Available online at www.sciencedirect.com
SCIENCE DIRECT
Infection, Genetics and Evolution 5 (2005) 167–175
Infection, Genetics and Evolution
www.elsevier.com/locate/meegid

First analysis of the proteome in two nematomorph species, *Paragordius tricuspidatus* (Chordodidae) and *Spinochordodes tellinii* (Spinochordodidae)

David G. Biron[a,*], Cécile Joly[a], Laurent Marché[b], Nathalie Galéotti[d], Vincent Calcagno[a], Andreas Schmidt-Rhaesa[c], Lionnel Renault[b], Frédéric Thomas[a]

[a] *GEMI, UMR CNRS/IRD 2724, IRD, 911 Av. Agropolis BP 64501, 34394 Montpellier Cedex 5, France*
[b] *INRA, UMR BiO3P, Domaine de la Motte, BP 35327, 35653 Le Rheu Cedex, France*
[c] *Department of Zoomorphology and Systematics, Faculty of Biology, University of Bielefeld, P.O. Box 100131, D-33501 Bielefeld, Germany*
[d] *LGF 2580-CNRS, 141 rue de la Cardonille, 34094 Montpellier Cedex 5, France*

Received 11 June 2004; received in revised form 10 September 2004; accepted 13 September 2004
Available online 5 November 2004

Abstract

The proteome of most parasite species is currently unknown. Hairworms (Nematomorpha), 300 species distributed around the world, are parasitic in arthropods (mainly terrestrial species) when juveniles, but they are free-living in aquatic environments when adult. Most aspects of their systematics and biology are currently unknown. The aim of this paper was (i) to report a novel and reproducible protocol for the analysis of the proteome of hairworms using two-dimensional gel electrophoresis (2-DGE) and mass spectrometry (matrix laser desorption ionization-time of flight mass spectrometry (MALDI-TOF)) and (ii) to determine the level of proteomic divergence between two sympatric but taxonomically unrelated nematomorph species in the adult stage, *Paragordius tricuspidatus* Dufour (Nematomorpha, Gordiidae) and *Spinochordodes tellinii* Camerano (Nematomorpha, Gordiidae). In total, 689 protein spots were observed for *P. tricuspidatus*, 575 for *S. tellinii*. Only 36.2% spots were shared between the two species. Quantitative analysis of the proteins which are common to both parasite species reveals substantial differences in the pattern of protein expression. These results suggest a rapid evolutionary divergence between these two nematomorph families. Also, to test the value of our MALDI-TOF protocol, we used Actin-2 (Act-2), a protein highly conserved in the course of evolution. Peptide mass fingerprint (PMF) data obtained for Act-2 of *P. tricuspidatus* and *S. tellinii* suggest a very high homology with Act-2 of different worms species belonging to the Bilateria phylum (Annelida and Nematoda) and more specifically to *Lumbricus terrestris* (Annelida, Lumbricidae) and *Caenorhabditis elegans* (Nematoda, Rhabditidae). We discuss our results in relationship with current ideas concerning the use of proteomics in systematics.

Keywords: Parasitoproteomics; Nematomorpha; Proteome; Systematics

1. Introduction

Understanding the biological mechanisms of parasitic strategies is not only of interest to parasitologists, but also to those involved in infectious diseases control strategies, evolutionary ecology and medicine (Thomas et al., 2002a). Among approaches that focus on proximate causes, the recent interest of scientists in proteomics is due to several factors, including firstly the fact that DNA sequences per se provide little information on the dynamic processes involved between a parasite and its host. In addition, although definition of the transcriptome using DNA array technology allows the detection of differential gene expression, the correlation between this information and levels of translated proteins often remains low (Anderson and Seilhaver, 1997; Gygi et al., 1999; Maniatis and Tasic, 2002). Because the proteome encompasses, at any given time, all proteins that are expressed in a biological system (cell, tissue, organ, etc.), proteomics is viewed as one of the main streams of science

* Corresponding author. Tel.: +33 4 67 41 63 18; fax: +33 4 67 41 62 99.
E-mail address: biron@mpl.ird.fr (D.G. Biron).

1567-1348/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2004.09.003

in the so-called 'post-genomic era' (Hochstrasser, 1998; Ashton et al., 2001; Barret et al., 2002; Fields, 2001). Finally, biochemical techniques like proteomics can also be particularly useful for the systematics of morphologically similar species, and/or when environmental factors influence classical taxonomic characters (Platzer, 1981; Fullaondo et al., 2001; Navas and Albar, 2004). Despite the promising perspectives offered by 'parasitoproteomics', the proteome of most parasite species is currently unknown. The 'parasitoproteomics' is the study of the reaction of the host's and parasite's genomes via the expression of the host's and parasite's proteomes (genome operating system) during their complex biochemical cross-talk.

In this paper, we report for the first time on a reproducible protocol for analysis of the proteome of hairworms (Nematomorpha) by two-dimensional gel electrophoresis (2-DGE) and mass spectrometry (matrix laser desorption ionization-time of flight mass spectrometry (MALDI-TOF)). We then analyse and compare the proteomes of two hairworm species in the adult stage, *Paragordius tricuspidatus* and *Spinochordodes tellinii*, just after they emerge from their host. The molecular (based on 18s rRNA) and morphological systematics suggest (i) monophyly for the Nematomorpha Order; (ii) a sister-groups relationship between Nematomorpha and Nematoda; (iii) a sister-groups relationship between the nematomorph marine genera and (iv) *P. tricuspidatus* and *S. tellinii* are not be closely related within the freshwater Nematomorpha (Gordiida) (Bleidorn et al., 2002). Based on these informations, we can predict that we will observe an important differential expression between the proteome of *P. tricuspidatus* and *S. telliniii*.

The Gordiida is a poorly studied taxonomic group (Schmidt-Rhaesa, 1997; Schmidt-Rhaesa et al., 2003). When juvenile, they are parasitic in arthropods, mainly terrestrial insects (Schmidt-Rhaesa, 1997, 2001). Hosts become infected when they ingest parasitic larvae, directly or indirectly through a paratenic host (Hanelt and Janovy, 1999; Schmidt-Rhaesa, 2001). During their development, the parasite grows from a microscopic cyst to a large worm whose size exceeds that of the host by a significant amount. When they reach this stage, nematomorphs must emerge into an aquatic habitat (e.g. streams) to reproduce. Previous studies have shown that mature hairworms manipulate the behaviour of their host, effectively making them jumping into water (Thomas et al., 2002b, 2003). After the emergence from the host, adult nematomorphs are free-living and aquatic, and gather to mate in tight masses called a 'Gordian knot'.

2. Materials and methods

2.1. Sampling

During July and August 2002, many crickets, *Nemobius sylvestris* Bosc (Orthoptera, Gryllidae), infected with *P. tricuspidatus* and many grassshoppers, *Meconema thalassinum* De Geer (Orthoptera, Tettignoidae), infected with *S. tellinii* Camerano (Nematomorpha, Gordiidae) were captured following published protocols (Thomas et al., 2003) at night between 22:00 and 01:00 h around a swimming pool (15 m × 10 m in area) located in Avenes les Bains (Southern France, 70 km north of Montpellier). This pool is located near a forest criss-crossed by small streams in which adult nematomorphs were commonly found during the summer. Between the pool and forest, a 5 m wide area allowed the direct observation and capture of behaviourally manipulated crickets and grasshoppers, which attempted to reach the pool (Thomas et al., 2003). Infected *N. sylvestris* and *M. thalassinum* were then placed individually in a glass of freshwater to cause the release of the parasitic worm. Immediately after emergence, worms of both species were preserved in liquid nitrogen until analysis. To avoid the possible effects of multiple infection, or host- and/or parasite-sex specific factors on the proteomic expressions of the two parasite species (Thomas et al., 2002a), only adult males in single infection and emerging from a male insect host were considered.

2.2. Protein extraction

For both nematomorph species, five worms were cut into fine equal pieces on an ice bath and under sterile conditions. The samples were put in a microcentrifuge 1.5 ml tube at 4 °C. To recover the water-soluble proteins and to avoid the risk of host contamination outside the parasite tissues, each sample was rinsed three times in a 10 mM Tris–HCl solution, pH 7.4. For each species, samples were crushed using an electric mini-grinder in 40 μl of an homogenizing solution (15 M urea, 10 mM Tris–HCl, pH 7.4, 5% (v/v) β-mercaptoethanol, 2% ampholytes, pH range 5–8) in an ice bath (Bossis and Mugniéry, 1993; Marché, 2003). The concentration of each protein sample was estimated by measuring the shift of extinction of Coomasie Blue G-250 at a wavelength of 595 nm (Bradford, 1976). Protein concentration was standardized at 2 μg/μl by the addition of the required volume of the homogenizing solution. The protein samples were stored at −70 °C prior to electrophoretic separation on 2-DGE.

2.3. First dimension

One (first)-dimensional BioRad IPG strips of pH 5–8 were rehydrated in passive conditions (0 V, 20 °C) during 10 min in a denaturation solution (7 M urea, 2 M thiourea, 4% Chaps, 0.24% Triton X100, 20 mM DTT, 4% carrier ampholytes pH 5–8). The extracted protein (40 μl) were added to this solution, resulting to 80 μg nematomorph proteins per gel. Following the passive rehydration and after recovering the IPG strips with mineral oil, an active rehydratation was performed at 0–200 V for 1 h, 1000 V for 1 h and 10 000 V, finally reaching a maximum 80 000 V/h.

After isoelectric focusing (IEF), the IPG strips were incubated at room temperature for 15 min in an equilibration buffer (6 M urea, 2% (w/v) SDS, 20% (w/v) glycerol, 375 mM Tris–HCl, pH 8.8, 130 mM DTT). A second equilibration step was perfomed for 20 min in the same buffer, except that DTT was replaced by 135 mM iodoacetamide. The IPG strips were rinsed with de-ionized water for 1–2 s. At least two IPG strips of pH 5–8 were run per hairworm species.

2.4. Second dimension

Following the two rehydratation steps, the IPG strips were laid on the top of the 1.5 mm vertical second-dimensional separating gel (BioRad vertical system) (10% T, 1.5 M Tris–HCl, pH 8.8, 10% SDS, acrylamide/PDA). Electrophoresis in the second dimension was performed at 30 mA/gel for 20 min, followed by 60 mA/gel. SDS–PAGE was halted when the bromophenol blue tracking dye had reached the bottom of the gel. Gels were stained using the tetrathionate–silver nitrate technique (Oakley et al., 1980; Rabilloud et al., 1994).

2.5. Analysis of 2D-gels

The 2D gels obtained for the two nematomorph species were digitized using a BioRad scanner. At least two replicated pictures were conserved per species. Replicated gels for the same treatment were compared using Melanie-IV software (GeneBio, Geneva, Switzerland). The best gels obtained for each treatment were then used to build a 2D synthetic gel for each nematomorph species. The selection criterion for these gels was the absence of significant distortion, a low background and good staining. The p*I* and MW scales of 2D gels were determined using a protein standard kit from BioRad (2D SDS–PAGE standards). These unique standards provide calibrated references for 2D SDS–PAGE applications and are analysed by Melanie IV to elaborate the p*I* and MW scales.

The Melanie IV software was also used to compare the protein patterns observed for *P. triscuspidatus* and *S. tellinii*. Melanie IV allows background subtraction, spot detection, quantification and spot matching across different gels. Three quantitative measures are available for each spot: 'Od' (the highest calibrated pixel intensity in the protein spot), 'Area' (protein spot's area in mm^2), and 'Volume' (integration of 'Od' over the spot's area. We used the normalized volume data for the common protein spots (groups). Melanie IV takes into consideration the gel variation in making a ratio for each common protein spots based on the sum of 'Volume' for all groups. By definition:

$$\mathrm{Vol}(\%) = \frac{\mathrm{Vol}}{\sum_{S=1}^{n} \mathrm{Vol}_S} \times 100,$$

where Vol_S is the volume of spot S in a gel containing n spots.

In order to analyze gel similarities between *P. tricuspidatus* and *S. tellinii*, a 2D scatter plot was done on common protein spots with the Melanie IV software. This approach is possible because 'spot-by-spot' quantitative comparison between two treatments may be summarized as 2D scatter plots characterized by a linear fit ($y = ax + b$) and a correlation coefficient, r (Salekdeh et al., 2002). In the case of the above scatter plot, variable x is the spot value (%Vol) from *S. tellinii* gel and y is the corresponding value in the *P. tricuspidatus* gel. For a high correlation (similarity) between the expression of the common proteins of the two hairworms, 'r' and 'a' should be closed to unity and 'b' to zero. A two-tailed Student's test was performed to compare the slopes (a), the intercept values (b) and correlation coefficients with theorical values, 1 for 'a' and 0 for 'b' and 'r'.

It is difficult to homologize loci among populations and/or species using 2DE, so the generally employed genetic distance methods could not be employed. Instead, we used the association coefficient $F = 2n_{xy}/(n_x + n_y)$ where n_x and n_y are the number of proteins spots scored in species x and y, respectively, and where n_{xy} is the total number of protein spots shared by both species x and y. The genetic divergence between species in this study is $1 - F$ (Thomas and Singh, 1992).

2.6. Protein identification by MALDI-TOF mass spectrometry

New gels with the candidate protein spots were run and were silver stained according to the procedure of Shevchenko et al. (1996). Candidate protein spots were excised manually and digested in gel using trypsin (sequencing grade, Promega, Madison, WI), as previously described (Shevchenko et al., 1996; Lee et al., 2002). Digest products were completely dehydrated in a vacuum centrifuge and resuspended in 10 μl formic acid (2%, v/v), desalted using Zip Tips C18 (Millipore, Bedford, MA), eluted with 10 μl acetonitrile:trifluoroacetic acid (80:0.1%) and concentrated to a 2 μl volume. Analyte solutions (0.3 μl) were mixed with the same volume of α-cyano-4-hydroxy-*trans*-cinnamic acid (10 mg/ml in acetronile:trifluoroacetic acid, 50:0.1%) and loaded on a MALDI target (384) using the Dry-droplet procedure (Karas and Hillenkamp, 1988). Analysis was performed using an UltraFlex MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) in a reflectron mode with an accelerating voltage of 20 kV and a delayed extraction of 70 ns. Mass spectra were acquired in an automatic mode using the AutoXecuteTM software from Flexcontrol™ (Bruker-Franzen Analytik, Bremen, Germany) (laser power from 35 to 75%, 900 shots).

Protein identification was obtained by conducting a database search of the peptide mass generated from MALDI analysis. Monoisotopic peak lists were imported into PeptIdent (http://www.expasy.org/tools/pepident.html) and

Protein Prospector (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) softwares with the following search parameters: (i) 'OTHER METAZOA' in species field for PepIdent and 'Codes Species' of all worms from SwissProt in species field of protein Prospector; (ii) p$I \pm 2.0$; (iii) Mw $\pm$ 20%; (iv) one missed cleavage; (v) tryptic digestion; (vii) carbamidomethylation as a cysteine modification; (viii) oxidation of methionine (Wilkins and Williams, 1997). Search tolerance was set at 100 ppm with a MH+ charge state. Taking into consideration the possibility of molecular crosstalk between the cricket and the hairworm via the synthesis of mimetic proteins or of host contamination, we performed protein searches with all categories of the host-parasite system. In result of interrogation, proteins with the highest score, a minimum of miss cleavages and a p*I*/Mw theorical near to the p*I*/Mw experimental were retained. Matching peptides with missed cleavages were considered as pertinent only when there were two consecutives basic residues or when arginine and lysine residues were followed by a proline or acidic residues inside the peptide amino acid sequence.

3. Results and discusssion

In our experimental conditions, at least 80 μg proteins were needed in order to produce 2D gel patterns. With the protocol of protein extraction used, enough proteins were extracted per sample to run five such gels. Fig. 1A shows a 2D gel for *P. tricuspidatus* while Fig. 1B shows the same for *S. tellinii*. In total, 689 protein spots were observed for *P. tricuspidatus*, 575 for *S. tellinii*. The numbers of detected spots are in a good range in comparison to proteome revealed for phytophagous nematode species, *Meloidogyne* spp. (Nematoda: Heteroderidae) at larval stages: ~700 protein spots (Navas et al., 2002; Marché, 2003).

In Fig. 2A, a synthetic gel obtained from comparison between the proteomes of *P. tricuspidatus* and *S. tellinii* is shown. A total of 928 protein spots are illustrated in this figure. Only 36.2% of total protein spots (336) were shared between the two hairworm species (Fig. 2A). Of the remaining spots, 353 are specific to *P. tricuspidatus* and 239 to *S. tellinii* (Fig. 2A). Based on our proteomic results, the genetic distance between *P. tricuspidatus* and *S. tellinii* is 0.47. The relationship between the volume of the spots common to both parasites is significant, but the correlation coefficient is low ($r = 0.42$ ($|t_{r=0;\ \text{d.f.}=334}| = 8.46$; $p < 0.0001$) suggesting a differential expression of many common spots between the two species studied (Fig. 2B). Moreover, the "a" and "b" values of the regression suggest an important differential expression of the proteome between both species in the adult stage since "a" value is far from unity ($|t_{a=1;\ \text{d.f.}=334}| = 18.55$; $p < 0.0001$) and "b" value far from zero ($|t_{b=0;\ \text{d.f.}=334}| = 124.98$; $p < 0.0001$) (Fig. 2B).

Actin is a protein which has from 374 to 379 amino acid residues. Its amino acids composition of actin has been highly conserved in the course of evolution (Sheterline et al., 1996). Also, it was demonstrated that Actin plays a role in innate immunity (phagocytosis process) (Ramet et al., 2002; Vierstraete et al., 2004). To develop and evaluate our MALDI-TOF protocol, firstly, we used the Actin-2 (Act-2) of the two nematomorph species. The protein spots A (*P. tricuspidatus*) and F (*S. tellinii*) were excised manually (Fig. 2A) and peptide mass fingerprint (PMF) (Table 1) data were obtained according to the protocol described in Section 2.

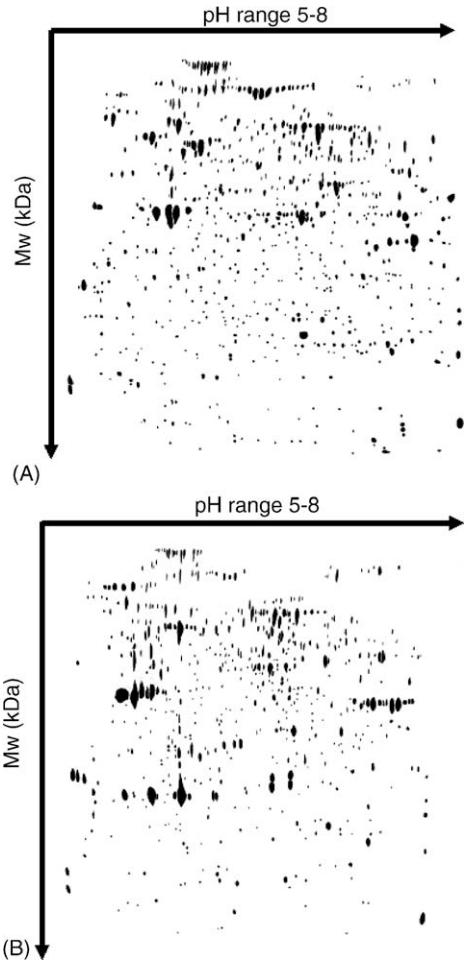
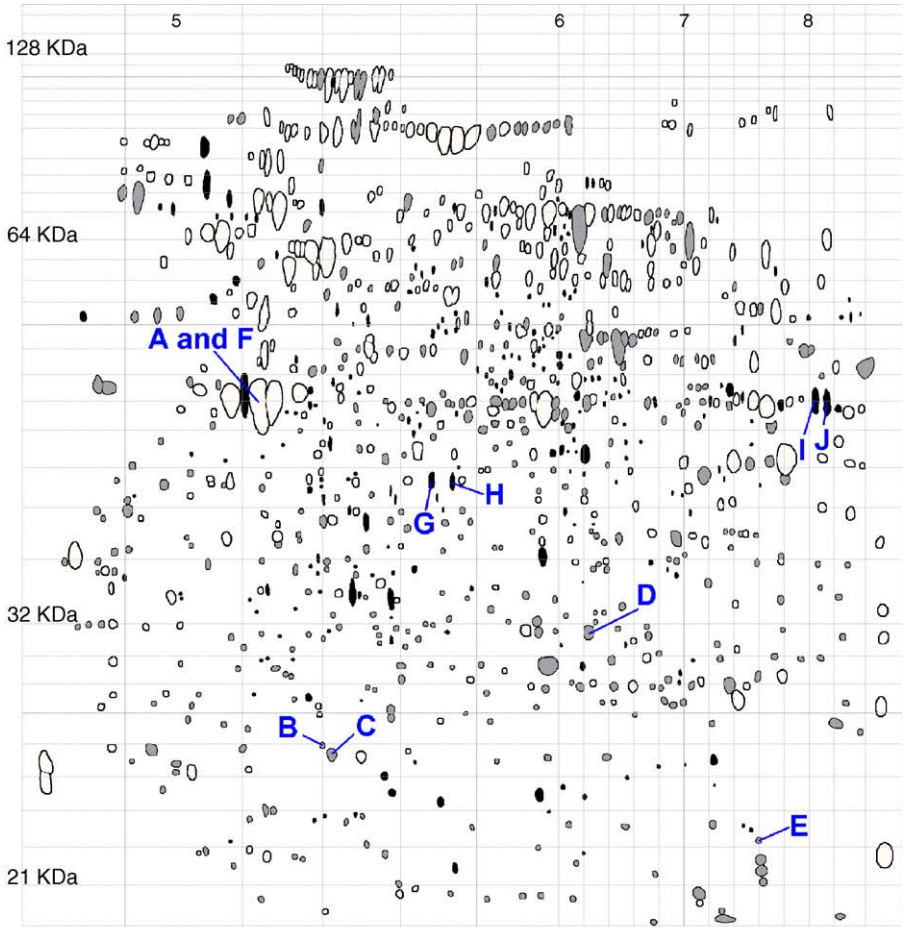
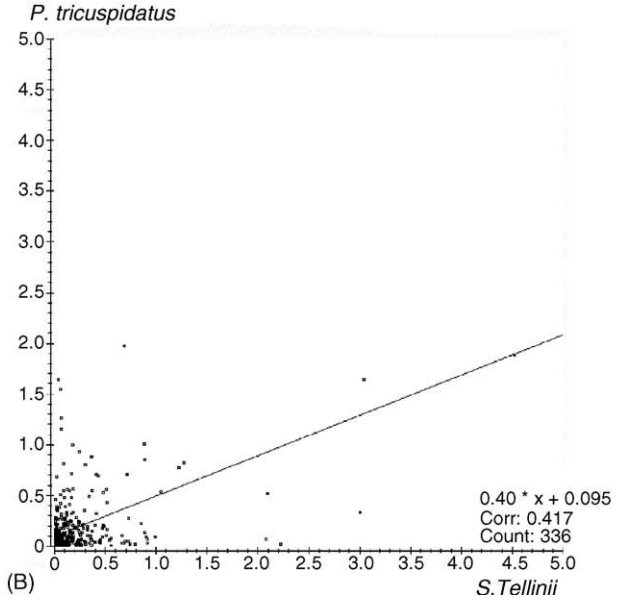


Fig. 1. Digitized two-dimensional gels of total soluble proteins extracted from two nematomorpha species: *P. tricuspidatus* (A) and *S. tellinii* (B).

Results of searches in Protein databases (NCBI, SwissProt and TrEMBL) with PepIdent and Protein Prospector confirmed that the protein spots A and F (see Fig. 2A) are the Act-2. All matchings found in protein databases were the Act-2 of different worm species from Bilateria phylum (Annelida and Nematoda) suggesting a high conservation of amino acid residues and a specific mass signals for the worm species. *P. tricuspidatus* (spot A) and *S. tellinii* (spot F) have 14 common peptides for Act-2. Also, nine and six specific peptides, respectively, for *P. trisupidatus* and *S. tellinii* (see Table 1). The two nematomorph species matched with Act-2 of *Lumbricus*



(A)



(B)

Fig. 2. (A) Two-dimensional synthetic gel issued from comparison between the protein patterns of *Paragoridus tricuspidatus* and *Spinochordodes tellinii* (a total of 928 spots). (○) Common protein spots ($n = 336$); specific protein spots to *P. tricuspidatus* ($n = 353$) (◍); specific protein spots to *S. tellinii* ($n = 239$) (●); (B) Scatter plots on groups for the %volume.

Table 1
Protonated mass signals observed in the MALDI fingerprint spectra for 10 protein spots

Species									
Paragordius tricuspidatus					*Spinochordodes tellinii*				
A	B	C	D	E	F	G	H	I	J
644.21[a]	1002.57	1019.62	931.49	900.51	644.36[a]	917.32	914.50	908.98	948.53
795.46	1005.54	1022.54	956.53	902.41	945.56[a]	950.47	917.27	992.59[b]	972.56
945.59[a]	1011.68	1332.81	964.49	916.50	976.47[a]	1219.72	956.56	1082.03	992.60[b]
976.50[a]	1022.56	1532.85	1035.58	947.56	1034.48	1332.80	1004.58	1219.69[b]	1129.65
1130.61[a]	1166.59	1640.00	1409.76	1233.62	1123.61	1378.68	1020.56	1248.67	1148.60
1188.63[a]	1184.63	1788.95	1477.77	1379.84	1130.59[a]	1390.82	1135.67	1332.76[b]	1190.73
1198.76[a]	1338.88	1987.09	1521.79	1424.81	1158.60	1410.79	1332.80	1338.86[b]	1203.72
1359.75[a]	1486.81	2003.09	1537.80	1523.85	1171.61	1424.85	1388.79	1424.79[b]	1219.71[b]
1499.74[a]	1506.82	2221.10	1539.83	1539.85	1188.60[a]	1650.95	1424.83	1482.81	1332.77[b]
1515.82[a]	1622.88	2224.09	1551.83	1563.86	1198.74[a]	1897.00	1446.82	1651.86[b]	1338.86[b]
1574.80[a]	1715.86	2478.24	1561.78	1649.92	1359.73[a]	1918.99	1652.94	1784.90	1424.79[b]
1590.87	1788.94		1563.83	1666.97	1499.72[a]	2831.05	1774.94	1793.79	1622.90
1639.92	1987.06		1568.87	1675.98	1515.77[a]		1832.96	1788.93[b]	1651.90[b]
1640.41			1570.88	1687.99	1557.74		1997.97	1987.09[b]	1666.03
1772.95			1634.95	1802.95	1573.77			2412.00	1758.97
1790.96[a]			1649.90	2794.05	1574.74[a]				1774.92
1848.98[a]			1666.92		1790.91[a]				1788.93[b]
1850.90			1675.96		1848.91[a]				1987.09[b]
1954.13[a]			1687.94		1954.06[a]				2224.13
2027.78			1720.91		2215.05[a]				2250.91
2069.04			1799.81						
2168.09			1907.02						
2215.07[a]			2210.01						
			2261.07						
			3046.13						

Alphabets A–J are assignation letters.
[a] Common peptides between A and F for the Actin-2.
[b] Common peptides between two isomorphs (I and J).

terrestris (Annelida, Lumbricidae) (code in SwissProt: LUMTE). Another search was performed with Mascott (http://www.matrix-science.com/search_form_select.html). The highest score obtained in protein databases for each nematomorph species confirm the results of PepIdent and Protein prospector softwares. Also, the score obtained for *P. tricuspidatus* (Act-2 LUMTE, score = 110) and *S. tellinii* (Act-2 LUMTE, score = 100) are significant ($p < 0.05$) (Fig. 3). The scores obtained in Mascott with Act-2 of *Caenorhabditis elegans* (Nematoda, Rhabditidae), belonging to a taxa nearest from the one of Nematomorpha, are 85 (34% of sequence coverage) and 71 (23% of sequence coverage), respectively, for *P. tricuspidatus* and *S. tellinii*. According to the scores obtained, our PMF results suggest that the Act-2 of *P. tricuspidatus* and *S. tellinii* show a higher similarity with the Act-2 of LUMTE.

To confirm the value of our MALDI-TOF protocol, we chose randomly four proteins spots for the nematomorph species: *P. tricuspidatus* (B–E) and *S. tellinii* (G–J) (see Fig. 2A). Table 1 gives the mass signals (peptides) obtained for each protein spots. The peptides obtained for two *S. tellinii* protein spots, I and J, suggest that these spots are probably two isoenzymes (isomorphs) (Table 2). Table 2 gives the results of searches in protein database of SwissProt and TrEMBL. As Act-2, all matches found were in the worm species within Bilateria phylum, more specifically in Nematoda, the nearest taxa of the Nematomorpha.

Few previous studies have been perfomed, which directly compare proteomes of species belonging to the same taxonomic group. Thomas and Singh (1992) compared the proteome of four *Drosophila* species, *D. simulans*, *D. melanogaster*, *D. sechellia* and *D. mauritania*, at larval and adult stages for many tissues (compartment) and they found a maximum of 10% of qualitative difference (presence/absence) between them for the majority of tissues except for the reproductive tissues with a maximum of 30%. Navas et al. (2002) compared the total proteome of three phytophagous nematode species of the *Meloidogyne* genus, *M. arenaria*, *M. incognita* and *M. javanica*, they found least than 10% of qualitative difference between the three *Meloidogyne* species. Zdobnov et al. (2002) compared the total proteome of two dipteran species, *Anopheles gambiae* s.s. Giles and *D. melanogaster* Meigen which diverged about 250 million years ago, and found about 44% of protein similarities. This indicates that these two insects diverged considerably faster than vertebrates (Zdobnov et al., 2002).

The two hairworm species considered in this study are distinct in several aspects. First, according to systematic analyses of morphological and molecular (18S ribosomal DNA (rDNA) gene) characters, *P. tricuspidatus* and *S.*

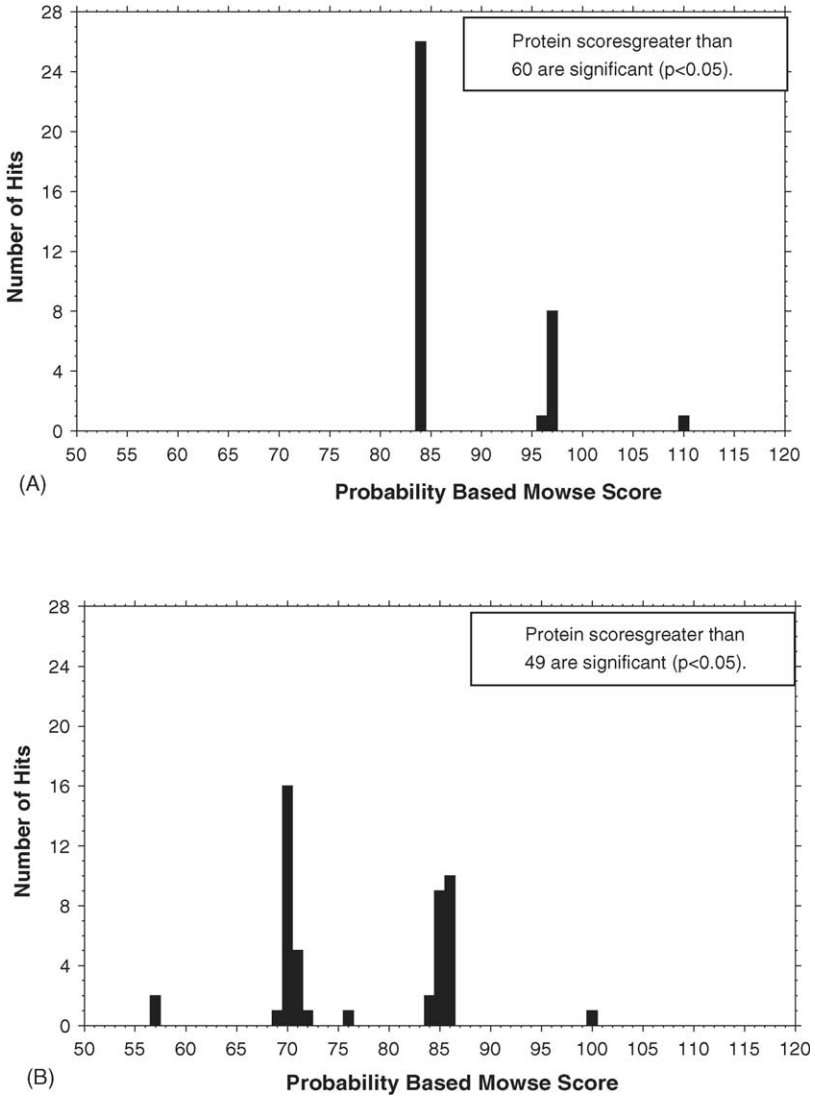


Fig. 3. Number of significant hits obtained with Mascot Server for Actin-2 of *P. tricuspidatus* (A) and *S. tellinii* (B).

tellinii are found to not be closely related within the freshwater Nematomorpha (Gordiida) (Bleidorn et al., 2002). The genus *Paragordius* is very basal in the tree (*Gordius* is the basal taxon, but *Paragordius* branches off next), while the genus *Spinochordodes* belongs to the derived Chordodinae. These two nematomorph species also display noticeable morphological differences. *S. telinii*, like all Chordodinae, is characterized by an undivided posterior end in males, whereas in all other Gordiida (including *Paragordius*), males have two tail lobes. Finally, host specificity also differs strongly between the two hairworm species: while *P. tricuspidatus* is found only in *N. sylvestris*, *S. tellinii* can occur in at least eight different species of grasshoppers (Bleidorn et al., 2002). Our comparison of the proteomes of the two nematomorph species in the adult stage and for a given range of p*I* (4.5–8.5) and Mw (21–128 kDa) reveals considerable differences. Some differences can be caused by the phosporylation of proteins but our proteomic results suggest, like the rDNA, ecological and morphological data, that *P. tricuspidatus* and *S. tellinii* are not be closely related.

The separation period of the nematomorph families used in the present study is unknown, but our proteomic data suggest a rapid divergence resulting in a differential expression of proteome in the adult stage. A total proteome analysis for each compartment (intestine, stomach, etc.) is, however, needed to confirm this view. Similarly, it would be necessary to verify in further studies whether the percentage of protein similarities obtained here (∼36%) remains stable when analysis is performed on other life stages, i.e. adult worms after several days of free-life or conversely, parasitic stages inside the insect host.

This preliminary study on proteome of hairworms will hopefully encourage future research on the systematics of this group, along with research on the biochemical interactions between such parasites and their arthropod

Table 2
Identification of protein spots with peptide mass fingerprint (PMF) data obtained with a MALDI-TOF

Species	Assignation letter (see Fig. 2A)	Protein name	Find in	Accession and identity codes	Family of protein	Number of peptides (%)	Matched peptides (%)	Sequence coverage (%)	Experimental pI/Mw	Theoretical pI/Mw
Paragordius tricuspidatus	A	Actin-2	Swiss-Prot	P92176; ACT2_LUMTE	Actin	23	48	38	5.13/46315	5.37/41840
	B	Y45F10B.9 protein	TrEMBL	O62463; O62463	Zinc Finger ring	13	38	18.9	5.27/27417	5.25/28641
	C	Clone ZZD1079 mRNA sequence	TrEMBL	Q86F62; Q86F62	Proteasome	11	36	17.7	5.30/27727	5.22/27379
	D	ZK1010.7 protein	TrEMBL	O18286; O18286	Nematode cuticle Collagen	25	20	12.4	5.77/31920	6.08/30260
	E	C37A5.2 protein	TrEMBL	062092; O62092	Unknown	16	31	26.7	6.34/23454	6.85/21235
Spinochordodes tellinii	F	Actin-2	Swiss-Prot	P92176; ACT2_LUMTE	Actin	20	45	33	5.13/46315	5.37/41840
	G	Hypothetical protein T10B5.6	TrEMBL	O76406; O76406	Unknown	12	42	16.9	5.65/37645	5.25/38407
	H	K10D3.3 protein	TrEMBL	Q21417; Q21417	Transcription regulation complex	14	29	18.7	5.70/37645	5.80/37593
	I	Hypothethical protein T27C10.4	TrEMBL	Q9TZM5; Q9TZM5	MATH-1	15	33	13.2	5.99/46852	5.44/42336
	J	Hypothethical protein T27C10.4	TrEMBL	Q9TZM5; Q9TZM5	MATH-1	20	25	15.9	6.08/49693	5.44/42336

hosts. Further research should include, for example, focus on the characterisation of the parasitic proteins involved in the defence against the host immune system and/or in the mechanisms involved in the behavioural manipulation exerted by nematomorphs on their hosts.

Acknowledgments

We thank Emmanuelle Demey-Thomas from the proteomic plateform of Montpellier Genopole® for their help concerning the PMF data. We thank Hugh Loxdale from Plant and Invertebrate Ecology Division, Rothamsted Research, Harpenden, Herts, UK, for his precious comments on this manuscript. This work was supported by an ACI CNRS grant to F. Thomas.

References

Anderson, L., Seilhaver, J., 1997. A comparison of selected mRNA and protein abundance in human liver. Electrophoresis 18, 533–537.

Ashton, P.D., Curwen, R.S., Wilson, R.A., 2001. Linking proteome and genome: how to identify parasite proteins. Trends Parasitol. 17, 198–202.

Barret, J., Jefferies, J.R., Brophy, P.M., 2002. Parasite proteomics. Parasitol. Today 16, 400–403.

Bleidorn, C., Schmidt-Rhaesa, A., Garey, J.R., 2002. Systematic relationships of Nematomorpha based on molecular and morphological data. Invert. Biol. 121, 357–364.

Bossis, M., Mugniéry, D., 1993. Specific status of six *Globodera* parasites of solonaceaous plants studies by means of two-dimensional gel electrophoresis with a comparison of gel profiles by a computed system. Fundam. Appl. Nematol. 16, 47–56.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Fields, S., 2001. Proteomics in genomeland. Science 291, 1221–1224.

Fullaondo, A., Vicario, A., Aguirre, A., Barrena, I., Salazar, A., 2001. Quantitative analysis of two-dimensional gel electrophoresis protein patterns: a method for studying genetic relationships among *Globodera pallida* populations. Heredity 87, 266–272.

Gygi, S.P., Rochon, Y., Franza, B.R., Aebersold, R., 1999. Correlation between protein and mRNA abundance in yeast. Mol. Cell. Biol. 19, 1720–1730.

Hanelt, B., Janovy, J., 1999. The life cycle of a horsehair worm, *Gordius robustus* (Nematomorpha, Gordiidea). J. Parasitol. 85, 139–141.

Hochstrasser, D.F., 1998. Proteome in perspective. Clin. Chem. Lab. Med. 36, 825–836.

Karas, M., Hillenkamp, F., 1988. Laser desorption ionization of proteins with molecular masses exceeding 10,000 Da. Anal. Chem. 60, 2299–2301.

Lee, K., Bae, D., Lim, D., 2002. Evaluation of parameters in peptide mass fingerprinting for protein identification by MALDI-TOF mass spectrometry. Mol. Cells 13, 175–184.

Maniatis, T., Tasic, B., 2002. Alternative pre-mRNA splicing and proteome expression in metazoans. Nature 418, 236–243.

Marché, L., 2003. Analyse différentielle du protéome de lignées virulentes et avirulentes du nématode Meloidogyne chitwoodi par électrophorèse bidimensionnelle. Mémoired'ingénieur du C.N.A.M., 68 pp.

Navas, A., Albar, J.P., 2004. Application of proteomics in phylogenetic and evolutionary studies. Proteomics 4, 299–302.

Navas, A., López, J.A., Espárrago, E., Camafeita, E., Albar, J.P., 2002. Protein variability in *Meloidogyne* spp. (Nematoda: Meloidogynidae) revealed by two-dimensional gel electrophoresis and mass spectrometry. J. Proteome Res. 1, 421–427.

Oakley, B.R., Kirsch, B.R., Moris, N.R., 1980. A simplified ultrasensitive silver stain for dectecting proteins polyacrylamide gels. Anal. Biochem. 105, 361–363.

Platzer, E.G., 1981. Potential use of protein patterns and DNA nucleotide sequences in nematode taxonomy. In: Zuckerman, B.M., Rhode, R.A. (Eds.), Plant Parasitic Nematodes, vol. 3. Academic Press, New York, pp. 1–21.

Rabilloud, T., Vuillard, L., Gilly, C., Lawrence, J.J., 1994. Silver-staining of proteins in polyacrylamide gels: a general overview. Cell. Mol. Biol. 40, 57–75.

Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., Ezekowitz, R.A., 2002. Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for *E. coli*. Nature 416, 644–648.

Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B., Bennett, J., 2002. A proteomic approach to analyzing drought- and salt-responsiveness in rice. Field Crop Res. 76, 199–219.

Schmidt-Rhaesa, A., Hanelt, B., Reeves, W.K., 2003. Redescription and compilation of nearctic freshwater nematomorpha (Gordiidae), with the description of two new species. Proc. Acad. Natl. Sci. Phila. 153, 77–177.

Schmidt-Rhaesa, A., 1997. Nematomorpha. In: Schwoerbel, J., Zwick, P. (Eds.), Sübwasserfauna Mitteleuropas 4/4. Gustav Fischer–Verlag, Stuttgart.

Schmidt-Rhaesa, A., 2001. The life cycle of horsehair worms (Nematomorpha). Acta Parasitol. 46, 151–158.

Sheterline, P., Clayton, J., Sparrow, J.C., 1996. Actins, third ed. Academic Press Ltd., London, p. 116.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chemv. 68, 850–858.

Thomas, F., Brown, S.P., Sukhedo, M., Renaud, F., 2002a. Understanding parasite strategies: a state-dependent approach? Trends Parasitol. 18, 387–390.

Thomas, F., Schmidt-Rhaesa, A., Martin, G., Manu, C., Durand, P., Renaud, F., 2002b. Do hairworms (Nematomorpha) manipulate the water seeking behaviour of their terrestrial hosts? J. Evol. Biol. 15, 356–361.

Thomas, F., Ulitsky, P., Augier, R., Dusticier, N., Samuel, D., Strambi, C., Biron, D.G., Cayre, M., 2003. Biochemical and histological changes in the brain of the cricket *Nemobius sylvestris* infected by the manipulative parasite *Paragordius tricuspidatus* (Nematomorpha). Int. J. Parasitol. 33, 435–443.

Thomas, S., Singh, R.S., 1992. A comprehensive study of genic variation in natural populations of *Drosophila melanogaster*. VII. Varying rates of genic divergence as revealed by two-dimensional electrophoresis. Mol. Biol. Evol. 9, 507–525.

Vierstraete, E., Verleyen, P., Baggerman, G., D'Hertog, W., Van den Bergh, G., Arckens, L., De Loof, A., Schoofs, L., 2004. A proteomic approach for the analysis of instantly released wound and immune proteins in *Drosophila melanogaster* hemolymph. Proc. Natl. Acad. Sci. U.S.A. 101, 470–475.

Wilkins, M.R., Williams, K.L., 1997. Cross-species identification using amino acid composition, peptide mass fingerprinting, isoelectric point and molecular mass: a theorical evaluation. J. Theor. Biol. 186, 7–15.

Zdobnov, E.M., Von Mering, C., Letunic, I., Torrents, D., Suyama, M., Copley, R.R., Christophides, G.K., Thomasova, D., Holt, R.A., Subramanian, G.M., Mueller, H.M., Dimopoulos, G., Law, J.H., Wells, M.A., Birney, E., Charlab, R., Halpern, A.L., Kokoza, E., Kraft, C.L., Lai, Z., Lewis, S., Louis, C., Barillas-Mury, C., Nusskern, D., Rubin, G.M., Salzberg, S.L., Sutton, G.G., Topalis, P., Wides, R., Wincker, P., Yandell, M., Collins, F.H., Ribeiro, J., Gelbart, W.M., Kafatos, F.C., Bork, P., 2002. Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster*. Science 298, 149–159.