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Application of a putative fatty-acid binding protein to discriminate serologically the two European quarantine root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax*, from other *Meloidogyne* species

Christophe Tastet, Florence Val, Michel Lesage, Lionel Renault, Laurent Marché, Michel Bossis and Didier Mugniéry
INRA/ENSAR – UMR BiO3P, Domaine de la Motte, B.P. 35357, 35653 Le Rheu Cedex, France (Phone: +33223485159; Fax: +33223485150; E-mail: tastet@rennes.inra.fr)

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Abstract

Two major proteins, Mcf-A67 and Mcf-B66, were identified by mini two-dimensional polyacrylamide gel electrophoresis in order to distinguish the two European quarantine root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax*, from eight other species. These 'quarantine proteinic markers' have been microsequenced after enzymatic digestion. The internal amino acid sequences exhibit similarities to members of a family of low molecular weight intracellular lipid-binding proteins. Moreover, to explore a simple, rapid, and inexpensive way to identify the two quarantine nematodes, dot blot hybridizations were performed using an antiserum (ΣA67) produced from the longest amino-acid sequence of the protein Mcf-A67. Although several proteins stained on the *M. chitwoodi* and *M. fallax* western blot membranes, the two nematodes were easily distinguished from other root-knot nematodes, on dot blot assays with soluble proteins extracted from a single female. Because of its specificity and sensitivity, the use of the ΣA67 antiserum to improve the diagnosis of the two European quarantine root-knot nematodes is discussed.

Abbreviations: CBB – Coomassie Brillant Blue; FABP – fatty-acid binding protein; i.d. – intra dermic; PDA – piperazine diacrylamide; 2-D – two-dimensional.

Introduction

Root-knot nematodes (*Meloidogyne* spp.) belong to the most damaging obligate plant parasites, causing an estimated yield loss of over 10% world-wide (Sasser and Freckman, 1987). They invade the host root as juveniles and induce the formation of highly specialized feeding structures within the vascular cylinder (Sijmons, 1993; Sijmons et al., 1994; Wyss et al., 1992). Galling of the cortical tissue enclosing the feeding site is observed in response to parasitism (Sijmons, 1993). The presence of numerous galls induces physiological disorders on host plants (Mateille, 1994) that generate both qualitative and quantitative losses.

Among the seventy or so described species, two species – *M. chitwoodi* and *M. fallax* – are particularly damaging, and they have been registered on the quarantine organisms list of the European Union. These nematodes reproduce on both mono- and dicotyledonous plants in temperate climates (Karssen and van Hoenselaar, 1998). Rare cases of resistant cultivars have been described as host race specific (Mojtahedi et al., 1988). Morphologically these species are very difficult to distinguish (Karssen, 1996), hence it is necessary to develop methods of nematode diagnosis.

In previous studies, we demonstrated the efficiency of two-dimensional electrophoresis to discriminate *Meloidogyne* species (Tastet et al., 2000) and notably

M. chitwoodi from *M. fallax* (Tastet et al., 1999). This paper reports the comparison of 2-D gels from the two European quarantine nematodes with those obtained from the four major *Meloidogyne* species, *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*, and from four other species *M. mayaguensis*, *M. arabicida*, *M. exigua* and *M. naasi*. The aim was to identify common proteins in *M. chitwoodi* and *M. fallax* which could be used as 'quarantine markers' in order to develop a serological kit of detection.

Little research has been devoted to the serological diagnostics of *Meloidogyne*. Lee (1965) applied the immunodiffusion technique to serologically discriminate species. An antiserum raised against *M. incognita* showed no precipitation arcs with *M. hapla*. As indicated by the author, this may have been due to the small number of individuals used. Indeed, later studies (Hussey, 1972, 1979; Hussey et al., 1972; Misaghi and McClure, 1974) indicated that other *Meloidogyne* species, e.g. *M. incognita*, *M. javanica* and *M. arenaria*, are serologically closely-related because of common precipitin bands. Later, Jones et al. (1988) developed monoclonal antibodies against *M. incognita* by immunizing Lou/Iap rats and Balb/c mice with soluble proteins extracted from adult female nematodes. Two hybridoma cell lines were isolated, producing antibodies with preponderant specificity for *M. incognita* antigens. Polyclonal and monoclonal antibodies have also been produced to supernatants of soluble proteins from homogenates of root-knot nematodes (Davies and Lander, 1992). The antibodies were used to investigate differences between second-stage juveniles and adults of the three major root-knot species by ELISA and immunoblotting. Most antigens revealed by the polyclonal antibody were conserved between species but several quantitative differences were observed (Davies and Lander, 1992). Finally, Ibrahim et al. (1996) used the esterase isoenzyme from *M. incognita* to raise monoclonal antibodies. Results showed that a monoclonal antibody to a diagnostic non-specific esterase allowed direct discrimination between *M. incognita* and *M. javanica* without a preceding electrophoresis step.

However, serology did not become a routine procedure in nematode diagnostics because the results were often complex and the antisera tended to cross-react. In this paper we report, for the first time, the combined use of several techniques (two dimensional gel electrophoresis, internal amino-acid sequencing and serology) to produce antisera which discriminate the two European quarantine root-knot nematodes *M. chitwoodi* and *M. fallax* from other root-knot nematodes.

Materials and methods

Meloidogyne *spp. isolates and protein sample preparation*

Meloidogyne incognita, *M. javanica*, *M. arenaria*, *M. mayaguensis, M. exigua, M. arabicida, M. hapla* race B, *M. chitwoodi* and *M. fallax* isolates were maintained in greenhouse pot cultures on tomato cultivar Saint-Pierre. *M. naasi* was reared on wheat, cultivar Arminda. The sources of the nematode isolates are presented in Table 1. Six to seven weeks after inoculation with second-stage juveniles, white adult females were dissected by hand from the infected root systems. Three total soluble protein samples per isolate were preparated (Tastet et al., 1999).

Mini 2-DE

Two-dimensional (2-D) electrophoresis was performed essentially as described by O'Farrell (1975) with slight modifications (Bossis and Mugniéry, 1993). Samples were submitted to 2-D gel electrophoresis on the Mini-Protean II 2-D cell system of Bio-Rad

Table 1. Isolates and sources of species of *Meloidogyne* used

Meloidogyne species	Isolate	Location	Source
M. incognita	INC-TRI	Trinidad	IRD[a]/ CIRAD[b]
M. javanica	JAV-AL1	Algeria	INA[c]
M. arenaria	ARE-ANT	French West Indies	IRD/ CIRAD
M. mayaguensis	MAY-SEN	Senegal	IRD/ CIRAD
M. exigua	EXI-NIC	Nicaragua	IRD/ CIRAD
M. arabicida	ARA-COS	Costa-Rica	IRD/ CIRAD
M. naasi	NAA-FRA	France	INRA[d]
M. hapla race B	HAP-MOL	France	INRA
M. chitwoodi	IPO-Ca	the Netherlands	PRI[e]
M. fallax	IPO-Fa	the Netherlands	PRI

[a]IRD: Institut de Recherche pour le Développement, Montpellier, France.
[b]CIRAD: Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Montpellier, France.
[c]INA: Institut National Agronomique, Alger, Algeria.
[d]INRA: Institut National de la Recherche Agronomique, Rennes, France.
[e]PRI: Plant Research International, Wageningen, the Netherlands.

(Richmond, CA, USA). 2-D electrophoresis of proteins with carrier ampholytes and silver staining of slab gels was performed (Bossis and Mugniéry, 1993). In order to control experimental variations, at least three 2-D gels were run for each protein sample. Protein loads of 5 μg were applied to each gel.

For amino acid sequencing, twelve 2-D gels were run and stained with Coomassie Brillant Blue R-250; 37 μg protein were loaded on each gel. After migration, the 2-D gels were soaked twice in 450 ml of methanol/acetic acid/deionized water solution (50/10/40, v/v/v) for 30 min. The fixing solution was then replaced with 450 ml of staining solution containing 0.003% w/v CBB R-250 in methanol/acetic acid/deionized water (45/10/45, v/v/v). When the staining was sufficient to locate the proteins of interest, the 2-D gels were washed 10 times with deionized water, in order to remove methanol, acetic acid and the excess of dye.

Preparation of the protein spots for enzymatic digestion

After CBB R-250 staining, the proteins of interest were excised precisely with a scalpel to minimize the amount of polyacrylamide matrix. For each protein, twelve spots were pooled in a single Eppendorf and dried at room temperature. The protein spots were then stored at −70 °C until enzymatic digestion.

Determination of amino acid sequences

The protein spots excised from the CBB-stained gels were rehydrated in 150–200 μl of 0.1 M Tris–HCl, pH 8.6, 0.01% Tween 20 (Pierce, Rockford, IL, USA) and digested for 18 h at 35 °C, with endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer, Mannheim, Germany) at a final concentration of 2 μg ml^{-1}.

The supernatant was recovered and the pellet was rinsed with 60% acetonitrile. The acetonitrile rinse was added to the supernatant and acetonitrile was removed in a Speed-Vac. The sample was injected on to a DEAE–HPLC column linked to a C18 reverse phase HPLC column and eluted with a 0–45% acetonitrile, 0.1% TFA gradient (Kawasaki and Suzuki, 1990). Peaks recorded at 210 nm were collected manually and frozen (−20 °C) until sequencing. Sequencing was performed on Applied Biosystems 473 and 494 sequencers.

Sequence database searching

Protein identifications by search of sequence homologies were performed with the FASTA program (Pearson and Lipman, 1988). The software is available via the Internet at http://bioweb.pasteur.fr/seqanal/interfaces/fasta-simple.html. Complementary information from the accessions corresponding to the two higher homologies scores was searched on the National Center for Biotechnology Information (http://www3.ncbi.nlm.nih.gov), the SwissProt and the TrEMBL databases (http://expasy.hcuge.ch/sprot). For each protein, two internal amino acid sequences were obtained.

Preparation of antigen

A synthetic peptide (Mcf-A67^{pep}) was produced from the longest amino-acid sequence (20 aa) of the protein Mcf-A67 (see results). The synthetic peptide (3.5 mg) was conjugated with 5 mg of keyhole limpet hemocyanin (KLH) using MBS (m-maleinidobenzoyl-N-hydroxysuccinide ester). Mcf-A67^{pep}-KLH was used as antigen.

Immunization of rabbits

Two rabbits (race New Zealand) were individually immunized intradermically (i.d.) with 200 μg of McfA67^{pep}-KLH in 1 ml of a 1 : 1 mixture of conjugate in PBS (10 mM sodium phosphate buffer containing 150 mM NaCl) and Freund's complete adjuvant. The rabbits were boosted four times at two week intervals by i.d. (same mixture as above) and usually bled one week after the third and subsequent injections. According to dates of taking a blood sample, antisera ΣA67 (ΣA67 #1, #2 and #3) were obtained after centrifugation at 3500 *g* (1 h, 37 °*C*). A last sample (ΣA67 #4) was taken during the bleeding of the rabbits. Antisera were stored in 1 ml fractions at −20 °C without preservative.

Dot-Blots

Serum titration from total soluble protein extracts
Antisera ΣA67 #1, #2 and #3 were tested against *M. incognita*, *M. javanica*, *M. arenaria*, *M. mayaguensis*, *M. hapla* race B, *M. fallax* and *M. chitwoodi* total soluble protein extracts. Protein concentrations ranged between 500 and 8 ng μl^{-1} (500, 250, 125, 62, 31,

16 and 8 ng μl^{-1}). Proteins were applied to nitrocellulose membranes (Bio-Rad, Richmond, USA; 0.2 μm pore size) as 1 μl spots (in duplicate). Protein spots were air-dried at room temperature and the membranes incubated overnight at 4 °C on a shaker in a saturation buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, 1% milk powder). Then they were washed three times with TNT (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) and TN buffers (10 mM Tris, 150 mM NaCl, pH 7.5). The blocked antigens were incubated for 2 h with diluted test ΣA67 #1, #2 and #3 antisera (1 : 4000, 1 : 8000 and 1 : 16000) in saturation buffer at 37 °C on a shaker at 50 rpm. Afterwards, the nitrocellulose membranes were thoroughly washed with the two washing buffers. The membranes were then incubated for 2 h at 37 °C with an optimal dilution (1 : 1000) of affinity purified alkaline phosphatase-labelled goat anti-rabbit IgG (Sigma, Saint Louis, USA) in saturation buffer and then washed in TNT followed by TN. Bound antibody was visualized by incubating the membranes at room temperature in an alkaline phosphatase solution comprising 0.48 mM nitro blue tetrazolium, 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate supplemented with 59.3 mM $MgCl_2$ and 10 mM Tris–HCl, pH 9.2. The substrate solution was decanted when spots were visible but before excessive background staining occurred. Dot-blots were washed and dried between sheets of filter paper. Assays were performed three times.

Serum titration from soluble proteins extracted at the individual level

M. incognita, *M. javanica*, *M. arenaria*, *M. mayaguensis*, *M. hapla* race B, *M. fallax* and *M. chitwoodi* females were individually crushed in an ice bath in 3 μl of a homogenizing solution (Tastet et al., 1999). Proteins were applied to nitrocellulose membranes (Bio-Rad, Richmond, USA; 0.2 μm pore size) as 2 μl spots (in duplicate). PBS was used as a negative control. ΣA67 #2 and ΣA67 #3 antisera were tested at dilutions of 1 : 4000, 1 : 8000, 1 : 16000, 1 : 24000 and 1 : 32000.

Electroblotting

Following SDS-PAGE, proteins were electrophoretically blotted onto 0.2 μm pore size nitrocellulose sheets (7 × 8.4 cm), using the Bio-Rad cell system, with a transfer buffer of 288 mM glycine and 37.5 mM Tris mixed in a ratio of 3 : 1 with methanol. Transfer was performed at a constant voltage of 80 V for 55 min at 4 °C. To visualize and confirm transfer of separated proteins, western-blotted membranes were stained by immersion for 10 min in a 5% acetic acid, 0.5% Ponceau S solution, followed by a brief rinse in TBS (150 mM NaCl solution containing 5% (v/v) 1 M Tris–HCl, pH 8). While the Ponceau S in the membrane background washes out, protein spots appear as red spots. Destaining was performed in large volumes of TBS. Immunodetection was carried out as described above with the antiserum ΣA67 #2 diluted at 1 : 1000 in the saturation buffer.

Results

Analysis of the 2-D PAGE profile of Meloidogyne *spp. proteins*

The results indicated that the polypeptides from the females could be separated and analysed by 2-D electrophoresis. More than 250 spots were detected on the gels when the total soluble proteins were electrophoresed; the isoelectric point (*I*p) ranged from 5.5 to 7.5 and the molecular weight (MW) from 14 to 94 kDa.

The 2-D profiles revealed a great variation in the proteins within the *Meloidogyne* species examined. 2-D gels from *M. chitwoodi* and *M. fallax* showed a high similarity. The same applied to the five tropical *Meloidogyne* species *M. incognita*, *M. javanica*, *M. arenaria*, *M. mayaguensis* and *M. arabicida*. Electrophoretograms from *M. exigua* and *M. naasi* were most distant from this group. The 2-D gel obtained from *M. hapla* was characterized by a larger number of protein spots when compared with all other *Meloidogyne* species.

Despite this protein variation, the comparison between the different species allowed the identification of two main protein spots that were present on the gels of the two quarantine nematodes *M. chitwoodi* and *M. fallax*, but absent on the 2-D gels from the eight other species (Figure 1). These proteins, labelled Mcf-A67 and Mcf-B66, were localized in a zone with a MW ranging from 14 to 20 kDa, and *I*p from 6.1 to 6.3 (rectangular boxes, Figure 1). This zone could be positioned with precision by the protein spot of actin, which was observed on all the electrophoretograms. Actin has been identified in the cyst nematode *Globodera rostochiensis* as a spot of MW 43000 Da and *I*p 6.2 (de Boer, 1996), corresponding to the spot indicated by the open arrow head in Figure 1.

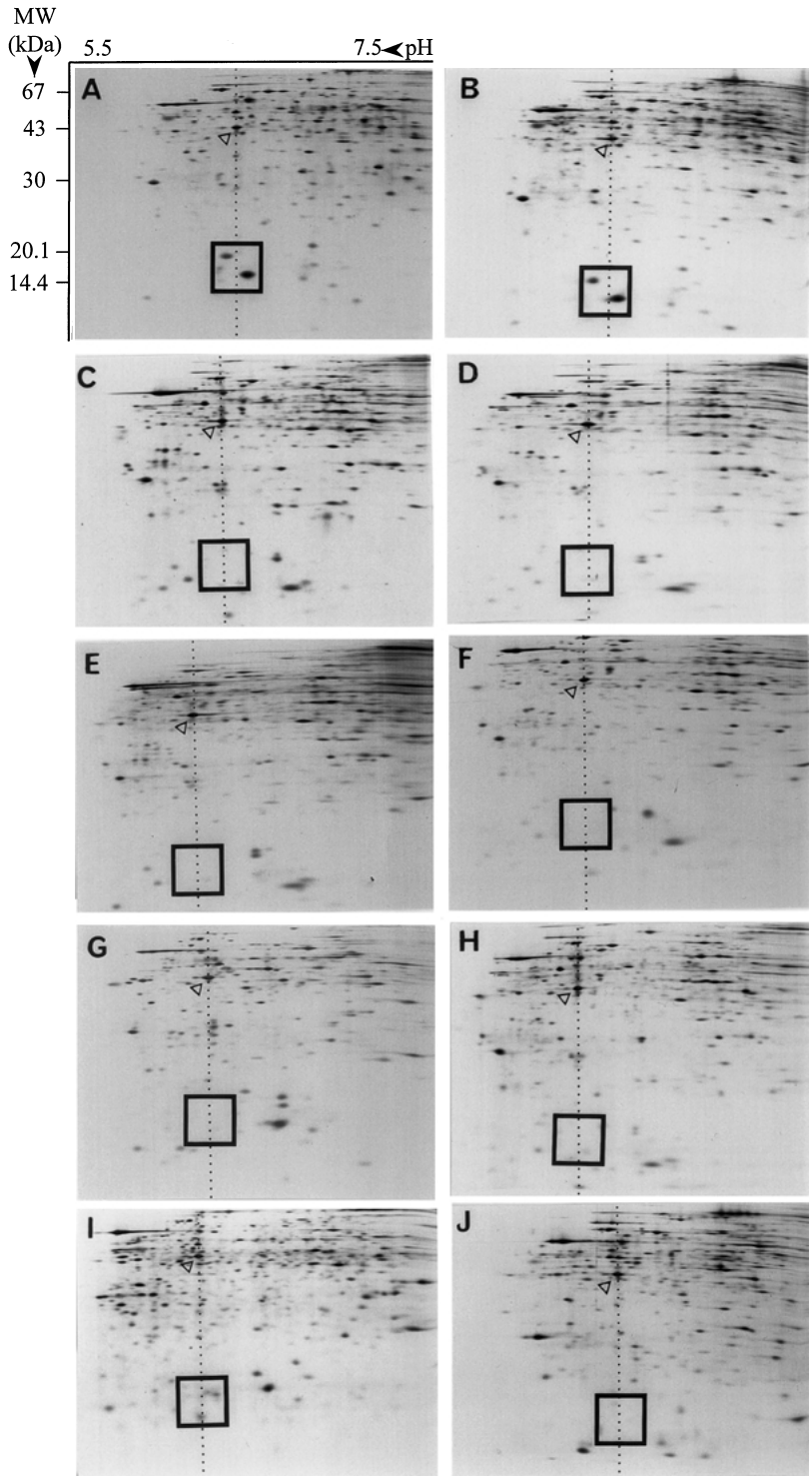

Figure 1. Typical protein patterns from two-dimensional gel electrophoresis of *Meloidogyne chitwoodi* (A), *M. fallax* (B), *M. incognita* (C), *M. javanica* (D), *M. arenaria* (E), *M. mayaguensis* (F), *M. exigua* (G), *M. arabicida* (H), *M. hapla* (I) and *M. naasi* (J). Open arrow head points to the proteinic spot of actin.

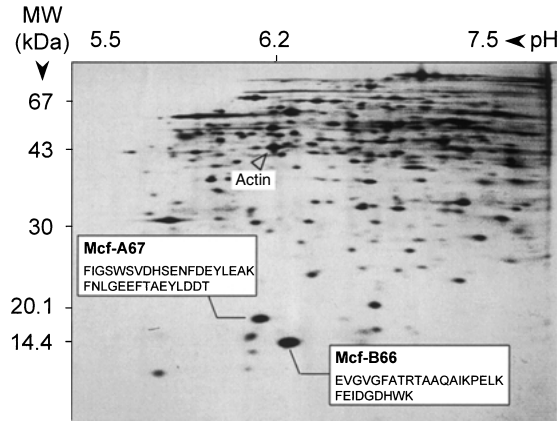

Figure 2. Internal amino acid sequences of the two protein markers, Mcf-A67 and Mcf-B66, for *M. chitwoodi* and *M. fallax*.

Identification of proteins by microsequencing

After enzymatic digestion, 21 and 24 peaks were obtained for the two common *M. chitwoodi*/*M. fallax* protein spots, Mcf-A67 and Mcf-B66, respectively. Two peaks per protein were microsequenced (Figure 2) and the sequences obtained were compared with sequences in available databases (Table 2). The two internal amino acid sequences from the protein spot Mcf-A67 showed homologies with fatty-acid binding proteins (FABPs). The two best scores from the first sequence matched with a FABP from the free living nematode *Caenorhabditis elegans*, whereas the second sequence corresponded to a FABP from *Schistocerca gregaria*. Spot Mcf-B66 had homology with different proteins. The first sequence corresponded to an allergen protein and an adipocyte FABP, respectively for the two best scores. The last sequence matched with a trans-cinnamate 4-monooxygenase from *Lycopersicon esculentum*, and a NRDH-redoxin from *Corynebacterium ammoniagenes* (Table 2).

Sensitivity and specificity of antisera ΣA67

The first sequence of the protein Mcf-A67 (20 aa, Table 2) was used as antigen after conjugation with KLH. The first experiment was conducted to test the obtained antisera (ΣA67 #1, #2 and #3) on total soluble protein extracts from adult females. Dot-blot assays results showed a positive reaction with the two quarantine species *M. chitwoodi* and *M. fallax* without significant cross reactions with the five other species examined (Figure 3). The rate of cross reactions decreased with increasing dilution factors (e.g. ΣA67 #1, Figure 3). Moreover, the antisera ΣA67 #2 and ΣA67 #3 were more specific than ΣA67 #1: nearly no cross reactions with all other species were observed (Figure 3).

In addition to the dilution of the antisera, a range of concentrations of the protein deposits from 8 to 500 ng μl^{-1} made it possible to determine the thresholds of detection of the antisera tested. For the antiserum ΣA67 #1, a minimal quantity of 8 ng of proteins was necessary to detect *M. chitwoodi* and *M. fallax* when the dilution factor of the antiserum was 1 : 4000, against 62 ng at 1 : 8000 and 1 : 16000 (Figure 3). Concerning antisera ΣA67 #2 and ΣA67 #3, regardless of the dilution (1 : 8000 or 1 : 16000), ΣA67 #2 appeared less sensitive than ΣA67 #3: the thresholds of detection were about 16 and 8 ng of proteins, respectively (Figure 3).

In an attempt to develop a reliable method of quarantine nematode diagnostic, antisera ΣA67 #2 and #3 were tested in a second experiment on soluble proteins from a single adult female. Although weak cross reactions were observed with all species examined, the spots corresponding to *M. chitwoodi* and *M. fallax* were the most strongly stained, regardless of the dilution of antisera (Figure 4). With the highest dilution factors 1 : 24000 and 1 : 32000, these cross reactions were nearly no longer visible (Figure 4).

Western blotting

The antiserum ΣA67 #2, tested for hybridization with total soluble protein extracts from females of *Meloidogyne* spp. showed no reaction on *M. javanica*, *M. incognita*, *M. arenaria* and *M. mayaguensis* western blots (Figure 5, only shown for *M. javanica*). However, it hybridized with several bands, with molecular masses of 43 kDa and more, on the *M. chitwoodi* and *M. fallax* western blots. A major protein band was stained with MW of 43 kDa and an approximate *I*p of 5.9. No hybridization was observed with Mcf–A67.

Discussion

The principal aim of this study was to detect proteins which discriminate the two quarantine nematodes species *Meloidogyne chitwoodi* and *M. fallax*, from other *Meloidogyne* species. The comparison of 2-D patterns from the ten *Meloidogyne* species revealed significant differences in protein expression. The species

Table 2. Homologous proteins identified after internal amino acid sequencing of two *M. chitwoodi/M. fallax* common proteins

Analysis of proteins from 2D gels		Homologous proteins	Score observed	Z score	E value	Identity (%)	Number of aa	Residues in identified proteins	MW (kDa)	pI	Organisms	Accession number
Protein	Sequence of peptide											
Mcf-A67	FIGSWSVDHSENFDEYLEAK	Similar to fatty acid binding protein and others of FABP/P2/CRBP/CRABP family of transporter proteins	100	222.4	4.9E−05	70.00	159	24–43	18.28	8.50	*Caenorhabditis elegans*	U40420
		Similar to fatty acid binding protein and others of FABP/P2/CRBP/CRABP family of transporter proteins	100	222.3	5.0E−05	70.00	161	26–45	18.84	6.22	*Caenorhabditis elegans*	U40420
	FNLGEEFTAEYLDDT	Fatty acid-binding protein, muscle (M-FABP)	56	141.9	1.60	69.23	133	66–78	14.94	6.20	*Schistocerca gregaria*	P41496
		Fatty acid binding protein	56	141.9	1.60	69.23	133	66–78	14.94	6.20	*Schistocerca gregaria*	M95918
Mcf-B66	EVGVGFATRTAAQAIKPELK	Allergen	78	172.7	2.9E−02	55.00	130	21–41	14.79	5.62	*Blomia tropicalis*	U58106
		Adipocyte fatty acid binding protein	78	172.7	2.9E−02	68.42	131	22–41	14.57	7.92	*Rattus norvegicus*	P70623
	FEIDGDHWK	Trans-cinnamate 4-monooxygenase (fragment)	53	134.7	4.00	66.66	67	32–40	8.05	9.60	*Lycopersicon esculentum*	Q42895
		NRDH-redoxin	53	134.0	4.40	71.42	75	55–61	8.29	5.15	*Corynebacterium ammoniagenes*	O69271

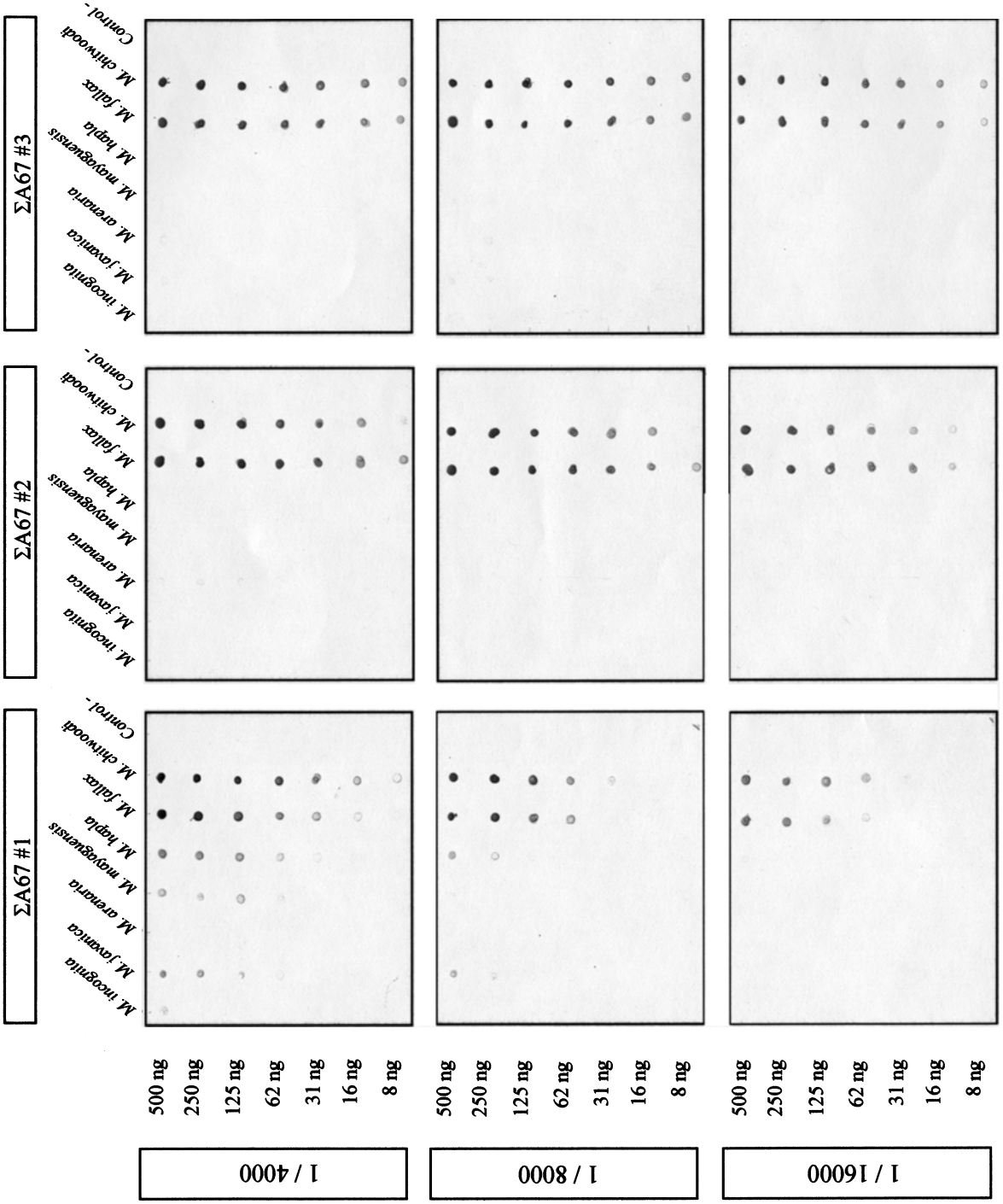

Figure 3. Specificity and sensitivity of the antisera ΣA67: determination of detection limit for *Meloidogyne* spp. isolates with different antisera dilutions and total soluble protein concentrations (ng μl^{-1}).

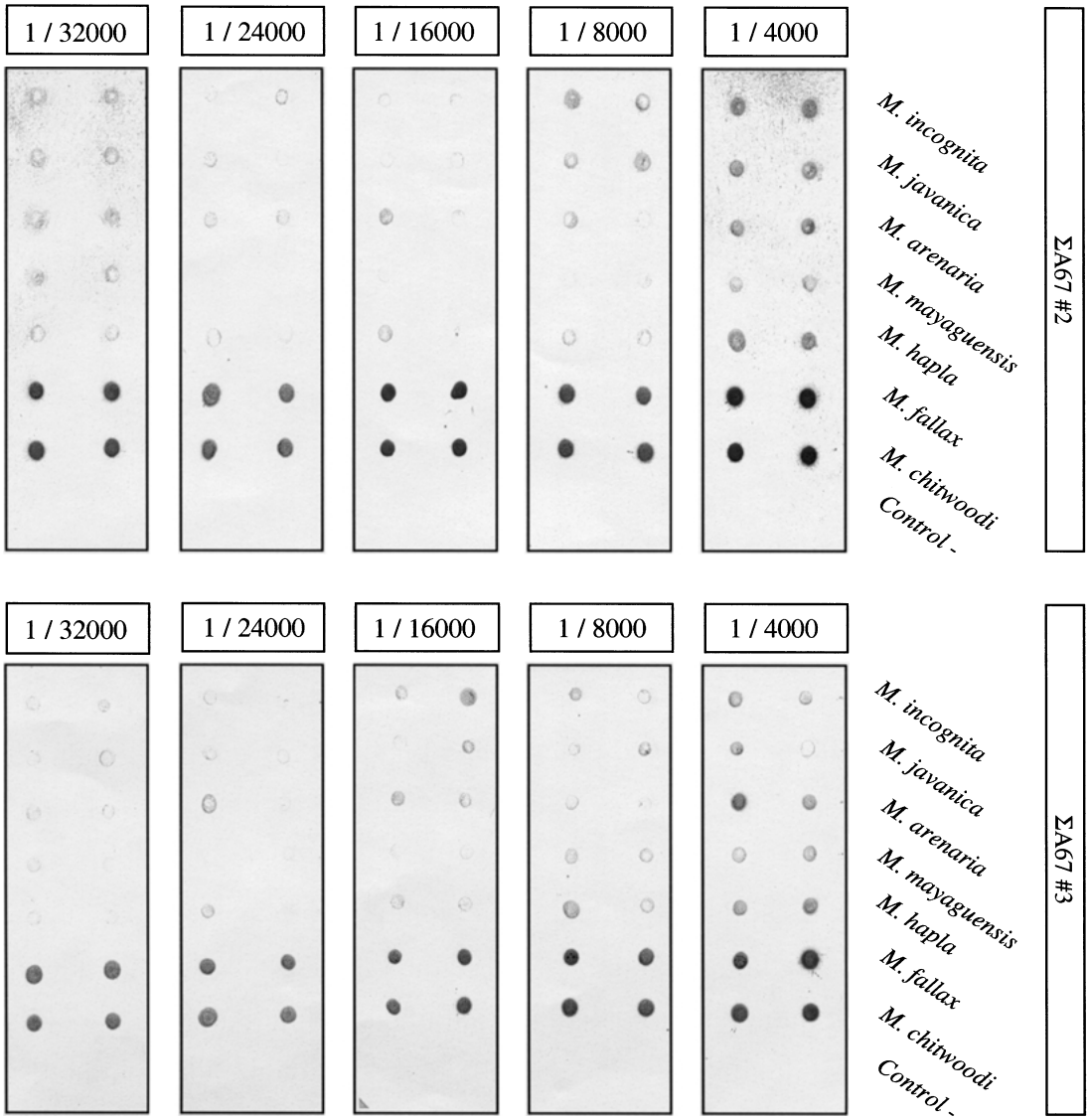

Figure 4. Specificity and sensitivity of the antisera ΣA67 #2 and #3 on protein extracts from a single *Meloidogyne* spp. female. Different antisera dilutions were used.

can be grouped according to 2-D pattern similarities. The similarities coincide with the mode of reproduction and geographical distribution. *M. fallax* and *M. chitwoodi*, which are facultative meiotic parthenogenetic nematodes and which occur in temperate climates, showed high similarity. These results confirm observations by van der Beek et al. (1998) and Tastet et al. (1999). Also the 2-D patterns of *M. incognita*, *M. javanica*, *M. arenaria*, *M. mayaguensis* and *M. arabicida*, which reproduce by mitotic parthenogenesis (Triantaphyllou, 1985) and are more prevalent in warm climates, were similar.

The 2-D protein profiles of *M. exigua*, *M. naasi* and *M. hapla*, on the other hand, were very different from all the others. Those of *M. exigua* and *M. naasi* revealed a distribution of the protein spots that prevent them being assigned them to either the *M. chitwoodi* or *M. incognita* groups. These differences are particularly evident for the low molecular weight proteins. *M. exigua* differs from the five other tropical species

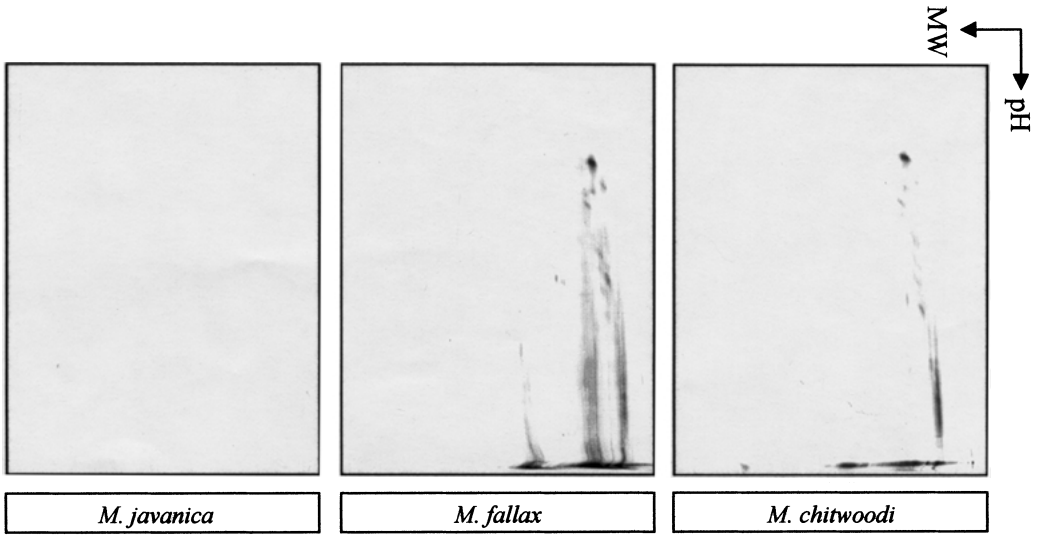

Figure 5. Two-dimensional western blots of total soluble protein extracts from females of *M. chitwoodi*, *M. fallax* and *M. javanica* stained with the ΣA67 #2 antiserum (dilution: 1 : 1000).

(*M. incognita*, *M. javanica*, *M. arenaria*, *M. mayaguensis* and *M. arabicida*) in that it reproduces by meiotic parthenogenesis (Triantaphyllou, 1985).

M. naasi, *M. chitwoodi* and *M. fallax* are sympatric species with the same mode of reproduction. However, *M. naasi* is clearly a distinct species; it has a narrow host range, reproduces preferentially on monocotyledons and has an unique ITS size higher than the other *Meloidogyne* species (Mugniéry, pers. com.). *M. hapla*, known as the northern root-knot nematode, is distinguishable from the nine other species examined by a greater number of protein spots. *M. hapla* race B is often geographically associated with *M. chitwoodi* and *M. fallax*, but reproduces by obligatory mitotic parthenogenesis (Triantaphyllou, 1985). In spite of these protein expression differences, the *M. fallax* and *M. chitwoodi* patterns revealed two proteins, Mcf-A67 and Mcf-B66, which can be used as 'quarantine markers'.

Based on internal amino acid sequencing, Mcf-A67 and Mcf-B66 showed homologies with members of a family of low molecular weight lipid binding proteins, which include fatty-acid binding proteins (FABP) (Ockner, 1990), adipocyte P2 and myelin P2 proteins, and cellular retinol/retinoic acid binding proteins (Kaikaus et al., 1990).

The two internal amino-acid Mcf-A67 sequences allow the identification of FABPs regardless of the sequence submitted on databases. For Mcf-B66, the first internal amino-acid sequence showed homologies with an allergen from *Blomia tropicalis* and an adipocyte FABP from *Rattus norvegicus*. With exception of the allergen match, more than thirty scores on databases show homologies with different FABP (data not shown). The second Mcf-B66 peptide sequence is too short (9 amino-acid residues) to obtain significant homologies scores on databases. Not enough amino-acids were submitted to validate the trans-cinnamate 4-monooxygenase and NRDH-redoxin identification.

The exact function of cytosolic FABPs is not yet clear. They are involved in the transport and the metabolism of long-chain fatty acids (Glatz and van der Vusse, 1996), but FABPs may have more diverse functions, like modulation of the cell growth (Kaikaus et al., 1990) and differentiation (Glatz et al., 1993). Such proteins have been identified in mammalian and non-mammalian species (Di Pietro et al., 1999), including the parasitic nematodes *Ascaris suum* (Mei et al., 1997) and the trematodes *Schistosoma mansoni* (Moser et al., 1991) and *Fasciola hepatica* (Rodriguez-Pérez et al., 1992). Because FABPs are ubiquitous and involved in cell physiology, these proteins should also be present on the 2-D gels of non quarantine species. On these gels, proteins with molecular weights similar to those of Mcf-A67 and Mcf-B66, but with different isoelectric points that may have resulted from amino acid substitutions or post-translational modifications, were highly expressed. In order to confirm that Mcf-A67 and Mcf-B66 are FABPs and identify similar proteins in the non quarantine species, labelled free fatty acids could be

used as reporters of the binding site of these proteins (Kim and Storch, 1992; Wootan et al., 1993; Hsu and Storch, 1996).

Among the family Heteroderidae, which includes the genus *Meloidogyne*, the immunological differentiation of the two potato cyst nematodes *Globodera pallida* and *G. rostochiensis* has been well developed (e.g. Schots et al., 1987, 1989). Our serological results allowed for the first time the discrimination of the two European quarantine *Meloidogyne* species *M. chitwoodi* and *M. fallax* from other root-knot nematodes. These results are particularly interesting for the development of a serological detection kit. The specificity and sensibility of the ΣA67 antisera can be explained by the fact that a synthetic peptide coupled to KLH, and not a whole protein, was used for immunization. However, the protein Mcf-A67 was not detected on the *M. chitwoodi* and *M. fallax* western blots. This may be explained by the characteristics of the immunogenecity with carrier. The produced antibodies may recognize only particular conformations which can be modified after electrophoresis and electroblotting. Nevertheless, the cross reactions observed on the *M. chitwoodi* and *M. fallax* western blots cannot be explained.

The use of serological diagnostics to identify the two quarantine nematodes, *M. chitwoodi* and *M. fallax*, appears to be an attractive solution. Traditional identification, based on morphometric characters (e.g. Eisenback and Hirschmann, 1981) and differential hosts (Sasser and Carter, 1985) are lengthy and time consuming, and require highly trained personnel. In addition, 2-D electrophoresis cannot be used as a fast and a routine quarantine diagnostic method. Thus, the advantage of serological diagnosis lies in the low technicality of the technique, its rapidity and low cost. Combined with the specificity and the sensitivity of the antiserum ΣA67, these criteria are important for efficient and accurate quarantine diagnosis. Moreover, in spite of 2-D protein profiles differences according to developmental stages, preliminary results (not shown) indicate that the antiserum ΣA67 could be used to discriminate *M. chitwoodi* and *M. fallax* second stage juveniles from other *Meloidogyne* species. However, in this case, the experimental procedures will have to be improved.

The challenge now is to produce an antiserum in order to make it possible to distinguish *M. chitwoodi* from *M. fallax*. In a previous study, nine discriminative proteins for each of the two species have been identified (Tastet et al., 1999). Among these, some proteins might be used in the future as antigens to produce specific detection kits.

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