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Cell proteome variability of protistan mollusc parasite *Perkinsus olseni* among regions of the Spanish coast

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ABSTRACT: We evaluated the proteome variability of *in vitro*-cultured *Perkinsus olseni* cells deriving from 4 regions of the Spanish coast: the rías of Arousa and Pontevedra (Galicia, NW Spain), Carreras River in Huelva (Andalusia, SW Spain) and Delta de l'Ebre (Catalonia, NE Spain). *P. olseni in vitro* clonal cultures were produced starting from parasite isolates from 4 individual clams from each region. Those clonal cultures were used to extract cell proteins, which were separated by 2-dimensional (2D) electrophoresis. Qualitative comparison of *P. olseni* protein expression profiles among regions was performed with PD Quest software. Around 700 protein spots from parasites derived from each region were considered, from which 141 spots were shared by all the regions. Various spots were found to be exclusive to each region. Higher similarity was found among the proteomes of *P. olseni* from the Atlantic regions than between those from the Mediterranean and the Atlantic. A total of 54 spots were excised from the gels and sequenced. Nineteen proteins were annotated after searching in databases, 13 being shared by all the regions and 6 exclusive to 1 region. Most of the identified proteins were involved in glycolysis, oxidation/reduction, metabolism and response to stress. No direct evidence of *P. olseni* variability associated with virulence was found within the protein set analysed, although the differences in metabolic adaptation and stress response could be connected to pathogenicity.

KEY WORDS: *Ruditapes decussatus* · *Ruditapes philippinarum* · Mollusc · 2D electrophoresis · *In vitro* culture

INTRODUCTION

The genus *Perkinsus* includes protistan parasites infecting a wide range of molluscs over the world (Villalba et al. 2011). The species *P. olseni* was first described infecting abalones *Haliotis ruber* in Australia (Lester & Davis 1981). Since then, *P. olseni* has been reported from gastropod and bivalve molluscs of Australasia, Europe, Asia and South America (Villalba et al. 2011). The name *P. atlanticus*, given to a parasite of carpet shell clams *Ruditapes decussatus* from Portugal (Azevedo 1989), is considered a synonym of *P. olseni* (Murrell et al. 2002). It is likely that *P. olseni* was introduced into Europe by commercial trade of Manila clams *R. philippinarum* from Asian coasts (Hine 2001, Elandaloussi et al. 2009a, Vilas et al. 2011). It has spread through Italy (Da Ros and Canzonier 1985, Abollo et al. 2006), Portugal (Azevedo 1989), Spain (Casas et al. 2002a, Elandaloussi et al. 2009b) and France (Goggin 1992, Arzul et al. 2012). Most studies on the genus *Perkinsus* have concentrated on *P. marinus*, which has been causing mass mortalities in Eastern oyster *Crassostrea virginica* populations of the Atlantic and Gulf coasts of the USA for more than 50 yr (Andrews 1996, Burreson & Ragone Calvo 1996, Ray 1996). The World Organisation for Animal Health (OIE) has included *P. marinus* and *P. olseni* in the list of notifiable diseases (www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2014/).

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Variability in virulence and other physiological aspects among populations of *P. marinus* has been demonstrated (La Peyre et al. 1995, Bushek & Allen 1996, Chu & Lund 2006), which is consistent with the occurrence of genetic variability among geographic strains (Reece et al. 1997, 2001, Kotob et al. 1999, Thompson et al. 2011). The existence of races that vary in virulence or environmental tolerance has important management implications and the spreading of virulent races should be avoided. Spreading races with varying environmental tolerances can also be harmful, producing epidemic outbreaks in areas that are inhospitable to indigenous parasitic races (Bushek & Allen 1996). Genetic variability of *P. olseni* populations along the Spanish coast has been demonstrated using microsatellite markers (Pardo et al. 2011, Vilas et al. 2011). Analysis of protein profiles is another way to search for variability and could be valuable for understanding biological processes including development, evolution and pathogenicity of these organisms. Proteomics involves the systematic analysis of gene expression at the protein level (Sperling 2001, Lee et al. 2005). Proteomic techniques are powerful tools for comparing different isolates of the same species (Shin et al. 2005, Regidor-Cerrillo et al. 2012) or morphospecies (Chan et al. 2005), and assessing genetic variability (Mosquera et al. 2003) and differences in virulence among isolates or strains of the same species (Regidor-Cerrillo et al. 2012). Recently, differences in protein expression among 3 *Perkinsus* spp. (*P. marinus*, *P. olseni* and *P. chesapeaki*) have been reported (Fernández-Boo et al. 2014).

A proteomic approach using 2-dimensional electrophoresis (2DE) coupled to mass spectrometry (MS) was performed to compare the protein expression profiles of *P. olseni* clonal cultures deriving from 4 regions of the Spanish coast, as a way to search for variability that could be the basis of geographical differences in virulence and environmental adaptation of *P. olseni*.

MATERIALS AND METHODS

Production of *in vitro* clonal cultures

Parasitic *Perkinsus olseni* were isolated from 12 carpet shell clams *Ruditapes decussatus*, 4 each from Ría de Arousa, Ría de Pontevedra in Galicia (NW Spain), and Carreras River in Huelva (Andalusia, SW Spain), and from 4 Manila clams *R. philippinarum* from Delta de l'Ebre in Catalonia (NE Spain) (Fig. 1).

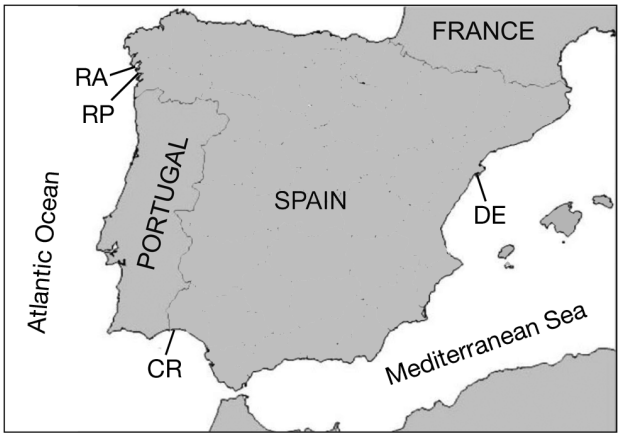

Fig. 1. The 4 Spanish locations where infected clams were collected: Ría de Arousa (RA) and Ría de Pontevedra (RP) in Galicia, Carreras River (CR) in Huelva (Andalusia) and Delta de l'Ebre (DE) in Catalonia

The parasites were isolated from the gills and were allowed to proliferate *in vitro* as described by Casas et al. (2002b). The *in vitro* cultures (1 per host) were cloned by limited dilution plating in 96-well culture plates (Casas & La Peyre 2009). One monoclonal derivate of each isolate was expanded *in vitro* and used in the study. After 2 mo, when the cultures were in the exponential growth phase, parasites were harvested from each clonal culture. The viable cells were counted in a Malassez chamber after staining with 50 mg l^{-1} neutral red. After centrifugation (800 × *g*, 10 min, 25°C), 5×10^6 cells were reseeded and the remaining cells were stored at −80°C for proteomic analysis. This process was repeated 5 more times until 150×10^6 cells had been collected from each clone. Species identification of the cells in each clonal culture was performed by PCR followed by restriction fragment length polymorphism assay as described by Abollo et al. (2006), thus confirming that the cells from each clone corresponded to *P. olseni*.

Protein extraction

A total of 150×10^6 frozen cells from each clonal culture were resuspended in 1 ml of lysis buffer (8 M urea, 2 M thiourea, 2% CHAPS detergent, 1% dithiothreitol [DTT], 0.8% ampholites pH 3 to 10) and incubated for 2 h 30 min for protein extraction. The protein concentration was determined by the Lowry assay using the RC/DC Protein Assay kit (Bio-Rad) and measured in a microplate reader Expert 96 (Asys Hitech). Then 400 µg of protein were purified using

the 2D CleanUp kit (Bio-Rad) and resuspended in 1 ml of rehydration solution (7 M urea, 2 M thiourea, 4 % CHAPS, 0.3 % DTT, 0.5 % immobilised pH gradient [IPG] buffer).

Two dimensional electrophoresis (2DE) and image analysis

Preliminary trials were performed to optimise the experimental conditions for separation of *P. olseni* proteins by 2DE. Each sample was processed in quadruplicate. For the first dimension, aliquots of 100 µg of protein were diluted to a final volume of 250 µl in rehydration solution. Samples were centrifuged for 1 min at maximum speed to remove bubbles and loaded onto the IPG-strips (11 cm, pH 5 to 8, Bio-Rad) by in-gel rehydration. Isoelectric focusing (IEF) was performed using a Protean®IEF System (Bio-Rad) at 20°C, as follows: 50 V were applied during the rehydration step for 12 h, then the IEF proceeded through 5 steps, 150 V for 30 min, 300 V for 30 min, 1000 V for 60 min, 8000 V for 180 min and 8000 V until 35000 Vh. After IEF, the strips were incubated for 20 min in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30 % glycerol, 2 % sodium dodecyl sulphate [SDS]) with 10 mg ml^{-1} DTT to reduce the proteins and then incubated for 20 min in this mix with iodoacetamide at 45 mg ml^{-1} to alkylate proteins. The second dimension involved separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out at 15°C through 12.5 % polyacrylamide homemade gels, using a Protean®II xi cell (Bio-Rad) in 2 steps: 15 mA per gel for 15 min and 50 mA per gel until separation was finished (ca. 3.5 h). Molecular markers from 250 to 10 kDa (Bio-Rad) were run in the second dimension next to the sample for protein size determination. Four gels were produced from each clonal culture. The gels were silver stained with a protocol compatible with MS analysis: (1) incubation in 50 % (v/v) methanol:5 % (v/v) acetic acid for 1 h or overnight; (2) incubation in 50 % methanol for 30 min; (3) wash with Milli-Q H_2O for 15 min (×2); (4) incubation in 0.02 % (w/v) sodium thiosulfate for 1 min; (5) wash with Milli-Q H_2O for 1 min (×2); (6) incubation in 0.1 % (w/v) silver nitrate for 30 min; (7) wash with Milli-Q H_2O for 1 min (×3); (8) incubation in 0.04 % (v/v) formaldehyde:2 % (w/v) sodium nitrate until complete appearance of the spots; (9) incubation in 5 % (v/v) acetic acid for 5 min to stop staining. Once stained, the gels were digitised with a GS-800 densitometer (Bio-Rad) and analysed with PD Quest V.7.4.0 software (Bio-Rad). Fig. 2 shows a scheme of the process of image analysis and comparison between geographic regions, which involved discarding the worst gel replicate from each clonal culture, thus using the best 3 gel replicates for the analysis. Highly reproducible replications were observed in gels of each clonal culture. The analysis of the gels was made region by region, producing a master gel of each region including just the spots shared by all the gels of the region. The master gels of each region were then compared to produce a final master gel in which spots shared by regions and spots exclusive to each region were revealed. Percentages of similarity between regions were calculated as the number of common spots shared by 2 regions with regard to the total number of spots:

$$PS = [C \times 2 / (T1 + T2)] \times 100$$

where PS is the percentage of similarity, C is the

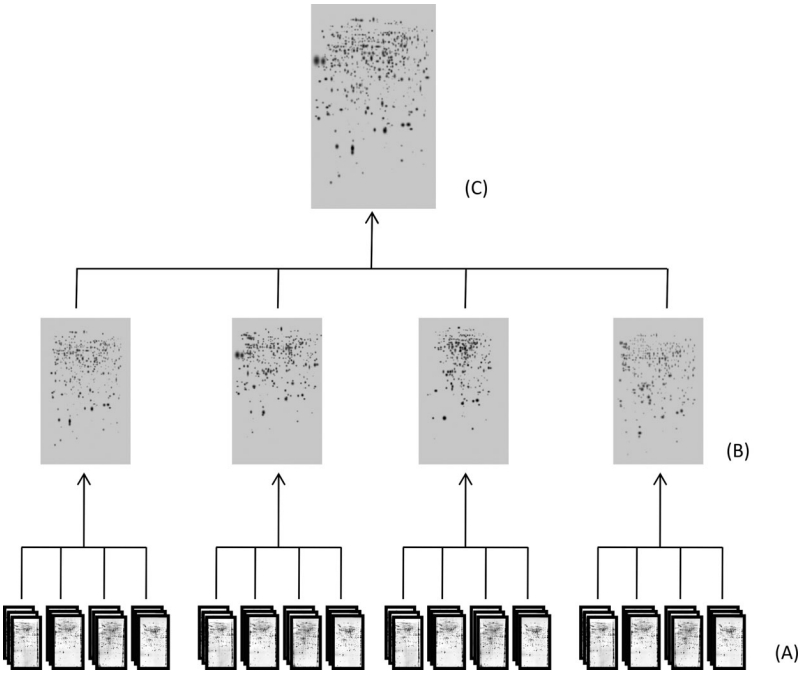

Fig. 2. Scheme of gel analysis and comparison between regions. (A) Analysis of 4 *Perkinsus olseni* clonal cultures from each region in triplicate to obtain a master gel of each region including just the spots shared by all the gels. (B) Comparison among master gels from each region. (C) Final master gel revealing the spots shared by regions and the spots exclusive to each region

number of the common spots shared by the 2 regions, and T1 and T2 are the total number of spots in regions 1 and 2, respectively.

Protein identification and database searching

A total of 19 spots shared by gels from every region and 35 spots that were exclusive to 1 region were selected for protein identification. The number of spots selected for identification was limited by funding availability, thus the selection criterion was to choose the most intense spots among the common and the regionally exclusive ones. The selected spots were excised manually within a laminar flow cabin with sterile scalpel blade. Protein identification by liquid chromatography (LC)-MS/MS analysis and *de novo* sequencing were carried out in the LP-CSIC/UAB Proteomics Laboratory (Barcelona, Spain), a member of the ProteoRed network. Details of the LC-MS/MS procedure are provided in the Supplement at www.int-res.com/articles/suppl/d113p245_supp.pdf. All sequence tags obtained from *de novo* sequencing were manually confirmed and were submitted to a homology search using the pBLAST algorithm (National Center for Biotechnology Information; NCBI). Data were contrasted against the non-redundant NCBI database using Alveolata as search category. Theoretical relative molecular weight (M_r) and isoelectric point (pI) values were obtained with the compute pI/Mw tool at the ExPASy Proteomics Server (www.expasy.org/tools/pi_tool.html).

Statistical analysis

In vitro proliferation of each *P. olseni* clone was measured as the number of cells counted in the clonal cultures after 2 mo growth of a starting culture of 5×10^6 cells. As explained above, 6 successive replicates of each clone were produced to harvest enough cells for proteomic analyses. Differences in *in vitro* proliferation were analysed by a 2-factor nested ANOVA (geographical origin as main factor and clone nested under origin). The number of cells was root-squared transformed because of statistical requirements. As no significant effect due to clone was detected, the clones were grouped by origin and paired comparisons between origins were performed with Fisher's test. Statistical analyses were performed using MINITAB 16 statistical software. The significance level was established at $p \leq 0.05$.

RESULTS

In vitro proliferation of *Perkinsus olseni* clones

Table 1 shows measures of *in vitro* proliferation of the 4 *P. olseni* clones from each geographic origin. No significant effect due to clone was detected but differences between origins were significant. The highest proliferation was observed in *P. olseni* from Delta de l'Ebre and Carreras River and the lowest in those from Ría de Arousa and Ría de Pontevedra, although the differences between Ría de Pontevedra and Carreras River were not significant.

Protein expression patterns of *P. olseni*

Fig. 3 shows representative 2DE gels corresponding to *P. olseni* cultures from every region. High similarity between gels was observed. A total of 831 different spots were found in gels of cultures from Ría de Arousa, 730 in those from Ría de Pontevedra, 629

Table 1. *Perkinsus olseni in vitro* proliferation corresponding to 4 clonal cultures from each of 4 locations. Proliferation was measured as the number of cells counted in the cultures 2 mo after starting with 5×10^6 cells. The mean values corresponding to 6 successive replicates performed for each clone are shown. Different superscript letters denote significant differences

Location	Clone no.	Mean no. cells in each clone ($\times 10^6$)	Mean no. cells for each location ($\times 10^6$)	Mean increment in no. cells (%)
Ría de Arousa	1	25.9	36.6[a]	733
	2	39.4		
	3	49.5		
	4	31.7		
Ría de Pontevedra	1	39.2	46.1[ab]	938
	2	51.8		
	3	43.5		
	4	49.8		
Delta de l'Ebre	1	68	64.3[c]	1287
	2	63.1		
	3	64.4		
	4	62.0		
Carreras River	1	54.9	58.7[bc]	1137
	2	65.3		
	3	57.3		
	4	57.1		

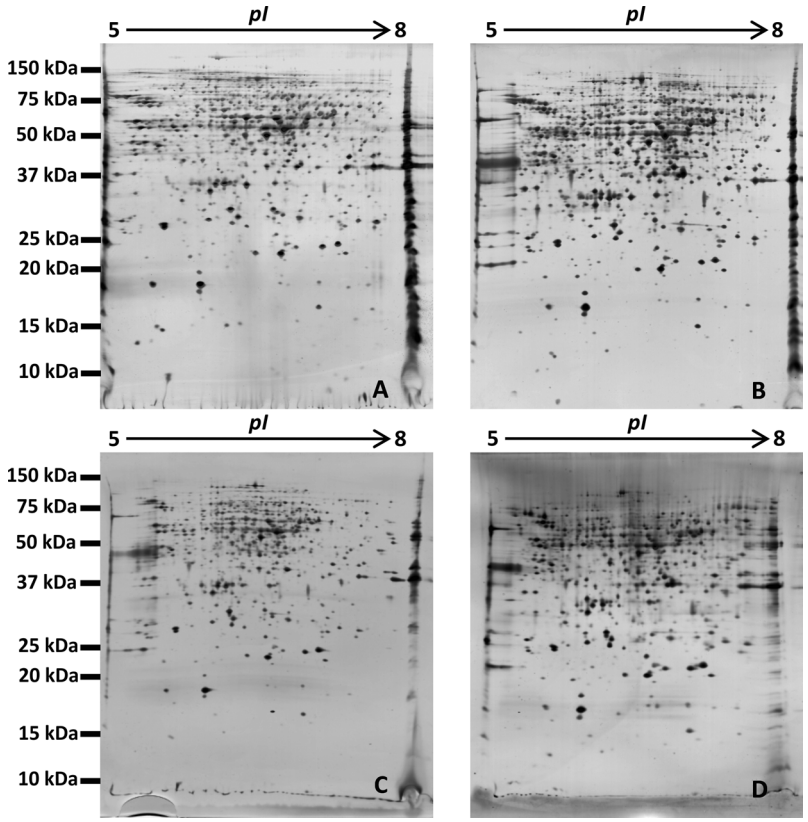

Fig. 3. Digitised images of silver-stained gels produced by 2D SDS-PAGE of proteins extracted from *Perkinsus olseni* clonal cultures deriving from isolates from (A) Ría de Arousa, (B) Ría de Pontevedra, (C) Delta de l'Ebre and (D) Carreras River

from Carreras River and 660 from Delta de l'Ebre. Comparison of gels between clonal cultures from within each population showed percentages of similarity higher than 70% in each pair comparison except in 2 cases from Ría de Pontevedra. The highest mean percentage of similarity was detected in cultures from Carreras River and the lowest in those from Ría de Pontevedra (Table 2).

Master gels for *P. olseni* were built for each region which included the spots shared by every gel from that region (417 spots for Ría de Arousa, 326 for Ría de Pontevedra, 346 for Carreras River and 315 for Delta de l'Ebre). Comparison of the master gels from each region showed that 141 spots were shared by all the regions; 39 (9%) spots of the master gel of Ría de Arousa were exclusive to that region (did not occur in the master gels of the other regions), 57 (17%) were exclusive to Ría de Pontevedra, 37 (11%) to Carreras River and 47 (15%) to Delta de l'Ebre. The percentages of similarity between regions (range: 55.5 to 71.9%; mean: 80.1; Table 3) were lower than those between clonal cultures within each region (range: 64.6 to 87.7%; mean: 65.7; Table 2). Higher percentages of similarity were observed between *P. olseni* from the Atlantic regions (Carreras River, Ría de Arousa and Ría de Pontevedra) than between parasites from Delta de l'Ebre and those from Atlantic regions (Table 3).

Protein identification

A total of 54 spots, including 19 spots shared by *P. olseni* from all the regions and 35 spots exclusive to *P. olseni* from 1 population, were excised for sequencing and identification. The percentage of shared spots that could be sequenced was higher than that of the exclusive spots. Some of the sequenced spots did not show a significant match with any protein in the screened databases (Table 4, Figs. 4 & 5). Table 5 shows the spots that were annotated.

DISCUSSION

The results showed that there is a remarkable variability in the protein expression of *in vitro* cultured *Perkinsus olseni* cells depending on the geographic origin. Genetic and physiological variability depending on geographic origin have also been found in *P. marinus* (Reece et al. 1997, 2001, Kotob et al. 1999, Thompson et al. 2011). The variability between regions was higher than between *P. olseni* clones within each region; the percentages of similarity between clones from the same region was high (above 80% on average) in 3 locations and slightly lower in the other one. The number of spots shared by *P. olseni* from all the regions was higher than the numbers of spots exclusive to *P. olseni* from each region. This result contrasts with the comparison of protein expression between 3 *Perkinsus* spp. (*P. olseni*, *P. marinus* and *P. chesapeaki*), in which the numbers of spots exclusive to each species was much higher than the number of spots shared by the 3 species (Fernández-Boo et al. 2014). However, lower variability between clones from the same species than between clones from different species was expected. Higher similarity was found among the proteomes of *P. olseni* from the

Table 2. Comparison of protein expression patterns between *Perkinsus olseni* clonal cultures from the same geographical location. For each location, diagonal cells (bold, underlined) show the number of spots shared by the 4 gel replicates from each clonal culture, the number of spots shared between pairs of clonal cultures is shown in cells below the diagonal, and the percentage of similarity between pairs of clonal cultures is shown in cells above the diagonal. The mean percentage of similarity of the paired comparisons within each location is also shown

Location	Clone no.	Clone no. 1	2	3	4	Mean percentage of similarity
Ría de Arousa	1	**623**	84.6	79.7	84.5	81.1
	2	499	**557**	74.0	82.3	
	3	523	461	**689**	81.5	
	4	532	491	540	**636**	
Ría de Pontevedra	1	**725**	64.6	79.3	83.6	75.3
	2	352	**364**	77.5	66.3	
	3	496	345	**526**	80.6	
	4	582	342	481	**668**	
Delta de l'Ebre	1	**490**	87.7	79.7	77.9	80.6
	2	418	**463**	78.3	79.8	
	3	373	356	**446**	80.9	
	4	403	402	401	**545**	
Carreras River	1	**437**	83.1	85.6	81.7	83.5
	2	382	**482**	83.8	82.2	
	3	376	387	**441**	84.7	
	4	413	434	430	**574**	

Atlantic regions than between the Mediterranean location and the Atlantic ones. Nevertheless, the highest similarity did not occur between the 2 closest locations (Arousa and Pontevedra) but between Carreras River and the other 2 Atlantic locations. This could be due to the fact that the movement of clams from the SW region of the Iberian Peninsula (Algarve and Huelva) to be immersed or depurated in the NW (Galicia) is much more frequent than movements of clams from the NE (Catalonia) to the SW and NW or from the NW to the NE. Considering that the host source (*Ruditapes decussatus*) of the *P. olseni* isolates from the Atlantic locations was a different species from the host source (*R. philippinarum*) of the isolates from the Mediterranean location, the extent to which the host difference influenced the variability remains unknown. Differences in *P. olseni in vitro* proliferation due to geographic origin were also detected.

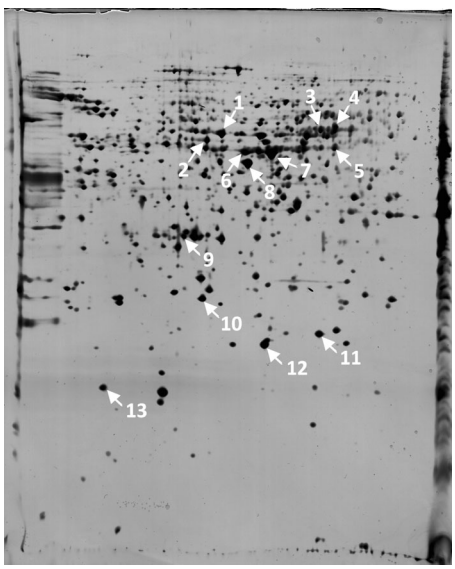

Fig. 4. Digitised image of a silver stained gel of proteins extracted from a *Perkinsus olseni* clonal culture deriving from Ría de Arousa. The spots shared by parasites from all the regions that were annotated are numbered in the image

Table 3. Number of spots shared between pairs of regions (below diagonal) and percentage of similarity between pairs of regions (above diagonal)

	Ría de Arousa	Ría de Pontevedra	Delta de l'Ebre	Carreras River
Ría de Arousa	–	68.9	63.7	71.9
Ría de Pontevedra	255	–	55.5	71.8
Delta de l'Ebre	231	179	–	62.2
Carreras River	271	242	205	–

Table 4. Total number of spots and number of spots excised, sequenced and annotated, corresponding to the group of spots shared by *Perkinsus olseni* from all the regions and the groups of spots exclusive to *P. olseni* from each region

	Shared by all regions	Exclusive to Ría de Arousa	Ría de Pontevedra	Carreras River	Delta de l'Ebre
Total	141	39	57	47	32
Excised	19	7	11	8	9
Sequenced	15	3	1	1	2
Annotated	13	3	1	1	1

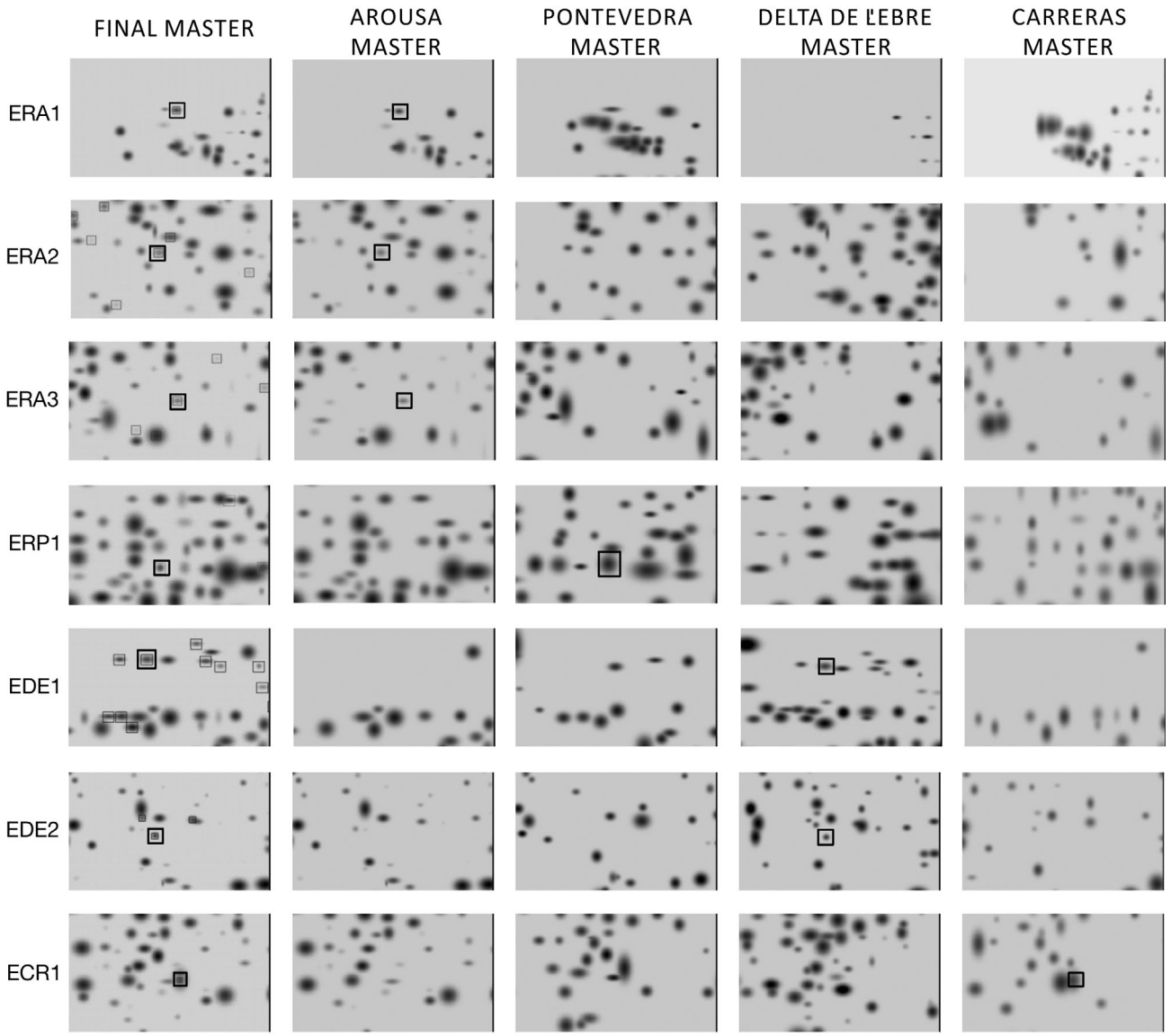

Fig. 5. Fragments of master gels showing the spots exclusive to *Perkinsus olseni* from 1 region that were annotated. Left column corresponds to the areas of the final master gel where the exclusive spots occur. The remaining columns correspond to the same areas in the master gel of each region. Each file corresponds to an exclusive spot labelled with the spot code (the same as in Table 5). ERA: exclusive to Ría de Arousa; ERP: exclusive to Ría de Pontevedra; EDE: exclusive to Delta de l'Ebre; ECR: exclusive to Carreras River

It was lower in clones from Ría de Arousa and Ría de Pontevedra and higher in those from Delta de l'Ebre and Carreras River, which could be the result of local adaptation to environmental conditions. Interestingly, the water is warmer during summer in the latter 2 locations. A previous report using microsatellites to analyse genetic variability of *P. olseni* among the same locations as in this paper showed no correlation between geographic and genetic distances (Vilas et al. 2011).

In the case of the spots shared by *P. olseni* from all of the regions, a high proportion of the annotated proteins were involved in sugar-associated metabolism, which could be due to the fact that the parasite cells were collected during the exponential growth phase. The occurrence of more than 1 spot in gels corresponding to glucose 6-P isomerase and enolase-2 suggests that several isoforms of these proteins occur in the parasite. In the case of the glucose 6-P isomerase gene, different alleles have been found

Table 5. Peptides annotated after searching in the BLAST database against the Alveolata group in the non-redundant proteins database. Spot codes 1 to 13 correspond to spots shared by *Perkinsus olseni* from all the regions, 'ERA' corresponds to spots exclusively found in *P. olseni* from Ría de Arousa, 'ECR' to spots exclusive to Carreras River, 'EDE' to spots exclusive to Delta de l'Ebre and 'ERP' to spots exclusive to Ría de Pontevedra. pI theor and pI obs: the theoretical (Swiss Prot, www.expasy.ch/tools) value of the isoelectric point and that observed in gels, respectively. M_r theor and M_r obs: the theoretical molecular weight and that observed in gels, respectively. Identified protein: protein name of identified peptide in the database. Organism: the species to which the protein recorded in the database corresponds. Acc. no.: accession number of protein in the database. Biological function: function of the protein according to Gene Ontology Biological Process. Peptide sequence: peptide sequence with the highest individual ion scores indicating identity or extensive homology ($p < 0.05$). Score: Score of the peptide match with proteins in the NCBI database after BLAST searching; the higher the value of the score, the better the peptide match. E-value: expected value, which describes the number of hits expected by chance when searching a database of a particular size; the lower the E-value, the better the peptide match

Spot code	pI Obs/theor	M_r (kDa) Obs/theor	Identified protein; organism	Acc. no.	Biological function	Peptide sequence	Score	E-value
1	6.25/6.00	62/56.55	Pyruvate kinase; *Perkinsus marinus*	XP_002788066.1	Glycolysis	VPSFQGTDHIIQSAINYGK	1080	0.0
2	6.15/8.35	54.5/17.08	Adenosylhomocysteinase; *P. marinus*	XP_002781556.1	One carbon metabolic process	NNAIVGNIGHFDNEIQmER	66.8	3.00×10^{-13}
3	7.03/6.20	60/61.04	Glucose-6-phosphate isomerase; *P. marinus*	XP_002787676.1	Glycolysis	FVAHIQQLDMESNGK	52.4	2.00×10^{-8}
4	7.12/6.20	62/61.04	Glucose-6-phosphate isomerase; *P. marinus*	XP_002787676.1	Glycolysis	FVAHIQQLDMESNGKR	55.8	2.00×10^{-9}
5	7.12/6.04	54/47.33	Formate dehydrogenase; *P. marinus*	XP_002782414.1	Oxidoreductase	DVEGMHLGTVA	32.5	0.057
6	6.50/5.23	49/48.63	Enolase-2; *P. marinus*	XP_002785647.1	Glycolysis	SGETEDTFIADIVVGLGTGQIK	70.2	2.00×10^{-14}
7	6.70/5.23	50/48.63	Enolase-2; *P. marinus*	XP_002785647.1	Glycolysis	VNQIGSVTESIEANNK	52.4	1.00×10^{-10}
8	6.50/5.82	48/44.77	Phosphoglycerate kinase 1; *P. marinus*	XP_002778580.1	Glycolysis	AGATSIIGGGDTAAmVEQQGK	65.5	2.00×10^{-12}
9	6.05/5.58	32/25.05	Malate dehydrogenase; *P. marinus*	XP_002784022.1	Carbohydrate metabolic process	ImGLmSLDVTR	39.7	1.00×10^{-6}
10	6.25/5.58	25/24.35	Conserved hypothetical protein; *P. marinus*	XP_002776039.1	Unknown	VEPATQP	21	0.76
11	7.05/5.86	22/21.55	Peroxiredoxin-2; *P. marinus*	XP_002765329.1	Oxidation-reduction process / antioxidant activity	VLDSIIETDEHGVVcPANWK	69.4	5.00×10^{-14}
12	6.5/6.03	20.5/22.11	Iron-dependent superoxide dismutase; *P. marinus*	XP_002788749.1	Superoxide metabolic process / oxidation-reduction process	MSAETLQYHYG	37.1	1.00×10^{-2}
13	5.58/6.28	17.5/17.00	Peroxiredoxin V; *P. chesapeaki*	ABV22156.1	Oxidation-reduction process / antioxidant activity	ALGVDFDVTPVLGNVR	52.4	2.00×10^{-8}
ERA1	5.65/5.39	81/81.53	Heat shock protein 90; *P. marinus*	XP_002773238.1	Protein folding / response to stress	LVNSPAVVTSAISPQMR	55.8	2.00×10^{-9}
ERA2	6.50/5.23	47/42.8	Galactokinase; *P. marinus*	XP_002788912.1	Galactose metabolic process	SGQLVHLDcR	35.4	0.005
ERA3	7.28/5.51	41.5/141.49	Conserved hypothetical protein; *P. marinus*	XP_002773742.1	Unknown	SLLDQDPENAEIK	44.3	9.00×10^{-6}
ERP1	6.15/5.42	63.1/56.36	Pyruvate kinase; *P. marinus*	XP_002788067.1	Glycolysis	HVAVMLDTK,	32.5	0.038
EDE1	6.8/5.71	100/30.55	Malate dehydrogenase; *P. marinus*	XP_002787252.1	Malate metabolic process	LSLYVAcAGINPGR	46.9	1.00×10^{-6}
ECR1	6.85/5.79	36.5/36.38	Glyceraldehyde 3 phosphate dehydrogenase; *P. marinus*	XP_002788755.1	Oxidation-reduction process	VIISAPPKDDTPMFVMGVNNK	72.3	7.00×10^{-15}

in the oomycete *Phytophthora infestans* (Ospina-Giraldo & Jones 2003) as well as in *Entamoeba histolytica* (Razmjou et al. 2006). Several stage-specific isoenzymes have been found in the apicomplexan parasite *Toxoplasma gondii*, which undergoes metabolic and morphological changes during infection. These isoenzymes have different biochemical properties that maintain the major parasitic metabolic processes such as glycolysis in pace with the stage-specific requirements of carbohydrate or polysaccharide biosynthesis, and include isoforms of glucose 6-P isomerase and enolase-2 (Tomavo 2001). The isoforms in *P. olseni* could have similar functions, regulating metabolism according to the progress of the infection. The strictly conserved plant enolase pentapeptide EWGWC insertion that has been found in *P. marinus* (Joseph et al. 2010) was also found in *P. olseni* enolase-2 in this study. This domain has previously been found in other apicomplexan parasites, such as *Plasmodium falciparum* (Read et al. 1994) and *T. gondii* (Tomavo 2001). Glucose 6-P isomerase and pyruvate kinase have been proposed as antiparasite drug targets in humans (Rigden et al. 1999, Tomavo 2001, Muñoz & Ponce 2003). A better understanding of these proteins in *Perkinsus* would help in the search for tools to fight the disease. The Perkinsozoa group, which includes the genus *Perkinsus*, is considered to be the earliest group diverging from the lineage leading to dinoflagellates, branching close to the node shared by dinoflagellates and apicomplexans (Saldarriaga et al. 2003, Bachvaroff et al. 2011, Fernández-Robledo et al. 2011). Members of both the dinoflagellates and apicomplexans, as well as *P. olseni*, possess plastids and recent analysis of newly identified photosynthetic members of the apicomplexan lineage have shown that these plastids evolved from a single common secondary endosymbiosis with a red alga (Teles-Grilo et al. 2007, Moore et al. 2008, Janouskovec et al. 2010, Fernández-Robledo et al. 2011). This suggests that non-photosynthetic relatives of both lineages, including *Perkinsus*, evolved from photosynthetic ancestors, raising the possibility that these lineages retain cryptic organelles (Keeling 2010). This common lineage ancestor could explain the existence of proteins derived from a plant origin, such as enolase-2, as well as the superoxide dismutase and peroxiredoxin proteins that are discussed in the following paragraph.

Enzymes linked to protection from the host immune reaction were also well represented in the group of spots shared by all the regions that were annotated. A high constitutive expression of antioxidant proteins was detected. Proteins like peroxiredoxin (Prx) II and V and superoxide dismutase (SOD) were expressed in large quantities, even in culture medium. These proteins have been found in 3 *Perkinsus* spp., *P. olseni*, *P. marinus* and *P. chesapeaki* (Fernández-Boo et al. 2014), which suggests the importance of these proteins as a defence mechanism. Their detoxifying role seems to be essential for infecting the host and eluding host defences. *P. marinus* inhibits the production of reactive oxygen intermediates when it is phagocytosed by host haemocytes, thus preventing oxidative damage (Fernández-Robledo et al. 2008). During the immune reaction of venerid clams against *P. olseni*, clam haemocytes encapsulate the parasite (Montes et al. 1995, Villalba et al. 2004) and, according to our results, *P. olseni* also produces enzymes allowing it to neutralise the reactive oxygen intermediates released from host haemocytes. S-adenosylhomocysteinase is a cytosolic enzyme that has important functions in the cytosolic metabolism linking processes of transmethylation, transsulphuration and purine metabolism (Kloor et al. 2000). It cleaves S-adenosylhomocysteine and provides homocysteine for the synthesis of cysteine and the regeneration of methionine (Takata & Fujioka 1983). Methionine is also the source of cysteine, the limiting reagent for the synthesis of glutathione (GSH), one of the most important antioxidants in organisms (Company et al. 2011). S-adenosylhomocysteinase has been proposed as a target for chemotherapy in other protozoan parasites, such as *Trichomonas vaginalis* (Bagnara et al. 1996).

In the case of spots exclusive to 1 region, the annotated proteins were classified into 4 categories according to Gene Ontology: metabolism (galactokinase in Ría de Arousa and malate dehydrogenase in Delta de l'Ebre), glycolysis (pyruvate kinase in Ría de Pontevedra), response to stress (heat shock protein 90 in Ría de Arousa), and oxidation/reduction (glyceraldehyde 3-P dehydrogenase in Carreras River). All these proteins are involved in processes related to the Kreb's cycle, except heat shock protein 90. Malate dehydrogenase, which was found among the common proteins as well as a Delta de l'Ebre-specific protein, is involved in the metabolism of the intermediaries of the Kreb's cycle. This enzyme catalyses the transformation of L-malate to pyruvate together with the coenzyme $NADP^+$ (Sánchez et al. 1996). Pyruvate kinase catalyses the transformation of phosphoenolpyruvate to pyruvate, the previous step to the generation of acetyl coenzyme A (CoA). Two pyruvate kinases were detected in *P. olseni* from Ría de Pontevedra, one shared by all the regions and the other exclusive to this region. The specific protein

from the Ría de Pontevedra population seems to be the same protein but with a post-translational modification which raises its pI by 0.1 units. Galactokinase takes part in the Leloir route involving the transformation of galactose to glucose. It is an ATP-dependent enzyme catalysing the phosphorylation of galactose to form galactose-1-phosphate (Chu et al. 2009). Glyceraldehyde 3-P dehydrogenase is a key enzyme in glycolysis and catalyses the oxidative phosphorylation of glyceraldehyde-3P into 1,3-bisphosphoglycerate in the presence of NAD^+ and inorganic phosphate (Daubenberger et al. 2000). Heat shock protein 90 has been studied in *P. marinus* due to its involvement in infection progression. This protein is necessary for the adaptation of the parasite to the host environment. *P. marinus*, like many other organisms, uses heat shock proteins as part of its adaptive survival repertoire (Tirard et al. 1995).

In contrast with the spots shared by *P. olseni* from all the regions, none of the annotated spots that were exclusively found in 1 region could be considered directly linked to virulence, which would have supported the occurrence of virulence differences between *P. olseni* geographic strains. However, the detected differences (proteins involved in metabolism and stress response) could offer a better adaptation to local environmental conditions that would favour cell division/growth and thus possibly pathogenicity. Local adaptation of *Perkinsus* spp. to different ranges of salinity and temperature could explain their adaptability to new environments, and differences in their proliferation and virulence (La Peyre et al. 2008, Thompson et al. 2014). Obviously, sequencing more spots would have allowed a deeper analysis of *P. olseni* variability, but limited funding impeded going further.

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