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Contribution to the understanding of the cycle of the protozoan parasite *Marteilia refringens*

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Abstract:

The paramyxean parasite *Marteilia refringens* infects several bivalve species including European flat oysters *Ostrea edulis* and Mediterranean mussels *Mytilus galloprovincialis*. Sequence polymorphism allowed definition of three parasite types 'M', 'O' and 'C' preferably detected in oysters, mussels and cockles respectively. Transmission of the infection from infected bivalves to copepods *Paracartia grani* could be experimentally achieved but assays from copepods to bivalves failed. In order to contribute to the elucidation of the *M. refringens* life cycle, the dynamics of the infection was investigated in *O. edulis, M. galloprovincialis* and zooplankton over one year in Diana lagoon, Corsica (France). Flat oysters appeared non-infected while mussels were infected part of the year, showing highest prevalence in summertime. The parasite was detected by PCR in zooplankton particularly after the peak of prevalence in mussels. Several zooplanktonic groups including copepods, Cladocera, Appendicularia, Chaetognatha and Polychaeta appeared PCR positive. However, only the copepod species *Paracartia latisetosa* showed positive signal by *in situ* hybridization. Small parasite cells were observed in gonadal tissues of female copepods demonstrating for the first time that a copepod species other than *P. grani* can be infected with *M. refringens.* Molecular characterization of the parasite infecting mussels and zooplankton allowed the distinguishing of three *Marteilia* types in the lagoon.

Keywords : bivalves ; Copepods ; *Marteilia refringens* ; *Paracartia latisetosa* ; parasite life cycle; zooplankton

INTRODUCTION

In France, aquaculture is mainly based on oyster production including production of Pacific cupped oyster, *Crassostrea gigas,* and in a lesser concern of flat oyster, *Ostrea edulis*. This last species is native from Europe where it occurs along the western European coast, the Mediterranean Sea and into the Black Sea. It is presently in the OSPAR (Oslo and Paris Conventions for the protection of the marine environment of the North-East Atlantic) list of threatened and / or declining species and habitats (OSPAR agreement 2008-6) notably because it has suffered from two protozoan diseases, marteiliosis due to *Marteilia refringens* and bonamiosis due to *Bonamia ostreae* (Meuriot and Grizel 1984; Goulletquer and Héral 1997). Considering their impact on flat oyster populations, these two diseases have been included in the list of notifiable diseases to the World Organisation for Animal Health (OIE 2011).

The knowledge of parasite life cycle is crucial for control, eradication and prevention of the disease emergence. Investigations carried out during these last thirty years have contributed to improve our knowledge on the parasite *Marteilia refringens* (Comps 1970; Grizel *et al.* 1974; Comps *et al.* 1982; Villalba *et al.* 1993; Le Roux *et al.* 1999; Berthe *et al.* 2000; Audemard *et al.* 2002; Lopez-Flores *et al.* 2004). However, its life cycle remains partly unelucidated. Several authors have suspected the involvement of intermediate host in the transmission of the parasite from infected to naive oysters (Berthe *et al.* 1998). During studies performed in natural oyster ponds named “claires”, the calanoïd copepod *Paracartia grani* appeared as a potential intermediate host of *Marteilia refringens*. Nevertheless, experimental transmission of the parasite from infected copepods to naive flat oysters failed suggesting that additional or other species could be involved in *M. refringens* transmission (Audemard *et al.* 2002; Carrasco *et al.* 2008a). Some work carried out in open bays in the Ebre Delta, Spain, allowed detection of parasite DNA by nested PCR targeting the IGS region in several zooplanktonic

81 species including *Acartia discaudata*, *A. clausi*, *A. italica*, *Oithona* sp., *Euterpina acutifrons*
82 and larval stages of brachyura (Carrasco *et al.* 2007a and b).

83 The parasite *Marteilia refringens* not only infects flat oysters *Ostrea edulis* but also mussels
84 *Mytilus edulis* and *M. galloprovincialis*. Several mussel mortality events associated with *M.*
85 *refringens* have been reported in Spain and in France including in Corsica (Villalba *et al.*
86 1993; REPAMO 2005). The parasite infecting mussels called *Marteilia maurini* was
87 previously considered as a distinct species from *M. refringens* infecting flat oysters. However,
88 ultrastructural criterions on which species distinction was based were considered dubious by
89 some authors (Villalba *et al.* 1993; Longshaw *et al.* 2001). Moreover, the analysis of the ITS-
90 1 and IGS sequences suggested that these two parasites were conspecific and that *M.*
91 *refringens* was able to infect either flat oysters or mussels (Le Roux *et al.* 2001; Lopez-Flores
92 *et al.* 2004). Based on a dimorphism in the locus of endonuclease *Hha*I in the ITS-1 sequence,
93 two types O and M were defined and can be detected by PCR-RFLP (Le Roux *et al.* 2001).
94 Although *M. refringens* type O preferentially infects flat oysters and type M is mainly found
95 in mussels, several cases of co or cross infections have been reported (Le Roux *et al.* 2001;
96 Lopez-Flores *et al.* 2004; Novoa *et al.* 2005; Balseiro *et al.* 2007). Recently, a new type called
97 *Marteilia* sp. type C was described in cockles *Cerastoderma edule* suffering mortality in the
98 Spanish Mediterranean coast (Carrasco *et al.* 2012).
99
100 Thus, two main questions remain unsolved regarding the parasite *Marteilia refringens*: (1)
101 which is or which are the intermediate host(s) involved in its life cycle ? (2) which are the
102 relative taxonomic positions of *M. refringens* type O and type M ?
103 In order to answer these questions, we have investigated the infection dynamics in flat oysters
104 *Ostrea edulis*, mussels *Mytilis galloprovincialis* and zooplanktonic species cohabiting in an
105 endemic site for marteiliosis: Diana Lagoon in Corsica off the southern coast of France.

Diana lagoon is located on northeastern of Corsica. With a yearly production of 400 tons of mussels (*Mytilus galloprovincialis*), 150 tons of Pacific cupped oysters (*Crassostrea gigas*) and 12 tons of flat oysters (*Ostrea edulis*), it represents the main shellfish culture area of the island. Although infection with *Marteilia refringens* is endemic in the lagoon, flat oyster reproduces naturally and has been exploited since the Roman period. Spat of mussels *Mytilus galloprovincialis* is imported from the Venice Lagoon and grows in Corsica until being marketed. The parasite *Marteilia refringens* was first detected by histology in Diana Lagoon in July 1984 in 58% of flat oysters collected from the natural bed and in 1986 in 8% of mussels originating from Italy and cultivated in Corsica. Since that time, the parasite has regularly been detected in flat oysters and mussels.

In this context, the monitoring of the parasite *Marteilia refringens* was undertaken by histology in flat oysters and mussels collected monthly between June 2007 and June 2008 in Diana Lagoon. Additionally, zooplankton was collected every two weeks around the mussels' production site and screened by PCR. Samples found positive by PCR were selected for further PCR and *in situ* hybridization tests at the species or group level in order to discriminate between true parasitism and presence of the parasite in the digestive tract or on the body surface. Lastly, diversity of *Marteilia* types was investigated by PCR-RFLP and sequencing.

MATERIAL AND METHODS

Sampling, temperature and salinity monitoring

Collection of flat oysters *Ostrea edulis* and mussels *Mytilus galloprovincialis* was performed by diving in Diana Lagoon every month between June 2007 and June 2008. Sampling sites within the lagoon are shown in Figure 1. Each sampling consisted of 30 adults bigger than 6 cm in length for oysters and 5 cm in length for mussels.

Zooplankton samples were collected at least once a month during one year using an Acthyd net (25 cm of diameter x 50 cm in length) with a 100 µm mesh towed for 5 minutes at an average speed of 2.5 knots at 3 to 4 meters in depth (depth at which mussels are cultivated) around the mussel ropes and near the oyster beds.

Temperature and salinity were recorded every two weeks using a multi 340i WTW probe at 1 meter in depth which represents the depth at which mussels were collected. These data were compared with data collected between 2001 and 2011 through REPHY (REseau de surveillance du PHYtoplancton et des PHYcotoxines) and REMI (REseau de controle MIcrobiologique) networks.

Biological material processing

For bivalves, a section of tissue including gills, mantle, gonad and digestive gland was cut and fixed in 10% Formalin in 0.22 µm filtered sea water (FSW) for histological observation and pieces of digestive gland were fixed in 95% ethanol for subsequent DNA extraction.

Each zooplankton sample was divided in two equal subsamples. One was fixed in 95% ethanol for molecular analyses and the other one was fixed in 10% Formalin in FSW for later

147 identification to group or species level and *in situ* hybridization assays. For each sample,
148 individuals of the main groups were sorted based on morphological characteristics.

149

150

151 **Histology**

152 Bivalve soft tissues were maintained in 10% Formalin in FSW until they were dehydrated
153 and embedded in paraffin for histology according to standard procedures (Howard and Smith
154 1983). Paraffin blocks were cut in 2-3 µm sections and stained with hematoxylin and eosin.
155 Infection level (from 0 to 5) was estimated according to Villalba, A. *et al.* (1993).

156

157 **DNA extraction**

158

159 Bivalves found infected by histology were selected for molecular analyses. For these
160 individuals, DNA was extracted from digestive gland using the QIAamp DNA minikit
161 (Qiagen) according to the manufacturer's instructions. DNA was eluted and resuspended in a
162 final volume of 50 µl of sterile deionised water and then diluted at a final concentration of
163 100 ng μl^{-1}.
164 DNA was extracted from bulk or sorted zooplankton samples according to the same protocol
165 after two baths in Phosphate Buffered Saline (PBS) 1X.

166

167

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

169 In order to detect and characterize parasites isolated during this study, two PCR assays
170 targeting sequences of rDNA internal transcribed spacer region (ITS) and inter genic sequence
171 (IGS) were used according to Le Roux, F. *et al.* (2001) and Lopez-Flores, I. *et al.* (2004)

172 respectively. In addition, for zooplankton samples, a PCR assay using eukaryotic "universal"
173 primers CS1-CAS1 and amplifying about 780bp of the 18S ribosomal DNA was used to
174 verify the absence of PCR inhibitors according to Le Roux, F. *et al.* (1999). Negative PCR
175 controls consisting of water were included with every 10 tested samples in order to check for
176 potential contamination. Positive PCR controls corresponding to DNA extracted from oysters
177 and mussels previously found infected with *M. refringens* type O and type M were included in
178 each PCR test.

179 Parasite type was firstly determined using the PCR-RFLP approach developed by Le Roux,
180 F.*et al.* (2001).

181 Representative PCR products of different PCR-RFLP profiles were cloned using the original
182 TOPO TA cloning kit (Invitrogen) according to manufacturer's recommendations and positive
183 clones were then selected for plasmid DNA purification by FastPlasmid® Mini (Eppendorf).
184 Some plasmidic DNA suspensions were bidirectionally sequenced using the Big Dye V3
185 sequencing kit (Applied Biosystem) and standard M13 forward and reverse primers. Obtained
186 sequences were compared with those included in GenBank using the BLASTn algorithm
187 (Atschul *et al.* 1997).

188

189 ***Sequence analyses***

190 Some available ITS1 sequences from *Marteilia* spp. were downloaded from GenBank
191 (DQ426611, DQ426550, and JN820085 as representatives of *Marteilia refringens* types O, M and C
192 respectively) and included in the phylogenetic analysis with sequences obtained in the present
193 study. Alignments were performed using Clustal W (Thompson *et al.* 1994) including in
194 MEGA 5 with open and extend gap penalties of seven and three, respectively.

Phylogenetic analysis was performed using the Neighbor-Joining method (Saitou and Nei 1987) and the evolutionary distances were computed based on the Tajima-Nei distance (Tajima and Nei 1984) under MEGA5 package (Tamura *et al.* 2011). The analysis involved 15 nucleotide sequences and a total of 362 positions in the final dataset. Bootstrap values were calculated over 1000 replicates.

Genetic distance corresponding to the number of base substitutions per site from averaging over all sequence pairs was estimated using the Tajima-Nei model (Tajima and Nei 1984) in MEGA 5 (Tamura *et al.* 2011).

In situ hybridization (ISH)

After species sorting, PCR positive zooplankton samples were tested by ISH according to a protocol adapted from Le Roux, F. *et al* .(1999). Five µm thick tissue sections on silane-prep™ slides (Sigma, France) were dewaxed, rehydrated, and treated with proteinase K (100 µg ml^{-1} in TE buffer [Tris 50 mM, EDTA 10 mM]) at 37°C for 5 min. Slides were dehydrated by immersion in an ethanol series and air-dried. Sections were then incubated with 100 µl of hybridization buffer (50% formamide, 10% dextran sulfate, 4× SSC [0.06 M Na_3 Citrate, 0.6 M NaCl, pH 7], 250 µg ml^{-1} yeast tRNA and 10% Denhardt's solution) containing 10ng $µl^{-1}$ of the digoxigenin-labelled probe, called SMART2 specific for the 18S rRNA gene of *M. refringens* (Le Roux *et al.* 1999). Target DNA and digoxigenin-labelled probe were denatured at 95 °C for 5 min and the hybridization was carried out overnight at 42 °C. Sections were washed in 2× SSC at room temperature (RT) (2×5 min), in 0.4× SSC at 42 °C (10 min) and in solution I (100 mM maleic acid, 0.15 M NaCl, pH 7.5) for 5 min. Tissues were then blocked for 30 min at room temperature with blocking reagent (Amersham Life Science) (1% w/v) in solution I. Specifically bound probe was detected using an alkaline phosphatase-conjugated mouse IgG antibody against digoxigenin diluted at 1.5 U ml^{-1} in solution I (1 h, RT). Excess

of antibody was removed by two washes in solution I (1 min) and one wash in solution II (0.1 M Tris pH 8, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5). Slides were incubated in NBT/BCIP, a chromogenic substrate for alkaline phosphatase, diluted in solution II (20 µl ml^{-1}) in the dark until the parasitic cells were completely stained black-purple. The reaction was stopped with solution III (100 mM Tris, 1 mM EDTA, pH 8). Slides were counterstained for 1 min with Bismarck brown yellow (5 mg ml^{-1}), dehydrated with ethanol and mounted in Eukitt resin. Negative controls included samples without digoxigenin-labeled probe in hybridization mixture or without antibodies during colour development. Positive control consisted of sections from flat oysters *Ostrea edulis* infected with *Marteilia refringens* originating from Thau lagoon (France).

Statistical analyses

Statistical analyses were performed using the rcmdr package under R (R Core Development Team, 2009). Linear regression model was used to test the potential effect of environmental parameters including temperature and salinity on prevalence. Pearson's product-moment correlation coefficient was also calculated between prevalence and salinity or temperature.

RESULTS

Temperature and salinity monitoring

Data obtained between June 2007 and June 2008 are shown in Figures 2A and B.

Water temperature fluctuated between 9.3°C (in December 2007) and 28.4°C (in July 2007). Temperature was maximum in July-August 2007 and then decreased until January 2008. In wintertime, temperature stagnated around 10°C before increasing between March and June

2008 (Figure 2A). Temperatures recorded in the context of this study generally appeared in the range of values recorded between 2001 and 2011 but sometimes appeared higher or lower than the first or third quartiles especially when temperature was recorded late in the month.

Due to the permanent connection with the sea and the low contributions of fresh water, salinity presented small fluctuations between 34.4 and 39.5 (Figure 2B).. However, three exceptional salinity decreases related to important rainfalls were recorded in November 2007, January and April 2008. Salinity measured between June 2007 and June 2008 was within the data range over the period 2001-2011 except in January and June 2008.

***Marteilia refringens* dynamics in bivalves and zooplankton samples**

Histological examination of flat oysters did not allow detecting *Marteilia refringens* in all the tested oysters.

Histological examination of 358 mussels revealed the presence of the parasite in 55 individuals in samples collected from June 2007 to March 2008. Mussels collected between April and June 2008 did not appear infected with *M. refringens* (Figure 3A). Depending on the sampling date, detection frequency fluctuated between 0 and 83% (Figure 3A).

Parasite detection was higher during summertime (June-August 2007) compared to autumn and winter. Transition between these two periods was associated with a sudden decrease of detection frequency from 83% in August to 3% in September 2007.

Neither temperature, nor salinity appeared to significantly influence prevalence (p=0.096 and p= 0.144, respectively); however, Pearson's product-moment correlation coefficients were positive and moderate, 0.45 and 0.5 between prevalence and salinity or temperature, respectively.

Infection intensity in mussels, estimated according to Villalba, A. *et al.* (1993), was high (levels 4 and 5) between June and August 2007 and then in March 2008, whereas it was moderate (level 3) or low (levels 1 and 2) between September 2007 and February 2008 (Figure 3B).

Developmental stages of the parasite were also recorded (Figure 3C). When intensity level was high, first maturation stages and zoosporangiosorous were detected in most of the digestive diverticula and a few parasites including primary and secondary parasite stages were observed in the epithelium of the stomach (Figures 4A and B). Conversely, when infection intensity was low, mainly young stages were located in the stomach epithelium.

Zooplankton samples were first tested by PCR using the eukaryotic universal primers. All the 23 samples yielded positive results and were subsequently tested by the nested PCR targeting the IGS region of the parasite genome. On the 23 tested samples seven showed PCR products at the expected size (358 bp). Four of these positive samples were collected between mid-August and mid-October while the other positive samples were collected in springtime (Figure 3A).

Zooplankton diversity in Marteilia refringens positive PCR samples and parasite detection in sorted zooplankton samples

PCR positive formalin fixed zooplankton samples were selected in order to identify and sort the main abundant taxa. Different zooplankton groups were observed in the PCR positive samples (Table I): Copepods, Appendicularia, Cladocera, Chaetognatha, Polychaeta (larvae), Decapoda (larvae), Echinoderm larvae, Cirripedia nauplii and Fish eggs.

All the sorted taxa were then processed for DNA extraction and tested by PCR using universal primers and *Marteilia refringens* specific PCR primers.

Except for one sample of Decapoda larvae (30/04/2008), all tested samples yielded amplification products using the universal primer pair.

Marteilia refringens specific PCR results are summarized in Table I. For three taxa, positive results were obtained for all the tested samples (*Paracartia latisetos*a, *Oithona* sp. and *Evadne* sp.) and for three other taxa, negative results were obtained for all tested samples (nauplii of Cirripedia, larvae of Echinoderm and anchovy eggs). Copepods *Centropages typicus*, Appendicularia, larvae of Polychaeta, Cladocera *Penilia avirostris* and Chaetognatha *Sagitta* sp. presented at least one positive sample each. Zooplankton organisms that were positive for *M. refringens* by PCR were selected for *in situ* hybridisation. Several sections from each paraffin block were tested each time. Positive specific labelling was observed in two individual *Paracartia latisetosa*. Positive cells were numerous, small (1-2 µm) and located within the gonadal tissues of female copepods (Figures 5A, B and C). Some copepod ovocyte appeared full of these multiple small cells.

Molecular characterization of Marteilia refringens detected in mussels and zooplankton samples

Mussels found to be infected by histology and bulk zooplankton samples found positive by nested PCR were selected for further molecular characterization work.

All the 55 mussels found infected by histology appeared positive by PCR using PR4-PR5 primers. Digestion of PR4-PR5 PCR products (Le Roux *et al.* 2001) produced two types of RFLP profiles in 53 mussels (two PCR products were too faint to be digested): type M was observed in 51 mussels while profiles of both types were observed in two mussels collected in June 2007. PCR products from these two mussels and one mussel showing type M profile were cloned. Two to three clones per mussel were sequenced. A total of eight sequences were

315 obtained among which five displayed 99 to 100% homology with *Marteilia refringens* type M
316 (DQ426550) and three showed 99% homology with *M. refringens* type O (DQ426611).

317 For zooplankton, on the seven samples found positive by nested PCR, twoappeared positive
318 by PCR using PR4-PR5 primers. These two PCR products were too faint to be directly
319 digested and were ligated in Topo A vector for cloning. Four clones obtained from
320 zooplankton samples were sequenced among which two had 99% homology with *M.*
321 *refringens* type M (DQ426550) and two sequences showed 96% homology with *Marteilia* sp.
322 type C (JN820088).

323 Alignment of obtained sequences with three sequences downloaded from Genbank is shown
324 in Figure 6. *Hha*I restriction sites are highlighted in yellow. As expected, restriction sites used
325 to discriminate between types O and M are present in the sequences showing maximum
326 homology with *M. refringens* types O and M. However sequences close to *Marteilia* sp. type
327 C (JN820085) show restriction sites close to type M ones. An additional site is observed in
328 07/69 clone 1 due to a substitution of a "A" by a "G".

329 Overall mean distance among all tested sequences was 0.069 whereas distances within each
330 type sequences were below 1%: 0.004 for types M and O and 0.005 "type C" sequences. Most
331 of the polymorphism consisted in point substitutions observed in only one clone or one
332 downloaded sequence. Distance between types M and O sequences (0.018) was lower than
333 between type C and other types (0.07 and 0.107 with types M and O respectively).

334 The phylogenetic analysis using the Neighbor-Joining method showed three distinct groups
335 corresponding to types M, O and C (Figure 7).

336

337 **DISCUSSION**

Considering the simultaneous presence of flat oysters, mussels and the parasite in Diana lagoon and the probable involvement of zooplankton in its transmission, the study presented herein investigated *M. refringens* dynamics in bivalves and zooplankton in order to better understand the parasite cycle.

None of the 360 tested flat oysters appeared infected with the parasite *M. refringens* during the period of study. Considering the small sample size (30 animals per month), the lack of detection of the parasite might be due to a very low prevalence of the disease. However, the parasite was regularly detected in the oyster population between 1984 and 1988 and then in 2002 and 2005 (Pichot *et al.* 2002; REPAMO 2007). Detection frequencies reported in the eighties ranged between 32 and 100% while in 2002 and 2005 they were between 0 and 13% (REPAMO, unpublished data). Considering that the oyster production in the lagoon relies only on natural reproduction and that no change in culture practices was reported, this apparent prevalence decrease could be due to the development of natural resistance to marteiliosis in the oyster population. However this hypothesis would need further investigations.

In contrast to flat oysters, mussels appeared infected with *Marteilia* sp. between June 2007 and March 2008 with detection frequencies fluctuating seasonally up to 83% in August 2007. These results suggest a lack of parasite transmission between mussels and flat oysters which can be due to distance between mussel and flat oyster sampling sites or to a stricter host specificity of the parasite than expected.

Previous studies carried out in locations where both flat oysters *O. edulis* and mussels *M. galloprovincialis* were present showed differing situations regarding infection with *M. refringens*. In Galicia, Spain, flat oysters cultured in the same raft as *Marteilia*-infected mussels, with prevalences up to 73%, remained free of the parasite (Figueras and Robledo 1993). Similarly, in Croatia, the parasite was only detected in mussels with low prevalence

(5%) while flat oysters were not found infected (Zrncic *et al.* 2001). In the Thermaikos gulf in Northern Greece, a study performed on bivalves including flat oysters and Mediterranean mussels showed infection with *M. refringens* in both species with 60 and 21% of detection frequency respectively (Virvilis *et al.* 2003). Lastly, sporadic detection of the parasite was reported in both species from different geographic origins in Alfacs and Fangar bays in Ebre Delta in Catalonia Spain (Carrasco *et al.* 2008b).

Seasonality of *Marteilia* sp. dynamics in mussels in Diana lagoon appeared weakly positively related to temperature and salinity. In summer, between June and August 2007, the number of infected mussels increased as did infection intensity. The increase of infected mussels was probably due to transmission of the parasite with more mussels exhibiting young parasite stages. During this period, the number of infected mussels showing high infection levels and mature parasite stages increased suggesting a development of the infection in these bivalves. These highly infected mussels displayed more young parasite stages not only in the stomach epithelium but also in the epithelium of digestive ducts suggesting that these primary stages could migrate along the digestive system. In September 2007, only one mussel was found infected. Considering that mussel sampling was performed on the same cohort and that no mortality was reported in this population (Bouchoucha, pers. comm.), the dramatic decrease of detection frequency observed between August and September suggests that the parasite, all stages included, was successfully degraded by mussels or released outside the mussels. Similarly, in a study carried out in different sites in Galicia, the variation in the prevalence of the infection was better explained by an elimination of the parasite via the action of the mussel's defence mechanisms rather than by death of infected mussels (Robledo and Figueras 1995). In Diana lagoon, after the important decrease of prevalence, the number of infected mussels remained low with variable infection intensity. Refringent stages, which contain refringent granules and spores, were observed in December, January and then March.

388 These results suggest that the parasite sporulates all year long but more importantly when
389 temperature is highest. Seasonality in the parasite dynamics was previously reported for
390 example in mussels in the Ebre Delta where a positive correlation between detection
391 frequency and temperature was observed (Carrasco *et al*. 2007a), and in flat oysters in France
392 where increased infection was associated with sporulation of the parasite and a peak in oyster
393 mortalities (Grizel and Tigé 1973; Alderman 1979; Balouet *et al*. 1979; Grizel 1985).
394 However, no clear temporal pattern of infection was observed in mussels in Galicia (Villaba
395 *et al.* 1993; Robledo and Figueras 1995) where prevalence of the parasite changed with site
396 and year.
397 Zooplankton samples collected between August and October 2007 and then between April
398 and June 2008 were positive by *Marteilia refringens* specific PCR. Detection by PCR
399 indicates presence of *M. refringens* DNA and does not necessary mean true infection
400 (Burreson 2008). However, the first period of detection in zooplankton coincided with
401 precipitous decline in prevalence of the parasite in mussels and could correspond to a
402 transmission of the parasite from mussels to zooplankton. This first period is followed by a
403 lack of detection in zooplankton and low detection frequency in mussels. Berthe, F. *et al.*
404 (1998) hypothesized that the parasites could mature in the sediment after being released from
405 oysters to the water column. The detection of *M. refringens* in zooplankton from April 2008
406 could be related to an increase of water temperature, above 17°C, allowing new zooplankton
407 infection or parasite development in zooplankton reaching an infection level detectable by
408 PCR. Contrary to a previous study carried out in the Ebre Delta (Carrasco *et al.* 2007a), no
409 positive signal was obtained in zooplankton collected in Diana lagoon before the peak of
410 prevalence observed in mussels in August in our study. Our results support the hypothesis of
411 the transmission of the parasite from mussels to zooplankton but not from zooplankton to
412 mussels. They are in agreement with previous experiments which succeeded in transmitting

413 *Marteilia refringens* from infected flat oysters and infected mussels to the copepod
414 *Paracartia grani* but failed in transmitting the parasite from infected copepods to naive
415 bivalves (Audemard *et al.* 2002; Carrasco *et al.* 2008a).
416 Zooplankton samples found positive by *Marteilia refringens* specific PCR were selected for
417 further work in order to identify and sort the main abundant species present in these samples.
418 The quality of DNA extracted from the different sorted taxa was confirmed with conserved
419 primers except in one sample of Decapoda larvae. For some taxa, all the tested samples
420 appeared negative by PCR specific to *M. refringens*. These taxa, including Cirripedia,
421 Echinoderm larvae and Anchovy eggs might not be involved in the parasite cycle. On the
422 contrary, taxa for which positive results were obtained for all the tested samples like
423 *Paracartia latisetosa*, *Oithona* sp. and *Evadne* sp. might more probably be involved in the
424 parasite transmission. Some similar taxa were previously found to be positive by PCR in
425 other ecosystems including decapod larvae in claire ponds (Audemard *et al.* 2002) and
426 *Oithona* sp. in the Ebre Delta (Carrasco *et al.* 2007b).
427 All the zooplankton species and groups found positive by PCR for the detection of *M.*
428 *refringens* were tested by *in situ* hybridization in order to discriminate between true
429 parasitism and presence of *M. refringens* in the digestive tract or on the body surface. Several
430 sections from each paraffin block were tested for the detection and localization of the
431 parasite. One species, *Paracartia latisetosa* presented specific labelling in the female gonadal
432 tissues. Other paramyxean species are known to target gonads in marine invertebrates:
433 *Marteilioides chungmuensis* in *Crassostrea gigas* (Comps *et al.* 1986) and *Paramarteilia*
434 *orchestiae* in *Orchestia gammarellus* (Ginsburger-Vogel and Desportes 1970). These
435 parasites could be released outside their host through the gonadal duct or be released with
436 eggs during spawning. The numerous small cells observed inside or instead of *P. latisetosa*
437 ovocytes suggest that the parasite develops and multiplies within the ovocytes. A similar

438 observation was reported in copepods *P. grani* collected from claire ponds (Audemard *et al.*
439 2002) where large numbers of small parasite cells were detected in ovarian tissue. Copepods
440 *P. grani* experimentally infected with *Marteilia refringens* from mussels displayed positive
441 labelling in the digestive epithelium while when infected with *M. refringens* from oysters, the
442 parasite was firstly found in the digestive epithelium and then in the gonad (Carrasco *et al.*
443 2008a). Our results demonstrate for the first time a true infection with *M. refringens* in a
444 zooplankton species other than *P. grani*. In addition, these results suggest, but do not
445 demonstrate, that *P. latisetosa* may act as an intermediate host for *M. refringens*. Members of
446 the genus *Paracartia* appear as key actors in the parasite cycle however further experimental
447 work would be necessary to fully confirm this hypothesis. The lack of visualization of the
448 parasite in other tested species found positive by PCR is probably due to the presence of *M.*
449 *refringens* DNA in the digestive tract or on the body surface. The numerous positive results
450 obtained by PCR suggest that the parasite was widely present in the lagoon at the sampling
451 dates. Some of these results might be explained by the feeding behaviour of the zooplankton
452 species. For example, appendicularians are filter feeders which can thus capture parasites
453 when filtering while chaetognaths are carnivorous, preying on other zooplanktonic organisms
454 including copepods that can be infected with the parasite.
455
456 Molecular characterization efforts were done in order to identify *Marteilia refringens* types
457 circulating in Diana lagoon.
458 The phylogenetic analysis of the 12 obtained sequences confirmed the distribution of the
459 tested clones in three groups corresponding to types M, O and C. Genetic distance was low,
460 below 1% within each of these groups of sequences. Type C appeared genetically more distant
461 from other types: 7 and 11% from types M and O respectively whereas overall distance
462 between types M and O sequences was below 2%. These results support the hypothesis that

types M and O represent the same unique species, *Marteilia refringens*. However, the relative position of type C would require further molecular work.

In the present study, *Marteilia refringens* type M appeared predominant in mussels and zooplankton. However, it was possible to demonstrate that type O was also present and might circulate between zooplankton and mussels. In addition, *Marteilia* sp. close to *Marteilia* sp. type C was also detected in one sample of zooplankton collected in September suggesting that this *Marteilia* type is also circulating in the lagoon. *Cerastoderma edule* has never been reported whereas several cockle species including *Acanthocardia paucicostata*, *Anadara inaequivalvis* and *Parvicardium exiguum* are present at low density in Diana lagoon (Casabianca *et al.* 1973; Andral and Sargian 2010). The detection of *Marteilia* sp. type C in zooplankton raises some questions concerning the status of these different cockle species regarding the parasite.

Although *Marteilia refringens* types O and M are preferentially detected in flat oysters and mussels respectively, host affinity is not strict (Le Roux *et al.* 2001; Lopez-Flores *et al.* 2004; Novoa *et al.* 2005; Carrasco *et al.* 2008b). Carrasco, N. *et al.* (2008b) hypothesized that when parasitic loads are high in the environment, a predominant type could infect both flat oysters and mussels. In our study, flat oysters appeared free of *Marteilia refringens* although both types were detected in mussels and also in zooplankton.

CONCLUSIONS

To conclude, our results show that *Marteilia refringens* is able to accomplish its life cycle in Diana lagoon in Corsica through mussels *Mytilus galloprovincialis*. The decrease of detection frequency in mussels coincides with the detection of parasite DNA in zooplankton samples which suggests that *M. refringens* is transmitted from mussels to zooplankton. Multiple taxa

488 of zooplankton yielded positive results by PCR whereas only one species showed positive
489 and specific labelling by *in situ* hybridization. The parasite was probably abundant in the
490 water but did not truly infect these taxa. Another interesting point is that *Paracartia grani*
491 was not identified in zooplankton samples tested in the present study. Moreover, the parasite
492 was detected in gonadal tissue of *Paracartia latisetosa* collected in September 2007.
493 Therefore, depending on its ecological context, the parasite seems to target different species
494 of the *Paracartia* genus to accomplish its life cycle. Both *Paracartia* species seem not able to
495 co-occur as *P. grani* is present in Claire ponds and in Thau lagoon (Audemard *et al.* 2001,
496 Boyer *et al.* 2012) and *P. latisetosa* in Diana lagoon and in Thau lagoon before the arrival of
497 *P. grani* in the lagoon (Boyer *et al.* 2012). Ecology and interactions between both species are
498 therefore to be studied to better understand the role of zooplankton species in *Marteilia* life
499 cycle. *M. refringens* type M appeared predominant in the lagoon during the studied period
500 but was not unique. Indeed, other types including type O and *Marteilia* sp. type C were also
501 detected in mussels or zooplankton samples. These results raise some questions regarding the
502 environmental conditions favouring one or another type.
503

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652

653 **TABLES**

		2007				2008		
Groups	Species or Genus	15-Aug	27-Aug	15-Sept	15-Oct	30-Apr	15-May	30-Jun
Copepods	*Paracartia latisetosa*			**P**				
	Oithona sp.				**P**	**P**	**P**	
	Centropages typicus	**P**	**N**		**N**		**N**	**P**
Appendicularia					**N**	**P**		
Polychaeta larvae				**N**	**P**			
Chaetognatha	*Sagitta sp.*		**N**	**P**				
Decapada larvae			**N**			**U**		**N**
Cladocera	*Penilia avirostris*	**P**	**N**	**P**				
	Evadne spp	**P**						
Fish eggs	Anchovy eggs	**N**	**N**	**N**				**N**
Cirripedia nauplii							**N**	
Echinoderm larvae						**N**		

654

655 Table I. Zooplankton taxa identified in the seven samples (one sample = one column)
656 found PCR positive. When a taxa is present, the cell is coloured in grey. PCR results
657 are indicated using letters: U (Undetermined) when PCR using universal primers was
658 negative; N (Negative) when PCR using universal primers was positive and PCR using
659 specific primers was negative; P (Positive) when PCR assays using universal and
660 specific primers were positive.

661

Description of tested samples	RFLP profiles	Code of obtained sequences	Maximum identity (Blastn)	Genbank Accession numbers
Mussel 1 collected in June 2007 (Mgallo1)	co detection type	Mgallo1_1 Mgallo1_2 Mgallo1_3	99% DQ426611 99% DQ426611 100% DQ426550	KC462435 KC462434 (= Mgallo2_2) Not deposited
Mussel 2 collected in June 2007 (Mgallo2)	co detection type	Mgallo2_1 Mgallo2_2 Mgallo2_3	99% DQ426550 99% DQ426611 100% DQ426550	KC462432 KC462434 (= Mgallo1_2) Not deposited
Mussel collected in August 2007 (Mgallo3)	type M	Mgallo3_1 Mgallo3_2	99% DQ426550 100% DQ426550	KC462433 Not deposited
Zooplankton collected in September 2007 (Zoopl1)	not done	Zoopl1_1 Zoopl1_2 Zoopl1_3	99% DQ426550 96% JN820088 96% JN820088	KC462430 KC462436 KC462437
Zooplankton collected in May 2008 (Zoopl2)	not done	Zoopl2_1	99% DQ426550	KC462431

Table II. RFLP profiles and sequences obtained on three mussels and two zooplankton samples. The third column presents codes of sequences used in the alignment and phylogenetic analyses. The fourth column shows the maximum identity with sequences available in GenBank using Blastn algorithm (Atschul *et al.* 1997). The last column indicates genbank accession numbers of the sequences obtained in this study.

670 **FIGURES**

671 Figure 1. Study site : Diana lagoon, North-East of Corsica, France

672

673 Figure 2A. Temperature (°C) in Diana lagoon between June 2007 and June 2008 (dark
674 triangles). Box plots indicate data collected at the same location between 2001 and
675 2011 through REPHY (REseau de surveillance du PHYtoplancton et des
676 PHYcotoxines) and REMI (REseau de controle MIcrobiologique) networks. Open
677 circles indicate outliers

678

679

680 Figure 2B. Salinity in Diana lagoon between June 2007 and June 2008 (dark
681 triangles). Box plots indicate data collected at the same location between 2001 and
682 2011 through REPHY (REseau de surveillance du PHYtoplancton et des
683 PHYcotoxines) and REMI (REseau de controle MIcrobiologique) networks. Open
684 circles indicate outliers

685

686

687 Figure 3A. Detection frequency of the parasite *Marteilia refringens* by histology in
688 mussels (histograms). No sampling was performed in October 2007 because of bad
689 weather conditions (the lack of data is indicated with the question mark). Arrows
690 indicate zooplankton samples found positive by nested PCR.

691

692

693

694 Figure 3B. I

695 nfection intensity (1-5) in infected mussels using the scale of Villalba, A. *et al.* (1993).
696 No sampling was performed in October 2007 (the lack of data is indicated with the
697 question mark).

698

699 Figure 3C. Evolution of parasite stages in infected mussels. No sampling was
700 performed in October 2007 (the lack of data is indicated with the question mark). I, II
701 and III correspond to primary, secondary and tertiary stages respectively. R
702 corresponds to refringent stages.

703

704 Figures 4. Hematoxylin & eosin stained histological section from mussels *Mytilus*
705 *galloprovincialis* infected with *Marteilia refringens*. (A) Low infection level :
706 primary-secondary stages (arrows) are observed in the epithelium of the stomach. (B)
707 High infection level: young stages (arrows) are observed in the epithelium of a
708 digestive duct and mature stages (stars) are located in digestive diverticula epithelium.

709

710

711 Figures 5. *In situ* hybridization analysis of copepods *Paracartia latisetosa*. (A)
712 Positive labeling is observed in small cells (1-2 µm) located in the gonadal tissue of a
713 female copepod. (B) All the gonad seems concerned by the presence of these positive
714 cells. (C) Positive cells are arranged in clusters and can be observed in a copepod
715 ovocyte (arrow).

716

717

718 Figure 6. Alignment of sequences obtained in the present study (corresponding
719 accession numbers are given in Table 2) with three sequences from Genbank
720 (DQ426550 for *Marteilia refringens* type M; DQ426611 for *Marteilia refringens* type
721 O; JN820085 for *Marteilia* sp. type C). *Hha*I restriction sites are boxed in yellow

722

723 Figure 7. Phylogenetic tree performed using the Neighbor-Joining method based on
724 the Tajima-Nei distance deduced from 12 rDNA ITS1 sequences of *Marteilia*
725 *refringens* obtained from three mussels and two zooplankton samples collected in
726 Diana lagoon and three sequences downloaded from Genbank (DQ426550
727 representative of type M, DQ426611 representative of type O and JN820085
728 representative of type C). Numbers at nodes indicate bootstrap values. Corresponding
729 accession numbers for sequences obtained in the present study are given in Table 2.

730
731
732
733

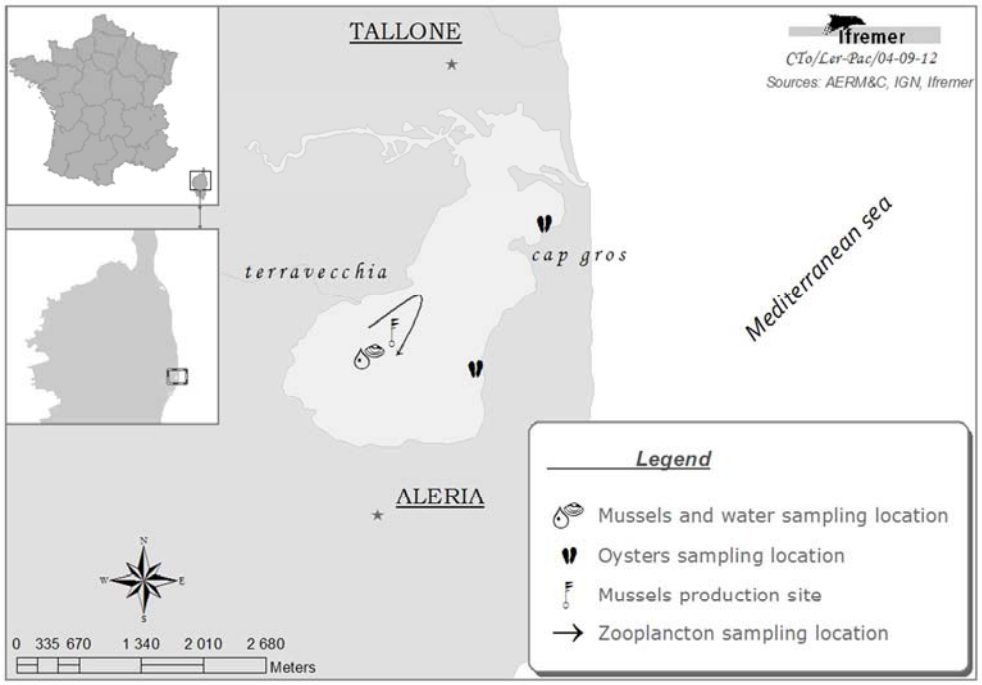
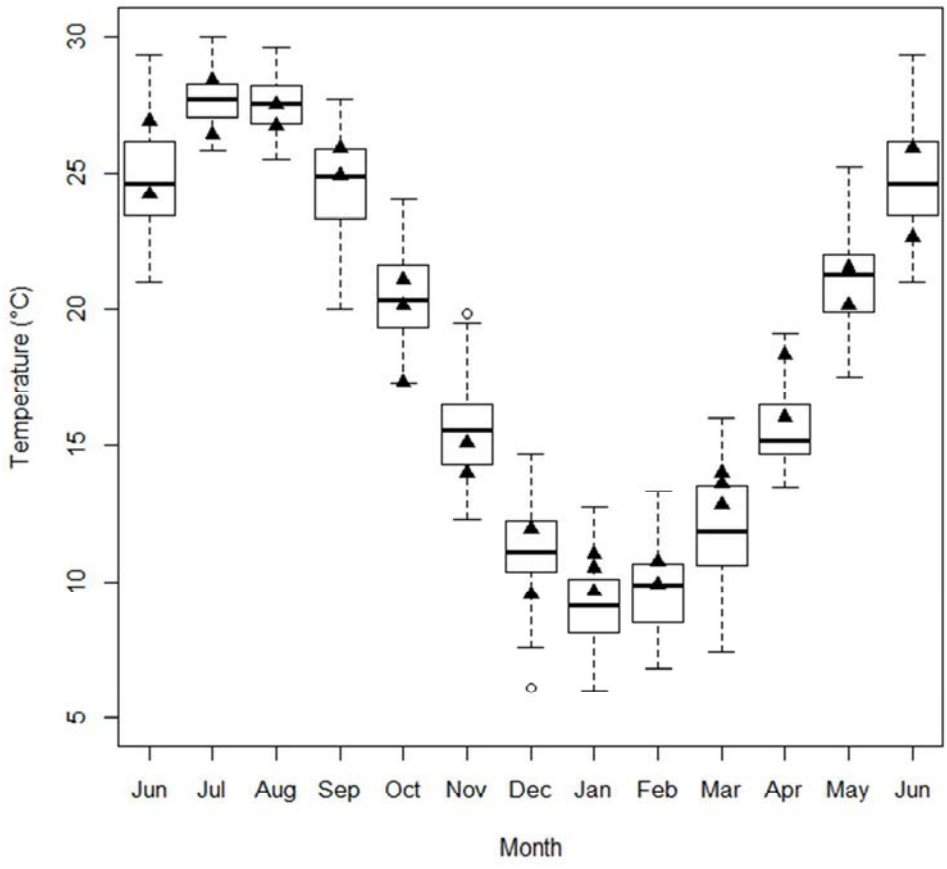
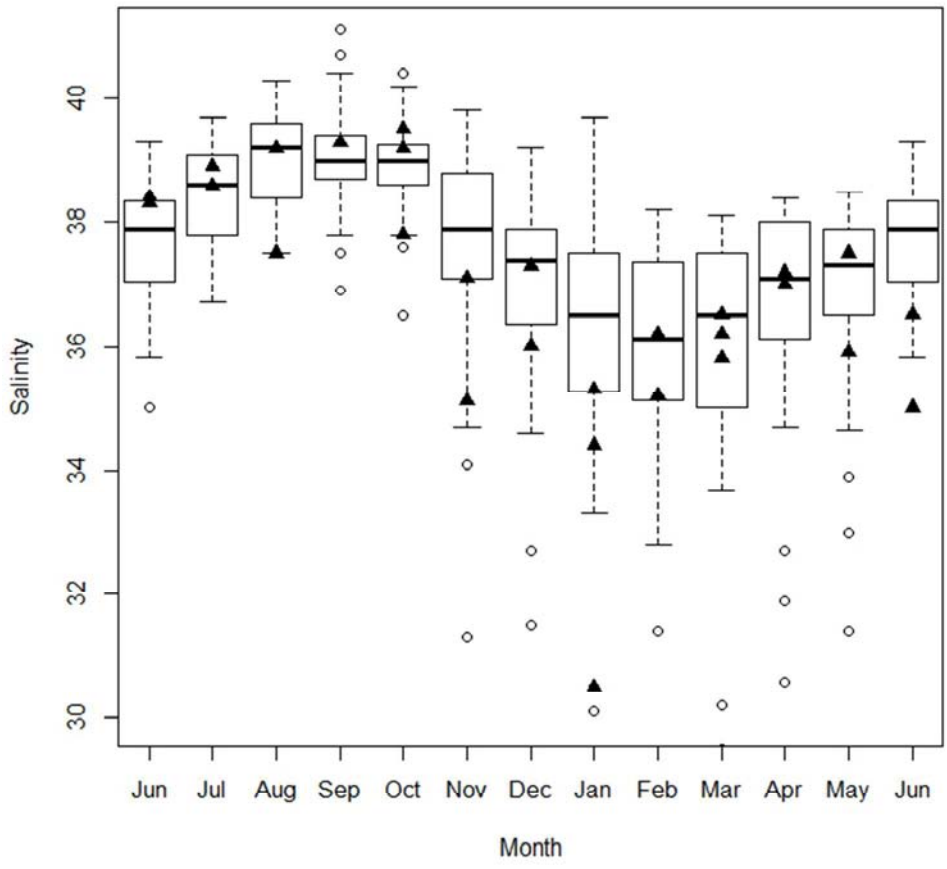


Figure 1. Study site : Diana lagoon, North-East of Corsica, France
296x210mm (96 x 96 DPI)



Temperature (°C) in Diana lagoon between June 2007 and June 2008 (dark triangles). Box plots indicate data collected at the same location between 2001 and 2011 through REPHY (REseau de surveillance du PHYtoplancton et des PHYcotoxines) and REMI (REseau de controle MIcrobiologique) networks. Open circles indicate outliers
237x236mm (72 x 72 DPI)



Salinity in Diana lagoon between June 2007 and June 2008 (dark triangles). Box plots indicate data collected at the same location between 2001 and 2011 through REPHY (REseau de surveillance du PHYtoplancton et des PHYcotoxines) and REMI (REseau de controle MIcrobiologique) networks. Open circles indicate outliers
237x236mm (72 x 72 DPI)

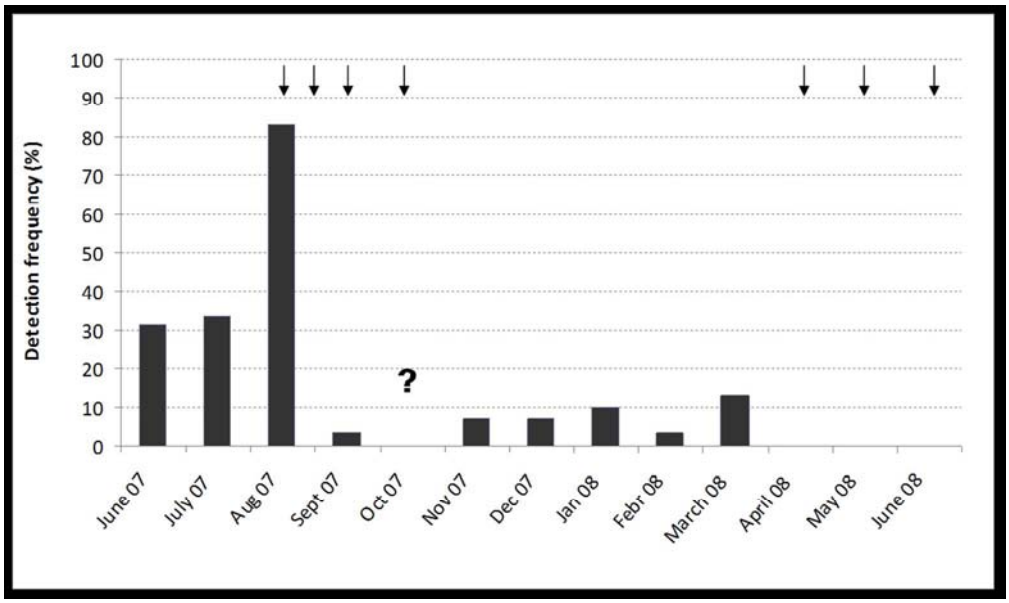


Figure 3A. Evolution of detection frequency of the parasite Marteilia refringens by histology in mussels. No sampling was performed in October 2007 (the lack of data is indicated with the question mark). Arrows indicate zooplankton samples found positive by nested PCR.
200x118mm (150 x 150 DPI)

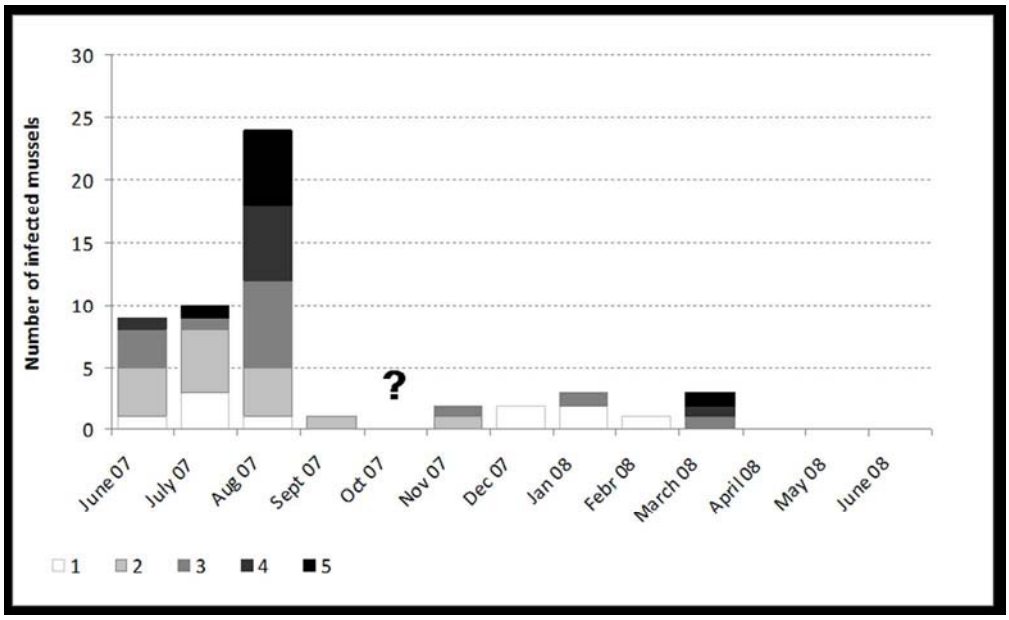


Figure 3B. Evolution of infection intensity (1-5) in infected mussels using the scale of Villalba, A. et al. (1993). No sampling was performed in October 2007 (the lack of data is indicated with the question mark).
160x98mm (150 x 150 DPI)

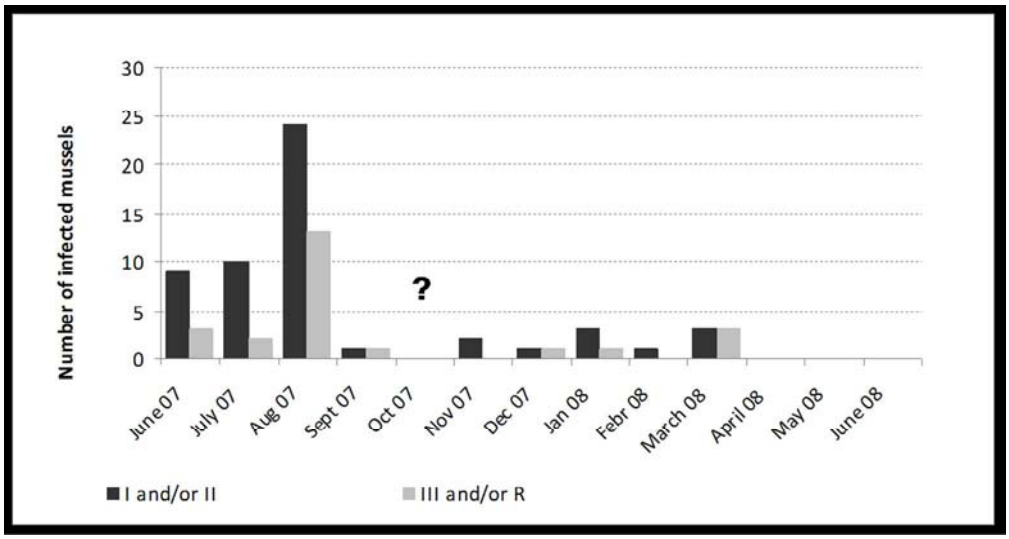
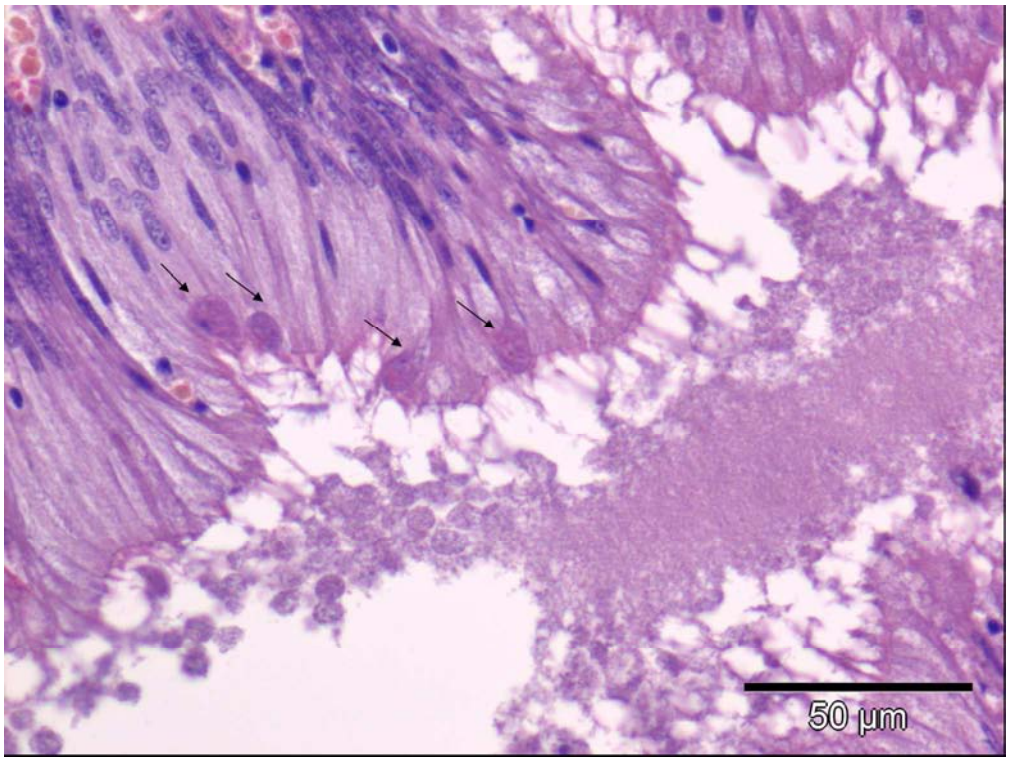
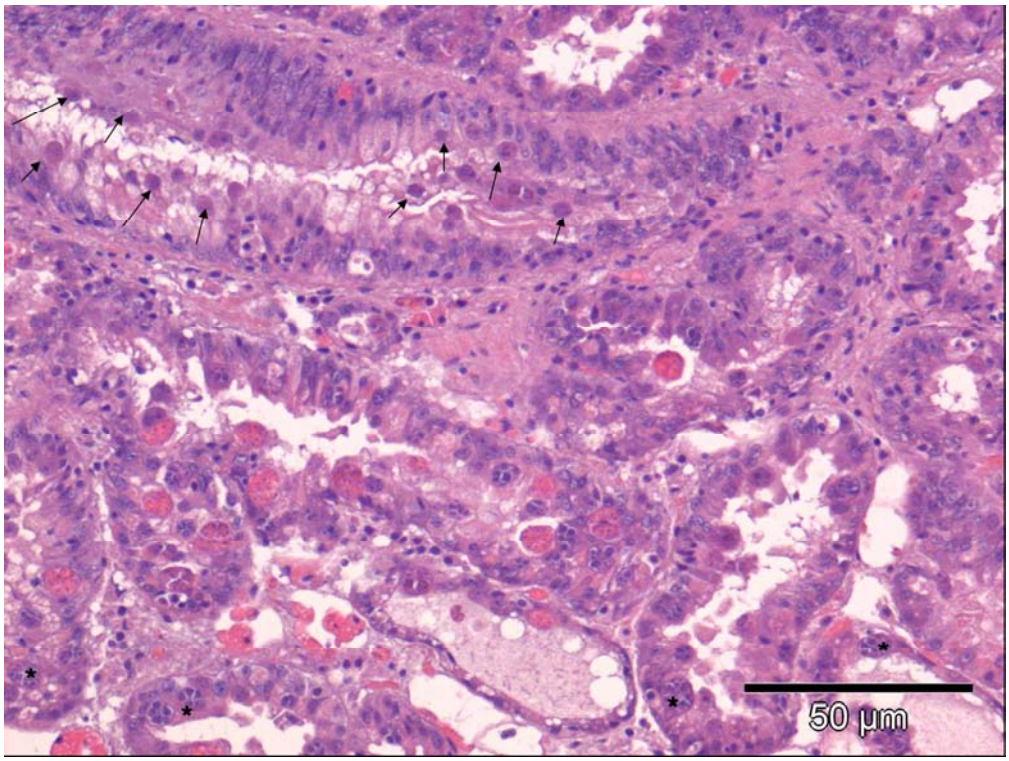


Figure 3C. Evolution of parasite stages in infected mussels. No sampling was performed in October 2007 (the lack of data is indicated with the question mark). I, II and III correspond to primary, secondary and tertiary stages respectively. R corresponds to refringent stages.
202x107mm (150 x 150 DPI)

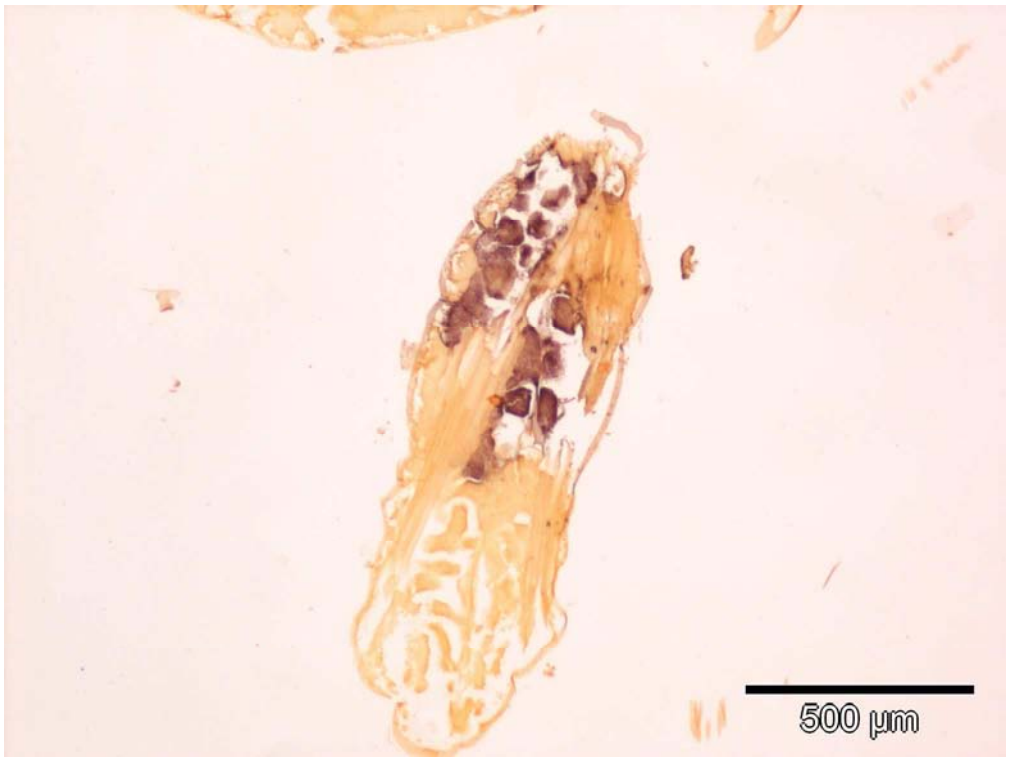


Figures 4. Hematoxylin & eosin stained histological section from mussels Mytilus galloprovincialis infected with Marteilia refringens. (A) Low infection level : primary-secondary stages (arrows) are observed in the epithelium of the stomach.
216x162mm (150 x 150 DPI)

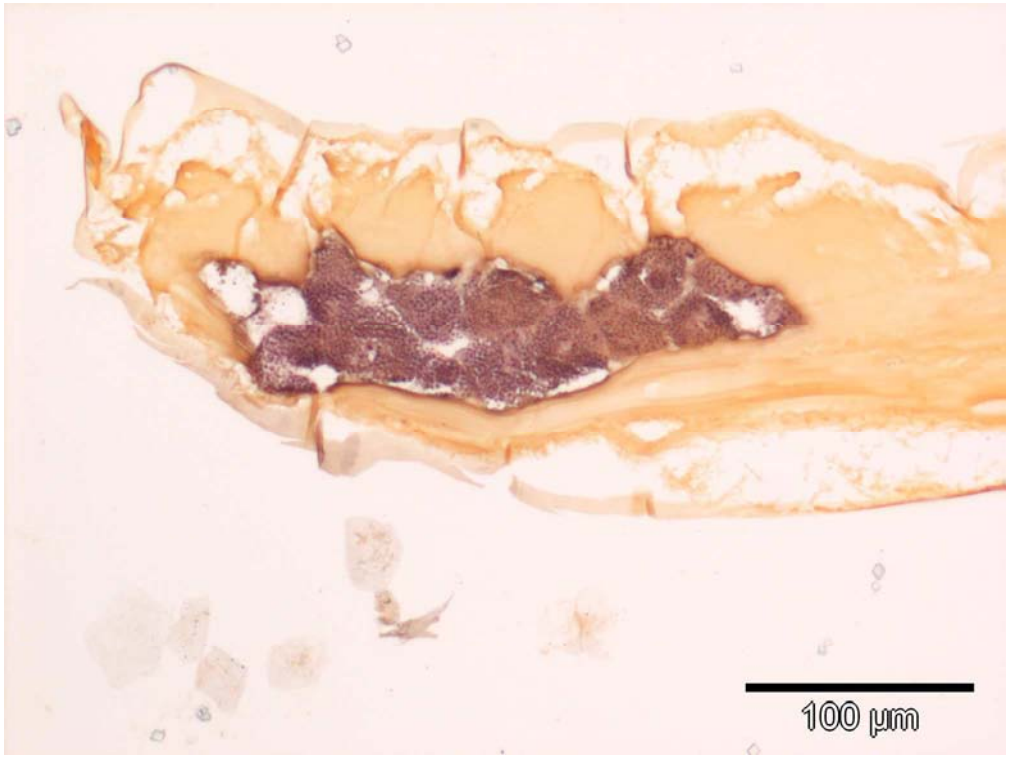


Figures 4. Hematoxylin & eosin stained histological section from mussels Mytilus galloprovincialis infected with Marteilia refringens.
(B) High infection level: young stages (arrows) are observed in the epithelium of a digestive duct and mature stages (stars) are located in digestive diverticula epithelium.

218x163mm (150 x 150 DPI)

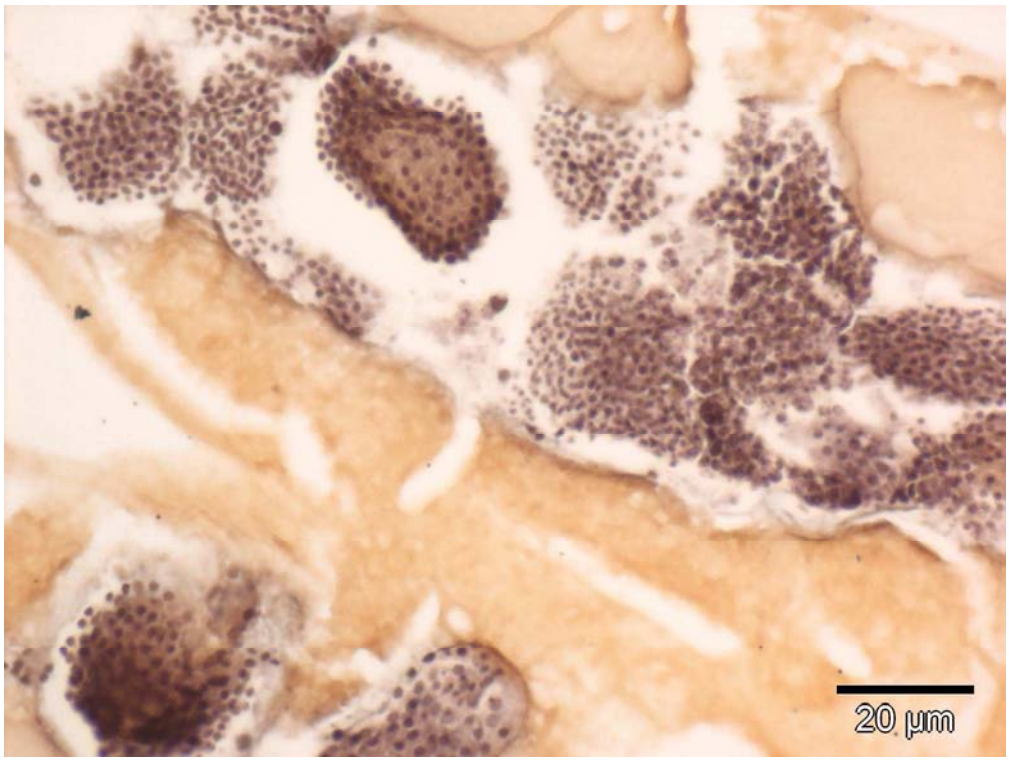


Figures 5. In situ hybridization analysis of copepods Paracartia latisetosa. (A) Positive labeling is observed in small cells (1-2 µm) located in the gonadal tissue of a female copepod.
327x245mm (200 x 200 DPI)



Figures 5. In situ hybridization analysis of copepods Paracartia latisetosa.
(B) All the gonad seems concerned by the presence of these positive cells.

327x245mm (200 x 200 DPI)



Figures 5. In situ hybridization analysis of copepods Paracartia latisetosa.
(C) Positive cells are arranged in clusters and can be observed in a copepod ovocyte (arrow).

327x245mm (200 x 200 DPI)

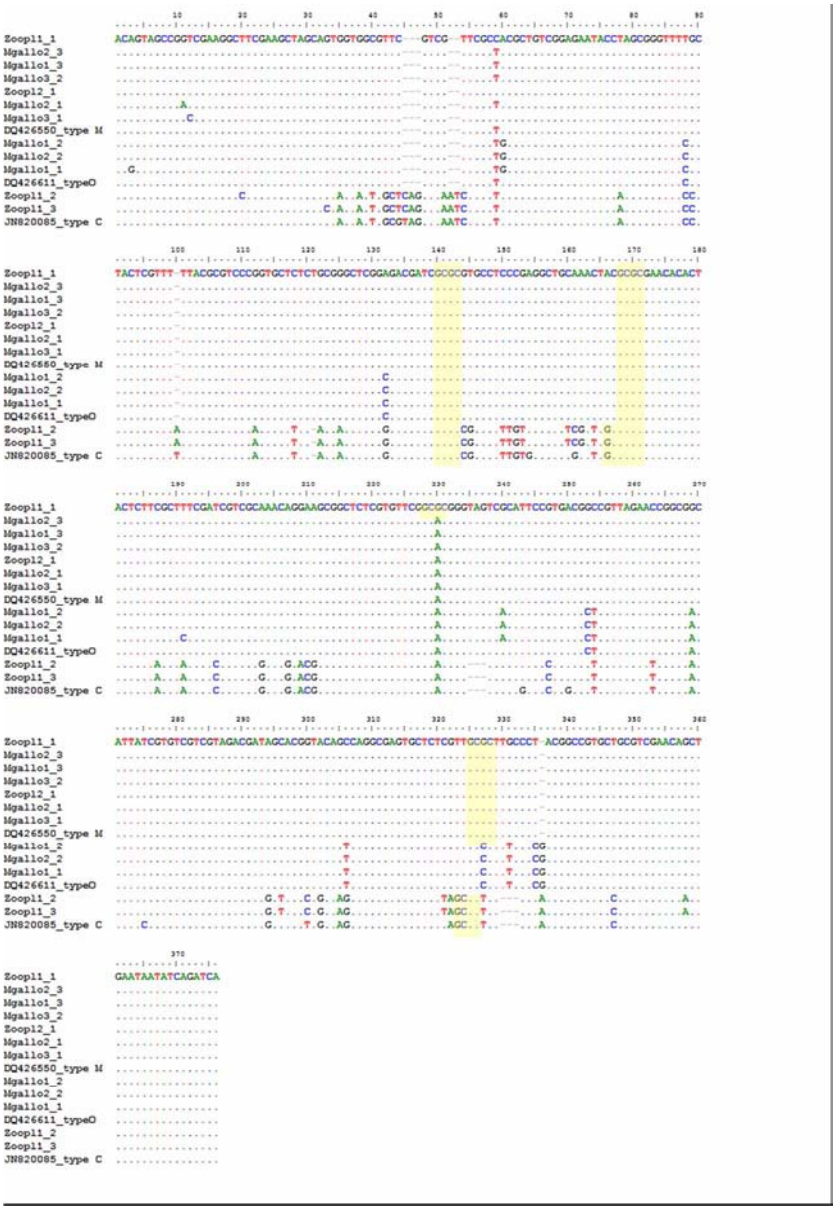


Figure 6. Alignment of sequences obtained in the present study (corresponding accession numbers are given in Table 2) with three sequences from Genbank (DQ426550 for Marteilia refringens type M; DQ426611 for Marteilia refringens type O; JN820085 for Marteilia sp. type C). HhaI restriction sites are boxed in yellow
235x341mm (150 x 150 DPI)

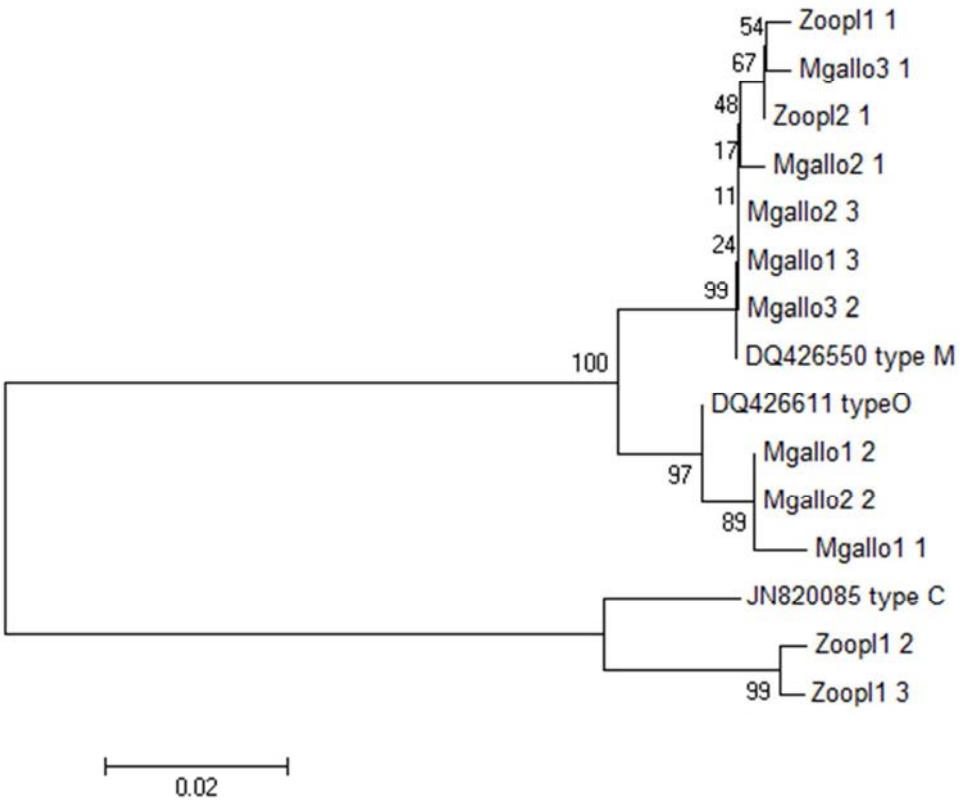


Figure 7. Phylogenetic tree performed using the Neighbor-Joining method based on the Tajima-Nei distance deduced from 12 rDNA ITS1 sequences of Marteilia refringens obtained from three mussels and two zooplankton samples collected in Diana lagoon and three sequences downloaded from Genbank (DQ426550 representative of type M, DQ426611 representative of type O and JN820085 representative of type C). Numbers at nodes indicate bootstrap values. Corresponding accession numbers for sequences obtained in the present study are given in Table 2.

174x146mm (72 x 72 DPI)