detected by two-dimensional electrophoresis. The number of *loci* detected also provide an efficient method for the screening of genetic changes. In a previous two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry study[10] on protein variability in isolates of these three species, several positions were selected as carrier for discriminating among *Meloidogyne* populations. Although the peptide mass fingerprints analyzed by MALDI-TOF were listed, no identified protein was proposed. Thus, the purpose of this study was to identify those proteins suitable for used as reliable diagnostic characters in order to complement other existing molecular and biochemical approaches to diagnostic or taxonomic.

## 2. Materials and Methods

As proteins may be stage-specific, only mature females were used for protein extractions. Two-dimensional electrophoresis and analysis of protein patterns are described in the previous variability study.[10] Selected spots were those which are labeled in reference master gel presented in such a study. These positions were selected in the process of classification by parsimony approach as discriminant for *M. incognita* (spots 18, 29, 31, 67, and 69) and *M. arenaria/M. javanica* (spots 17 and 50). Position 26 was not analyzed in this study because of reversions along the tree, even thought this spot appears within the root of the cluster in isolates from both, *M. arenaria* and *M. javanica* species. Protein spots were manually excised from the original two-dimensional dried gels. Analysis of proteins pattern was performed by the Image Quant software (Computer densitometer, Molecular Dynamic) to record the optical density and coordinates of each spot.

Protein spots were incubated for several minutes in ultrapure water solution and digested in accordance with the protocol of

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10.1021/pr0500298 CCC: $30.25 



Published on Web 05/18/2005

Schevchenko et al.[11] with some minor variations. Gel pieces were equilibrated in 50 mM ammonium bicarbonate (ABC) prior to reduction with 10 mM dithiothreitol and alkylation with 100 mM iodoacetamide, both in 50 mM ABC. Modified porcine trypsin (sequencing grade, Promega, Madison, WT) at a final concentration of 16 ng/$\mu$L in 50 mM ABC was added to dried spots and the digestion took place at 37 °C overnight. Finally, tryptic peptides were extracted with 0.5% trifluoroacetic acid. The total digestion solution was vacuum-dried and redissolved in 20 $\mu$l of A solution. (A = 5% ACN and 0.5% acetic acid in water).

**Nano-Liquid Chromatography and Ion-Trap Tandem Mass Spectrometric Analysis of Tryptic Peptides.** The resulting tryptic peptides were injected *on line* onto a C-18 reversed-phase self-packing nano-column (Discovery BIO Wide pore, Supelco, Bellafonte, PA) and analyzed in a continuous acetonitrile gradient consisting of 0−50% B in 45 min, 50−90% B in 1 min. A flow rate of ca. 300 nL/min was used to elute peptides from the reversed-phase nano-column to a PicoTip emitter nano-spray needle (New Objective, Woburn, MA) for real-time ionization and peptide fragmentation on an Esquire HCT ion-trap (Bruker-Daltoniks, Bremen, Germany) mass spectrometer. Every 1 s, the instrument cycled through acquisition off a full-scan mass spectrum and one MS/MS spectrum. A 3 Da window (precursor $m/z$ ±1.5), an MS/MS fragmentation amplitude of 0.90 V and a dynamic exclusion time of 0.30 min were used for peptide fragmentation. Nano-liquid chromatography was automaticaly performed on an advanced nano-gradient generator (Ultimate nano-HPLC, LC Packings, Amsterdam, The Netherlands) coupled to an autosampler (Famos, LC Packings). Hystar 2.3 software was used to control the whole analytical process.

**Database Analysis.** MS/MS spectra corresponding to contaminants keratins tryptic peptides were removed and the filtered MS/MS spectra were batch-processed using DataAnalysis 5.1 SR1 and BioTools 2.0 software packages and searched against the MSDB protein database using Mascot software (Matrix Science, London, UK). MS BLAST at EMBL (http://dove.embl-heidelberg.de/Blast2/msblast.html) search with default settings was used for spot 29 in order to identify this protein by sequence alignment from the obtained de novo tentative sequence.

## 3. Results

The distribution of selected spots which appeared as discriminants between *M. incognita* and M. *arenaria−M. javanica−Meloidogyne sp.* in Navas et al., 2002[10] are displayed in Figure 1. Protein identification for *M. incognita* (positions 18, 29, 31, 67, and 69) and *M. arenaria−M. javanica−Meloidogyne sp.* (positions 17 and 50) was performed by nano-liquid chromatography coupled to ion-trap mass spectrometry. Proteins identified by tryptic peptide fragmentation include six out of the seven tested spots whose sequenced matched peptides are shown in Table 1. For all positions, at least, two peptides were properly fragmented and sequenced for protein identification except in sample 29 (identified as enolase) where only a single peptide, apart of derived from trypsin or keratins, was detected, although the alignment of the analyzed sequence leads unambiguously to a single identified protein. All identified proteins have a high degree of homology with nematode proteins (*Caenorhabditis elegans, Anisakis simplex, Onchocerca volvulus*, and *Trichinella spiralis*) except the protein CG3752-Pa similar to aldehyde dehydrogenase (spot 31) homologous to *Drosophila melanogaster*. Spot 50 contains a tryptic peptide homologous to HSP-60 of *Meliothis virescens.* Statistics for each

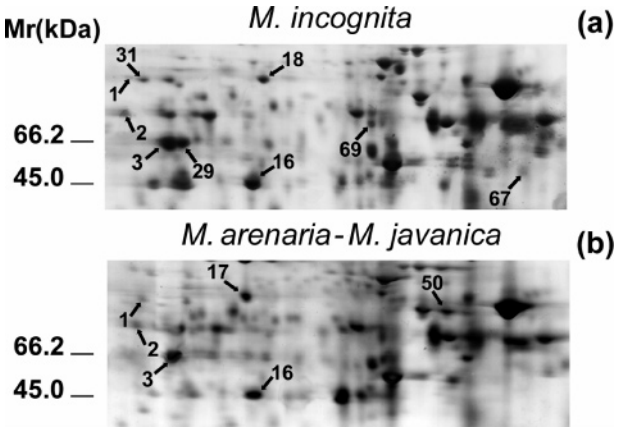


**Figure 1.** Distribution of protein positions in *M. incognita* (a) and *M. arenaria−M. javanica* (b). Spots 18, 29, 31, 67, and 69 are present only in gel (a), and spots 17 and 50 are present in gel (b). Spots 1, 2, 3, and 16 are labeled as reference points according to master gels of Navas et al. 2002.[10]

identification is referred as RMS error and the Mascot Scores (Table 1). Fragmentation spectra corresponding to some of the sequenced tryptic peptides in each spot are displayed in Figure 2. As can be observed, all higher intensity mass peaks belong to one of two main fragmentation series (b − amino and y − carboxy series). Spot 18 was analyzed twice in two independent experiments but none fragmentation spectrum was clearly obtained, so consequently this marker has not yet identified. MS2 spectrum from spot 29 corresponding to doubly charged ion at $m/z$ 936.9 did not reach a satisfactory identification when it was automatically submitted to protein database. This ion corresponds to monoisotopic singly charged peptide at 1872.97, which was previously observed in the same position by MALDI-TOF mass spectrometry in Navas et al., 2002.[10] Because of the high quality of the fragmentation spectrum we could reconstruct the primary sequence by exhaustive analysis of the main fragmentation series as shown in Figure 3a. This spot was finally identified as enolase by homology alignment with the BLAST software when tentative sequence was compared with *Anisakis simplex* and *Onchocerca volvulus* enolase. Interestingly, as shown in Figure 3b, three changes within the primary sequence in the peptide analyzed in position 29 (enolase) were identified which can be assumed to be mutations, whose phylogenetic or evolutionary value will be subject of further studies. One substitution, from threonine (T) to lysine (K), was observed when the peptide sequence of *M. incognita* was aligned with the homologous sequence in *A. simplex*. This punctual change might be explained by existence of an SNP (Single Nucleotype Polymorphism) along the DNA sequence in which the second nucleotide in codon changes from cytosine (C) to adenosine (A), Figure 3b. Two different substitutions, from glutamic acid (E) to aspartic acid (D), and from threonine (T) to asparagine (N) were observed when the peptide sequence from *M. incognita* was aligned with the homologous sequence in *O. volvulus*. These changes could be explained by existence of other two SNPs (the third nucleotide in codon changes from A/G to T/C, and the same second nucleotide which changes in *A. simplex* when compared with *M. incognita*), Figure 3b.

## 4. Discussion

Value of proteins as phylogenetic markers has been recognized[12−14] in conjunction with their structural and functional

**Table 1.** Identified Protein Markers[a]

| spot | protein | accession | taxonomy | sequence | RMS error (ppm) | score |
|---|---|---|---|---|---|---|
| 17 | Actin protein 4, isoform c | Q8I9k0 | *M. javanica* (is also present in about other 60 Metazoa) | GYSFTTTAER<br>EITALAPSTMK<br>LDLAGRDLTDYLMK | 95 | 163 |
| 31 | CG3752-PA | Q9VLC5 | *D. melanogaster* | IYDEFVER<br>RVTLELGGK | 112 | 101 |
| 50 | Chaperonin protein HSP-60 | S11035<br>AAA28077<br>Q8MZM9 | *Meliothis virescens*[1]<br>*C. elegans*[2]<br>*Trichinella spiralis*[3] | GANPIEIR[1,3]<br>VGGSSEVEVNEK[3]<br>NVIIEQSWGSPK[2,3]<br>TNEQAGDGTTCATVLAR[2,3]<br>AAVEEGIVPGGGVALLR[2]<br>TLEDELEIIEGMK[1] | 157 | 343 |
| 67 | Chaperonin protein HSP-60 | | AAA28077<br>Q8MZM9 | GANPIEIR[1,3]<br>NVIIEQSWGSPK[2,3]<br>AAVEEGIVPGGGVALLR[2] | 120 | 130 |
| 69 | Translation initiation factor eIF-4A | S26281 | *C. elegans* | DVIAQAQSGTGK<br>MFVLDEADEMLSR | 119 | 106 |
| 29 | Enolase[b] | Q8MU59<br>Q7YZX3 | *Anisakis simplex*<br>*Onchocerca volvulus* | ---LEQTESIEAATLAR | | 65[c]<br>60[c] |

[a] Peptide sequences obtained by analysis of the corresponding MS2 spectrum were processed and searched against the protein databases for identification using the MASCOT program. The unique sequence obtained in the spot 29 was de novo sequenced and the protein identification in this case was performed by BLAST alignment. Uncertainty in 2−3 first residues in sample 29 fragmented peptide is labelled as "---". [b] Identification obtained by MS BLAST at EMBL alignment: http://dove.embl-heidelberg.de/Blast2/msblast.html. [c] High and total scores present in the BLASTP2 result report. First sequence from *Anisakis simplex* was aligned with red color and the second sequence had green color. Superscripted numbers indicate the presence of the corresponding sequences in taxa.

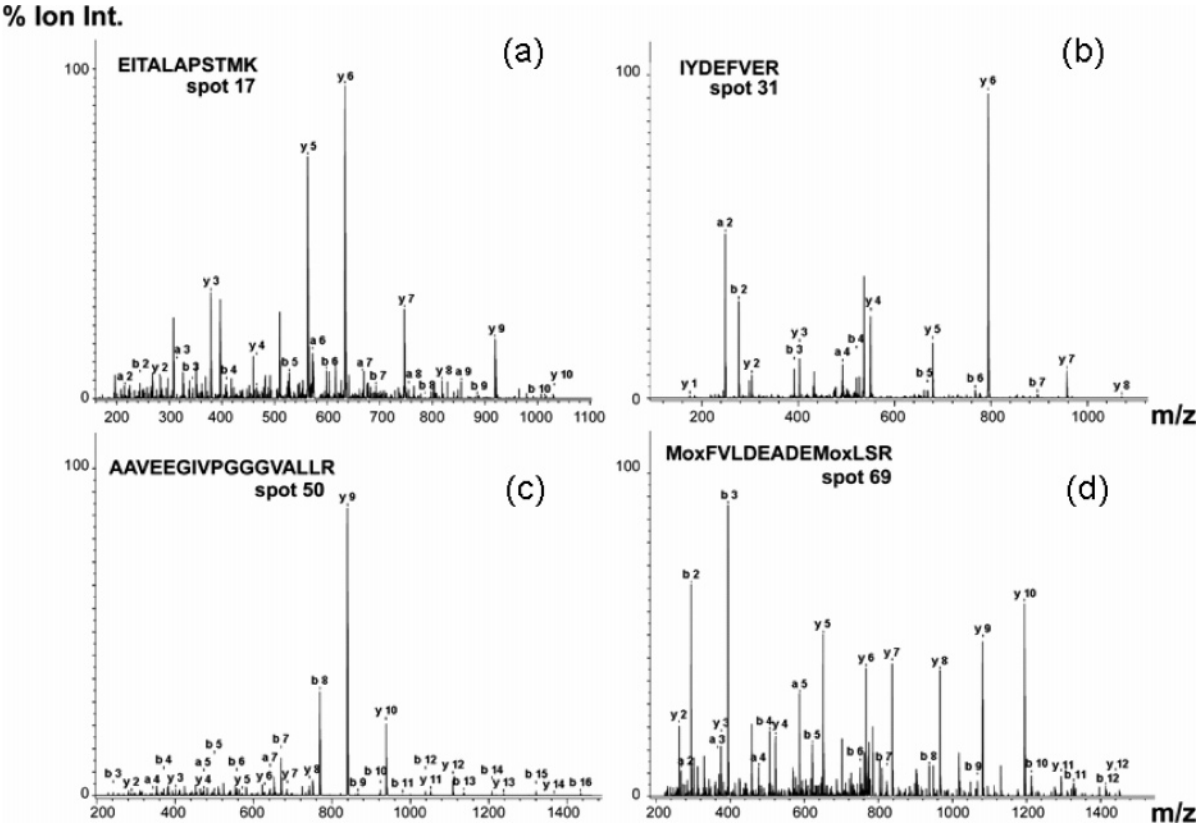


**Figure 2.** Fragmentation spectra. MS2 spectra displaying the main fragmentation series (b-amino and y-carboxy series) from doubly charged parent ions from the following sequences: EITALAPSTMK (a), IYDEFVER (b), AAVEEGIVPGGGVALLR (c), and MoxFVLDEADEMoxLSR (d) corresponding to samples 17, 31, 50, and 69, respectively.

importance along the evolutionary tracks.[15,16] In nematodes, most proteins that are characterized are related to the host−parasite interaction.[3−6] The proteins chosen for identification in this experiment have a clear expression and confirmable shared positions within the set of assayed nematode isolates. Due to protein variability, genetic distance among species and isolates was estimated to be similar to those obtained for other species of *Meloidogyne* (*M. chitwoodi* and *M. fallax*).[17,18] This means that, probably, evolution has already fixed the identified proteins (Actin, enolase, protein CG3752-PA similar to Aldehyde dehydrogenase, HSP-60, and Translation initiation factor elF-4A) as stable features. The comparison of two-dimensional gel

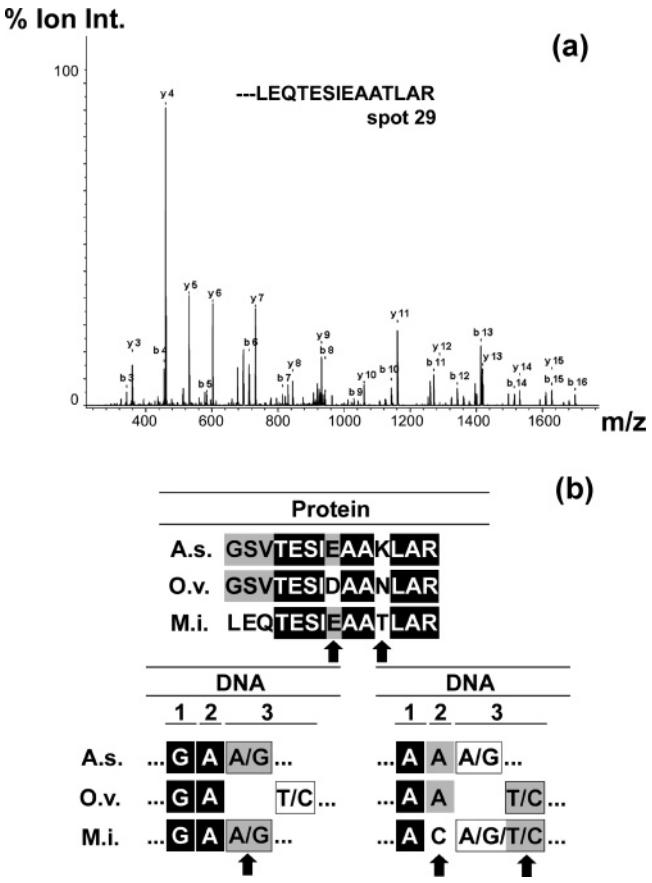


**Figure 3.** MS2 spectrum corresponding to de novo sequenced peptide LEQTESIEAATLAR present in *M. incognita* spot 29 (Figure 3a) which is similar to *Anisakis simplex,* and *Onchocerca volvulus* enolase. As shown in Figure 3b, at least three residue substitutions were observed along the enolase protein sequence that could be consequence of the three posible SNPs pointed by black arrows (A to C) along the nucleotidic sequence.

electrophoresis revealed significant differences in protein expression, particularly evident within the range of 70 to 45 kDa and 8 to 5 p*I*. The three internal amino acid Actin sequences (from spot 17) shared significant homologies in the databases with *M. javanica* and other six nematode species including *O. volvulus, C. elegans, Plectus acuminatus, Panagrellus redivivus, Wuchereria bancrofti,* and *Brugia malayi*. The six (even the shortest eight amino acid residues) HSP-60 sequences shared significant homologies with nematodes *C. elegans*, and *T. spiralis*, and the insect *Meliothis virescens* in the databases referring as diagnostic characters for *M. arenaria*−*M. javanica* (positions 50 and 67). The rest of the peptides were also unambiguously identified and shared homologies with *C. elegans* (translation initiation factor elF-4A in position 69), *A. simplex* and *O. volvulus* (enolase in position 29) and *D. melanogaster* (Protein CG3752-PA similar to Aldehyde Dehydrogenase in position 31) were considered to be diagnostic characters for *M. incognita*.

Actins are an important group of proteins whose conventional functions are related to motility, nematode cuticle, and cytoskeletal proteins.[20,21] Because these proteins are ubiquitous and highly conserved troughout evolution within eukaryotes[22,23] they are present as a multigene family whose products are represented by multiple isoforms. This would explain why the actin characterized in species of *Meloidogyne* (43 kDa, p*I* 6.2)[7] falls outside the range we have identified.

Heat shock proteins are phylogenetically highly conserved proteins and within the HSP-60 family the sequence identity between proteins from organisms as different as mammals and mycobacteria is aproximately 50%.[24] They are present in almost all the phylla and, based on sequence homology, are divided into two groups. Group I proteins are found in Bacteria and eukariotic organelles, while group II proteins are present in Archaea and cytosol of eukaryotes.[25] Of both, the groups found in Archaea and cytosol eukariotes are less well-known. HSP-60 appears to have a major role in the folding of actin and tubulin, although they may also act on other substrates.[26] The disposition in gels of *M. arenaria*−*M. javanica*−*Meloidogyne sp.* (position 50) and *M. incognita* (position 67) of different molecular weight might be related to the fact that the cytosolic chaperonin complex (CCT) in eukaryotes is a completely hetero-oligomeric complex, with eight distinct CCT subunit genes evolved by duplication in eukaryotes.[25,27] Because of their role as cellular key components in dealing with stress from evolutionary forces (environmental, and/or genetic), they have been considered to be suppliers of magnificent clues for elucidating phylogenies,[28,29] highlighting the findings herein as good markers or targets for detection and species identification.

Enolases are key enzymes involved in glycolysis, found in all organisms, as members of the enolase gene superfamily.[30] The sequence gene of enolase, highly variable, has been used for the phylogenetic reconstruction of a wide range of taxa belonging to several domains, from Eubacteria, Archaea, and Eukaria. Use of enolase as a marker has resolved the disposition of Parabasilia by considering as ancient[31] what constitutes a monophyletic group in the root of the eukariotes.[32] Distinct enzymatic and antigen properties have been reported during the development stage of parasite *Toxoplasma gondii*,[33] which shows considerable homologies with plant counterparts.[34] The potential roles of these proteins in *Meloidogyne*−plant interactions have to be addressed.[35] In the present study, we report the possible existence of three SNPs which can explain three different residue substitutions along the short sequence identified as enolase in the spot 29. Two of these three SNPs (the change from C to A in the second nucleotide, and from T/C to A/G in the third nucleotide of the codon displayed in Figure 3b, right bottom) might play an important role in the establishment of possible diagnostic marker (lysine in *A. simplex*, Asparagine in *O. volvulus*, and threonine in *M. incognita*).

Aldehyde dehydrogenases play an important role in the detoxification pathway for aldehyde metabolism in its oxidation to carboxylic acids. Their importance becomes evident from the known polymorphims (i.e., in human).[36,37] In our results, the protein CG3752-PA similar to Aldehyde Dehydrogenase peptides shows an strong homology with *D. melanogaster*, while little is known of this enzyme in nematodes. As isoenzymes, they are frequently used in classical population genetics studies, because of their importance in the reduction of the cytotoxic and genotoxic effects of aldehydes, aldehyde dehydrogenase can also be considered to be an important mechanism in plant−nematode interaction as it is for other host-parasite systems.[38,39]

Position 69 is homologous with the translation initiation factor eIF-4A of *C. elegans*. Initiation of protein synthesis is regulated by many translation initiation factors, as it is important in the process of controlling cell proliferation under normal physiological conditions.[40] These kinds of proteins have been demonstrated to be related to the avirulent determinants in

plant pathogens or to be host factors controlling recessive resistance in plant hosts.[41]

None identified proteins are specific for any of the tested species, and the presence/absence remains unknown for most species of the genus *Meloidogyne*. However, it is a matter of fact that the identified proteins in this study have a value for discrimination between sets of assayed isolates or populations. In this study, peptide sequences are provided for the first time for what are important plant pathogens (*Meloidogyne*) which also can be valuable for taxonomic diagnosis complementing others molecular and biochemical approaches. An understanding of the structural bases for the observed diversity of proteins and sequences through proteomics application may provide the basis for the discovery of the functions of proteins in these or other nematode genomes, as well as guidance for resolving phylogenies.

**Acknowledgment.** This study was supported by Project AGF-971143 from MEC, Spain. Thanks are due to two anonymous reviewers whose criticism and suggestions improved the manuscript.

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PR0500298