Vol. 44: 143–148, 2001 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published March 9

First record of *Marteilia* sp. in mussels *Mytilus galloprovincialis* in Croatia

Snježana Zrnčić[1,*], Frédérique Le Roux[2], Dražen Oraić[1], Branko Šoštarić[1], Franck C. J. Berthe[2]

[1]Croatian Veterinary Institute, Department of Fish Diseases and Aquaculture, Savska 143, 10000 Zagreb, Croatia
[2]IFREMER, Laboratoire de Génétique et Pathologie, BP 133, 17390 La Tremblade, France

ABSTRACT: Marteiliosis is a disease of molluscs caused by *Marteilia refringens* in Europe and *M. sydneyi* in Australia. During routine examination of cultured mussels *Mytilus galloprovinciallis* in the northern Adriatic, the occurrence of *Marteilia* sp. was recorded with a prevalence of 5%. This parasite was not detected in flat oysters reared in the same area. The affiliation of the detected parasite in *M. galloprovinciallis* was confirmed by *in situ* hybridization using a *M. refringens* probe, specific at the genus level. DNA of these infected mussels originating from the same area will be used to clarify the taxonomic position of this species within the genus *Marteilia* using a molecular approach.

KEY WORDS: *Marteilia maurini* · Parasite · *Mytilus galloprovincialis* · Detection · Taxonomy

INTRODUCTION

Marteiliosis is a disease of molluscs caused by protistan parasites of the genus *Marteilia*, phylum Paramyxea (Desportes & Perkins 1990, OIE 1997, Berthe et al. 2000). *Marteilia refringens* is the etiological agent of the 'Aber disease' (Grizel et al. 1974) and this pathogen has caused mass mortalities in the European flat oyster *Ostrea edulis* industry (Alderman 1979, Robert et al. 1991). Similarly, a related organism, *Marteilia sydneyi*, is responsible for mass mortalities of *Saccostrea commercialis* in Australia (Perkins & Wolf 1976, Adlard & Ernst 1995). The diagnosis of these 2 species can easily be achieved by classical histological methods. *M. sydneyi* may be distinguished from *M. refringens* by the number of secondary and tertiary cells in the characteristic cell-within-cell structure of these parasites (Table 1).

In Europe, *Marteilia refringens* was observed in *Ostrea edulis* (Grizel et al. 1974), in *Mytilus edulis* and *M. galloprovincialis* (Tigé & Rabouin 1976, Villalba et al. 1993). *M. refringens* cells were also found accidentally in the Pacific cup oyster *Crassostrea gigas* (Cahour 1979). Another species of the genus *Marteilia*, *M. maurini*, was described in mussels *M. galloprovincialis* imported to France from Venice lagoon in Italy (Comps et al. 1982) as well as in *M. edulis* from France (Auffret & Poder 1985). *Marteilia* sp. was observed in cockles *Cardium edule*, clams *Tapes rhomboides* and *T. pullastra* (Comps et al. 1975, Poder et al. 1983, Figueras et al. 1996), and in mussels *Modiolus modiolus*, *Mytilus edulis*, and *M. galloprovincialis* (Comps

Table 1. Number of secondary, tertiary and sporoplasm cells in tertiary cells of paramyxean genera and species. ND: not determined

Species	No. of secondary cells	No. of tertiary cells	No. of sporoplasm cells
Marteilia refringens	8	3–4	3
Marteilia sydneyi	8–16	2(3)	3
Marteilia lengehi	8	?	ND
Marteilia maurini	8	3–4	3
Marteilia christenseni	8	4	3

*E-mail: zrncic@rudjer.irb.hr

et al. 1975, Poder et al. 1983, Auffret & Poder 1985, Ceschia et al. 1991, Figueras et al. 1991). Lastly, the species *Marteilia christenseni* was described in *Scrobicularia piperata* P. (Comps 1983) from the Atlantic coast of France.

The differential diagnosis of the 2 main species of *Marteilia* reported in Europe (*M. refringens* and *M. maurini*) was based on ultrastructural characteristics and host specificity (Grizel et al. 1974, Perkins 1976, Comps et al. 1982, Figueras & Montes 1988). Because *M. maurini* had been reported only from mussels, it has been proposed that *M. maurini* should be considered as the species parasitizing mussels. However, host specificity was disputed by Villalba et al. (1993), who found *Marteila refringens* in *Mytilus galloprovincialis*. Moreover, a recent study (Longshaw pers. comm.) has challenged the criteria used for the identification of species within the genus *Marteilia* (Perkins 1976, Perkins & Wolf 1976, Comps et al. 1982, Auffret & Poder 1985). It was concluded that *M. maurini* from *Mytilus edulis* and *M. galloprovincialis* cannot be separated from *M. refringens* from *Ostrea edulis* using current ultrastructural criteria. Sequencing genes of taxonomic value may help to resolve the taxonomic position of *M. refringens* and its relatives. Therefore this may assist in determining if *M. maurini* really is a different species from *M. refringens* (Berthe et al. in press).

In this paper we document the first record of marteiliosis in mussels *Mytilus galloprovincialis* from Croatian shellfish farms. Taxonomic implications of this finding will be discussed.

MATERIALS AND METHODS

The origin of the molluscs included in the study. The 2 shellfish farms included in the study are located on the northern Adriatic peninsula of Istra. The first farm is located on the eastern coast of Istra in Raša Bay, and the second is on the western coast of Istra in Limski Bay (Fig. 1). Both farms cultivate mussels on rafts, while in the Limski Bay mussels and oysters are cultivated on the same rafts.

One hundred and fifty *Mytilus galloprovincialis* mussels were sampled from the first shellfish farm and 150 *M. galloprovincialis* mussels and 100 *Ostrea edulis* oysters were sampled from the second farm at the end of August 1998 and 1999, respectively.

Cytological procedures. Molluscs from each batch were checked for the presence of *Marteilia* spp. by means of digestive gland imprints. Imprints were stained using a Giemsa-modified staining method (Hemacolor Kit, Merck). Results were confirmed by histology and *in situ* hybridization.

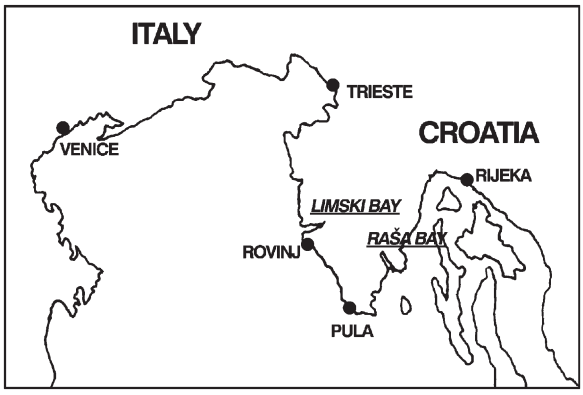

Fig. 1. Shellfish farms included in the study are situated on the eastern and western coast of the Istra peninsula in the northern Adriatic, close to the Venice lagoon, where *Marteilia maurini* was first described in *Mytilus galloprovincialis*

Histological procedures. The visceral masses of the mussels were cut along the sagittal plane and placed in Davidson's fixative AFA (10% glycerine, 20% formalin, 30% 95% ethanol, 30% dH_2O, 10% glacial acetic acid) for at least 24 h. The sections were subsequently treated by conventional histological procedures. Sections were cut 2 µm thick and stained with hematoxylin and eosin.

Preparation of the probe for *in situ* hybridization. The probe was labelled with digoxigenin by PCR using primers designed in the SSU rDNA gene specifics for *Marteilia refringens* (SS2/SAS1) and DNA of *Marteilia* sp.-infected *Mytilus galloprovincialis* from Croatia as the target (Le Roux et al. 1999). DNA from infected mussels was extracted according to standard protocols using Proteinase K, phenol chloroform and a precipitation of ethanol. PCR was performed in 50 µl with about 10 ng of purified DNA mixed with 5 µl of PCR buffer 10X, 5 µl of $MgCl_2$ 25 mM, 5 µl of dNTP 2 mM, 1 µl of DIG-dUTP 25 mM, 0.5 µl of each primer 100 µM and 0.25 µl (1 unit) of Taq DNA polymerase (Promega). Samples were overlaid with mineral oil, denatured for 5 min at 94°C and amplified by 30 cycles: 1 min at 94°C for denaturation, 1 min at 55°C for primers annealing, and 1 min at 72°C for elongation in a thermal cycle apparatus (MJ Research). Polymerisation at 72°C was then extended for 10 min to ensure complete elongation of the amplified products. The labelling of the probe was confirmed after electrophoresis of the PCR products in 1% agarose gel because labelled fragments migrate slower than unlabelled (same PCR reaction without DIG-dUTP).

***In situ* hybridization.** For *in situ* hybridization, sections were cut at 5 µm and placed on aminoalkylsilane coated slides (Silane-Prep Slides, Sigma) and then baked overnight in an oven at 60°C. The sections were

dewaxed by immersing in xylene for 10 min. This was repeated once and then the solvent was eliminated by immersion in 2 successive absolute ethanol baths followed by 10 min air-drying.

The sections were treated with Proteinase K (100 µg ml^{-1}) in TE buffer (Tris 50 mM, EDTA 10 mM), at 37°C for 30 min. Slides were dehydrated by immersion in absolute ethanol and air-dried. Sections were pre-hybridized in a humid chamber for 1 h at 42°C in 500 µl of 4× SSC, 50% formamide, 1× Denhardt's solution, 250 µg ml^{-1} yeast tRNA, 10% dextran sulphate. The solution was then replaced with 50 µl hybridization mix and the buffer described above containing 10 ng (1 µl of the PCR reaction) of the digoxigenin-labelled probe. Sections were covered with *in situ* plastic coverslips and placed on heating block at 94°C for 5 min. Slides were then cooled on ice for 1 min before overnight hybridization at 42°C. Sections were washed twice for 5 min in 2× SSC at room temperature, and once for 10 min in 0.4× SSC at 42°C. The detection steps were performed according manufacturer's instructions (Dig Nucleic Acid Detection Kit, Boehringer Mannheim).

RESULTS

No mortality of *Mytilus galloprovincialis* mussels or *Ostrea edulis* oysters has been noted at either farm.

The presence of different developmental stages of the parasite was observed in stained imprints of the mussel's digestive gland at a prevalence of 5% at both sites included in the study during 1998 and 1999 (Fig. 2).

Haematoxylin-eosin stained histological sections of the parasite within visceral mass of the mussels revealed different stages characterising the life cycle of the genus *Marteilia*. A variable number of presporulation vegetative stages with ovoid primary cells including 1 or more secondary cells were observed between epithelial cells of the digestive tubules (Figs. 3 & 4).

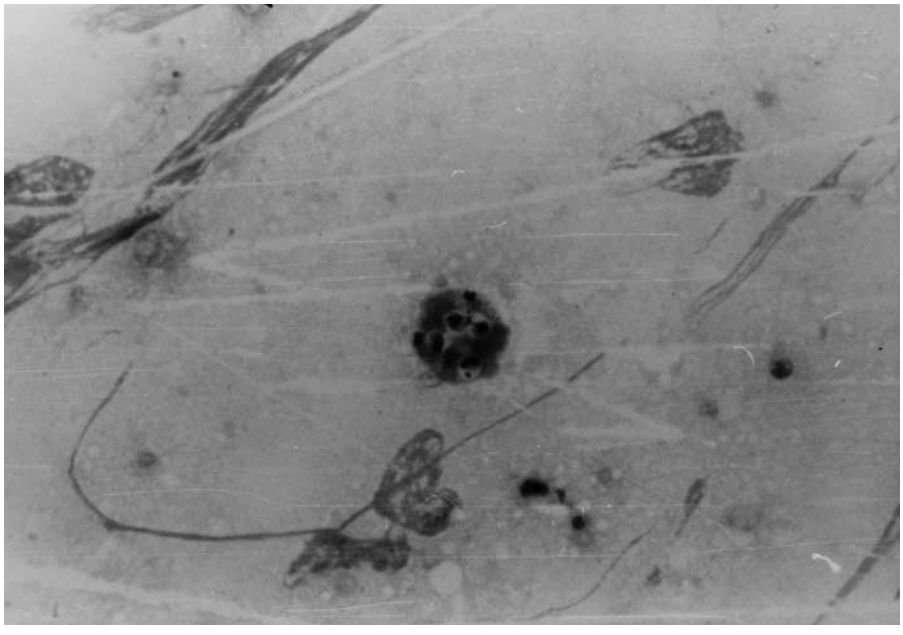

Fig. 2. *Marteilia* spp. in the digestive gland imprints of *Mytilus galloprovincialis*. Giemsa-modified staining (Hemacolor Kit, Merck), ×1000

In situ hybridization experiments performed with *Marteilia* sp.-infected tissue of *Mytilus galloprovincialis* gave a consistently strong reaction with all *Marteilia* sp. stages but not with the host cell nuclei (Fig. 5).

Both histological approaches (haematoxylin-eosin and *in situ* hybridization) showed the same prevalence of 5% for *Marteilia* sp. infection similar to the digestive gland imprints. Furthermore, it has to be noted that the infection was focused in only 1 region of the digestive gland. *Marteilia* sp was not detected in *Ostrea edulis* oysters from this region.

DISCUSSION

Since the late sixties, *Marteilia* spp. have presented a major problem for cultured oysters in Europe (*Ostrea edulis*) and Australia (*Saccostrea commercialis*) (Alderman 1979, Wolf 1979).

In Europe, *Marteilia refringens* has been reported from different species of oysters and mussels. The existence of another species within the genus *Marteilia maurini*, based on ultrastructural analysis and host specificity, has been proposed (Comps et al. 1982). The validity of this species is still controversial because a parasite can have a different morphology depending upon the host. Furthermore, when tested on a large number of samples, ultrastructural criteria failed to discriminate the 2 species, *M. refringens* and *M. maurini* (Longshaw pers. comm.).

The pathogenicity of *Marteilia refringens* for flat oysters is well documented (Alderman 1979, Balouet

1979, Grizel 1985, Berthe et al. 1998) whereas for other bivalves and other species of the genus *Marteilia* the relationships between the host and the parasite are less clear.

The control of marteliosis is based mainly on the development of programs to prevent the transfer of infected stocks into areas free of the disease. With this in mind, diagnostic tools are of utmost importance. In this respect a clarification of the taxonomy is needed. For this, 3 fundamental questions are still unresolved: (1) Are there different species in the genus *Marteilia* in Europe (i.e. assessment of *M. maurini*)? (2) Is there any tropism and/or specificity of a parasite for a host species or geographical repartition? (3) Are there different pathogenesis of the parasite taxa?

In this paper we present the first record of marteiliosis in the Croatian shellfish cultivation facilities in a region neighbouring the site of original description of *Marteilia maurini* (Comps et al. 1982). Histological findings and *in situ* hybridization show that this parasite belongs to the genus *Marteilia*. Prevalence of the parasite in mussels was low, 5%, and was constant for 1998 and 1999. We did not detect the parasite in the oyster *Ostrea edulis*. The very same situation was observed in Spain where, although a high prevalence (75%) of *M. refringens* in mussels was reported, only 1 oyster was detected with a single *M. refringens* primary cell in the digestive epithelium. This might be ascribed to an accidental infection (Figueras & Robledo 1993). This host distribution could reside in the fact that: (1) There are 2 types of *Marteilia* with a tropism for a specific host; (2) An intermediate host is believed to be involved in the *M. refringens* life cycle (Balouet 1979, Van Banning 1979) and, therefore, the transmission of *Marteilia* from mussels to oysters requires a distinct intermediate host not present in this area; (3) Oysters from the 2 samples examined could be resistant to *M. refringens* infection. However, the spat of the same species, which was obtained in a hatchery from oysters from Croatia, was transplanted and further cultivated in France (Thau pond). As a result, cultivated oysters became infected with *M. refringens* and, indeed, had a high

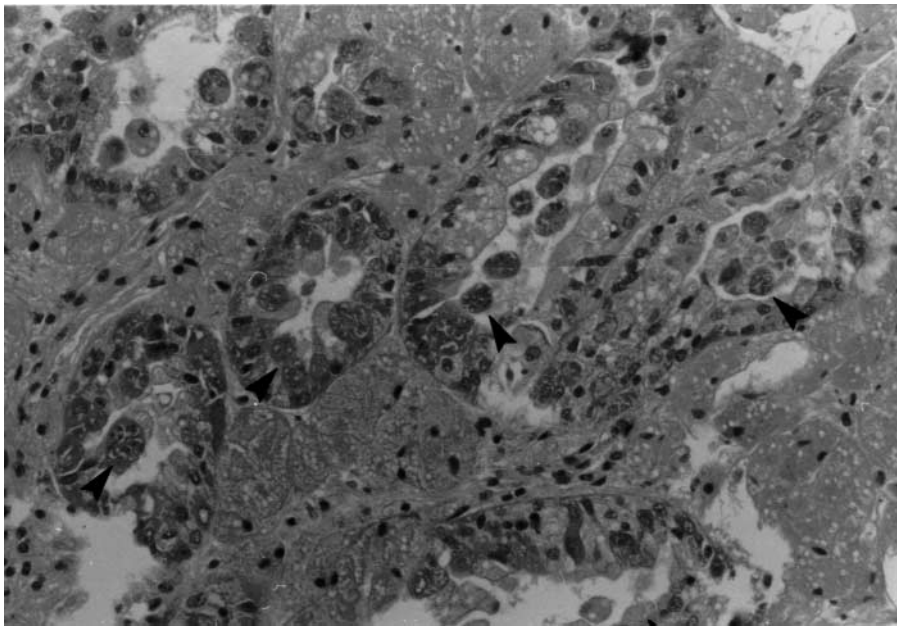

Fig. 3. Presporulation stages (arrowheads) of the genus *Marteilia* between epithelial cells of the digestive tubules of *Mytilus galloprovincialis*. H&E staining, ×400

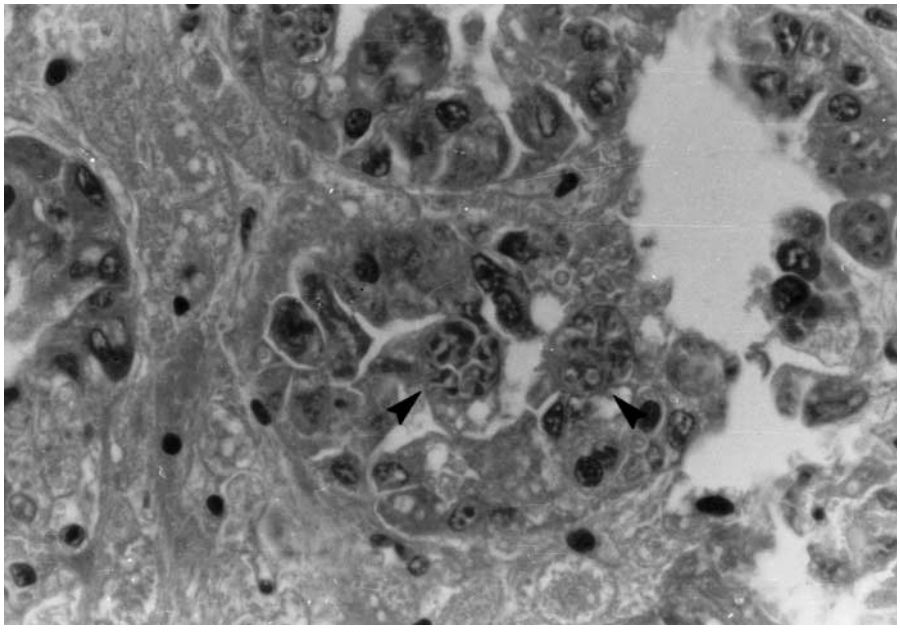

Fig. 4. Vegetative stages of *Marteilia* spp. showing ovoid primary cells (arrowheads) including 1 or more secondary cells. H&E staining, ×1000

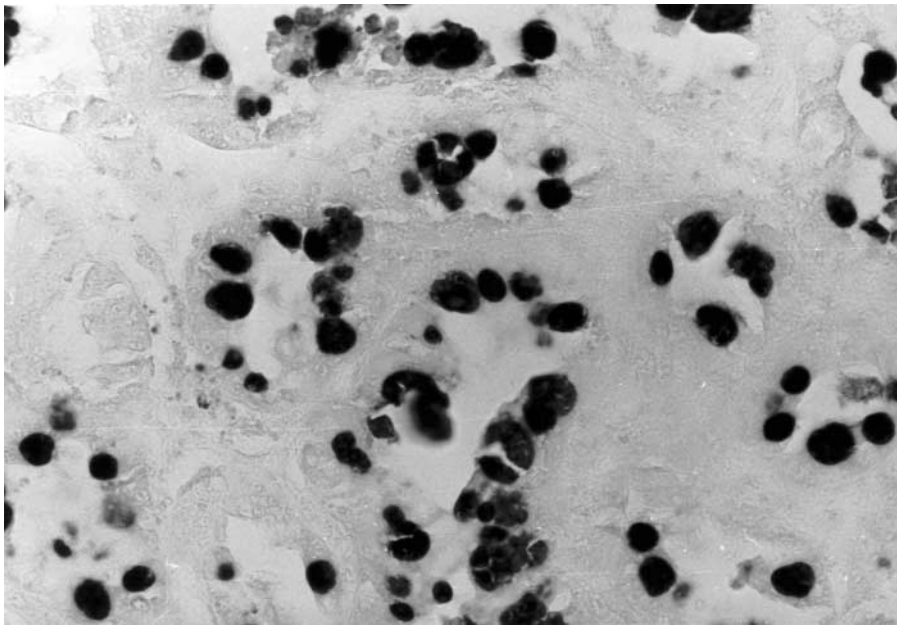

Fig. 5. Detection of the *Marteilia* spp. by *in situ* hybridization performed with infected tissues of *Mytilus galloprovincialis*. ×400

prevalence of this disease. Based on these facts, we presume that flat oysters originating from Croatia are more sensitive to this parasite than flat oysters from France (Y. Pichot pers. comm.).

Determination of the species within the genus *Marteilia* detected in Croatia will require additional research. Because ultrastructural data has recognised limitations (i.e. morphology depending on the host), DNA sequencing of genes with taxonomic value will help clarify the controversial taxonomy of *M. refringens* and its relatives. Sequence comparison of ribosomal genes has given new insights into molecular and cellular evolution. These genes contain sufficient evolutionary information to allow measurement of both close and distant phylogenic relationships (Sogin & Gunderson 1987). In a previous work the small ribosomal gene subunits (SSU rDNA) of *Marteilia*, purified from infected *Mytilus edulis* mussels, and *Ostrea edulis* oysters, were sequenced and shown to be identical (Berthe et al. in press). Therefore, on the basis of the SSU rDNA sequence it is impossible to distinguish *Marteilia* spp. parasiting oysters and mussels. However, doubt persists regarding the existence of a second species of *Marteilia*. Namely, *M. maurini* was first described in *Mytilus galloprovincialis* originating from the Venice lagoon (Comps et al. 1982). Therefore, *M. maurini* could be truly indigenous to this geographic area. To answer this question, the sequence of the SSU rDNA gene of *Marteilia* sp. infecting *M. galloprovincialis* from the Adriatic Sea is needed. Therefore, the parasite detected in Croatia will provide a precious source of parasite that can be used in a molecular approach to determine if this parasite is truly a different species.

Acknowledgements. This study was partially funded by the EU DG XIV (contract FAIR CT: PL97-3640) and the EU DG VI through the Community Reference Laboratory for Mollusc Diseases, Ifremer, La Tremblade. The authors thank Yves Pichot (Laboratoire Conchylicole de Méditerrannée) for his unpublished information.

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Editorial responsibility: Albert Sparks, Seattle, Washington, USA

Submitted: July 5, 2000; Accepted: October 18, 2000
Proofs received from author(s): January 22, 2001