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Infection of Manila clams *Ruditapes philippinarum* from Galicia (NW Spain) with a *Mikrocytos*-like parasite

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ABSTRACT: The name 'microcells' is frequently used to refer to small-sized unicellular stages of molluscan parasites of the genera *Bonamia* (Rhizaria, Haplosporidia) and *Mikrocytos* (Rhizaria). Histological examination of Manila clams *Ruditapes philippinarum* revealed microcells in the connective tissue of adductor muscle, foot, mantle, gills, siphon and visceral mass. The clams had been collected from 4 beds on the coast of Galicia, Spain. The prevalence of these microcells ranged from 73 to 93% in surface clams and from 3 to 33% in buried clams. However, the detection of brown ring disease signs in clams from every bed prevented us from making the assumption that the microcells alone were responsible for clam mortality. PCR assays using primer pairs designed to detect *Bonamia* spp. and haplosporidians gave negative results, whereas positive results were obtained with primers for the genus *Mikrocytos*. A consensus sequence of 1670 bp of the ribosomal gene complex of the microcells was obtained. It contained a section of the 18S region, the whole first internal transcribed spacer, the 5.8S region, the second internal transcribed spacer and a section of the 28S region. Comparison of this sequence with those of *M. mackini* infecting *Crassostrea gigas* and *Mikrocytos* sp. infecting *Ostrea edulis* showed that the microcells of Galician clams were the most divergent among the compared parasites. This is the first report of a *Mikrocytos*-like parasite infecting Manila clams. Care must be taken to avoid the spread of this parasite through Manila clam transfers.

KEY WORDS: *Ruditapes philippinarum* · Manila clam · Microcells · *Mikrocytos* · PCR · Ribosomal gene

INTRODUCTION

The Manila clam *Ruditapes philippinarum* (Adams & Reeve 1850) is a bivalve mollusc species native to Asia. It was introduced unnoticed, together with *Crassostrea gigas* oyster seed imported to the Pacific coast of North America during the 1930s, spreading from California (USA) to British Columbia (Canada). The Manila clam was also introduced into Europe in 1972, through French hatchery production, following the overfishing and irregular yields of the European native clam *R. decussatus*. Subsequently, it has been introduced into other European countries such as Italy, the UK, Ireland, Portugal and Spain, becoming the major contributor to clam landings in Europe (Gosling 2003). The Manila clam is among the major aquacultured species, with a global production of 3.6 million t through aquaculture and 40 000 t from fisheries in 2010 (www.fao.org/fishery/species/3543/en). Two diseases are the most relevant pathological threats for the Manila clam industry both in Asia and Europe: brown ring disease (BRD), caused by *Vibrio*

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tapetis (Paillard et al. 1989, Borrego et al. 1996, Paillard 2004, Park et al. 2006), and infection with *Perkinsus olseni* (Navas et al. 1992, Hamaguchi et al. 1998, Park & Choi 2001, Wu et al. 2011).

The protozoan parasite *Mikrocytos mackini* (Farley et al. 1988) is the aetiological agent of Denman Island disease, blamed for severe mortalities of oysters *C. gigas* in British Columbia (Canada) (Quayle 1982, Bower 1988, Bower et al. 1994). Recently, it has also been detected in Washington State (USA) (Bower 2010, Abbott et al. 2011) and has been found to infect *C. sikamea* from California (USA) (Elston et al. 2012). *M. mackini* is included in the group of microcell parasites of molluscs due to its small size (2–4 µm), together with haplosporidian parasites of the genus *Bonamia*. The position of this parasite within the global eukaryotic phylogeny was uncertain until it was recently assigned to Rhizaria (Burki et al. 2013). The microcell parasite originally described as *M. roughleyi*, which infects oysters *Saccostrea glomerata* in SE Australia, is considered closely related to *B. exitiosa* and should not be included in the genus *Mikrocytos* (Cochennec-Laureau et al. 2003, Carnegie & Cochennec-Laureau 2004, Hill et al. 2010). *Mikrocytos*-like parasites have been described infecting *Ostrea lurida* from California (Friedman et al. 2005); *O. edulis* from Nova Scotia (Canada) (Gagné et al. 2008), and *C. gigas* from China (Wang et al. 2010). *Mikrocytos* sp. infecting *C. gigas* from British Columbia, reported by Abbott et al. (2011), has now been fully described as the new species *Mikrocytos boweri* (Abbott et al. 2014, this DAO Special). Various challenges involving exposure to the parasite were performed to evaluate the susceptibility of different mollusc species to *M. mackini*; the oysters *C. virginica*, *O. edulis* and *O. lurida* became infected with *M. mackini*, whereas the clams *Panope abrupta* and *R. philippinarum* did not (Bower et al. 1997, 2005, Meyer et al. 2008). Those results suggested that these clam species are resistant to *M. mackini* (Bower et al. 2005, Meyer et al. 2008) and perhaps the parasite is restricted to oysters. However, a parasite closely related to *Mikrocytos*, with 79–80% homology with *M. mackini,* has recently been reported infecting clams *Donax trunculus* from French beds affected by high mortality (Garcia et al. 2012); that is the first report of a parasite related to the genus *Mikrocytos* in Europe. *M. mackini* has been removed from the list of notifiable diseases of the World Organisation for Animal Health (OIE), but the European regulation on aquatic animal health includes *M. mackini* on the list of exotic diseases that must be under surveillance (Council of the European Union 2006).

Microcell parasites were observed in Manila clams *R. philippinarum* collected from 4 beds located in Galicia (NW Spain), where high mortality had been claimed by clam farmers in late 2007 and early 2008, although abnormal mortality was not technically certified. Because *R. philippinarum* was thought to be resistant to *M. mackini*, the hypothesis that the microcells were related to *Bonamia* was considered the most probable and, thus, tested with PCR-based diagnostic assays in 2008. However, the results did not confirm the hypothesis. When Garcia et al. (2012) reported that a *Mikrocytos*-like parasite had been found infecting clams *D. trunculus*, the hypothesis that the microcells of Galician clams were related to *Mikrocytos* seemed sound. DNA sequence analyses performed with stored materials established a close relationship of those microcells with the genus *Mikrocytos.* Here we characterise the microcells found in the Manila clams.

MATERIALS AND METHODS

Clams

Samples of adult (≥30 mm in length) clams *Ruditapes philippinarum* were collected from 4 sites in Galicia (NW Spain): 1 natural bed located in Camariñas and 3 farm sites located in Espasante (Ría de Ortigueira), Abanqueiro (Ría de Arousa) and Carril (Ría de Arousa; Fig. 1). Fifteen buried clams and 15 surface clams all deriving from natural recruitment

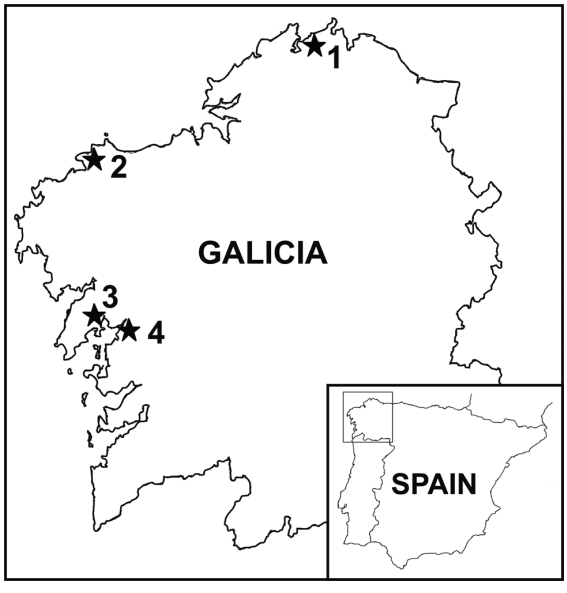

Fig. 1. Location of sampled beds in Galicia (NW Spain). 1: Espasante; 2: Camariñas; 3: Abanqueiro; 4: Carril

were collected from Camariñas in February 2008. Clams grown-out in Espasante had been imported from a foreign country and seeded in the bed at a size range of 9–11 mm in length; 30 buried and 19 surface clams were taken from this location in November 2007. Clams that had been produced in a local hatchery and clams that had been imported from a foreign country were being grown-out in Abanqueiro at the time of sampling (December 2007) and could not be discriminated by origin; 30 buried clams and 30 surface clams were taken from this location. The only available information on the origin of clams grown-out in Carril was that clam seed in some plots had been imported from a foreign country and clam seed produced in a Spanish hatchery had been set in other plots; 15 buried and 15 surface clams from each origin were collected from this farming site. The seawater temperature range of clam beds on the Galician coast is 10 to 24°C. All clam samples and the information on clam origin were provided by fishermen and clam farmers. Once in the laboratory, the clams were placed in tanks with filtered seawater with aeration overnight to allow sediment expulsion before being processed.

Macroscopic observations and histological analysis

The specimens were examined for macroscopic anomalies and measured for length and weight. The condition index of each clam was calculated as follows: (wet meat weight × 100)/shell weight. The inner surface of the shell was examined to detect signs of BRD, i.e. the occurrence of characteristic brown conchiolin deposits (Paillard & Maes 1994).

A transverse section (ca. 5 mm thick) of each clam containing gills, visceral mass, mantle lobes and foot plus pieces of adductor muscle and siphon were fixed in Davidson's solution, dehydrated in an ethanol series and embedded in paraffin. Histological sections (5 µm thick) were stained with Harris' haematoxylin and eosin (Howard et al. 2004) and examined with a light microscope to diagnose pathological conditions.

Genomic DNA extractions and detection of microcells by PCR

Small pieces of gill, visceral mass, mantle lobes and foot were preserved in 96% ethanol for molecular analysis. DNA extractions were performed employing the commercial Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol. DNA quality and quantity were checked with a spectrophotometer Nanodrop® ND-1000 (Nanodrop Technologies).

PCR assays were performed using primers described for the genus *Bonamia*, for haplosporidian parasites and for the genus *Mikrocytos* (Table 1) to identify the microcells observed by histological analysis. The PCR assays for the genus *Bonamia* were

Table 1. Sequences of the PCR primers used in this study

Primer	Sequence 5'–3'	Purpose in this study	Reference
Bo	CAT TTA ATT GGT CGG GCC GC	Detection	Cochennec et al. (2000)
Boas	CTG ATC GTC TTC GAT CCC CC	Detection	Cochennec et al. (2000)
HAP-F1	GTT CTT TCW TGA TTC TAT GMA	Detection	Renault et al. (2000)
HAP-F2	GCC RTC TAA CTA GCT S	Detection	Renault et al. (2000)
HAP-R1	CTC AWK CTT CCA TCT GCT G	Detection	Renault et al. (2000)
HAP-R2	GAT GAA YAA TTG CAA TCA YCT	Detection	Renault et al. (2000)
HAP-R3	AKR HRT TCC TWG TTC AAG AYG A	Detection	Renault et al. (2000)
MIKROCYTOS-F	AGA TGG TTA ATG AGC CTC C	Detection	Carnegie et al. (2003)
MIKROCYTOS-R	GCG AGG TGC CAC AAG GC	Detection	Carnegie et al. (2003)
Mm18SF1	GAC GGC AGG AGT ATT GTT TGA CGA	Detection/DNA sequence analysis	Abbott et al. (2011)
Mm_1450R	TGA CGG ACA GTG TGA ACA AGT C	Detection/DNA sequence analysis	Abbott et al. (2011)
Mm18S_1435F	GTT CAC ACT GTC CGT CAT ACA C	DNA sequence analysis	Abbott et al. (2011)
pro28S-R	TAC TTG TTY GCT ATC GGT CTC	DNA sequence analysis	Abbott et al. (2011)
MikrocGal-1F	ATC CGC GCT AAA TTC TGG T	DNA sequence analysis	This study
MikrocGal-358R	TAC CCC AAC TTT GGT CCT TG	DNA sequence analysis	This study
MikrocGal-811F	GGT AGG TCC GTG ATG CAT TT	DNA sequence analysis	This study
MikrocGal-1284R	ATT AGG CCA TCG GGA GTT CT	DNA sequence analysis	This study

performed using Bo/Boas primers (Cochennec et al. 2000) according to the cycling protocol described by Ramilo et al. (2013). The PCR assays for haplosporidians were performed using the set of primers HAP-F1/HAP-R1, HAP-F1/HAP-R2, HAPF1/HAP-R3, HAP-F2/HAP-R1, HAP-F2/HAP-R2 and HAP-F2/HAP-R3 as described by Renault et al. (2000). In both *Bonamia* and haplosporidian PCR assays, a positive control (DNA from *Ostrea edulis* infected with *B. ostreae*) and a negative control (no DNA) were used. The PCR assays for the genus *Mikrocytos* were performed with the primers MIKROCYTOS-F/MIKROCYTOS-R (Carnegie et al. 2003) and Mm18SF1/Mm18S_1450R (Abbott et al. 2011). All PCR reactions were carried out in a total volume of 25 µl containing 1 µl of genomic DNA (200 ng), PCR buffer at 1× concentration, 1.5 mM $MgCl_2$, 0.2 mM nucleotides (Roche Applied Science), 0.3 µM of each primer and 0.025 U μl^{-1} *Taq* DNA polymerase (Roche Applied Science). A positive control (DNA from an oyster *Crassostrea gigas* infected with *M. mackini*) and 2 negative controls (no DNA and DNA from *R. philippinarum* non-infected with microcells) were used. All PCRs were carried out in a TGradient thermocycler (Biometra). PCR products (10 µl) were analysed by electrophoresis on 2% agarose gels, in 1% Tris acetate EDTA buffer, stained with ethidium bromide and scanned in a GelDoc XR documentation system (BioRad).

Molecular characterisation of microcells by DNA sequence analysis

PCR amplification

The pair of primers Mm18S_1435F/pro28S-R (Abbott et al. 2011) (Table 1) were used to amplify a sequence including a fragment of 18S rDNA, the whole first internal spacer transcribed (ITS1), 5.8S rDNA, the second internal spacer transcribed (ITS2) and a partial fragment of 28S rDNA of the microcells infecting *R. philippinarum* from Galicia. In order to amplify the 18S gene, the pair of primers Mm18SF1/Mm18S_1450R (Abbott et al. 2011) and the primers MikrocGal-1F/MikrocGal-358R designed in this study were used. Likewise, to ensure a correct overlapping of 18S and ITS-1 regions, the pair of primers MikrocGal-811F/MikrocGal-1284R was designed based on the 3′ end of the sequence amplified with Mm18SF1/Mm18S_1450R and 5′ end of the sequence amplified with Mm18S_1435F/pro28S-R using the program Primer 3 (Rozen & Skaletsky 2000). PCR mixtures were prepared as described above. A negative control (no DNA) and a positive control (DNA from an oyster *C. gigas* infected with *M. mackini*) were used in each PCR. The PCRs were performed under the following reaction parameters: 94°C for 2 min, 35 cycles at a melting temperature of 94°C for 1 min, an annealing temperature of 50°C for 1 min, an extension temperature of 72°C for 1 min 30 s, followed by a final extension period of 72°C for 7 min.

Cloning and sequencing

The PCR products obtained were ligated into the cloning vector pCR2.1 at 14°C overnight and transformed into *Escherichia coli* One Shot Top 10F′ Chemically Competent cells (Invitrogen Life Technologies™). Transformed cells were screened by PCR using the pair of primers M13 forward (5′ GTA AAA CGA CGG CCA G 3′) and reverse (5′ CAG GAA ACA GCT ATG AC 3′). PCR products from 4 positive clones of the ITS1-5.8S-ITS2-28S region and 2 clones of each fragment of the 18S regions were cleaned, before sequencing, with ExoSap-It (Usb) for 15 min at 37°C, followed by an inactivation of 15 min at 80°C. Sequencing was performed by the company Secugen (Madrid) using M13F and M13R primers and the chromatograms were analysed using ChromasPro v.1.41 (Technelysium). All generated sequences were searched for similarity using the basic local alignment search tool (BLAST) through web servers of the National Center for Biotechnology Information (USA) and were then aligned using the Clustal W multiple alignment in the MEGA program to produce the consensus sequence of rDNA.

Phylogenetic distances

To estimate of evolutionary divergence between sequences of the ribosomal gene complex, a pairwise distance estimation was calculated using the maximum composite likelihood method in MEGA 4 software. The compared sequences were those of the *Mikrocytos* parasite infecting clams from Galicia, *M. mackini* infecting *C. gigas* (HM563060), *Mikrocytos* sp. infecting *O. edulis* from Canada (*Mikrocytos* sp.-Atl, DQ237912), and *Mikrocytos* sp. infecting *C. gigas* from British Columbia (*Mikrocytos* sp.-BC, HM563061). The analysis of the 18S, 5.8S and ITS2-28S rDNA involved 472, 122 and 387 nucleotides, respectively.

Statistical analysis

Differences in length, weight and condition index between buried and surface clams from each sampling site were analysed with the Mann-Whitney test, using MINITAB 15 software. Comparisons of prevalence of BRD signs and infection with microcells between buried and surface clams from each sampling site were performed using a critical ratio (Z), which calculates a 95% confidence interval on the difference between the 2 groups and a 2-sided p-value, using EpiCalc 2000 software Version 1.02 (Gilman & Myatt 1998).

RESULTS

Biometric measurements and BRD diagnostics

The mean length, mean weight and mean condition index of the clams from each sampled bed are shown in Table 2. Differences in length between buried and surface clams were not significant at any sampled location; differences in weight between buried and surface clams were significant only in the case of clams of foreign origin collected in Carril; surface clams showed significantly lower condition index than buried ones except those of foreign origin collected in Carril and those from Camariñas, where differences were not significant. Signs of BRD were detected in clams from every sampled location (Table 2); differences in prevalence between buried and surface clams were significant only in Abanqueiro ($p < 0.001$) and in clams with Spanish origin grown-out in Carril ($p = 0.01$); in both cases, prevalence was higher in surface clams.

Histopathology

Histopathological analysis disclosed uninucleated, round to ovoid microcells, 2.4–3.8 µm in diameter (mean = 3.0; SD = 0.34; N = 25), with a basophilic, round to ovoid, central nucleus ca. 1–2 µm in diameter, occurring in the connective tissue of most organs, including adductor muscle, foot, mantle lobes, siphon, gills and visceral mass. Some microcells appeared within haemocytes and others showed an extracellular location (Fig. 2). Haemocytic infiltration was observed in the tissue areas where microcells occur, which caused disorganisation of muscle fibres in the adductor muscle, foot

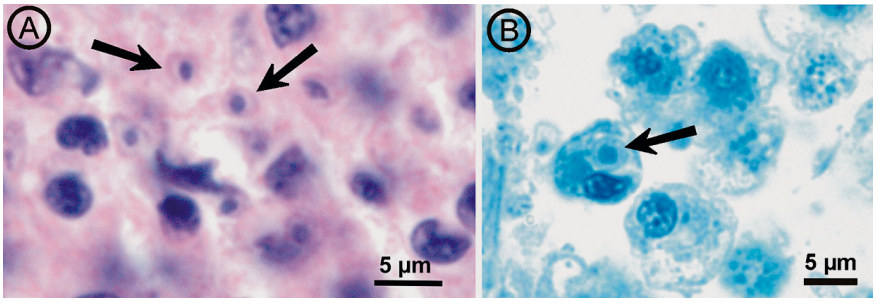

Fig. 2. Micrographs of sections of a Manila clam *Ruditapes philippinarum* infected with microcells corresponding to a *Mikrocytos*-like parasite. (A) Histological section showing the parasite (arrows); Harris' haematoxylin and eosin. (B) Semithin section showing the parasite (arrow) within a clam haemocyte; toluidine blue

Table 2. Mean (±SD) values of length, weight and condition index and prevalence of brown ring disease (BRD) signs in the study samples of Manila clam *Ruditapes philippinarum*. B: buried clams; S: surface clams

Site	B or S	Origin	N	Length (mm)	Weight (g)	Condition index	Prevalence (%) of BRD signs
Camariñas	B	Local recruitment	15	39.3 ± 1.4	14.74 ± 2.01	39.45 ± 5.13	13.3
	S	Local recruitment	15	39.0 ± 2.5	14.29 ± 2.85	43.17 ± 4.91	40
Abanqueiro	B	Undetermined	30	44.5 ± 2.7	22.53 ± 4.31	43.68 ± 8.71	7
	S	Undetermined	30	43.7 ± 3.5	21.03 ± 6.45	34.08 ± 6.57	87
Carril	B	Foreign	15	39.08 ± 4.7	14.46 ± 4.33	35.33 ± 6.83	46.7
	S	Foreign	15	38.7 ± 1.4	10.88 ± 2.50	31.87 ± 5.48	53.3
	B	Spanish	15	30.6 ± 1.1	6.05 ± 0.72	36.93 ± 5.21	20
	S	Spanish	15	30.7 ± 4.2	5.55 ± 1.99	31.66 ± 4.30	73.3
Espasante	B	Foreign	30	35.3 ± 2.7	10.96 ± 3.08	38.48 ± 4.28	46.7
	S	Foreign	19	36.5 ± 2.4	10.53 ± 1.68	32.81 ± 8.22	42.1

and siphon. The microcells were found at each sampling site, with significantly ($p < 0.001$) higher prevalence in surface than in buried clams (Table 3). Additionally, other parasites were detected in the clams (Table 3).

Analysis of rDNA sequences

Regarding PCR assays for detection purposes, a positive PCR amplification was obtained with the pair of primers Mm18SF1 and Mm18S_1450R. PCRs carried out using the primers marked in Table 1 as used for DNA sequence analysis, allowed obtaining a consensus sequence of 1670 bp of the ribosomal gene complex of the microcells infecting the Galician clams. This sequence contained a section of 1166 bp of the 18S region, the whole ITS1 (29 bp), 5.8S region (122 bp) and a section of the ITS2-28S region (353 bp). The 18S region was amplified using 2 pairs of primers: (1) MikrocGal-1F/MikrocGal-358R yielded a PCR product of 377 bp, and (2) Mm18SF1/Mm18S_1450R yielded a PCR product of 763 bp. The cluster ITS1-5.8S-ITS2-28S was obtained with the primers Mm18S_1435F/pro28S, which yielded a product of 660 bp. The sequences were assembled to obtain the consensus sequence, which was deposited in GenBank under accession number KF548044. Likewise, the partial sequences obtained in this study were also deposited in GenBank under accession numbers KF548045–KF548052. Sequences of the microcells of Galician cells shared 92% nucleotide identity with *Mikrocytos* sp. from *Ostrea edulis* (DQ237912, 15% of the sequence compared) and 85% nucleotide identity with *M. mackini* (HM563060, 76% of the sequence compared) and *Mikrocytos* sp. from *Crassostrea gigas* (HM563061, 74% of the sequence compared). The pairwise distances calculated among the different regions of the ribosomal gene complex of the microcells of the Galician clams and those of *M. mackini* and the 2 *Mikrocytos* sp. are shown in Table 4. The pairwise distances corresponding to the ITS1 gene could not be calculated due to the high divergence among the sequence of the microcells of Galician clams and those of the *Mikrocytos* spp. For every region, the microcells of the Galician clams were the most divergent among the compared parasites.

Table 4. Genetic divergence (%) between sequences corresponding to the ribosomal gene complex of the microcells of Galician clams (*Mikrocytos*-like-Gal, GenBank accession no. KF548044), *M. mackini* infecting *Crassostrea gigas* from Canada (accession no. HM563060), *Mikrocytos* sp. infecting *C. gigas* from British Columbia, Canada (BC, accession no. HM563061), and *Mikrocytos* sp. infecting *Ostrea edulis* from Atlantic Canada (Atl, accession no. DQ237912)

	M. mackini	*Mikrocytos* sp.-BC	*Mikrocytos* sp.-Atl
18S rDNA			
Mikrocytos-like-Gal	18.2	16.4	16.8
M. mackini		4.5	4.3
Mikrocytos sp.-BC			0.5
5.8S rDNA			
Mikrocytos-like-Gal	25.4	24.1	
M. mackini		3.4	
ITS2-28S rDNA			
Mikrocytos-like-Gal	34.4	34.8	
M. mackini		16.7	

Table 3. Mean prevalence (%) of parasites in Manila clam *Ruditapes philippinarum* samples from 4 sites. The *Mikrocytos*-like parasite investigated in this study is highlighted in **bold**. B: buried clams; S: surface clams; For.: foreign clams; Sp.: spanish clams

Pathological condition	Camariñas		Abanqueiro		Carril				Espasante	
	B	S	B	S	B (For.)	S (For.)	B (Sp.)	S (Sp.)	B	S
Rickettsia-like organisms in digestive gland	33.3	13.3	23	20	6.7	6.7	6.7	6.7	10	10.5
Rickettsia-like organisms in gills	0	0	7	0	0	0	0	0	0	42.1
Ciliates in gills	0	6.7	10	13	13.3	6.7	13.3	16.7	13.3	52.6
Nematopsis sp.	0	6.7	3	3	20	6.7	0	0	0	0
Undetermined gregarine	20	0	3	0	0	0	0	0	6.7	5.3
Perkinsus sp.	0	0	23	10	0	0	0	0	3.3	0
***Mikrocytos*-like parasite**	**33.3**	**93.3**	**3**	**37**	**13.3**	**73.3**	**33.3**	**86.7**	**10**	**73.7**
Turbellarians	0	6.7	6	3	6.7	0	6.7	0	13.3	0
Trematode metacercariae	0	6.7	0	0	0	0	0	0	40	15.8
Copepods	0	0	0	0	0	0	0	0	3.3	0

DISCUSSION

Infection with microcells was detected at all 4 sampling sites, with significantly higher prevalence in surface than in buried Manila clams. The rise to the surface of infaunal molluscs can be considered a precursor to death (Desclaux et al. 2002, Le Grand et al. 2010) when they are heavily stressed. In fact, surface clams showed either lower condition index or lower weight than buried clams while differences in size (length) were not significant, which suggests higher stress in surface clams. However, microcells could not be categorically blamed for the rise of the clams to the sediment surface or for mortality because BRD signs were also detected at every site. BRD can be lethal (Paillard 2004), and previous studies have reported higher prevalence of BRD in surface than in buried clams (Paillard et al. 1994, Figueras et al. 1996, Park et al. 2006). Nevertheless, there were some sites in our study at which the BRD prevalence was not significantly different between buried and surface clams. The other parasites found in this study should not be considered as the main cause of the rise of clams to the sediment surface or clam mortality because of their limited pathogenicity or low prevalence. The most productive commercial clam beds in Galicia (including *Ruditapes philippinarum*) are sampled each year, and the specimens are analysed by histology within a shellfish health monitoring programme performed by INTECMAR. Only 2 out of ca. 500 clams analysed in 2009 showed microcells but with low intensity. Since then, microcells have not been detected in Galician clams by histology (data not shown).

The morphological (light microscopy) characteristics of the microcells were similar to those described for *Mikrocytos mackini* parasitising *Crassostrea gigas* (Hervio et al. 1996, Carnegie et al. 2003, Meyer et al. 2005) and to the *Mikrocytos*-like parasite infecting *Donax trunculus* (Garcia et al. 2012). Those characteristics do not allow discrimination from the oyster parasite *Bonamia exitiosa*, whereas the central position of the nucleus in microcells infecting the Manila clams would allow distinguishing them from the oyster parasite *B. ostreae*, whose nucleus is eccentric (Abollo et al. 2008). Sequencing results showed that the microcells infecting Manila clams from Galicia were closely related to the genus *Mikrocytos.* Characterisation of the 18S-ITS1-5.8S-ITS2-28S regions of the ribosomal gene of the microcells of the Galician clams confirmed that they corresponded to a *Mikrocytos*-like parasite, closer to the *Mikrocytos* sp. infecting *C. gigas* from Canada than to *M. mackini*; higher similarity values with the *Mikrocytos* sp. infecting *Ostrea edulis* were recorded, but they were based on a shorter sequence fragment. The ITS1 region of the *Mikrocytos*-like parasite from Galicia was much shorter (29 nucleotides) than the ITS1 regions described by Abbott et al. (2011) for *Mikrocytos* sp. (fully described as the new species *Mikrocytos boweri* by Abbott et al. 2014) infecting *C. gigas* (159 nucleotides) and *M. mackini* (108 nucleotides); this important difference in the sequence length impeded further comparison regarding this region. Lack of sequences deposited in GenBank from *Mikrocytos* sp. infecting *C. gigas* from China (Wang et al. 2010) and the *Mikrocytos*-like parasite infecting *D. trunculus* from France (Garcia et al. 2012), precluded comparison with them. Pairwise distances pointed out the microcells of the Galician clams as the most divergent among the parasites involved. This fact, the lack of ultrastructural characterisation of the microcells of the Galician clams and the lack of a well developed taxonomic characterisation of the genus *Mikrocytos* prevent us from going beyond considering the microcells of the Galician clams as a *Mikrocytos*-like parasite in taxonomic terms.

This is the first report of *a Mikrocytos*-like parasite infecting *R. philippinarum*. Garcia et al. (2012) reported a *Mikrocytos*-like parasite infecting the clam *D. trunculus*, which was associated with high clam mortality in 3 wild beds from the French Atlantic coast during summer and autumn of 2010, reaching 80% prevalence. The reports of *Mikrocytos*-like parasites infecting *D. trunculus* (Garcia et al. 2012) and *R. philippinarum* support that parasites of this genus are not exclusive to oysters. The Manila clam is farmed in many countries worldwide, and transfers are frequent for farming purposes. Therefore, attention should be paid to avoid spreading *Mikrocytos*-like parasites to new areas through Manila clam transfers. In our study, the *Mikrocytos*-like parasite was detected in clams that had been introduced into cultured beds (Espasante, Carril, Abanqueiro) for growing-out and in clams deriving from natural recruitment in a natural bed (Camariñas), according to the information on the clam samples offered by fishermen and clam farmers. The recent reports of *Mikrocytos*-like parasites infecting new hosts and widening the geographic range of this group of parasites to Europe and China (Wang et al. 2010) together with the high levels of divergence among their sequences highlight that this group of parasites remains poorly known. More attention should be paid in looking for these microcells in new areas and hosts, which should contribute to improve the knowledge on this group of microorganisms.

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