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Detection of *Marteilia refringens* using nested PCR and *in situ* hybridisation in *Chamelea gallina* from the Balearic Islands (Spain)

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ABSTRACT: In the course of a histopathological survey performed to discover the cause of mass mortality of the striped clam *Chamelea gallina* in the Balearic Islands (Spain, Mediterranean Sea), we detected a *Marteilia*-like parasite in 3 clams. Molecular methods were applied to identify the parasite. DNA extracted from a paraffin block was used to carry out a PCR assay for *Marteilia refringens* detection based on a rDNA sequence of the parasite (the intergenic spacer of ribosomal genes, IGS). The nucleotide sequence of the IGS amplified fragment and the positive signal obtained by *in situ* hybridisation analysis with a *M. refringens*-specific probe allowed us to confirm the presence of this parasite in the digestive gland tissue of *C. gallina*.

KEY WORDS: *Marteilia refringens* · Molecular diagnosis · rDNA · Intergenic spacer · IGS · *Chamelea gallina*

INTRODUCTION

Paramyxeans are a group of protists responsible for parasitosis in marine invertebrates. After a number of different taxonomic affiliations were suggested for these organisms — including the fungal order Chytridiales, the algal genus Microspora, the lower fungi and the genus Labyrinthomyxa (reviewed by Berthe et al. 2004) — Desportes & Perkins (1990) proposed the phylum Paramyxea based on the organisms' development within the host. It is characterized by the formation of spores consisting of several cells enclosed inside one another that arise by a process of internal cleavage within a stem cell (Desportes & Perkins 1990). The number of spores and sporal cells differentiated in the sporonts, and the shape of the spores are the principal distinguishing characters at the genus level (Desportes & Perkins 1990, Larsson & Køie 2005). Subsequently, the availability of DNA sequences made possible the molecular confirmation of Paramyxea as an independent eukaryotic phylum not closely related to any single phylum whose SSU rDNA sequence was known (Berthe et al. 2000, 2004). The 9 paramyxean species described so far are currently divided into 5 genera (Table 1). Certain species belonging to the genera *Marteilia* and *Marteilioides* have been the main focus of research because of the harm they cause to farmed oysters. The species *Marteilia refringens* has caused recurrent mass mortalities in the European flat oyster *Ostrea edulis* over the last 4 decades (Grizel et al. 1974, Alderman 1979, Figueras & Montes 1988, Robert et al. 1991, Berthe et al. 2004), while *Marteilia*

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Table 1. Phylum Paramyxea (Desportes & Perkins, 1990) and host in which each species has been detected

Parasite	Host	First citation
Class Marteiliidea (Desportes & Ginsburger-Vogel, 1977)		
Genus *Marteilia*		
M. refringens	*Ostrea edulis*	Grizel et al. (1974)
	Mytilus edulis	Tigé & Rabouin (1976)
	Mytilus galloprovincialis	Comps & Joly (1980)
	Solen marginatus	López-Flores et al. (2008)
	Chamelea gallina	This study
M. sydneyi	*Saccostrea glomerata*	Perkins & Wolf (1976)
M. lengehi	*Saccostrea cucullata*	Comps (1976)
M. christenseni	*Scrobicularia plana*	Comps (1983)
Marteilia sp.	*Cardium edule*	Comps et al. (1975)
	Crassostrea gigas	Cahour (1979)
	Tapes pullastra	Poder et al. (1983)
	Tapes rhomboides	Poder et al. (1983)
	Modiolus modiolus	Auffret & Poder (1983)
	Ostrea chilensis	Grizel et al. (1983)
	Ostrea angasi	Bougrier et al. (1986)
	Ostrea puelchana	Pascual et al. (1991)
	Argopecten gibbus	Moyer et al. (1993)
	Tridacna maxima	Norton et al. (1993)
	Crassostrea virginica	Renault et al. (1995)
	Ensis minor	Ceschia et al. (2001)
	Ensis siliqua	Ceschia et al. (2001)
	Saccostrea forskali	Taveekijakarn et al. (2002)
	Tapes philippinarum	Itoh et al. (2005)
Genus *Marteilioides*		
M. chungmuensis	*Crassostrea gigas*	Comps et al. (1986)
M. brachialis	*Saccostrea glomerata*	Anderson & Lester (1992)
Marteilioides sp.	*Tapes philippinarum*	Lee et al. (2001)
Genus *Paramarteilia*		
P. orchestiae	*Orchestia gammarellus*	Ginsburger-Vogel et al. (1976)
	Orchestia mediterranea	Ginsburger-Vogel (1991)
	Orchestia aestuarensis	Ginsburger-Vogel (1991)
Class Paramyxidea (Chatton, 1911)		
Genus *Paramyxa*		
P. paradoxa	*Poecilochaetus serpens*	Chatton (1911)
Genus Paramyxoides		
P. nephtys	*Nephtys caeca*	Larsson & Køie (2005)

sydneyi has had similar effects on the Sydney rock oyster *Saccostrea commercialis* in Australia (Perkins & Wolf 1976, Adlard & Ernst 1995, Butt & Raftos 2007). *Marteilioides chungmuensis* affects the gonad of the Pacific oyster *Crassostrea gigas* in Korea and Japan, resulting in serious economic losses for the oyster industry (Comps et al. 1986, Ngo et al. 2003, Tun et al. 2006).

In Europe, 2 molecular types or species of *Marteilia* have been identified: *M. refringens* (or type O), infecting oysters, and *M. maurini* (or type M), infecting mussels (Le Roux et al. 2001). Recent data suggest that parasites infecting oysters and mussels constitute 2 different strain of the species *M. refringens* (López-Flores et al. 2004, Novoa et al. 2005, Balseiro et al. 2007). Therefore, the name *M. refringens* would have taxonomic priority and this is the nomenclature we use in this study. *M. refringens* has also been reported in other bivalves (see Table 1), although the accurate identification of the parasite species has only been achieved in the mussels *Mytilus edulis* and *Mytilus galloprovincialis* and in the razor clam *Solen marginatus* (Table 1) by using precise histological and/or molecular methods. Among these species, mortalities have only been documented in mussels (Villalba et al. 1993a,b, Fuentes et al. 2002). The presence of the parasite in different marine species is also interesting for the study of its life cycle, in which intermediate hosts are thought to be involved. So far, only the copepod *Paracartia granii* has been suggested as an intermediate host in the life cycle of the parasite (Audemard et al. 2002). In addition, the existence of closely linked dynamics between *M. refringens* in mussel populations and a group of 6 different zooplankton taxa has been suggested in the coastal northeast Mediterranean near Spain (Carrasco et al. 2007a,b). In histological studies on venerid clams the presence of *Marteilia* sp. has been reported in *Tapes rhomboides* and *T. pullastra* (Poder et al. 1983), and in *T. philippinarum* (Itoh et al. 2005), but identification of the exact species involved has not so far been documented.

The analysis of nucleotide sequence data has been widely used for species identification in different organisms, including *Marteilia*. The 18S, internal transcribed spacer-1 (ITS-1) and intergenic spacer (IGS) of the major ribosomal locus are the characterised sequences for *Marteilia refringens*, whereas only a partial 18S-ITS-1 sequence is known for *M. sydneyi* (Fig. 1). Nucleotide sequence of the 18S ribosomal gene is highly conserved throughout evolution, which makes it more useful for comparing relatively distant species, in evolutionary studies for example, than as a precise molecular marker for species identification. The IGS is under little or no selective pressure; therefore its nucleotide sequence evolves quickly and it results in an appropriate marker for differentiating

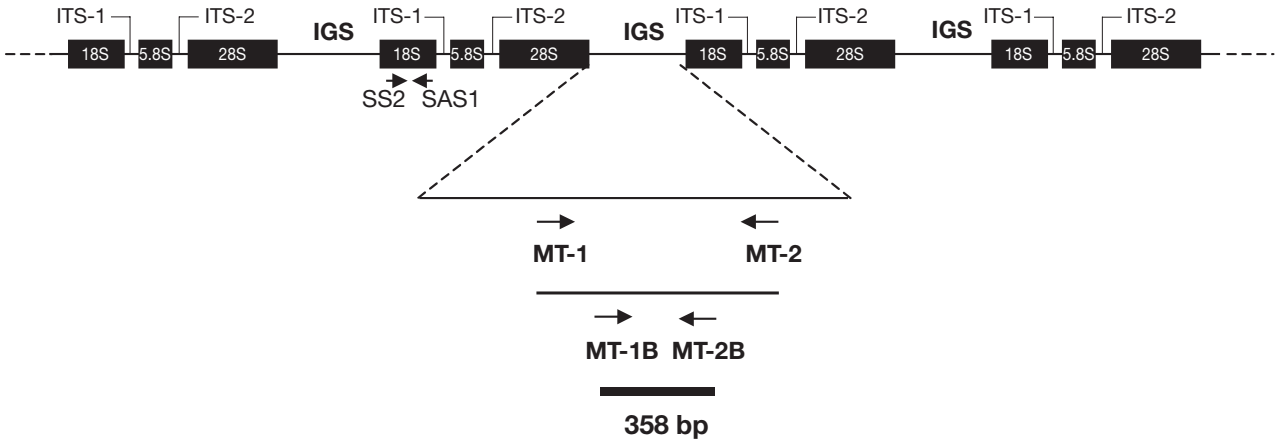

Fig. 1. Schematic representation of the ribosomal RNA gene complex containing the genes that encode rRNAs and the spacer regions. Locations of the primers used in this study are indicated

Table 2. Description of primers and their uses

Primer	Sequence (5′-3′)	Use	Size (bp)	Reference
MT-1	GCCAAAGACACGCCTCTAC	First-round PCR	525	López-Flores et al. (2004)
MT-2	AGCCTTGATCACACGCTTT			
MT-1B	CGCCACTACGACCGTAGCCT	Nested-PCR, probe synthesis for ISH	358	López-Flores et al. (2004)
MT-2B	CGATCGAGTAAGTGCATGCA			
SS2	CCGGTGCCAGGTATATCTCG	Probe synthesis for ISH	265	Le Roux et al. (1999)
SAS1	TTCGGGTGGTCTTGAAAGGC			

between closely related species (Hillis & Dixon 1991). The IGS of *M. refringens* has been used in both phylogenetic and taxonomic studies (Le Roux et al. 1999, 2001, Berthe et al. 2000, López-Flores et al. 2004, 2008 Novoa et al. 2005, Carrasco et al. 2007a,b).

In this study, we applied molecular methods for the identification of a *Marteilia*-like parasite found in *Chamelea gallina* (Bivalvia, Veneridae) in the course of a histopathological survey performed to find out the cause of a mass mortality of this clam species in the Bay of Palma (Mallorca, Balearic Islands, Spain, Mediterranean Sea). Histological and molecular analyses led to detection, for the first time, of *M. refringens* in the striped clam *C. gallina*.

MATERIALS AND METHODS

DNA extraction from the sample. Tissues of every sampled clam (69 individuals) were fixed in Lillie's seawater formalin solution (85% ethanol, 10% acetic acid, 5% formalin) for 48 h, dehydrated in an ethanol series and embedded in paraffin. Histological sections (4 µm thick) were stained with hematoxylin-eosin and observed under a light microscope for histological analysis. A *Marteilia*-like parasite was found in 3 of the 69 clams. Genomic DNA was obtained using twenty 5 µm thick sections from the paraffin block of a clam infected by a *Marteilia*-like parasite. They were collected into a 1.5 ml microcentrifuge tube and dewaxing was carried out as follows: 1 ml of sterile ultra pure water was added to the sections prior to vortexing and incubation at 65°C overnight. After centrifugation at 5000 × *g* for 5 min, the solid paraffin in the upper layer was removed and the procedure was repeated once, this time with a shorter period of incubation at 65°C (2 h). The pellet was then processed as tissue using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's protocol.

Detection of *Marteilia refringens* by nested PCR. The sequences and location of the primers used for first-round PCR and nested PCR are shown in Table 2 and Fig. 1. Both PCRs were done in a final volume of 50 µl using 100 ng of each primer (MT-1 and MT-2 for first-round PCR and MT-1B and MT-2B for nested PCR) and Ready-to-Go PCR beads (Amersham Biosciences), following manufacturer's recommendations. Different quantities of extracted DNA were used in the first ampli-

fication (100 ng to 200 pg). Thermal cycling was 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, plus 72°C for 10 min. For nested PCR, 2 µl of first-round PCR were used as template. Thermal cycling was 94°C for 5 min, 25 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, plus 72°C for 5 min. During each PCR (first-round and nested) several negative controls were used, including a non-template water sample and genomic DNA extracted from *Chamelea gallina* previously confirmed as non-infected by histological examination and PCR analysis. PCR products were electrophoresed in 1 × TAE buffer and visualised under UV light on a 1% (w/v) ethidium bromide-stained agarose gel. Amplified fragments obtained from 3 PCR reactions were excised from the gel and purified using a GFX™ PCR-DNA and Gel Band Purification kit (Amersham Biosciences). Nucleotide sequences were obtained using ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems). The protocols were carried out following the manufacturer's recommendations.

Comparison of IGS *Marteilia refringens* sequences purified from bivalves. To asses the genetic affinity of the sequence obtained from *Chamelea gallina*, IGS rDNA sequences of *Marteilia refringens* were downloaded from GenBank and used in a comparative analysis. Accession numbers of the sequences were as follows: *M. refringens* isolated from *Ostrea edulis,* AJ629352–AJ629356; *M. refringens* isolated from *Mytilus galloprovincialis,* AJ629357–AJ629376, AM748042, AM748043; *M. refringens* isolated from *Solen marginatus,* AM748037–AM748041. Multiple sequence alignment was done using the MegAlign program of the DNA package (Lasergene). Sequence variability within and between host species was calculated with the Kimura 2-parameter model distance matrix (Kimura 1980) using the MEGA package (Kumar et al. 2004).

***In situ* hybridisation.** The probes were generated by PCR using the PCR DIG probe Synthesis Kit (Boehringer Mannheim) according to the manufacturer's instructions, and *Marteilia refringens* DNA was purified from *Ostrea edulis* as a target for the amplification reaction. Primers MT-1B and MT-2B were used for amplifying *M. refringens* IGS probe as described above for nested PCR. Primers SS2 and SAS1 were used for the synthesis of the probe Smart2, located at the 18S ribosomal gene of the parasite and generic for paramyxean species, as described by Kleeman et al. (2002). See Table 2 and Fig. 1 for sequence and location of the primers. Serial sections were cut 4 µm thick, placed on treated slides (Sigma) and incubated for 30 min at 65°C. *In situ* hybridisation was performed as described elsewhere (López-Flores et al. 2008).

Negative controls, without the DIG-labelled probe in the hybridisation buffer and *Saccostrea commercialis* digestive gland tissue infected with *Marteilia sydneyi,* were included in the analysis. Positive control consisted of *Ostrea edulis* digestive gland tissue infected with *M. refringens*.

RESULTS

Amplified products by first-round PCR were not detectable on the agarose-stained gel with any of the DNA quantities used. However, nested PCR yielded the expected amplification product (358 bp in length) from the *Chamelea gallina* sample, whereas amplification was never observed in negative controls (data not shown). The sequence obtained from the amplified fragment was compared with those included in the public databases obtained from the hosts *Ostrea edulis*, *Mytilus galloprovincialis* and *Solen marginatus*. Nucleotide sequence of the *Marteilia* parasite obtained from *C. gallina* showed the closest identity with *Marteila refringens* sequences isolated from *O. edulis* (99.1% identity with sequences isolated from *O. edulis* vs. 97.4% identity with sequences isolated from either *Mytilus galloprovincialis* or *S. marginatus*; Table 3). The partial IGS sequence of *Marteilia refringens* found in *C. gallina* has been entered into the EMBL database with accession number AM292652.

Parasite multinucleated cells were observed using histology in Haematoxylin-eosin stained sections from the striped clam sample. In some of them, the characteristic structure of *Marteilia* sp. of 1 cell inside another was easily distinguished. Different stages of the life-cycle were found in the epithelium of the digestive diverticula: primary cells (young stem cells) that contained only 1 nucleus, stages containing 1 or more secondary cells and more advanced stages containing up to 8 sporonts (secondary cells) within which, up to 4 spores (tertiary cells) were also visible. In advanced stages sporonts contained refringent bodies in the cytoplasm (Fig. 2A). The parasite was not detected in other organs appearing in histological sections such as gills, mantle, gonad and foot.

Table 3. Divergence (percentage) among MT-1B/MT-2B fragments from *Marteilia refringens* infecting *Ostrea edulis*, *Mytilus galloprovincialis*, *Solen marginatus* and *Chamelea gallina*

	O. edulis	*M. gallo-provincialis*	*S. mar-ginatus*
O. edulis	1.4		
M. galloprovincialis	2.0	1.0	
S. marginatus	1.8	0.7	0.4
C. gallina	0.9	2.6	2.6

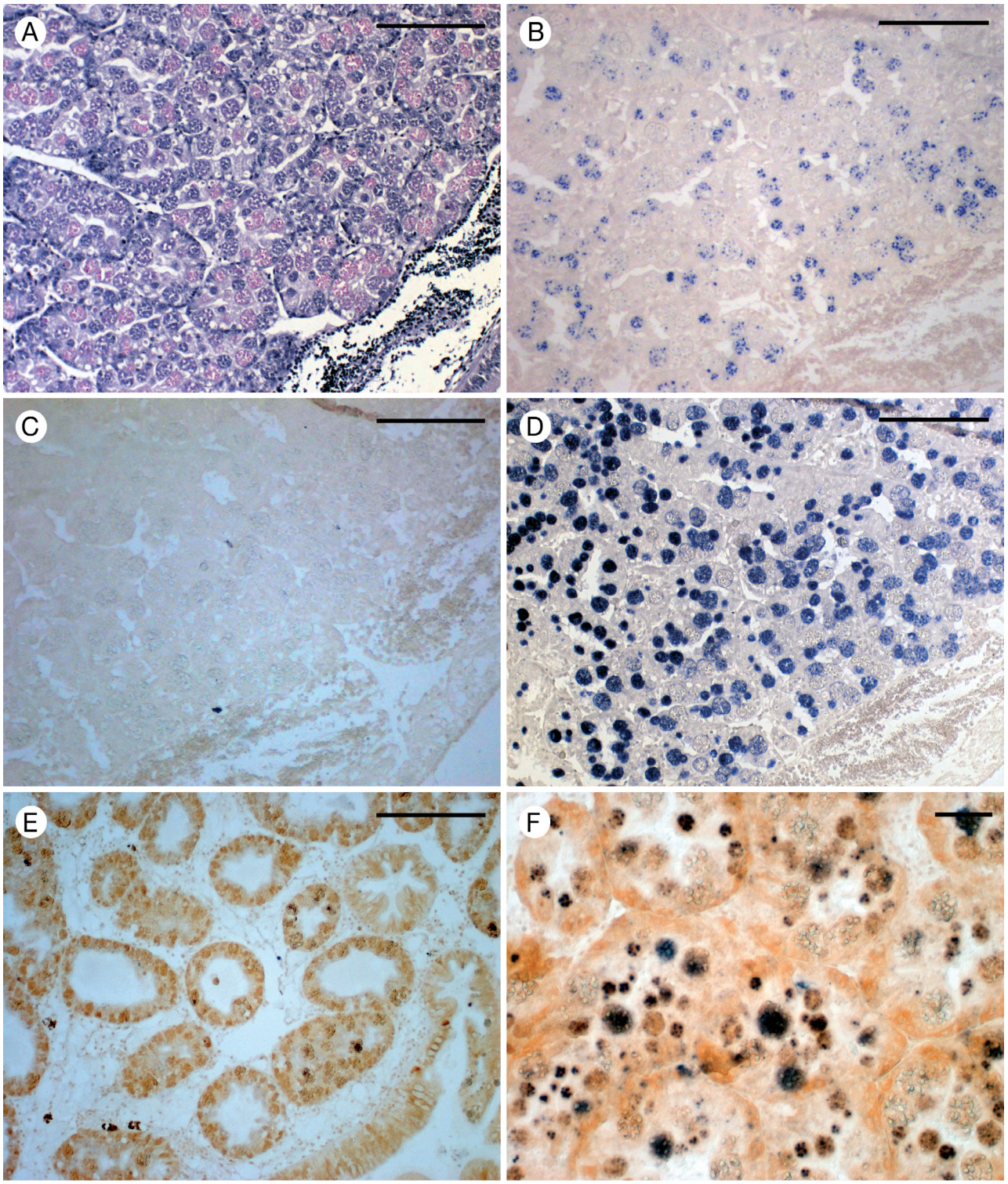

Fig. 2. *Marteilia refringens* cells in *Chamelea gallina* digestive gland sections by *in situ* hybridisation. (A) Classic histology with haematoxylin-eosin staining. (B) Positive hybridisation signal by digoxigenin-labelled MT-1B/MT-2B probe (*M. refringens* IGS probe). (C) Negative control (lack of DIG-labelled probe in the hybridisation buffer). (D) *In situ* control with digoxigenin-labelled Smart2 probe (*Marteilia* 18S ribosomal gene generic probe, Kleeman et al. 2002). (E) *Saccostrea commercialis* digestive gland tissue infected with *Marteilia sydneyi* (*in situ* negative control). (F) *Ostrea edulis* digestive gland tissue infected with *M. refringens* (*in situ* positive control). Scale bars = 200 µm

In situ analysis performed with a *Marteilia refringens* IGS probe supplied hybridisation signal with the different stages of the parasite (Fig. 2B). Some of the mature cells of the parasite showed a weak hybridisation signal or no signal at all. A stronger signal was obtained with the probe targeting the 18S ribosomal gene, used as a control. Equally, some cells showed no hybridisation (Fig. 2D). *Ostrea edulis*-infected tissue provided a positive control for *M. refringens in situ* detection (Fig. 2F). Cross hybridisation with host cells nuclei did not appear. Negative controls were used to ensure specificity and no signal was detected either in tissue sections carrying *M. sydneyi* infection (Fig. 2E) or in tissue section in where no probe was added (Fig. 2C).

DISCUSSION

Ethanol-fixed material was not adequately collected during routine sampling and the identification of the *Marteilia*-like parasite detected in *Chamelea gallina* had to be accomplished using paraffin-embedded tissues. However, the method we used, as discussed later in this section, was effective in identifying parasite species from fixed specimens. The methodological approach we described in this paper may have the potential to be conveniently applied for the identification of this parasite species from archival fixed tissues. Retrospective examination of archival samples by molecular techniques could enable retrospective diagnosis and provide valuable epidemiological data of *M. refringens* infection. It must be taking into account, however, that obtaining high-quality DNA from fixed paraffin-embedded tissues is often difficult and failure to extract amplifiable DNA from this kind of specimen usually prevents us from obtaining molecular confirmation of the precise parasitic species present (Greer et al. 1991). In our case, the method used for *M. refringens* genome detection included first-round and nested PCR. First-round PCR increased the primer binding sites for the second set of primers used in the nested PCR. Nested PCR therefore provides a higher sensitivity than standard PCR, since the second set of primers amplifies a secondary target present within the first amplified product. We used a combination of nested and short-length PCR to overcome decreased efficiency of amplification by the standard PCR, probably due to extensive DNA degradation and the presence of PCR inhibitors (both of which are typical with these kinds of samples) (Greer et al. 1991, Wilson 1997).

Thus, the identification of the *Marteilia*-like parasite infecting *Chamelea gallina* was possible after amplification of a fragment of the parasite's genome by nested PCR and the comparison of its DNA sequence with the homologous sequence of *M. refringens.* The sequence obtained from the paraffin block showed 97.4% identity with *M. refringens* sequences obtained from both *Mytilus galloprovincialis* and *Solen marginatus*. Identity with the sequences isolated from *Ostrea edulis* rose to 99.1%. This result supports the presence of *M. refringens* in the *C. gallina* sample. Moreover, sequences isolated from *O. edulis* correspond to the molecular type of *M. refringens* that parasitizes oysters, while sequences isolated from *M. galloprovincialis* and *S. marginatus* correspond to the parasite strain mainly found infecting mussels (López-Flores et al. 2004, 2008). This is confirmed by the lower genetic identification between molecular types (i.e. 2% divergence between *M. refringens* from oysters and *M. refringens* from mussels, and 1.8% divergence between *M. refringens* from oyster and *M. refringens* from razor clams) than within the same type (i.e. 0.7% divergence between *M. refringens* from mussels and *M. refringens* from razor clams). Therefore, the higher identification of the parasite sequence amplified from *C. gallina* with the sequences isolated from *O. edulis* (0.9% divergence) indicates that the molecular profile of *M. refringens* infecting *C. gallina* corresponds to *M. refringens* from oysters.

Detection of *Marteilia refringens* in new species is interesting for the study of this parasite's life cycle and it is also of great interest for the purpose of identifying particular molecular types responsible for infections in new hosts. Recently, transmission experiments between *M. refringens* from oyster and mussels and females of the copepod *Paracartia grani* (the species suspected of being an intermediate host of the parasite) showed that apparently *M. refringens* from oysters proliferate more rapidly than *M. refringens* from mussels in infected copepods (Carrasco et al. 2008). Therefore, characterization at molecular level of *M. refringens* infecting different species, together with complementary studies such as mortality data and transmission experiment between the hosts, would provide valuable information for the management of bivalve production systems and marteiliosis control programs.

Specific location of *Marteilia refringens* cells on *Chamelea gallina* tissues was determined by *in situ* hybridisation, using probes obtained from DNA from *M. refringens* infecting *Ostrea edulis*. One of the probes was Smart2, located in the 18S ribosomal gene of the parasite and considered to be specific at *Marteilia* genus level (Le Roux et al. 1999, Kleeman et al. 2002). We use this probe as a control in our *in situ* analysis because the morphological features of the parasite in histological sections were in agreement with those described for this genus, and the result confirmed the presence of a parasite belonging to the

genus *Marteilia* in *C. gallina* digestive gland tissue. The other probe used was the 358 bp IGS fragment of *M. refringens* included between primers MT-1B and MT-2B. The hybridisation signal obtained with the IGS probe was similar to that obtained with *O. edulis* parasitized tissue, whereas no signal was observed with cells of the related species *M. sydneyi* infecting *Saccostrea commercialis* tissues. Although the IGS rDNA sequence of *M. sydneyi* genome has not been characterized, and thus interspecific divergence between *M. refringens* and *M. sydneyi* cannot be determined based on this region, the result of the *in situ* hybridisation analysis in both sample and controls showed that the IGS probe can be useful for the specific identification of *M. refringens* among related species. This result, together with the identity of the sequence amplified by nested PCR, allowed us to identify the species of the genus *Marteilia* present in the digestive system of *C. gallina* as *M. refringens*. In addition, the weak signal of the IGS probe with respect to the probe targeting the 18S gene could be explained by the lower availability of the IGS target sequence (a non-transcribed spacer only present in the genomic DNA) compared with the 18S gene sequence (present also in the transcribed rRNAs), such as has been previously described for *M. sydneyi* ITS-1 probe with respect to the same Smart2 probe (Kleeman et al. 2002). The lack of hybridisation of the IGS probe with some of the parasite cells could suggest the possible presence of another species of the *Marteilia* genus in the sample that did not hybridise with the *M. refringens* probe (as occurred with *M. sydneyi* cells in the negative control). Nevertheless, this possibility seems inconsistent, due to the fact that hybridisation with the Smart2 probe, specific at genus level, also shows a lack of signal with some parasitic cells. Therefore we consider that this result must be due to some problem in the permeabilization process during the hybridisation protocol (insufficient proteolitic enzymatic treatment) causing only partial access of the probes to the target sequence. Alternative protocols, such as the use of oligo probes, will be considered for further analysis in order to minimize this possibility during *in situ* hybridisation analysis.

In clams, unidentified *Marteilia* sp. were reported in the digestive glands of *Tapes* [*Venerupis*] *pullastra* and *T. rhomboides* from the north of France (Poder et al. 1983) and *T. philippinarum* from the north of Spain (Figueras et al. 1996) and Japan (Itoh et al. 2005) using histological analysis. The first record in Spain was in *T. decussates* and *T. rhomboides* from Galicia (Villalba et al. 1993c). In the *Chamelea gallina* sample, morphological characteristics of the parasite found in the digestive tubules correspond to those of the *Marteilia* genus (Grizel et al., 1974) and the molecular analysis we performed identified it as *M. refringens*. To our knowledge, this is the first identified *Marteilia* parasite in the clam *C. gallina*. The Mediterranean Sea is considered an enzootic area for *M. refringens*, which has been reported infecting bivalves in the nearby region of Delta del Ebro (Durfort 1994, Bigas et al. 2000, Novoa et al. 2005, Carrasco et al. 2007b); *M. refringens* has also been identified, using histological techniques, in the digestive gland of the European flat oyster *Ostrea edulis* and the mussel *Mytilus galloprovincialis* from the Balearic coast (Port of Maó, Minorca) (A. Grau unpubl. data), as well as in samples from the coast of Italy, Croatia and Greece (Zrncic et al. 2001, López-Flores et al. 2004, Karagiannis & Angelidis 2007). The presence of the different known stages of *M. refringens* cells in *C. gallina*, including mature sporulation stages, suggests that the parasite is able to complete the infection in this mollusc species and, therefore, *C. gallina* should be considered as a new host of *M. refringens*. A clear association between infection by *M. refringens* and *C. gallina* mortality in the Bay of Palma could not be established because only 3 of the 69 clams were found to be infected. Nevertheless, the causes of this high mortality have yet to be discovered. Further research, including transmission experiments of the parasite, is required to assess whether *C. gallina* species has been identified as another host for *M. refringens* or whether it plays another role in the life cycle of the parasite.

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