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One *Perkinsus* species may hide another: characterization of *Perkinsus* species present in clam production areas of France

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Abstract :

Although clam populations in France are known to be infected with protozoans of the genus *Perkinsus*, no molecular characterization was previously performed on these parasites. Considering that several members of this genus have been associated with mortalities of molluscs worldwide, a study was undertaken in order to characterize these parasites in France. For that purpose, clams, *Ruditapes philippinarum* and *R. decussatus,* collected from different production areas and found to be infected with *Perkinsus* sp. in thioglycolate culture medium, were selected for PCR-RFLP tests and sequencing. *Perkinsus olseni* was detected in all the investigated areas and results also suggested the presence of *P. chesapeaki* in Leucate, a lagoon on the Mediterranean coast and in Bonne Anse in Charente Maritime, on the Atlantic coast. Clonal cultures from both detected species were produced in order to describe and compare *in vitro* stages. Differences in size between both *Perkinsus* spp. were noticed especially for schizonts and zoosporangia. Lastly, *in situ* hybridization tests allowed confirmation of the presence of both species in the same *R. decussatus* population and even in same clams. This is the first detection of *P. chesapeaki* in *Ruditapes* species and outside North America, which questions its introduction into Europe.

Keywords : clam parasites ; *Perkinsus chesapeaki* ; *Perkinsus olseni* ; *Ruditapes philippinarum* ; *Ruditapes decussatus* ; ribosomal RNA; actin; ISH

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48

49 **INTRODUCTION**

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52 Clam is among the ten most noticeable bivalves produced by European aquaculture.
53 This production mainly relies on two different species: the Japanese carpet shell clam
54 *Ruditapes philippinarum* and the grooved carpet shell clam *R. decussatus* with an
55 estimated production of 68 010 t and 7 994 t respectively in 2009 if we include
56 aquaculture and fisheries figures (FAO 2009). In France, clam production, estimated
57 at 1 263 t (FAO 2009), is the third most important bivalve one after *Crassostrea gigas*
58 and mussels. This production is mainly concentrated in Arcachon Bay and South
59 Brittany (D'Hardivillé *et al.* 2010a and b, Sanchez *et al.* 2010). However, several
60 natural beds are present along the French coasts including lagoons like Leucate
61 Lagoon or semi-closed bays like Gulf of Morbihan and Bonne Anse in Charente
62 Maritime (Figure 1). The Japanese carpet shell clam is mainly present in North and
63 West of France while the grooved carpet shell clam is dominant in lagoons from the
64 Mediterranean Coast (Garcia *et al.* 2006). In spite of being fairly well organised and
65 regulated, French clam industry is still facing some problems with economic
66 consequences for producers including diseases such as the brown ring disease due to
67 *Vibrio tapetis* (Paillard 2004) or the Brown Muscle Disease (Dang *et al.* 2008). In
68 addition, clam population dynamics may be affected by the presence of parasites
69 belonging to the genus *Perkinsus* (Lassalle *et al.* 2007).
70 Microorganisms of this genus have frequently been associated with important
71 mortality events in different mollusc species worldwide. The first described species in

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72 this genus was *Perkinsus marinus* in the 1940s in U.S.A during mass mortality of the
73 American oyster *Crassostrea virginica* (Ray 1996). *Perkinsus olseni* was then
74 reported and described in the abalone species *Haliotis ruber* and *H. laevigata* in
75 Australia (Lester & Davis 1981, Goggin & Lester 1995). A *Perkinsus* species initially
76 named *P. atlanticus* was described in the grooved carpet shell clam *R. decussatus*
77 following mortality events in Portugal (Azevedo 1989). Molecular investigations
78 demonstrated that *P. olseni* was a senior synonym of *P. atlanticus* (Murrell *et al.*
79 2002). More recently other *Perkinsus* species have been characterized: *P. chesapeaki*,
80 a parasite of the soft-shell clam *Mya arenaria* (McLaughin *et al.* 2000) which is the
81 senior synonym of *P. andrewsi* detected in the Baltic clam *Macoma balthica*
82 (Burreson *et al.* 2005) present on the eastern coast of U.S.A.; *P. qugwadi* associated
83 with mortalities of Japanese scallop *Patinopecten yessoensis* in Canada (Bower *et al.*
84 1998); *P. mediterraneus* described in flat oysters *Ostrea edulis* in Balearic Islands,
85 Spain (Casas *et al.* 2004); *P. honshuensis* described in the Japanese carpet shell clam
86 *R. philippinarum* in Japan (Dungan & Reece 2006) and *P. beihaiensis* characterized
87 from oyster species *Crassostrea ariakensis* and *C. hongkongensis* of Souhern China
88 (Moss *et al.* 2008). These characterizations are based on initial detection using non
89 specific assays like histology or culture in Ray's Fluid Thioglycolate Medium
90 (RFTM), geographic distribution, host range, parasite morphology especially the
91 description and comparison of different stages of several *in vitro* isolates and on
92 phylogenic analysis. In Europe, *Perkinsus* sp. parasites have been reported in France,
93 Spain, Portugal and Italy. However some of these reports are only based on genus-
94 specific assays. Molecular characterization of the 18S and ITS rDNA sequences
95 allowed concluding about the presence of *P. olseni* in *Ruditapes decussatus* from
96 Catalonia and Galicia in Spain and from Adriatic Sea in Italy (Elandaloussi *et al.*

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97 2009, Casas *et al.* 2002, Abollo *et al.* 2006) and about the presence of *P.*
98 *mediterraneus* in *Ostrea edulis* from Balearic Islands (Casas *et al.* 2004).
99 While high clam mortality associated with perkinsosis has been recorded in European
100 affected areas (Azevedo 1989, Ruano and Cachola 1986, Figueras *et al.* 1992,Villalba
101 *et al.* 1993), the actual impact of perkinsosis on clam production is thus still under
102 debate (Villalba 2008) and seems to be positively related to environmental factors
103 such as water temperature and salinity (Casas *et al.* 2002, Cigarria *et al.* 1997, Villalba
104 *et al.* 2005). Furthermore, Choi, K. *et al.* (2002) reported that *Perkinsus* sp. infection
105 levels relate significantly to sediment type: clams living on muddy flats tend to have
106 higher levels of infection than those on sandy tidal flats. Considering the lack of data
107 related to *Perkinsus* species in France and that infection with *Perkinsus olseni* is
108 notifiable to the World Organisation for Animal Health (OIE 2011), the study reported
109 here was undertaken to specifically identify parasites of this genus occurring in
110 different French clam growing areas. For that purpose, clams collected from different
111 areas and shown to be infected by RFTM assays were selected for PCR-RFLP tests
112 and sequencing works. *Perkinsus olseni* was detected in most of the investigated areas
113 and unexpected results were obtained in two locations: Leucate Lagoon in south of
114 France and Bonne Anse in Charente Maritime on the middle West coast of France
115 (Figure 1). Additional sampling was carried out in Leucate Lagoon. Clams were
116 treated in order to study intra- and inter-individual genetic variability of the parasite.
117 Moreover, parasites were cultivated in order to describe *in vitro* stages. Taken
118 together, results obtained during this study are indicative of the presence of two
119 *Perkinsus* species *P. olseni* and *P. chesapeaki* in two French clam production areas.
120 This is the first report of *P. chesapeaki* infections among clams outside of North
121 America, the first report of *P. chesapeaki* infections among *Ruditapes decussatus* and

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122 *R. philippinarum* clams, and the first report of *P. chesapeaki in vitro* isolates from
123 European clams.
124
125
126 **MATERIAL AND METHODS**
127
128
129 **Sampling**
130 Clams including *Ruditapes decussatus* and *R. philippinarum* were collected in
131 October 2004 and 2005 during an epidemiological survey aiming at establishing the
132 distribution, prevalence and infection rates of perkinsosis in main French clam
133 production areas (Figure 1). For some samples, gill tissues were processed for *in vitro*
134 parasite culture (see section "culture" below). Clams from Golfe of Morbihan,
135 Charente Maritime and Arcachon consisted of Japanese carpet shell clams *R.*
136 *philippinarum* while clams from Leucate consisted of grooved carpet shell clams *R.*
137 *decussatus*.
138 Additional samplings were carried out in Leucate Lagoon (60 clams *R. decussatus*) in
139 October 2008 and in Bonne Anse, Charente Maritime, in September 2010 (30 clams
140 *R. philippinarum*). Pieces of gills collected from these clams were ground and
141 processed for *in vitro* parasite culture in order to describe parasite stages (see below
142 section "culture"). Clams from Leucate were also processed in order to test them by *in*
143 *situ* hybridization.
144
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146 **DNA extraction**

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147

148 Sixty four samples of cryopreserved or fresh parasite cultures were used for DNA
149 extraction. For cultures obtained from clams collected before 2008, DNA was
150 extracted using the QIAamp DNA minikit (Qiagen) according to the manufacturer's
151 instructions. DNA was eluted and resuspended in a final volume of 50 µl of sterile
152 deionised water and then diluted at a final concentration of 100 ng μl^{-1}. For cultures
153 obtained from clams collected in 2008 or after, cells were concentrated by
154 centrifugation (150 g for 5 min) and 100 µl of cell suspension (minimum
155 concentration 10^5 cells ml^{-1}) were placed and stored on a QIAcard FTA spot (Qiagen)
156 according to the manufacturer's instructions. QIAcard FTA spot was used after 2008
157 to simplify the handling and processing of DNA from parasite cultures.

158

159

160 **PCR, PCR-RFLP, cloning and sequencing**

161 Three PCR assays targeting sequences of rDNA internal transcribed spacer regions
162 (ITS), large subunit rRNA genes (LSU), and actin-1 genes were used in order to detect
163 and characterize parasites isolated during this study. All the PCR reactions were
164 performed using the Go Taq Polymerase (Promega) in a final volume of 25 µl. DNA
165 (50-100 ng μl^{-1} or QIAcard FTA disk) was added to 24 µl of the PCR mix presented in
166 Table 1. Negative PCR controls consisting of water were included every 10 tested
167 samples in order to check potential contamination. Positive PCR control
168 corresponding to DNA extracted from culture of previously characterized *P. olseni*
169 from Arcachon Bay was included in each PCR test.

170 Parasite species was firstly determined using the PCR-RFLP approach developed by
171 Abollo, E. *et al.* (2006). Analyses were performed by separate digestions for 2 h at

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172 37°C of 10 µl of PerkITS750-85 with 0,25 units of *Rsa*I and *Hinf*I (Promega).
173 Enzymes were inactivated 20 min at 65°C and the resulting fragment patterns were
174 analysed electrophoretically on 2% agarose gel.

175 Some PCR products were cloned using the original TOPO TA cloning kit (Invitrogen)
176 according to manufacturer's recommendations and positive clones were then selected
177 for plasmid DNA purification by FastPlasmid® Mini (Eppendorf). Some plasmidic
178 DNA suspensions were bidirectional sequenced using the Big Dye V3 sequencing kit
179 (Applied Biosystem) and standard M13 forward and reverse primers. Obtained
180 sequences were compared with those included in GenBank using BLAST algorithm
181 (Atschul *et al.* 1997).

182

183 ***Phylogenetic analyses***

184 Available ITS, LSU and actin gene sequences from *Perkinsus* spp. and dinoflagellates
185 were downloaded from GenBank and included in phylogenetic analyses (Table 2) with
186 sequences obtained in the present study.

187 Alignments were performed using Clustal W (Thompson *et al.* 1994) including in
188 MEGA 5 with open and extend gap penalties of seven and three, respectively.
189 Parsimony analyses were also conducted using MEGA version 5 (Tamura *et al.* 2011)
190 with the close neighbour interchange (CNI) heuristic option. Bootstrap values were
191 calculated over 100 replicates and cut-off value for condensed tree was of 50 %.
192 Phylogenetic analysis of the translated actin gene sequences was performed using
193 deduced amino acid sequences.

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194 Genetic distance corresponding to the number of base substitutions per site from
195 averaging over all sequence pairs was estimated using the Tajima-Nei model (Tajima
196 & Nei 1984) in MEGA5 (Tamura *et al.* 2011).
197
198 ***In vitro culture of Perkinsus spp.* and cryopreservation**
199 Gill tissues were rinsed three times in sterile sea water (SSW) and then
200 decontaminated twice for 30 min in 30 ml of SSW supplemented with an
201 antimicrobial suspension (penicillin G 61,3 mg L^{-1}; streptomycin sulphate 131 mg L^{-1}; nystatin 50 000 U L^{-1}). Tissues were rinsed again 3 times in SSW and ground in
202
203 250 µl of the antimicrobial suspension using a disposable homogenizer. The
204 suspension was mixed and complemented with 750 µl of the antimicrobial suspension
205 before incubation for 15 min at room temperature. Homogenized tissues were
206 centrifuged at 12 000 *g* for 5 min, supernatants were discarded and pellets were
207 resuspended with 1 ml of SSW. Suspensions were then filtered at 100 µm and 50 µl of
208 the filtrate were finally inoculated into 6 wells per sample, each containing 1 ml of
209 antimicrobial and salt- supplemented Dulbecco's modified Eagle's/Ham's F12 (1:2)
210 medium (Gauthier & Vasta 1995, Ordas & Figueras 1998) in 24-well culture plates.
211 Culture plates were incubated at 26°C in humidified air atmosphere chamber and
212 regularly observed under an inverted microscope.
213 Some clonal cultures were established by limiting dilution of the above mentioned
214 cultures according to Gauthier, J.D. and Vasta, G.R. (1995). These clonal cultures
215 were used to describe *in vitro* parasite stages.
216 Parasites cultures were cryopreserved in medium consisting of 10% DMSO, 50% fetal
217 Bovine Serum and 40% DME: Ham's F12 (1:1) according to Gauthier, J.D. and
218 Vasta, G.R. (1995).

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219 Cells from four clonal cultures from two clams characterized as *Perkinsus olseni* and
220 cells from three clonal cultures from one clam characterized as *P. chesapeaki* were
221 observed and measured after 16 days of cultures. For that purpose, 20 µl of each
222 clonal culture were collected twice and observed microscopically using a Malassez-
223 cell haemocytometer. All the cells present in the Malassez-cell haemocytometer were
224 observed in order to estimate proportions of trophozoïtes, schizonts and zoosporangia.
225 Mean cell diameter of each cell type was determined by measuring 100 - 200
226 trophozoïtes and all the schizonts and zoosporangia using the Analysis software
227 (Olympus). Finally, for each *Perkinsus* species, we estimated the mean proportion
228 and cell diameter of each parasite stage by averaging data obtained from the different
229 conspecific clonal cultures.
230
231

232 **Histology and *in situ* hybridization (ISH)**

233 After 48 hours in Davidson's fixative, half clam soft tissues. were maintained in 70%
234 ethanol until they were dehydrated and embedded in paraffin for histology according
235 to standard procedures. Paraffin blocks were cut in 2-3 µm sections and stained by
236 hematoxylin and eosin.
237 The protocol followed for ISH was adapted from Moss *et al.* (2006) and Reece *et al.*
238 (2008). The digoxigenin-labeled probes consisted of a *Perkinsus olseni*-specific LSU-
239 rRNA gene probe (Moss *et al.* 2006) PolsLSU-464DIG (5'-
240 CTCACAAGTGCCAAACAACTG-3') and *Perkinsus chesapeaki*-specific LSU-
241 rRNA gene probe (Reece *et al.* 2008) PchesLSU-485DIG (5'-CAG GAA ACA CCA
242 CGC ACK AG-3').
243 Five µm thick tissue sections on silane-prep™ slides (Sigma, France) were dewaxed,
244 rehydrated, and treated with proteinase K (100 µg ml^{-1} in TE buffer [Tris 50 mM,

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245 EDTA 10 mM]) at 37°C for 5 min. Slides were dehydrated by immersion in an
246 ethanol series and air-dried. Sections were then incubated with 100 µl of hybridization
247 buffer (50% formamide, 10% dextran sulfate, 4× SSC [0.06 M Na_3 Citrate, 0.6 M
248 NaCl, pH 7], 250 µg ml^{-1} yeast tRNA and 10% Denhardt's solution) containing 7 ng
249 $µl^{-1}$ of digoxigenin-labeled probes (Eurogentec). Target DNA and digoxigenin-
250 labeled probe were denatured at 95 °C for 5 min and the hybridization was carried out
251 overnight at 42 °C. Sections were washed in 2× SSC at room temperature (RT) (2×5
252 min), in 0.4× SSC at 42 °C (10 min) and in solution I (100 mM maleic acid, 0.15 M
253 NaCl, pH 7.5) for 5 min. Tissues were then blocked for 30 min at room temperature
254 with blocking reagent (Amersham Life Science) (1% w/v) in solution I. Specifically
255 bound probe was detected using an alkaline phosphatase-conjugated mouse IgG
256 antibody against digoxigenin diluted at 1.5 U ml^{-1} in solution I (1 h, RT). Excess of
257 antibody was removed by two washes in solution I (1 min) and one wash in solution II
258 (0.1 M Tris pH 8, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). Slides were incubated in
259 NBT/BCIP, a chromogenic substrate for alkaline phosphatase, diluted in solution II
260 (20 µl ml^{-1}) in the dark until the parasitic cells are completely stained black-purple.
261 The reaction was stopped with solution III (100 mM Tris, 1 mM EDTA, pH 8). Slides
262 were counterstained for 1 min with Bismarck brown yellow (5 mg ml^{-1}), dehydrated
263 with ethanol and mounted in Eukitt resin. Negative controls included samples without
264 digoxigenin- labeled probe in hybridization mixture or without antibodies during
265 colour development. Positive control consisted of sections from *Ruditapes*
266 *philippinarum* infected with *Perkinsus olseni* originating from Arcachon Bay (France)
267 and *Mya arenaria* infected with *P. chesapeaki* (kindly provided by Dr R. Carnegie).
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272 **RESULTS**

273

274 **<u>Characterization of parasites of the genus *Perkinsus* detected in clam producing</u>**

275 **<u>areas in France</u>**

276 In total, 30 and 33 *in vitro Perkinsus* sp. isolate cultures were propagated from clams

277 *R. philippinarum* and *R. decussatus* respectively (Table 3).

278 Direct PCR-RFLP was performed on five cultures from Arcachon Bay, eight cultures

279 from Morbihan Gulf, 17 cultures from Bonne Anse, Charente Maritime, 33 cultures

280 from Leucate Lagoon (Table 3). Restriction profiles after *Rsa*I and *Hinf*I digestion

281 appeared similar to *Perkinsus olseni* ones (around 413 bp, 193 bp, 74 bp after

282 digestion with *Rsa*I and around 363 bp, 160 bp, 150 bp after digestion with *Hinf*I) for

283 all the tested samples except for seven cultures from five clams collected in Leucate

284 and for 17 cultures from five clams from Bonne Anse, Charente Maritime (Table 3).

285 Indeed, for these cultures, profiles similar to *P. chesapeaki* one were obtained after

286 *Rsa*I digestion (around 248 bp, 195 bp, 157 bp, 74 bp) (Figure 2).

287 Generally, when several cultures were tested for one clam, *Rsa*I digestion yielded

288 similar restriction profiles. However, for 3 clams collected in 2005 in Leucate

289 Lagoon, both *P. olseni* and *P. chesapeaki* RFLP profiles were obtained from replicate

290 cultures propagated from individual clams (Table 4).

291

292 **<u>Genetic variability of the parasite</u>**

293

294 Twenty four Perk ITS 750-85 PCR products were cloned and up to 27 clones per

295 sample were tested again using PCR-RFLP (Table 3). Generally, restriction profiles

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296 obtained on clones were concordant with restriction profiles observed after direct
297 PCR-RFLP. However, in some cases, restriction profiles were different from the
298 profiles described by Abollo, E. *et al.* (2006) (noted ambiguous in Table 3) or
299 corresponded to *P. olseni* restriction profiles whereas the other clones tested for this
300 culture showed *P. chesapeaki* profiles.
301 These clones (which have given ambiguous or unexpected restriction profiles) were
302 selected for sequencing as well as up to 3 clones per culture which have yielded
303 concordant PCR-RFLP results before and after cloning.
304 In total, 28 sequences were obtained for clones showing *Perkinsus olseni* restriction
305 profiles as well as ambiguous ones and displayed between 99 and 100% of identity
306 with *P. olseni* (U07697). The genetic distance within these sequences was low (0.4%).
307 These sequences showed between 1 and 3 point nucleotide modifications (mainly
308 substitutions) and concerned one clone. However, four substitutions could be noticed
309 in more than one clone. These substitutions were located in the ITS-2: A instead of G
310 in positions 40 and 54, G instead of A in position 158 and C instead of T in position
311 225. Five types of sequence (POa; POb; POc; POd and POe) were finally obtained and
312 deposited in GenBank under accession numbers JQ669641- JQ669645. No correlation
313 could be observed between sequences and individual or geographic origin.
314

315 In total, 21 sequences were obtained for clones showing *Perkinsus chesapeaki*
316 restriction profiles and displayed 96-97% of identity with *P. chesapeaki* (AF091541).
317 The overall mean distance between these sequences was 1.6%. Some sequences
318 showed point nucleotide substitutions. However, some modifications could be
319 observed in more than one clone. One substitution (T instead of A) could be noticed in
320 the ITS-1 (80 bp before the end of the fragment) in three clones obtained from clams

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321 collected in Charente Maritime (Bonne Anse). In positions 143-145 of the ITS-2, 13
322 sequences showed an insertion of ATA: eight of those were obtained from clams
323 collected in Charente Maritime, and four were from Leucate clams. Finally, four types
324 of sequence (PCa; PCb; PCc and PCd) were obtained and deposited in GenBank under
325 accession numbers JQ669646- JQ669649. Similarly to *P. olseni*, no correlation could
326 be observed between sequences and individual or geographic origin.
327
328
329 **Phylogenetic analyses of *Perkinsus olseni* and *P. chesapeaki* strains isolated**
330 **during this study**
331
332
333 *ITS region sequence analysis*
334
335 To determine the taxonomic affiliation of *Perkinsus* species reported in the present
336 study with other congeneric species, phylogenetic analysis was first performed on the
337 rRNA- ITS region.
338 The different sequence types (9) obtained on the rRNA-ITS region were included in a
339 maximum parsimony (MP) analysis (Figure 3). As expected, the five sequences
340 showing maximum identity with *Perkinsus olseni* were in the *P. olseni* clade and the
341 four sequences showing maximum identity with *P. chesapeaki* were in the *P.*
342 *chesapeaki* group. However, the MP analysis revealed with 95 % bootstrap support
343 the existence of a sub clade including only French strains within the *P. chesapeaki*
344 clade (Figure 3).
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347 *LSU and actin-1 sequence analyses*

348 In order to complete the data obtained on the rRNA-ITS region, one culture of the
349 *Perkinsus olseni* clade and one culture from *P. chesapeaki* clade were selected for
350 amplification using primers targeting the actin-1 gene region and the LSU region.
351 Both cultures were successfully amplified using PerkActin1-439R and
352 PerkActin1130F. After cloning, three 330 pb-PCR products were sequenced. Two of
353 them were identical and showed maximum identity (98%) with *P. chesapeaki* actin-1
354 (AY876361). The GenBank accession number for this sequence is JQ669650. Last
355 PCR product showed 100% identity with *P. olseni* (AY876352).
356 Both cultures were amplified using LSU A and LSU B primers. PCR products were
357 cloned and five 970 bp-clones were sequenced. Three clones showed maximum
358 identity (99%) with *Perkinsus olseni* LSU (AY876332) whereas the two other ones
359 showed maximum identity (99%) with *P. chesapeaki* (AY876347). These five
360 sequences were deposited in GenBank under the following accession numbers :
361 JQ669651- JQ669655.

362 Phylogenetic analyses performed on the LSU and actin-1 genes supported results
363 obtained on the ITS region: some cultures showing maximum identity on the ITS region
364 with *P. olseni* and *P. chesapeaki* grouped with the same conspecific strains (Figures 4
365 and 5).

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370 **Description of *in vitro* stages of *Perkinsus olseni* and *P. chesapeaki* strains**
371 **isolated from Leucate Lagoon**

372

373 Cells from four clonal cultures from two clams characterized as *Perkinsus olseni* and
374 cells from three clonal cultures from one clam characterized as *P. chesapeaki* were
375 observed and measured after 16 days of cultures.
376 Cultures of both species were propagated in the DME/Ham's F-12 culture medium,
377 where they proliferated predominantly by schizogony, and to a lesser extent by
378 zoosporulation. Trophozoïtes from both species showed a typical signet ring
379 morphology with a large vacuole and eccentric nucleus with a prominent nucleolus
380 (Figures 6A & B) and presented comparable mean cell diameter (Table 5). However,
381 *P. chesapeaki* trophozoites could enlarged up to 41 µm while *P. olseni* ones showed
382 maximum cell diameter of 27 µm.
383 *Perkinsus olseni* showed more but smaller schizonts than *P. chesapeaki* (Table 5,
384 Figure 6A). Finally, only three zoosporangia (0.2%) could be observed for *P. olseni*
385 while at the same time, *P. chesapeaki* presented about 18% of zoosporangia (Table 5,
386 Figures 6B & C).

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391 **<u>Histology and *in situ* hybridization</u>**

392

393

394 Sixty *Ruditapes decussatus* clams collected during 2008 from Leucate Lagoon were
395 analyzed histologically and by ISH assays with the *Perkinsus chesapeaki* probe.
396 Twenty of the same clams were also analyzed by ISH assays with the *P. olseni* probe.
397 Fifty one clams were found infected by histology and showed mature trophozoïtes in
398 connective tissue of different organs including gills, mantle, digestive gland, gonad,
399 muscle, heart, kidney and palps. Mature trophozoïtes were characterized by an
400 eccentric vacuole and a signet ring (Figure 7). Several trophozoïtes were observed
401 inside haemocytes or encapsulated in an eosinophylic acellular matrix (Figures 7 and
402 8) and haemocytes infiltration could be observed closed to the trophozoïtes (Figure 9).
403 Among 60 tested clams, 83% (50/60) showed specific labeling of parasite cells with
404 the *P. chesapeaki* probe. All (20/20 = 100%) tested clams showed labeling of parasite
405 cells by the *P. olseni* probe. Ten clams (10/20 = 50%) that showed dual infections
406 through labeling of parasite cells by one of both probes, were selected for
407 comparisons of *in vivo* tissue tropisms, relative abundances, and morphologies among
408 cells of the co-infecting pathogen species.
409 Connective tissues of all the organs appeared infected with both probes. Gills, mantle
410 and then digestive gland were more often found infected than kidney palps and heart.
411 Gonad and muscle appeared positive for both assays in one clam. Generally in clams
412 with dual infections, *P. olseni* cells were more abundant and widely distributed than
413 *P. chesapeaki* cells. Indeed, *P. olseni* was observed in more organs than *P.*

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414 *chesapeaki*. Moreover, 1.3 up to 17 times more *P. olseni* were counted than *P.*
415 *chesapeaki* in co infected clams except in one clam for which this last was twice
416 more abundant than *P. olseni*.
417 Both *Perkinsus* species could appear as isolated trophozoïtes or in clusters (Figures
418 10A and B and 11A and B). Haemocytic infiltration was observed more frequently
419 associated with *P. olseni* cells (Figure 10A) than with *P. chesapeaki* cells. In
420 histological material, no consistent size differences were detected between cells of the
421 two parasite species (9, 1 ±2,8 µm, n = 161 for *P. olseni* labeled cells; 9,8 ± 2,9 µm, n
422 = 58 for *P. chesapeaki* labeled cells).
423
424
425 **DISCUSSION**
426
427 Parasites of the genus *Perkinsus* have been associated with mortalities of molluscs
428 around the world, including oysters, clams, abalones and scallops (Perkins 1996,
429 Villalba *et al.* 2004). Among these parasites, *Perkinsus olseni* affects the clams
430 *Ruditapes decussatus and R. philippinarum* (Casas *et al.* 2002), two bivalves with
431 important commercial value in Europe where mortalities associated with this
432 parasite have been reported (Figueras *et al.* 1992, 1996). Although parasites of
433 the genus *Perkinsus* are known to infect clams in France (Garcia *et al.* 2006,
434 Goggin 1992, Lassalle *et al.*, 2007, Dang *et al.* 2010), no molecular
435 characterization was previously performed on these parasites.

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436 The main objective of the current study was to characterize at the species level,
437 *Perkinsus* sp. parasites that were detected by genus-specific histological or RFTM
438 assays. For that purpose, parasite cultures obtained from infected clams collected in
439 various French locations were screened by PCR-RFLP according to Abollo, E. *et al.*
440 (2006). In order to be able to detect potential co infection (infection with several
441 species or strains), PCR products were cloned and several clones were screened again
442 by PCR-RFLP. This work obtained *P. olseni*-like restriction profiles in all the
443 investigated areas and also *P. chesapeaki* like restriction profiles in two locations:
444 Leucate Lagoon in southern France and Bonne Anse in Charente Maritime on the
445 middle Atlantic coast of France. In these locations, replicate cultures from individual
446 clams presented different PCR-RFLP profiles; indicating co-infections by multiple
447 pathogen species. Some clones obtained from clams collected in different places and
448 displaying *P. olseni* and *P. chesapeaki* or ambiguous restriction profiles were selected
449 for sequencing. Phylogenetical analyses confirmed RFLP results and showed that
450 parasites of the genus *Perkinsus* present in France belong either to *P. olseni* or *P.*
451 *chesapeaki* clades. However, based on the ITS region parsimony analysis, French *P.*
452 *chesapeaki* strains appeared slightly different from other conspecific strains and
453 grouped together (95% bootstrap support). Genetic distances within our isolate strains
454 were low: 0,6% and 1,6% for *P. olseni* and *P. chesapeaki* sequences respectively.
455 These values are in the range of the intraspecific variations observed within the
456 currently accepted *Perkinsus* species (Moss *et al.* 2008). During the present study, five
457 and four different ITS sequences were obtained for *P. olseni* and *P. chesapeaki*
458 respectively. In a previous study, Reece, K. *et al.* (2001) showed that allelic and
459 genotypic frequencies differed significantly among *Perkinsus marinus* strains isolated
460 from three regions of the U.S.A. and those genotypic differences could be related to

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461 differences in virulence. Based on the analysis of the NTS domain of *P. marinus*,
462 sequence type frequencies varied according to the geographic origin of the samples
463 (Robledo *et al.* 1999). In a study on *P. olseni* cultures, different level of microsatellite
464 polymorphism varied with the geographic origin of the parasites (Vilas *et al.* 2011).
465 More specifically, Japan and New-Zealand parasites appeared much more variable
466 than those from Spain (Vilas *et al.* 2011). In our study, we could not observe any
467 relationship between sequence type and geographic origin. This lack of correlation
468 could partly be explained by a low number of sequences to support regional
469 comparison.

470 The detection of several sequence types (up to 3 different ones) in a same clam can
471 reflect the presence of different *P. olseni* and *P. chesapeaki* strains in a same
472 individual clam as it has been reported for *P. marinus* (Reece *et al.* 1997, 2001) and
473 for *P. olseni* (Vilas *et al.* 2011). It can also reflect variability in the rRNA locus or
474 between the rRNA repeats in a same *Perkinsus* sp. strain. Indeed, previous studies
475 suggested that *in vitro P. marinus* cells are diploids (Reece *et al.* 1997) and that
476 distinct rRNA units exist in *P. andrewsi* -newly called *P. chesapeaki* (Pecher *et al.*
477 2004).

478 In order to confirm and support results obtained on the ITS region, one *Perkinsus*
479 *olseni* and one *P. chesapeaki* cultures were selected for further molecular
480 characterization on the LSU and actin 1 genes. Maximum Parsimony analyses
481 performed on both regions confirmed our previous results and allowed us to conclude
482 that *P. olseni* and *P. chesapeaki* are present in France.

483 The propagation of monoclonal cultures of *Perkinsus olseni* and *P. chesapeaki*
484 produced all parasite stages previously described for these parasite species. Mature

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485 trophozoïtes of both isolates showed vacuolated, signet ring morphology with
486 eccentric nuclei bearing prominent nucleoli. These trophozoïtes presented
487 simultaneous schizogonic and zoosporogonic proliferative cycles. However, in our
488 conditions, zoosporulation was less frequent in *P. olseni* compared to *P. chesapeaki.*
489 Schizonts and zoosporangia of *P. chesapeaki* appeared bigger than those of *P. olseni.*
490 Such differences can also be deduced from data available in the literature (Casas *et al.*
491 2002, Burreson *et al.* 2005, Dungan & Reece, 2006). Compared to these previous
492 observations, *P. olseni* isolated during this study looks very similar in size to other *P.*
493 *olseni* isolates (Casas et al. 2002, Dungan & Reece 2006); while the *P. chesapeaki*
494 isolates of the current investigation showed larger schizonts and smaller zoosporangia
495 than those reported for North American isolates (Coss *et al.* 2001a, Dungan *et al.*
496 2002, Burreson *et al.* 2005).

497 Histological examination of infected *R. decussatus* clams from Leucate Lagoon
498 revealed the presence of spherical trophozoïtes in the connective tissue of many
499 different organs, occasionally isolated but more often in clusters. Haemocytic
500 infiltration was sometimes observed around infected zones. Parasites could be
501 observed inside haemocytes or encapsulated. ISH allowed distinguishing *P.*
502 *chesapeaki* and *P. olseni* in same infected clams. Generally *P. olseni* appeared more
503 spread out and more abundant. These observations were supported by molecular
504 works. Indeed, direct PCR-RFLP allowed detecting *P. olseni* more frequently than *P.*
505 *chesapeaki* in Leucate Lagoon.

506 Infections of different mollusc hosts at the same site by different *Perkinsus* species
507 and strains have been previously reported (Reece *et al.* 1997, Dungan & Reece
508 2006, Reece *et al.* 2008). More specifically, a molecular epizootiology study

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509 revealed the presence of *P. marinus* and in a lesser extent *P. chesapeaki* in
510 oyster samples from several sites located in Chesapeake Bay (Reece *et al.*
511 2008). In our study, *P. olseni* and *P. chesapeaki* appeared sympatric in two
512 distinct locations: in South of France along the Mediterranean sea (Leucate)
513 and in Charente Maritime on the middle West coast of France (Bonne Anse).
514 In the first case *P. olseni* appeared more abundant while in Bonne Anse, PCR-
515 RFLP results suggested that *P. chesapeaki* was more abundant than *P.*
516 *olseni*. These apparent abundance differences could be due to the difference
517 of host species. Indeed, during this study, clams collected from Leucate were
518 *Ruditapes decussatus* whereas clams collected from Charente Maritime
519 (Bonne Anse) were *R. philippinarum*. The apparent abundance difference
520 could also reflect competition between both parasite species. However, the
521 distribution of these parasite species among the organs appeared identical
522 which does not support the hypothesis of interspecific competition.

523 Respective impact of these both parasite species on clams is difficult to assess from
524 our results. No mortality and no population decline were reported in the investigated
525 locations during this study and through the French mollusc health surveillance
526 network (REPAMO). However, considering that mortality has already been reported

527 in association with both parasite species, their prevalence and infection intensity in
528 French clam populations should be monitored.

529 Although the presence of *Perkinsus olseni* was suspected in France because of the
530 presence of this parasite in other closed European country (Elandaloussi *et al.* 2009,
531 Casas *et al.* 2002, Abollo *et al.* 2006), the detection of *P. chesapeaki* was more
532 surprising. Indeed, until now this parasite has only been reported in North America
533 and more especially in the soft-shell clam *Mya arenaria* in Chesapeake Bay
534 (McLaughlin & Faisal 2000, McLaughlin et al. 2000, Dungan et al. 2002) and the
535 razor clam *Tagelus plebeius* in Chesapeake and Delaware bays (Dungan et al. 2002,
536 Bushek et al. 2008). The synonymization by Burreson, E. *et al.* (2005) of *P.*
537 *chesapeaki* and *P. andrewsi* previously characterized in *Macoma balthica* from Rhode
538 River, Maryland (Coss *et al.* 2001b) has consequently enlarged the geographic and
539 host ranges of this parasite. Other clam hosts for *P. chesapeaki* include: *Macoma*
540 *mitchelli*, *Mercenaria mercenaria*, *Mulinia lateralis*, *Rangia cuneata* and *Cyrtopleura*
541 *costata* (Burreson *et al.* 2005, Reece *et al.* 2008). This is the first description of *P.*
542 *chesapeaki* in *Ruditapes decussatus* and *R. philippinarum* and in Europe. Its
543 introduction might have occurred through introduction of susceptible species
544 including *Mya arenaria* or *Mercenaria mercenaria* from North America. *Mercenaria*
545 *mercenaria* was first introduced to the Atlantic coast of France in 1861 and then to the
546 Mediterranean coast in 1965 (Lambert 1947-1949, Ruckebusch 1947-1949, Bascheri
547 *et al.* 1965, Goulletquer *et al.*, 2002). In Europe *Mya arenaria* occurs widely; but it is
548 believed to have been extinct in Pleistocene times and reintroduced by man in
549 historical times (Petersen *et al.* 1992).

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550 The work presented herein would require testing the presence of *P. chesapeaki* in
551 other bivalve species including *Mya arenaria* or *Mercenaria mercenaria* and in other
552 European locations, in particular areas where clams were introduced from North
553 America. Apparent co-habitation of *P. olseni* and *P. chesapeaki* in two distinct
554 locations in France also raises interesting questions regarding relationships between
555 these two parasite species as well as parasite-host interactions.

556

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756 **Villalba, A., Casas, S.M., Lopez, C. and Carballal, M.J.** (2005). Study of
757 perkinsosis in the carpet shell clam *Tapes decussatus* in Galicia (NW Spain). II.
758 Temporal pattern of disease dynamics and association with clam mortality. *Diseases of*
759 *Aquatic Organisms* **65**, 257–267.
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767 ## Figure caption

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770 Figure 1. Locations where clams *Ruditapes decussatus* or *R. philippinarum* were
771 collected for the current study.

772

773 Figure 2. Restriction profiles obtained after *Rsa*I digestion of Perk ITS750-85 PCR
774 products from clams collected in 2005 in Leucate Lagoon. Lanes 2-7 and 10 and 11
775 show *Perkinsus olseni-P. mediterraneus* restriction profiles (around 413 bp, 193 bp
776 and 74 bp) while lanes 8 and 9 show *P. chesapeaki* restriction profiles (around 248 bp,
777 195 bp, 157 bp, and 74 bp). Lanes 1 and 12 show a 100-bp ladder (Smartladder,
778 Eurogentec).

779

780 Figure 3. Maximum Parsimony analysis showing the taxonomic position of *Perkinsus*
781 ITS sequences obtained in the present study. Numbers at branch nodes indicate
782 bootstrap confidence values in percent. The analysis included 57 nucleotide sequences
783 and was conducted using 841 aligned nucleotide positions. Sequences (Accession
784 numbers) obtained in this study were : POa (JQ669641); POb (JQ669642); POc
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790 Figure 4. Maximum Parsimony analysis showing the taxonomic position of *Perkinsus*
791 Actin 1 gene sequences obtained in the present study. Numbers at branch nodes
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793 sequences and was conducted using 88 aligned amino acid positions. (*) sequences
794 obtained in this study.

795
796

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798 large subunit (LSU) rRNA gene sequences obtained in the present study. Numbers at
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800 32 nucleotide sequences and was conducted using 984 aligned nucleotide positions.
801 (*) sequences obtained in this study.

802

803 Figures 6A, B and C. *In vitro Perkinsus olseni* (6A) and *P. chesapeaki* (6B-C)
804 cultures obtained from *Ruditapes decussatus* clams (Leucate Lagoon). 6A-
805 Trophozoïte (T) showing a typical signet-ring morphology with a large vacuole and
806 eccentric nucleus, schizont (S) containing numerous daughter cells, cluster (C) of
807 sibling daughter trophozoites. 6B- Trophozoïte (T) showing a typical signet-ring
808 morphology with a large vacuole and eccentric nucleus, zoosporangium (arrow) with
809 probable discharge tube (*). 6C- Zoosporangium (arrow) with extended discharge
810 tube (*) enclosing hundreds zoospores. Scale bars = 50 µm.

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812 Figure 7-9. H&E–stained tissue section showing *Perkinsus* trophozoïtes in tissues of
813 *Ruditapes decussatus* from Leucate Lagoon. 7 Cluster of mature trophozoïtes inside
814 an eosinophilic acellular matrix (*) showing an eccentric vacuole and a signet ring
815 located in the connective tissue of the mantle. 8. Isolated or grouped trophozoïtes
816 located in the connective tissue of the mantle. Parasite clusters are associated with
817 eosinophilic acellular matrix (*). 9. Isolated and grouped trophozoïtes associated with
818 an important infiltration of hameocytes in the connective tissue of the digestive gland.

819

820 Figures 10A and B. *In situ* hybridization assay using *Perkinsus olseni* probe on a clam
821 *Ruditapes decussatus* from Leucate Lagoon. A. Positive isolated and grouped
822 trophozoïtes in the connective tissue of gills. The presence of the parasites is
823 associated with a strong haemocytic infiltration. B. Positive isolated and grouped
824 trophozoïtes in the connective tissue of the digestive gland.

825

826 Figures 11A and B. *In situ* hybridization assay using *Perkinsus chesapeaki* probe on a
827 clam *Ruditapes decussatus* from Leucate Lagoon. A. Encapsulated positive
828 trophozoïtes in the connective tissue of the digestive gland. B. Positive isolated and
829 grouped trophozoïtes in the gonadal connective tissue.

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Tables

Table 1. Conditions and primer sequences used for the PCR assays

		ITS	LSU	Actin
PCR mix composition	MgCl2 (mM)	2.5	1.5	3
	BSA (µg/ml)	0.4	0.125	$2\ 10^{-4}$
	dNTP (mM)	0.25	0.2	0.1
	Primers (µM)	0. 2	1	1
	Taq polymerase (units/ml)	0.04	0.025	0.125
Amplification programme		4 min at 95°C 40 cycles (1 min at 95°C, 1 min at 65°C, 1 min at 72°C) 5 min at 72°C	4 min at 94°C 35 cycles (30 s at 94°C, 30 s at 55°C, 2 min at 65°C) 5 min at 65°C	5 min at 95°C 40 cycles (1 min at 95°C, 45 s at 58,5°C, 1 min at 68°C) 5 min at 68°C
Size of the amplicons		703 bp	900 bp	300 bp
Reference		Casas *et al.* 2002	Lenaers et al. 1989	Moss *et al.* 2008
Forward primer	Name	Perk ITS 750	LSU A	PerkActin1130F
	Sequence (5’ 3’)	ACATCAGGCCTTCTAATGATG	ACCCGCTGAATTTAAGCATA	ATGTATGTCCAGATYCAGGC
Reverse primer	Name	Perk ITS 85	LSU B	PerkActin1-439R
	Sequence (5’ 3’)	CCGCTTTGTTTGGATCCCC	ACGAACGATTTGCACGTCAG	CTCGTACGTTTTCTCCTTCTC

Table 2. GenBank accession numbers of rRNA-ITS, rRNA-LSU and actin-1 gene sequences used in phylogenetic analyses

Region of the parasite genome	*Perkinsus* species	GenBank accession number
rRNA-ITS	*Perkinsus chesapeaki*	AF091541, AF102171, AF252288, AF440464, AF440466, AF440468, AY876302-AY876307, AY305326, EU919470, EU919484
	Perkinsus olseni	AF140295, AF369967,AF369969, AF441211, AF441213, AF473840, AF522321, AY435092, UO7697
	Perkinsus marinus	AY295188, AY295189, AY295194, AF150987, AF091542, UO7700
	Perkinsus mediterraneus	EU068096, EU068098, DQ370482, DQ370483, AY487839
	Perkinsus beihaiensis	EF204015, EU068095, EF204050, EU068080
	Perkinsus honshuensis	Q516701, DQ516696, DQ516697, DQ516698, DQ516699, DQ516700
	Symbiodinium sp.	AF360576
	Prorocentrum micans	AY465117
	Perkinsus qugwadi	AF151528
rRNA-LSU gene	*Perkinsus chesapeaki*	AY876344- 49
	Perkinsus olseni	AY876330-32
	Perkinsus marinus	AY876319, AY876320, AY876322, AY876325, AY876328, AY876329
	Perkinsus mediterraneus	EF204095-98, EF204100
	Perkinsus beihaiensis	EF526448, EF526451
	Perkinsus honshuensis	DQ516680-82, DQ516684
	Prorocentrum micans	X16108
Actin-1	*Perkinsus chesapeaki*	AY876359-61
	Perkinsus olseni	AY876352, AY876355-57, EF204109-11
	Perkinsus marinus	U84287, U84288, AY876350
	Perkinsus mediterraneus	EF204112-15
	Perkinsus beihaiensis	EF526411-12
	Perkinsus honshuensis	DQ516686-89
	Prorocentrum micans	U84290
	Amphidinium carterae	U84289

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Table 3. Number of tested clams and cultures tested per clam for each locations included in the present study. The sixth column presents number of restriction profile types obtained by direct PCR-RFLP (PO= *Perkinsus olseni* and PC= *Perkinsus chesapeaki*). The following columns present results after cloning PCR products. The last column indicates the obtained sequence types (POa = JQ669641; POb = JQ669642; POc= JQ669643; POd= JQ669644; POe= JQ669645; PCa= JQ669646; PCb = JQ669647; PCc = JQ669648; PCd = JQ669)

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Area	Site	year	number of clams	number of cultures	PCR-RFLP	Number of cloned PCR products	Number of clones tested by RFLP	PCR-RFLP	Sequences
Arcachon (*Ruditapes philippinarum*)	Les Argiles	2004	3	3	3 PO	2	46	45 PO + 1 ambiguous	4 PO (2 POa ; 2 POc)
	Château Madère	2005	1	2	2 PO	2	28	27 PO+ 1 ambiguous	5 PO (4 POa ; 1 POb)
Leucate (*Ruditapes decussatus*)	Nord 2	2005	4	5	5 PO	2	19	19 PO	2 PO (2 POa)
	Nord 1	2005	6	12	9 PO 3 PC	8	68	44 PO 22 PC 2 ambiguous	10 PO (9 POa; 1 POe) 7 PC (4PCc ; 3PCd)
		2008	13	16	4 PC 12 PO	Not done			3 PO (3 POa) 4 PC (3 PCc ; 1PCd)
Golfe du Morbihan (*Ruditapes philippinarum*)	Ile Tascon	2005	3	3	3 PO	2	18	18 PO	1 PO (1 POd)

	Le Lern	2005	2	5	5 PO	2	14	14PO	1 PO (1 POc)
Charente Maritime (*Ruditapes philippinarum*)	Bonne Anse	2010	5	17	17 PC	6	80	77 PC 3 PO	10 PC (1 PCa ; 2PCb ; 1 PCc ; 6 PCd) 2 PO (1 POa ; 1 POe)

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Table 4. Results obtained for six clams *Ruditapes decussatus* collected in Leucate Lagoon in 2005. Between one and three cultures (noted I to III) were obtained for each clam. The third column presents restriction profile types obtained by direct PCR-RFLP (PO= *Perkinsus olseni* and PC= *Perkinsus chesapeaki*). The following columns present results after cloning PCR products. The last column indicates the obtained sequence types (POa = JQ669641; POe= JQ669645; PCc = JQ669648; PCd = JQ669649)

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Clam number	Culture number	PCR-RFLP	Number of cloned PCR products	PCR-RFLP	Sequences
1	I	PO	10	10 PO	1POa
	II	PC	2	2 PC	2PCc
	III	PO	Not done	Not done	
2	I	PO	Not done	Not done	
	II	PC	9	9 PC	1PCc, 1PCd
3	I	PO	6	6 PO	1POa
	II	PO	Not done	Not done	
4	I	PC	10	10 PC	2PCd, 1PCc

	II	PO	1	1 PO	1POa
5	I	PO	9	7 PO and 2 ambiguous	4POa, 1POe
	II	PO	Not done	Not done	
6	I	PO	20	20 PO	2POa

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Table 5. Mean diameters, diameter ranges and proportional abundances of the different parasite stages observed for the *in vitro* culture of *Perkinsus olseni* and *P. chesapeaki*

	Perkinsus chesapeaki				*Perkinsus olseni*			
	Mean diameter (µm)	Diameter range (µm)	Number	Abundance (%)	Mean diameter (µm)	Diameter range (µm)	Number	Abundance (%)
Trophozoïtes	10.7	3.5 - 41.4	936	63.3%	9.7	3.7 - 27.4	1,048	58.1%
Schizonts	34.5	10.8-62.9	277	18.7%	18.2	6.0-40.1	752	41.7%
Zoosporangia	39	21.1 - 63.9	265	17.9%	24.4	22.7 -27.4	3	0.2%

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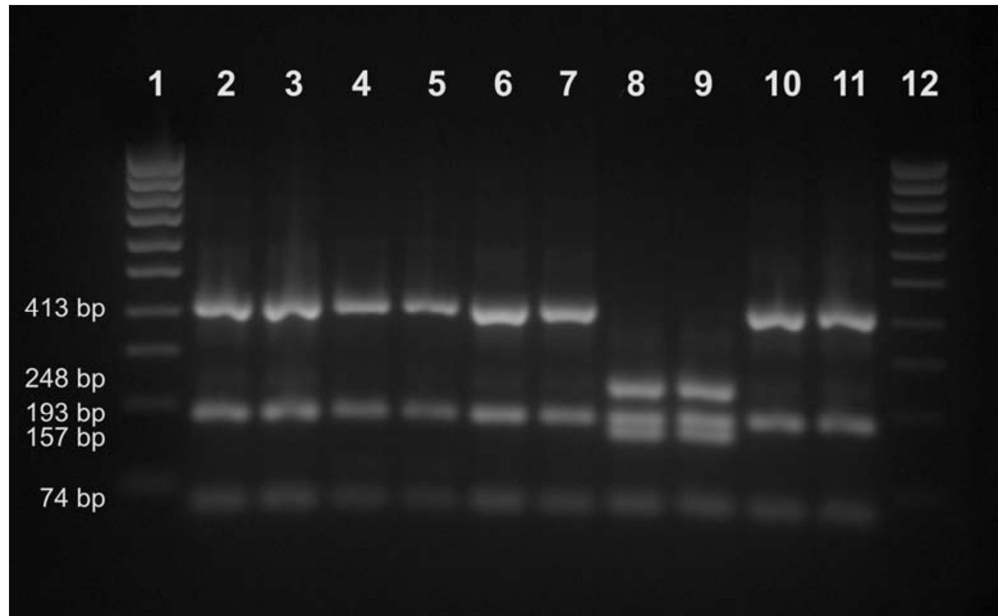
Figure 1. Locations where clams Ruditapes decussatus or R. philippinarum were collected for the current study.



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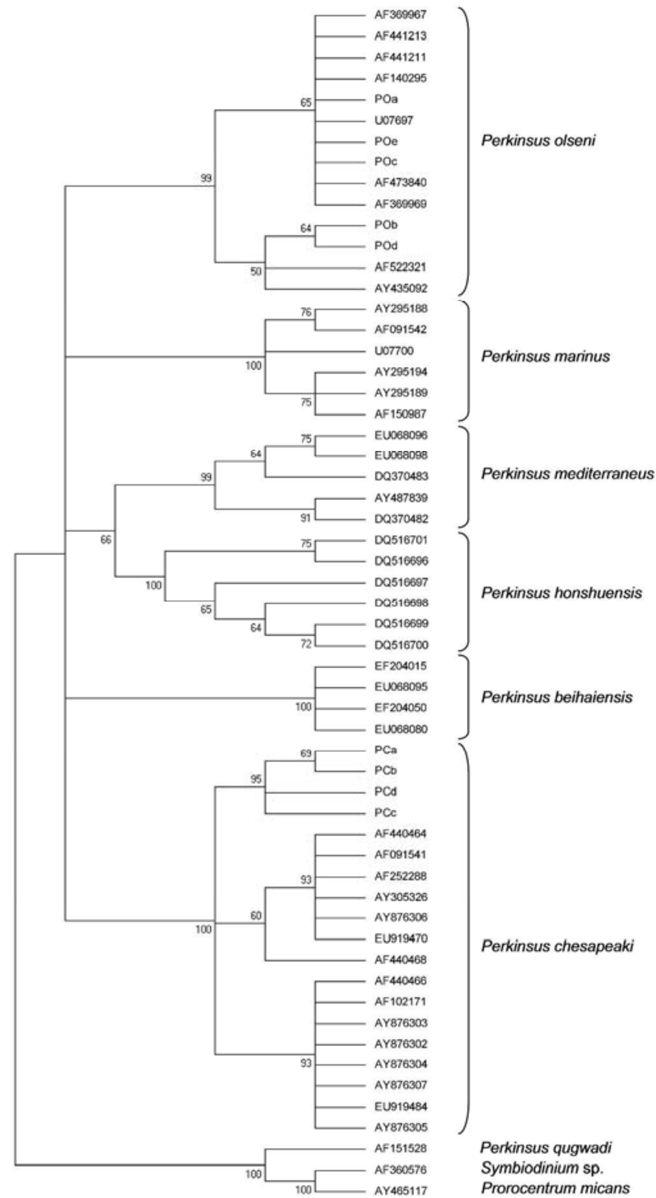


Figure 3. Maximum Parsimony analysis showing the taxonomic position of Perkinsus ITS sequences obtained in the present study. Numbers at branch nodes indicate bootstrap confidence values in percent. The analysis included 57 nucleotide sequences and was conducted using 841 aligned nucleotide positions. Sequences (Accession numbers) obtained in this study were : POa (JQ669641); POb (JQ669642); POc (JQ669643); POd (JQ669644); POe (JQ669645); PCa (JQ669646); PCb (JQ669647); PCc (JQ669648) and PCd (JQ669649). (*) sequences obtained in this study.

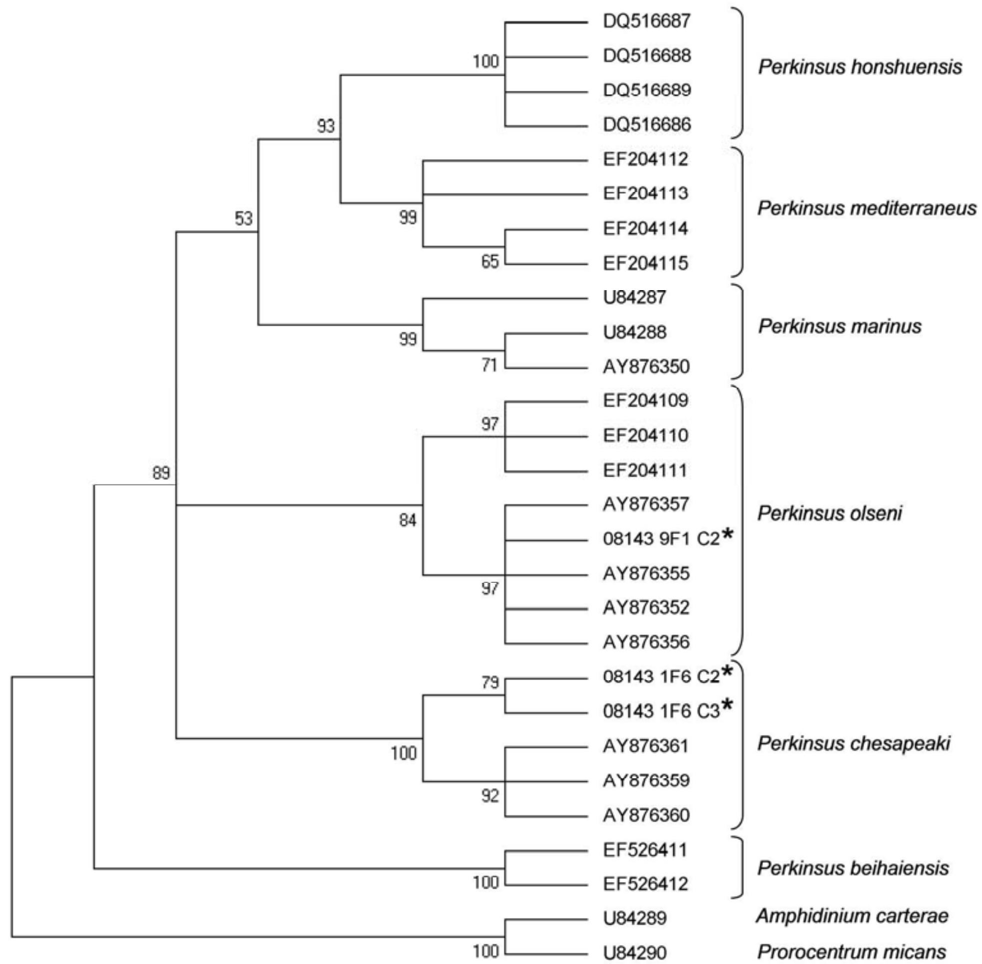
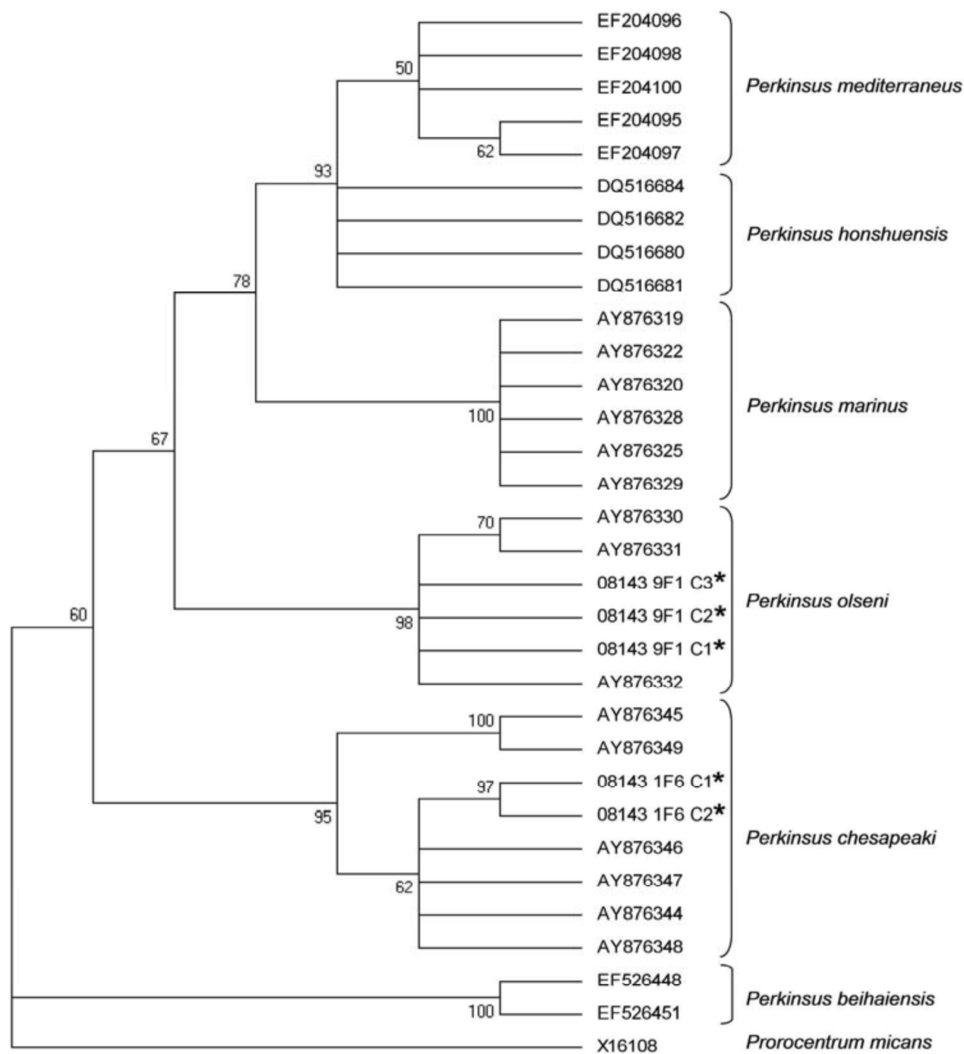


Figure 4. Maximum Parsimony analysis showing the taxonomic position of Perkinsus Actin 1 gene sequences obtained in the present study. Numbers at branch nodes indicate bootstrap confidence values in percent. The analysis included 28 amino acid sequences and was conducted using 88 aligned amino acid positions. (*) sequences obtained in this study.



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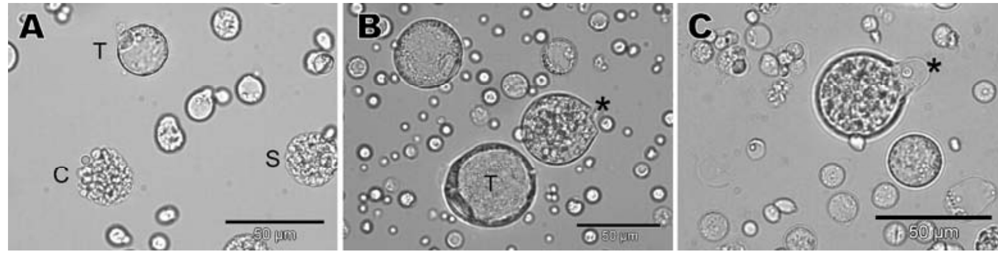

Figures 6A, B and C. In vitro Perkinsus olseni (6A) and P. chesapeaki (6B-C) cultures obtained from Ruditapes decussatus clams (Leucate Lagoon). 6A- Trophozoïte (T) showing a typical signet-ring morphology with a large vacuole and eccentric nucleus, schizont (S) containing numerous daughter cells, cluster (C) of sibling daughter trophozoites. 6B- Trophozoïte (T) showing a typical signet-ring morphology with a large vacuole and eccentric nucleus, zoosporangium (arrow) with probable discharge tube (*). 6C- Zoosporangium (arrow) with extended discharge tube (*) enclosing hundreds zoospores. Scale bars = 50 µm.

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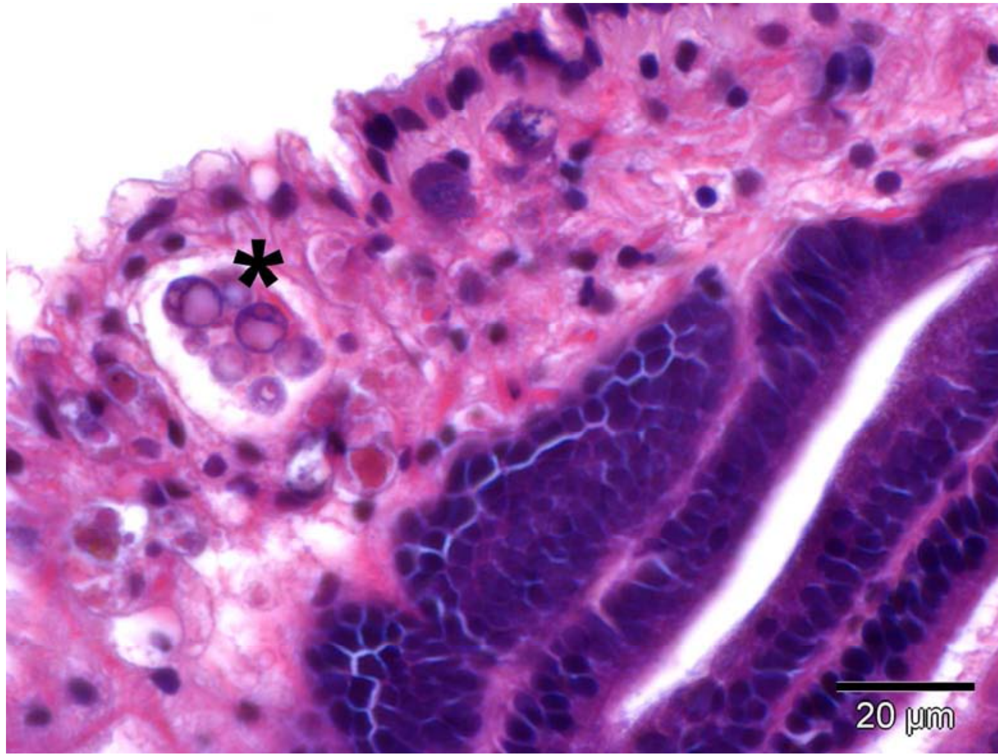

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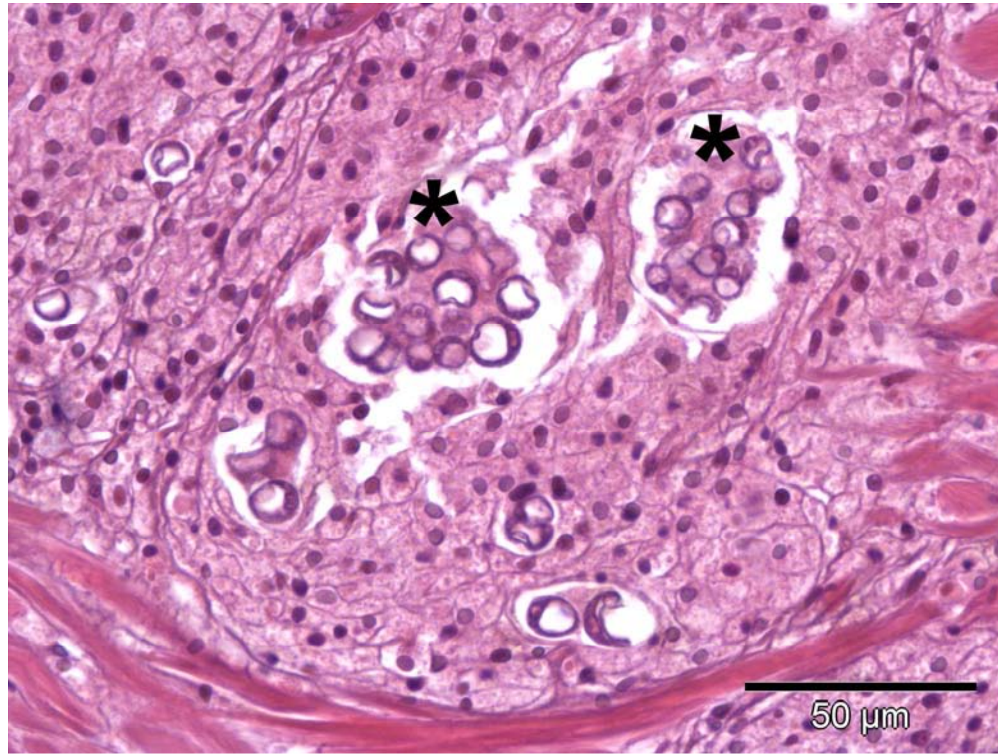
eview



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8. Isolated or grouped trophozoïtes located in the connective tissue of the mantle. Parasite clusters are associated with eosinophilic acellular matrix (*).

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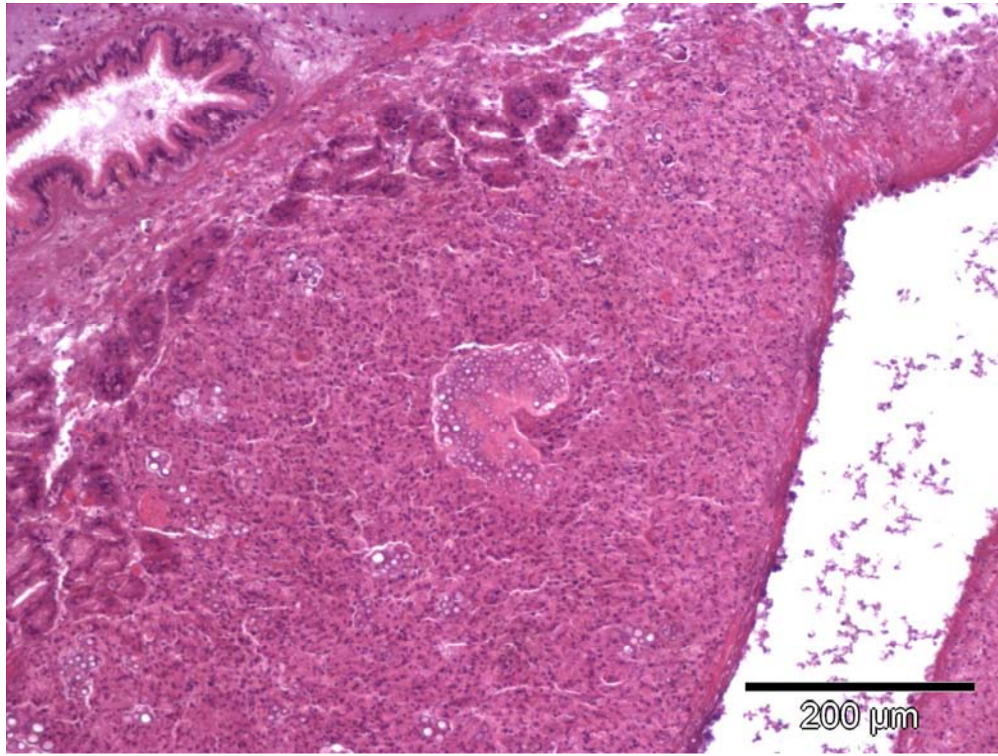

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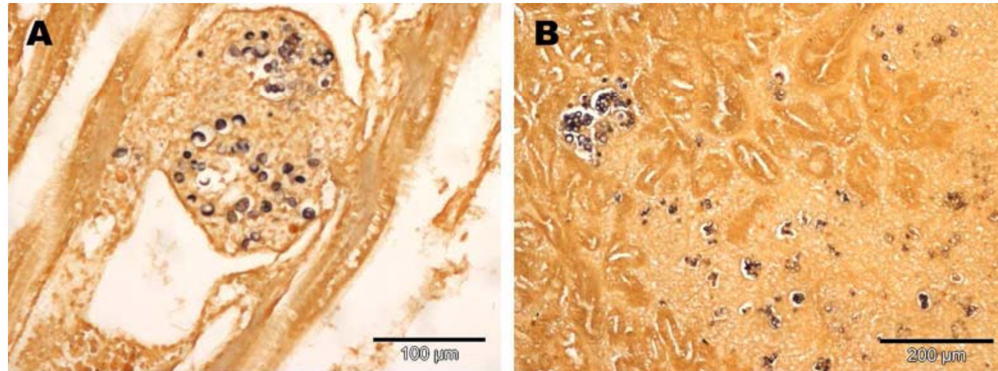
eview



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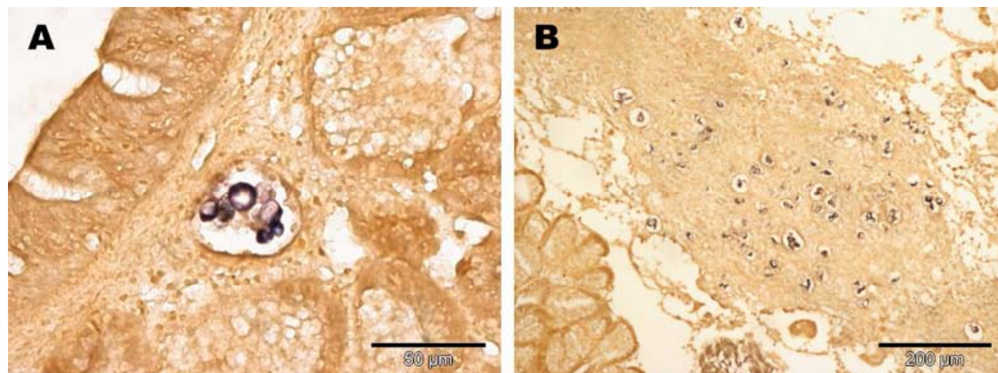

Figures 11A and B. In situ hybridization assay using Perkinsus chesapeaki probe on a clam Ruditapes decussatus from Leucate Lagoon. A. Encapsulated positive trophozoïtes in the connective tissue of the digestive gland. B. Positive isolated and grouped trophozoïtes in the gonadal connective tissue.

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