

Isolation and 18S ribosomal DNA gene sequences of *Marteilioides chungmuensis* (Paramyxea), an ovarian parasite of the Pacific oyster *Crassostrea gigas*

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ABSTRACT: To develop sensitive detection techniques with the aim of elucidating the life cycle of *Marteilioides chungmuensis*, an intracellular paramyxean infecting the ovary of the Pacific oyster *Crassostrea gigas*, we isolated the parasite at the sporont stage from infected oysters using a freeze-thaw procedure at -20°C and differential centrifugations in discontinuous sucrose and Percoll gradients. DNA was extracted from the isolated sporonts, and a PCR amplicon of 18S small subunit ribosomal RNA gene DNA was partially sequenced. *In situ* hybridization using 3 parasite-specific probes designed from the obtained sequence successfully detected parasite cells in infected oysters, and confirmed that the sequenced DNA was derived from *M. chungmuensis*.

KEY WORDS: *Marteilioides chungmuensis* · Paramyxea · Isolation · *Crassostrea gigas* · Pacific oyster · 18S ribosomal DNA gene sequence · *In situ* hybridization

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INTRODUCTION

Species of the genus *Marteilioides* are parasites of oysters, and 3 species are currently known: *Marteilioides chungmuensis* from the ovocyte of the Pacific oyster *Crassostrea gigas* (Comps et al. 1986, Park & Chun 1989, Imanaka et al. 2001, Itoh et al. 2002); *M. branchialis* from the gill of the Sydney rock oyster *Saccostrea glomerata* (= *S. commercialis*) (Anderson & Lester 1992); and *Marteilioides* sp. from the ovocyte of the Sydney rock oyster (Hine & Thorne 2000). *M. chungmuensis* poses a serious problem for oyster fisheries in Japan, because infected oysters display an abnormal appearance, resulting in the loss of marketability. In Korea, *M. chungmuensis* was first reported as an amoeba (Chun 1979), then identified as a new species in the genus *Marteilioides* by Comps et al. (1986). Although a parasite similar to *M. chungmuensis* had also been reported in Japan by several authors (Seki 1934, Matsusato et al. 1977, Imanaka et al. 2001), the parasite was not identified until this year, when

Itoh et al. (2002) confirmed it as the same parasite reported in Korea.

The sporulation stages of *Marteilioides chungmuensis* in the host cell have been studied by Park & Chun (1989), Imanaka et al. (2001) and Itoh et al. (2002), and it has been postulated that spores in the ovocyte are released from the oyster ovary via the genital canal into the environment (Itoh et al. 2002). However, developmental stages other than the sporulation stages, such as vegetative and infective stages, have not yet been detected. This is because an adequately sensitive detection method has so far not been developed.

Recently, the ribosomal DNA genes (18S rDNA) of 2 related paramyxean pathogens, *Marteilia refringens* and *M. sydneyi*, were sequenced (Anderson et al. 1995, Berthe et al. 2000). Molecular techniques were then employed for the detection of both these pathogens (Le Roux et al. 1999, Kleeman & Adlard 2000), the early developmental stages of *M. sydneyi* (Kleeman et al. 2002) and a possible alternative host of *M. refringens* (Audemard et al. 2002). Additionally,

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Berthe et al. (2000) postulated the phylogenetic position of *M. refringens*. However, diagnostic molecular techniques have not yet been developed for *M. chungmuensis*, as the presence of parasites in the host ova make isolation of the parasite difficult.

In the current study, we described an isolation method for this ovarian parasite, performed the partial sequence of the small subunit ribosomal gene and developed a molecular detection method using *in situ* hybridization.

MATERIALS AND METHODS

Infected oysters. For the isolation of the parasite, oysters infected with *Marteilioides chungmuensis* were collected at Ushimado, Okayama Prefecture, Japan, in November 2001, where *M. chungmuensis* is prevalent (Itoh et al. 2002). Each oyster was opened, and infection by the parasite was confirmed by gross and wet-mount slide observations. The infected ova were excised, then frozen at -20°C .

Isolation of parasite sporonts. Established isolation methods involving discontinuous gradients for other shellfish protozoan parasites, such as *Bonamia ostreae* (Mialhe et al. 1988), *Marteilia refringens* (Mialhe et al. 1985) and *Mikrocytos mackini* (Hervio et al. 1996), were not successful. We modified the usual isolation methods for the isolation of parasite sporonts. Frozen infected ova (weighing 27.3 g) were thawed and homogenized with a glass homogenizer in filtered ($0.22\ \mu\text{m}$) seawater (FSW) supplemented with 1% Tween 80 (FSWT), and then sieved successively through 100, 60 and $20\ \mu\text{m}$ nylon mesh to remove large tissue debris and fibrous materials. The homogenate was then sieved with $10\ \mu\text{m}$ nylon mesh to capture the sporonts ($18\ \mu\text{m}$ in diameter), while allowing host phagocyte cells to pass through the small nylon mesh. The mesh was washed with FSWT to suspend the sporonts, which were then collected in a petri dish.

The sporont suspension was dispensed into several 50 ml plastic centrifuge tubes (Iwaki) and centrifuged (10 min, $500 \times g$, 4°C) to concentrate the parasite cells. The pellets were resuspended in 5 ml FSWT, and layered on a discontinuous 30–40–50% (w/w) sucrose gradient and centrifuged (25 min, at $500 \times g$, 4°C). After centrifugation, the 40–50% interface was collected,

washed twice in FSW with centrifugation (10 min, $500 \times g$, 4°C) and pelleted to eliminate sucrose.

The resulting pellets were resuspended in 1 ml FSW and layered on a 14 ml Percoll (Pharmacia) 10–20% discontinuous gradient (v/v: Percoll/FSW supplemented with 0.55 M NaCl). After centrifugation (25 min, $500 \times g$, 4°C), the interface between 10 and 20% was collected, diluted in 15 ml FSW and centrifuged (10 min, $500 \times g$, 4°C) to eliminate Percoll. The isolated sporonts were stained with the fluorescent dye DAPI (4',6-diamino-2-phenylindole) to confirm the presence of parasite cells and DNA (Wesche et al. 1999). The pellets were suspended in 1 ml absolute ethanol and stored at -20°C until DNA extraction took place.

DNA extraction. DNA was extracted from the suspension of the isolated sporonts (1.18×10^7 cells in 3 ml) using a QIAamp DNA Mini Kit (QIAGEN) following the manufacturer's tissue protocol, and the purified DNA was used for polymerase chain reaction (PCR) amplification.

PCR amplification. A part of 18S rDNA was amplified by PCR using 2 oligodeoxynucleotide primers designed in conserved regions in the rDNA, 530f (Vossbrinck et al. 1993), and PD-18S-R (Iwashita 2000) (Table 1). PCR reactions were carried out in 50 μl volume according to the standard conditions of Takara Ex Taq (Takara Shuzo). The following temperature profile was employed using the GeneAmp PCR system 2400 (Perkin-Elmer): preheating at 94°C for 30 min, 35 cycles of denaturation (94°C , 30 s), annealing (52°C , 3 min) and extension (72°C , 3 min), and a further elongation step of 30 min at 72°C .

Amplified products were analyzed electrophoretically on 1.5% agarose gel, as follows: After purification of the amplicons with a PCR amplicon purification kit (QIAGEN), the PCR products were digested with the restriction enzyme *Sac* I for 2 h at 37°C , and run out on a 1.5% agarose gel to confirm that the amplicon was not derived from host cells.

DNA sequencing. PCR amplicons were cloned into a pt-7 Blue T-vector system (Takara Shuzo). Recombinant plasmids were sequenced by means of the dideoxy chain termination method (Sanger et al. 1977) using a BigDye™ Terminator v3.0 Cycle Sequencing Ready Reaction Kit, and 310 DNA sequencer (Applied Biosystems), according to the manufacturer's instructions. Sequences were aligned with Genetic-

Table 1. Primers used for PCR reaction

Primer	Direction	Sequence	Tm
530f (Vossbrinck et al. 1993)	Forward	5'-GTGCCAGC(C/A)GCCGCGG-3'	54 _
PD-18S-R (Iwashita 2000)	Reverse	5'-GATCCTTCTGCAGGTTACCT-3'	55 _

Mac/ATSQ 3.0 (Software Development), and compared for similarity with sequences lodged in GenBank using BLAST (Altschul et al. 1990).

In situ hybridization. To confirm that the obtained sequences were derived from the parasite, 3 oligonucleotide probes were designed for *in situ* hybridization studies (Table 2). The probes were labelled with digoxigenin using a Dig Oligonucleotide Tailing Kit (Boehringer Mannheim) according to Lee et al. (2000). Infected oysters were obtained in the Okayama Prefecture in October 2001 and February 2002, then fixed in 10% buffered formalin for at least 48 h, and embedded in paraffin wax. Paraffin blocks were sectioned at 5 μm thickness and placed on positive charged slides (Fisher Scientific). Hybridization reactions were performed with the Micro-Probe Staining System (Fisher Scientific) using manual capillary actions modified from the manufacturer's instructions. Briefly, after baking in a 45°C oven, the sections were rapidly deparaffinized, rehydrated with a Tris-HCl buffer, pH 7.4 (Universal Buffer, Research Genetics), and digested with pepsin (2.5 mg ml⁻¹; Research Genetics) for 3 min at 105°C. Probes diluted in formamide-free diluent were applied to the sections. The sections were heated to 105°C for 3 min, cooled for approximately 1 min at room temperature, and allowed to hybridize at 45°C for 60 min. The sections were then washed once with 2 \times SSC (standard saline citrate; 0.3 M NaCl, 0.03 M sodium citrate) at 45°C for 3 min and incubated with anti-DIG-AP antibody solution (1:500 in Tris-HCl buffer, pH 7.5) for 20 min at 50°C.

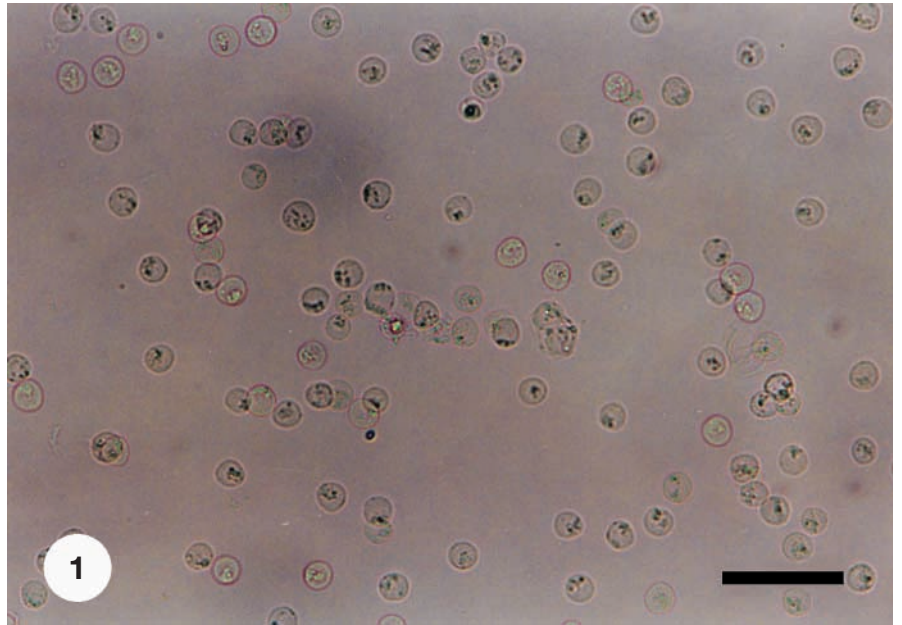


Fig. 1. *Marteilioides chungmuensis*. Isolated sporonts. Scale bar = 100 μm

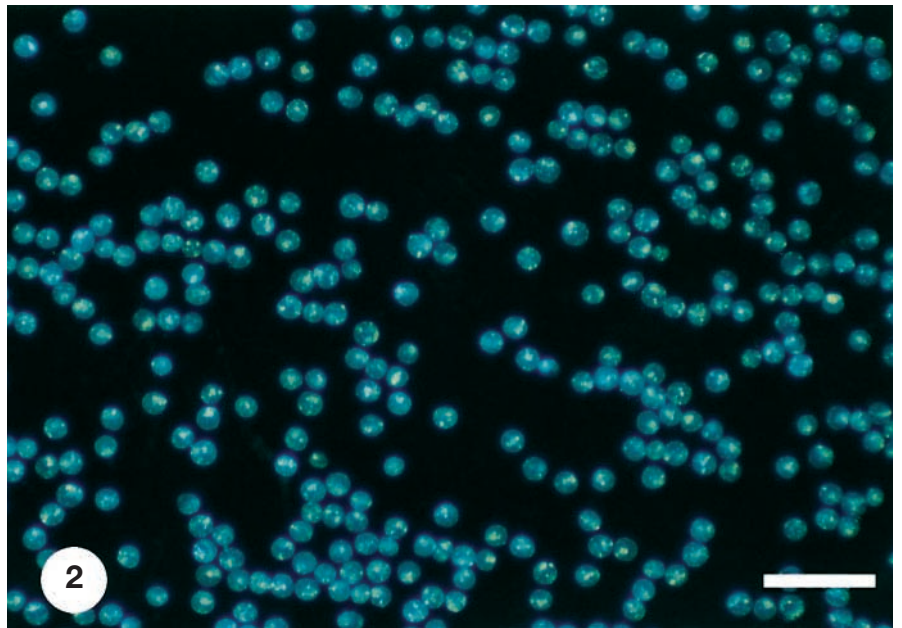


Fig. 2. *Marteilioides chungmuensis*. Isolated sporonts stained with DAPI (4',6-diamino-2-phenylindole), showing that they contain DNA. Scale bar = 80 μm

Table 2. Oligonucleotide probes used for the *in situ* hybridization

Primer	Direction	Sequence	T _m
MCSP-01	Forward	5'-AACGAGTCTCGTCTCGGAGCG-3'	53
MCSP-05	Forward	5'-GCACGAAAAGCCGGCCAGTC-3'	55
6-R	Reverse	5'-AGGTATTTCAGCCGGTGCTTCATCGGG-3'	58

<i>C. gigas</i>	1	GTGCCAGCAGCCGGGTAATTCAGCTCCAATAGCGTATAATAAAGTTGCTGCAGTTAAAAGCTCGTAGCTGGATCTCGGGTCCAGGCTGGCGGTCCGGCTCGTTGGGGTCACTGGCCTG
<i>M. chungmuensis</i>	1	<u>GTGCCAGCCGGCCGGGTAATTCAGCTCCAATAGCGTATAATAAAGTTGCTGCAGTTAAAAGCTCGTAGCTGGATCTCGGGTCCAGGCTGGCGGTCCGGCTCGTTGGGGTCACTGGCCTG</u>
<i>C. gigas</i>	121	TCCTGACCCAAACCTCTCGGTTGTAAACCCCTGGTCTCTGACTGAGTTCCTCGGTTGGCGGAAACTTTACTTTGAAAATAATAGAGTTCCTCAAACAGCAGGCGCTTCGCCTGTACAATG
<i>M. chungmuensis</i>	121	CTCCGCCACGACCGTTCGTTCCG-----CC-TCGGCG-----TGGCTCGGTCCACGCT--CCGTGAC-----AAACCAG-GTGATCAAGCCGAGCTCGT--GCTCG-AITGGTT
<i>C. gigas</i>	241	GTGCATGGAATAATGGAATAGGACCTCGGTTCTATTTTGGTTTCGGAACACGAGGTAATGATTAAGAGGACTGACGGGGGCAATTCGTATGGCGGGGTAGAGGTGAATTCCTTTGG
<i>M. chungmuensis</i>	212	TTGCATGGAATCGTAGAACGAACCTTCGCTCGGC-----GTTCCAAGAGCGC-AGGTGATGATCAACGGGAGCGGTCCGAGACGAGTGAACTGCAGGGCCGAGGGTAAATCTGATG
<i>C. gigas</i>	361	ATCCTCGCCAGACGGCCCTACAGGAAAGCATTTGCCAAGCATGTTTTCAITAGTCAAGAACGAAAGTCAGAGGTTCAAGAGCATCAGATACCGTCCGTAGTTCG-AACATAAACAGATGC
<i>M. chungmuensis</i>	324	ATCCTTGCAAGACAGCCGAAAGCGGAAGGCATCGTCTAGAGCGTTTCCGTCGATAAAGGACGGAACCCACCTAGTCAACAGGATTAGATACCCCGGTAGCGGTGCCCCCAAACTATGCG
<i>C. gigas</i>	480	CAACTAGCAATCCGCCGGAGTTGCTTCAAAGACTCGCGGAGCACCCGCCGGGAAACCAAAGTCCTTTGGGTTCCGGGGGAGTATGGTTGCCAAAGCTGAAAACCTTAAAGGAATTCACGGAA
<i>M. chungmuensis</i>	444	CGACCGCCCGACGCCCGGTTTACACTGTGAC-CGGTGTGGGTCC--GAGGAAGCTGGAGTGAATTTGGCTTCGGAGATTACGGCTGCAAGCTGAAAACCTTAAAGGAATTCACGGAA
<i>C. gigas</i>	600	GGGACACACAGGAGTGGAGCCCTGAGCTCAACACGGGAAACTCACCCGGCCCGAAACACTGTAAGGATTTGACAGATTGAGAGCTTTCTTGTGATTCGGTGGGTGGTGGT
<i>M. chungmuensis</i>	560	GGGCACCATCAGGTCGTGGATTAGCGGCTCAATTGGATTCAACGCCCGACACCCACAGGACAGGAGCG <u>CCCGATGAAGCACCCGGTGAATAC-CTTCTCCGATGGCGGTGGGGTGGT</u>
<i>C. gigas</i>	720	GCAATGGCCGTTCTTAGTTGGTGGAGGATTTGTCGGTAAATCCGATAACGAAACGAGACTTAACCTATAATA-----GTTCAACCGACTAATTTGCTCGGTTGAATCTTTTAGA-----
<i>M. chungmuensis</i>	679	GCAATGGCCGC-CGTAGTTCGTGAGCGGACTTGTCTGGTTAAATCCGACAAACGAAACCTAACCGCACTGCCCGAACCAATGGTGGCGCGCGTCCGGTGC-GTCCGCAAACTCGGGCAGACAA
<i>C. gigas</i>	833	-----GGGACAAAGTGGCGTFTT-----AGGCACACGAGATTG-AGCAATAACAGGCTCTGTGATGCCCTTAGATGTCGGGGCCGACGCGGCTACACTGAAAGAAITCAACAGGTTCTCTGTCTCT
<i>M. chungmuensis</i>	798	ATCCG <u>CAACGAGTCTCGTCTCGGAGCGGAAAGGAAAGCTACGGCGGATGGCAGGTCGTGATGCCCTTAGACGCCCTGGATCGCACCGCTAATACAATGCCCGCC-----CAAGCGCGCAGGTT</u>
<i>C. gigas</i>	945	GGCCCGAAAGGGTCGGGTAACCCGGTTGAACTCCTCGTGTAGGGATGGGGTGTATAATTCCTCCCATGAACGAGGAAATCCCAGTAAGCCGAGTCAATCAGCTCGCGTTGATTACGT
<i>M. chungmuensis</i>	914	CGCCCGAGAGGGTCTACAAAT <u>CCACGAAAAGCCCGCCCAAGTCHGGATCGAGGACTGCAACTCTCCTCGTGAACCGGAAACACTCGTAGTGGCGGTCATTAACGCCGCCACGAAATGAGT</u>
<i>C. gigas</i>	1066	CCCTGCCCTTTGTACACACCGCCCGTGCCTACTACCGAATGGATGGTTTGGACTGGTCCCGACGGCGCGCAACCGTTTTTCGTGGTGGTCCCGGGAAAGAGGCAA
<i>M. chungmuensis</i>	1034	CCCTGCTTTGTACACACCGCCCGTGCCTACAGCAATTAACGTGTCTCGTCAAG-----AGGCCGACCGGTTCCGCCCTC--G-----GGCGGATAATCGGAAGTCCCTGTA
<i>C. gigas</i>	1185	ACTGTACTTTCTAGAGGTCGTAACGTAACGAAAGTTCCTGTAGTGAACCTGCAGAGGATC 1249
<i>M. chungmuensis</i>	1134	ACGTCCGTACGAGGAGGGTGTAAGAGTCGTAACAGAGTATTTGTTGGTGAACCTGCAGAGGATC 1199

Fig. 3. Partial gene sequences of 18S ribosomal RNA of the Pacific oyster *Crassostrea gigas* and of the parasite *Marteilioides chungmuensis*, and designed primers annealing sites

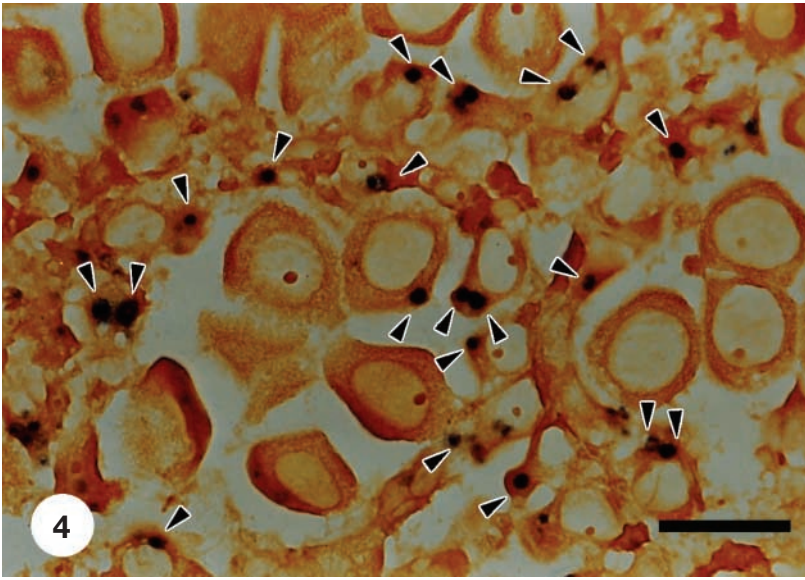


Fig. 4. *Crassostrea gigas*. *In situ* hybridization on the ovary of the Pacific oyster infected with *Marteilioides chungmuensis* sampled in February 2002. Note that immature sporogenic stages were presented in ova (arrowheads). Scale bar = 50 μ m

After washing 3 times in AP chromogen buffer pH 9.5 (Research Genetics) at room temperature, the sections were incubated in nitroblue tetrazolium/bromochloroindolyl phosphate (NBT/BCIP, in Tris-HCl based buffer, pH 9.5) for 30 min at 45°C to visualize the hybridized products. After *in situ* hybridization, the sections were counterstained with 0.05% Bismarck brown Y (Sigma).

RESULTS

The freeze-thaw procedure at -20°C ruptured the oyster ova and primary cells of the parasite, resulting in free sporonts (secondary cells) in homogenates. Without this treatment, parasite cells were retained in host ova after the isolation procedure (data not shown).

In the isolation of *Marteilioides chungmuensis* in the sucrose gradients, the 40–50% contained mainly sporonts; this interface also contained ova membranes and undigested diatom cells that oysters fed upon. In the discontinuous Percoll gradients, sporonts were isolated in the 10–20% interface, while oyster ova membranes were found in the 0–10% interface, and diatoms were

under the 20% Percoll phase. Approximately 1×10^7 sporonts were isolated from 30 g infected gonad tissue (Fig. 1); no microorganisms other than sporonts were observed microscopically, and DAPI staining showed that the isolated sporonts contained DNA (Fig. 2).

When PCR was applied to the DNA extracted from the isolated sporonts using the universal primers 530f and PD-18S-R, an amplicon of 1200 bp was obtained. When the PCR amplicons were digested with *Sac* I, 1000 and 200 bp fragments were obtained.

The DNA sequence of the parasite is shown in Fig. 3 and registered in GenBank (GenBank accession no. AB089819). Comparisons of this sequence with those in public databases using BLAST confirmed that it belongs to 18S rDNA and bears the closest similarity to *Marteilia refringens*.

In the *in situ* hybridization, early sporulation and mature stages of *Marteilioides chungmuensis* showed positive reaction with oysters obtained in February 2002 (Fig. 4) and in October 2002 (Fig. 5), respectively, and no reactions were found on any host tissue (Figs. 4 & 5).

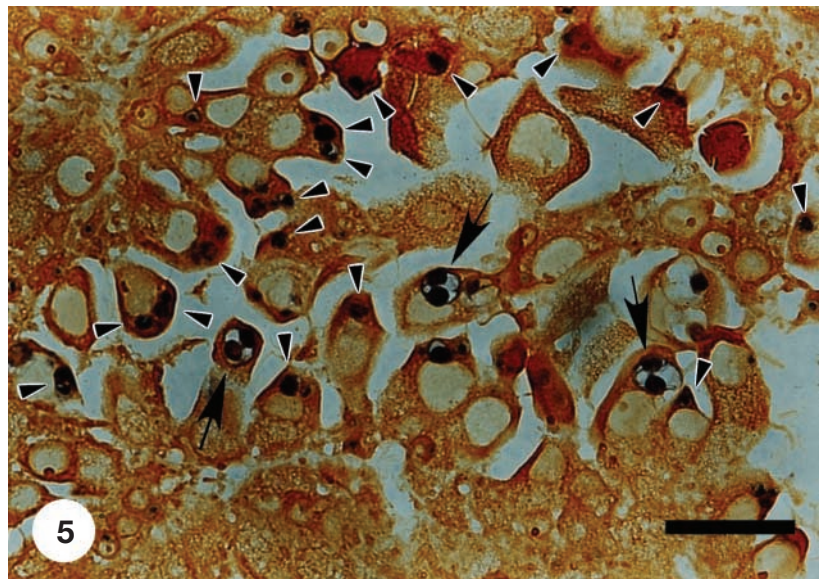


Fig. 5. *Crassostrea gigas*. *In situ* hybridization on the ovary of the Pacific oyster infected with *Marteilioides chungmuensis* sampled in October 2002. Note mature stages (arrows) and immature stages (arrowheads) present in ova. Scale bar = 50 μ m

DISCUSSION

Recently, sensitive molecular detection methods such as PCR and *in situ* hybridization have been developed for the diagnosis and understanding of the life cycle of several species of bivalve parasites. So far, however, such molecular techniques have not been applied to *Marteilioides chungmuensis*, because the isolation of the parasite has not been achieved. Usual isolation protocols for other protozoan parasites of bivalves such as *Bonamia ostreae* (Mialhe et al. 1988), *Marteilia refringens* (Mialhe et al. 1985, Robledo et al. 1995) and *Mikrocytos mackini* (Hervio et al. 1996) were not applicable to the *M. chungmuensis* harboured in the host ovocyte. The isolation protocol developed in the present study made it possible to rupture oyster ovocytes with the freeze-thaw procedure at -20°C , and to collect sporonts effectively using nylon mesh filtration and centrifugation. It was demonstrated by DAPI that the isolated sporonts contained DNA, and they were used for DNA analysis. We failed to isolate parasite cells from oysters obtained from December 2001 to February 2002, when mature sporonts were absent in infected oysters. The best period for isolating parasite cells was from late summer to early winter (August to November 2002), when mature sporonts were frequently observed in infected oysters.

Our previous work (Authors' unpubl. data) on the 18S rDNA sequence of the Pacific oyster (GenBank accession no. AB064942) showed that the rDNA has a single recognition site by *Sac* I between the annealing site of the used primer 530f and PD-18S-R, resulting in 694 and 556 bp fragments. On the other hand, the digestion of the present PCR amplicon by *Sac* I generated 1000 and 200 bp, indicating that the amplicon was not derived from the host oyster. The sequence of the amplicon bore a close resemblance to the 18S rDNA gene of *Marteilia refringens* (GenBank accession no. AJ250699). Moreover, the probes designed from the sequence showed a positive reaction to *M. chungmuensis* in the *in situ* hybridization. This demonstrates that the obtained sequence was the 18S rDNA of the parasite.

Only parasite cells showed positive signals in the *in situ* hybridization, indicating that this technique detects the parasite in oysters effectively. Interestingly, Klee-man et al. (2002) found previously unknown stages of a related pathogen, *Marteilia sydneyi*, in the Sydney rock oyster, which led to the establishment of a hypothetical life cycle. So far, only sporogonic stages in host ova have been described for *M. chungmuensis* (Park & Chun 1989, Imanaka et al. 2001, Itoh et al. 2002). We therefore expect that this newly applied technique will contribute to the discovery of previously undetected developmental stages of the parasite.

In the present study, once an isolation method for *Marteilioides chungmuensis* was established, its 18S rDNA was partially sequenced. This sequence will be helpful in the further development of molecular detection techniques such as PCR and *in situ* hybridization, which in turn will lead to the elucidation of the parasite's life cycle.

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