Vol. 44: 137–142, 2001 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published March 9

Ultrastructural characterisation of *Marteilia* species (Paramyxea) from *Ostrea edulis*, *Mytilus edulis* and *Mytilus galloprovincialis* in Europe

Matt Longshaw[1,*], Stephen W. Feist[1], R. Anthony Matthews[2], Antonio Figueras[3]

[1]Centre for Environment, Fisheries and Aquaculture Science (CEFAS) Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, United Kingdom

[2]Department of Biological Sciences, University of Plymouth, Drakes Circus, Plymouth, Devon PL4 8AA, United Kingdom

[3]Instituto de Investigaciones Marinas-CSIC, Departmento de Biologìa y Patologìa de Organismos Marinos, Eduardo Cabello 6, 36208 Vigo, Spain

ABSTRACT: A focused ultrastructural study of *Marteilia* spp. found in cultured *Ostrea edulis*, *Mytilus edulis* and *Mytilus galloprovincialis* from France and Spain was conducted with emphasis placed on haplosporosomes, striated plate-like inclusions and spore wall morphology. Two types of haplosporosome were identified, sphaeroid and oblate, which were common to the parasite in all 3 host species. A total of 492 haplosporosomes were measured; those from the *Marteilia* sp. in *Mytilus* spp. were marginally smaller than those in *Ostrea edulis*. Spore wall morphology was found to vary depending on the state of maturity of the parasite—the more mature the parasite, the thicker the wall surrounding it. It is suggested that the current criteria used to distinguish *M. maurini* from *M. refringens* are invalid and that *M. maurini* was relegated to a junior synonym of *M. refringens*.

KEY WORDS: *Marteilia refringens* · *Marteilia maurini* · Ultrastructure

INTRODUCTION

The paramyxeans are a group of protistan parasites that includes several species known to cause significant disease in cultured marine bivalve molluscs. Currently the phylum Paramyxea consists of 2 classes, Marteiliidea with 3 genera, *Marteilia* (Grizel et al., 1974) Perkins, 1976, *Paramarteilia* Ginsburger-Vogel & Desportes, 1979 and *Marteilioides* (Comps et al., 1986) Anderson & Lester, 1992; and Paramyxidea with a single genus, *Paramyxa* Chatton, 1911. These organisms, particularly those in the genera *Marteilia* and *Marteilioides*, have been extensively studied due to their detrimental effect on commercially exploited bivalves. One such species, *Marteilia refringens* (Grizel et al., 1974) Perkins, 1976, has caused recurring mortalities in flat oysters *Ostrea edulis* L. from France, Spain and Portugal since its discovery in 1968. The antipodean equivalent, *Marteilia sydneyi* Perkins & Wolf, 1976, the causative agent of QX disease, is responsible for mortalities of *Saccostrea commercialis* in Queensland, Australia. In addition, Comps et al. (1982) described *M. maurini* from *Mytilus galloprovincialis* Lmk. collected in Venice Lagoon and imported into France. It was subsequently reported in *M. edulis* collected from 3 bivalve culture areas in Brittany, northern France (Auffret & Poder 1985). The genus *Marteilioides* includes 2 recognised pathogens, *M. chungmuensis* Comps et al., 1986 from Japanese and Korean *Crassostrea gigas*, and *M. branchialis* Anderson & Lester, 1992 in *S. commercialis* from Australia. These pathogens cause focal lesions, reduced growth and frequently death.

There is concern about the translocation of these pathogens to naive, susceptible stocks; thus, strict guidelines have been adopted to ensure that only

*E-mail: m.longshaw@cefas.co.uk

healthy animals are transferred to areas where the diseases are known to be absent. This is the case with *Marteilia refringens* in Europe. The European Community Council Directive 91/67 (OJ No L46 19.2.1991) includes *M. refringens* in annex A, list II, as a serious pathogen of the susceptible species *Ostrea edulis*. The diagnosis of the 2 species of *Marteilia* found in Europe (*M. refringens* and *M. maurini*) currently requires analysis of the ultrastructural characteristics and consideration of the host specificity (Grizel 1974, Comps et al. 1982). Indeed, the only ultrastructural features selected by Comps et al. (1982) to distinguish the 2 species were subtle differences in haplosporosome shape and the 'existence of a multimembranous envelope next to the spore wall'.

The recent discovery of *Marteilia refringens* in *Mytilus galloprovincialis* (Villalba et al. 1993, Robledo & Figueras 1995) has raised some doubt that *Marteilia maurini*, which was described as the only *Marteilia* species parasitizing mussels, is a distinct species from *M. refringens*. Experimental transmission of *M. refringens* between members of the same or differing host species has been unsuccessful, although it has been attempted in a number of experiments under differing conditions (Figueras & Robledo 1993, Berthe et al. 1998).

Because flat oysters cultured in Europe suffer mortalities as a result of infections with *Marteilia refringens* and the questionable taxonomic status of the *Marteilia* sp. reported from mussels *Mytilus edulis* (described as *M. maurini* Comps et al., 1982) which has not been associated with mortalities, it is important to establish whether one or several European *Marteilia* species exist.

The objective of the current study was to re-evaluate the taxonomic features used to discriminate the *Marteilia* species in *Ostrea edulis*, *Mytilus galloprovincialis* and *Mytilus edulis*.

MATERIALS AND METHODS

Ostrea edulis and *Mytilus edulis* were collected from France in February and April 1995. These were screened for infections with *Marteilia* spp. by examining air-dried, methanol fixed, digestive gland imprints stained with methylene blue. Intensity of infection was classified as light (+), medium (++) and heavy (+++). Digestive gland material from parasitised specimens was fixed in 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer for 24 h at 4°C, washed twice in buffer, post fixed in 1% osmium tetroxide in 0.1 M buffer for 1 h and again rinsed in buffer. Samples were dehydrated through graded alcohols and propylene oxide and embedded in resin.

Similarly, samples of infected *Mytilus galloprovincialis* were collected in Ria de Vigo (North West Spain) during 1994. This material included infected digestive glands and *Marteilia maurini* from *M. galloprovincialis* purified as described by Robledo et al. (1995) embedded as above.

Semi-thin sections were cut at 1 µm thickness and stained with toluidine blue. Examination of these at light microscope level enabled the selection of heavily infected digestive glands containing a wide range of development stages. Ultrathin sections (80 to 85 nm) were cut on a Leica Ultracut S, floated onto copper EM grids and stained with uranyl acetate/Fahmys lead citrate (Lewis & Knight 1977). Stained sections were examined using a JEOL 1210 transmission electron microscope.

A limited ultrastructural investigation was undertaken of both *Marteilia refringens* from *Ostrea edulis* and *Marteilia maurini* from *Mytilus edulis* and *Mytilus galloprovincialis*. Particular attention was given to selected organelles, namely haplosporosomes and striated plate-like inclusions and to the spore wall, as these have previously been used for species discrimination by other workers. Measurements of selected organelles were done directly on the TEM using inbuilt calibrated image measuring software. A total of 492 haplosporosomes were examined including 243 from *M. refringens* and 249 from *M. maurini*. The terminology of Perkins (1979) was adopted in describing the haplosporosome and all other structures examined.

RESULTS

Fourteen infected *Mytilus edulis* from France were used in the ultrastructural analysis. Of these, 5 were classed as lightly infected, 5 were medium infected and 4 were heavily infected. Additionally, 6 *Ostrea edulis* were used in ultrastructural studies; of these, 2 had a medium infection level and 4 were heavily parasitised.

Ultrastructural studies confirmed the presence of stages previously described by Perkins (1976). The earliest identifiable stage was a primary cell with a single secondary cell within it (Fig. 1A). The parasite then proceeded through a series of divisions to produce 8 presporangia or secondary cells (Fig. 1B). After division these presporangia contained 4 spore primordia (tertiary cells), each of which cleaved internally to produce mature spores. The mature spore consisted of 3 sporoplasms, one inside the other (outermost, intermediate and innermost) (Fig. 1C). The outermost sporoplasm contained haplosporosomes. In mature *Marteilia refringens* the haplosporosomes were sphaeroid or oblate (Fig. 1D), as was the case with *M. maurini*,

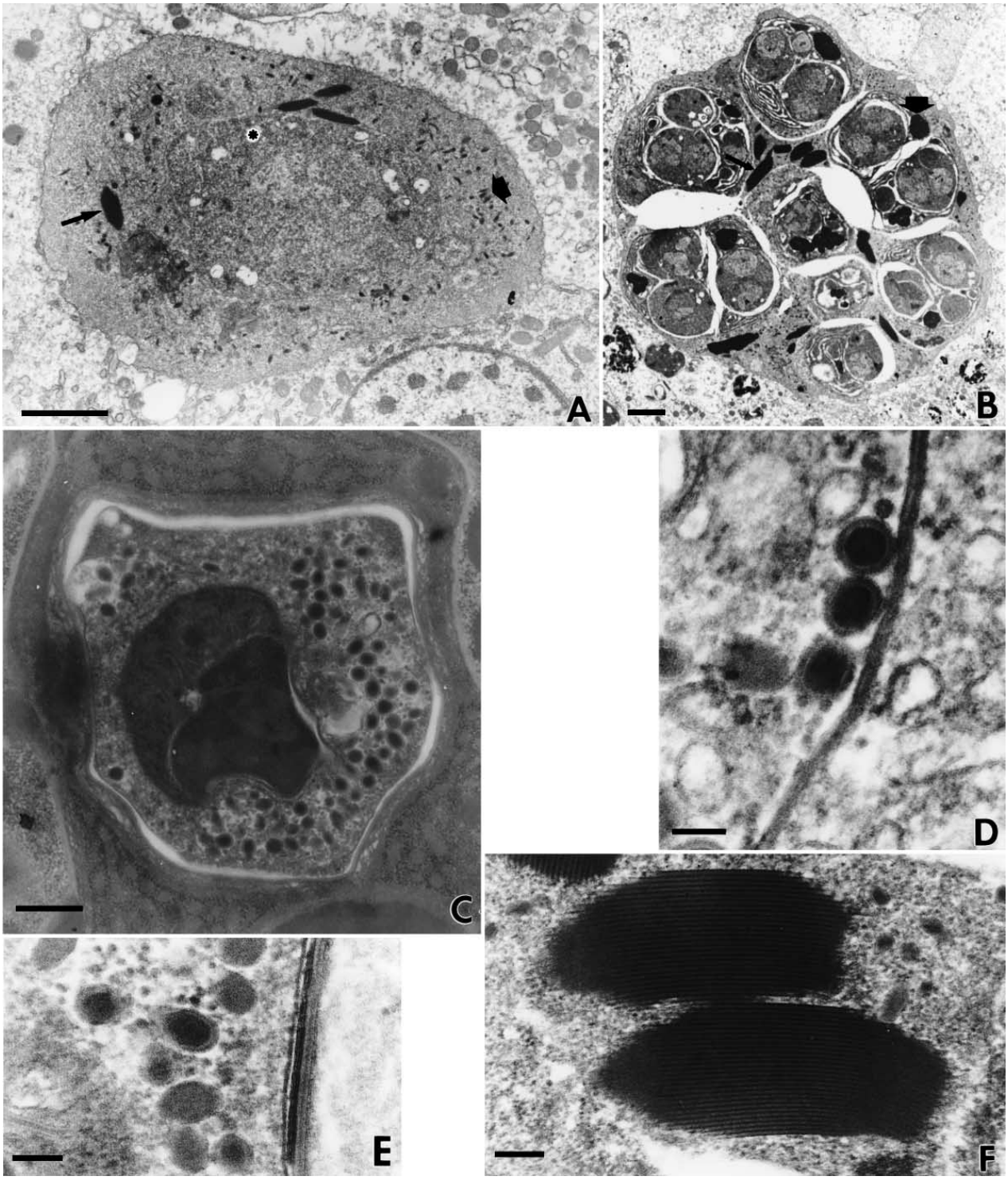

Fig. 1. (A) Primary cell of *Marteilia* sp. containing a single secondary cell within it. Note numerous mitochondria (*) within secondary cell, and striated plate-like inclusions (➞) and haplosporosomes (◀) in the cytoplasm of the primary cell. (B) Sporangiosorus with 8 presporangia and immature spores. Numerous striated plate-like inclusions (➞) are visible in the cytoplasm of the sporangiosorus and refringent granules (◀) are visible within presporangia. (C) Mature spore of *M. refringens* from oysters showing 3 sporoplasms; thickened wall characteristic of maturity and sphaeroid haplosporosomes. (D) Sphaeroid haplosporosomes of *M. refringens* from oysters clearly showing medulla and cortex. (E) Oblate sphaeroid haplosporosomes of *Marteilia* sp. from mussels. Note cortex and medulla and thickening of cell wall. (F) Striated plate-like inclusions in cytoplasm of developing sporangiosorus. Scale bars: (A) = 1 µm; (B) = 2 µm; (C) = 500 nm; (D) = 100 nm; (E) & (F) = 200 nm

Table 1. Measurements of haplosporosomes from *Marteilia* spp. parasitic in *Ostrea edulis*, *Mytilus edulis* and *M. galloprovincialis* taken during the current study and from published data. All measurements are in nm

Species	Host	Oblate haplosporosomes Length × Width (mean)	Sphaeroid haplosporosomes Length × Width (mean)	Source
M. refringens	*O. edulis*	106–191 (137) × 71–117 (95)	87–137 (111)	Present study
M. refringens	*O. edulis*	230 × 140	–	Grizel et al. (1974)
M. refringens	*O. edulis*	175–203 (189) × 71–158 (111)	98–196 (113)	Perkins (1976, 1979)
Marteilia sp.	*M. edulis*	107–296 (230) × 41–86 (65)	72–141 (90)	Present study
Marteilia sp.	*M. edulis*	260 × 120	130–160	Auffret & Poder (1985)
Marteilia sp.	*M. galloprovincialis*	92–312 × 40–150	–	Villalba et al. (1993)
Marteilia sp.	*M. galloprovincialis*	130–400 × 130–200	130–200	Robledo & Figueras (1995)
Marteilia sp.	*M. galloprovincialis*	230–320 × 70–90	80–120	Comps et al. (1982)

although the latter were marginally smaller (Fig. 1E) (Table 1). There was some variation in the structure of the spore wall associated with the stage of development. With the breakdown of haplosporosome and sporangiosorus cytoplasm there was an increasing build up of myelin whorls around the spore.

Striated plate-like inclusions (Fig. 1F) were only found in those stages of development which contained haplosporosomes, including earlier stages of the sporangiosorus (primary cell) and in stages containing spores. They consisted of alternating electron-dense and electron-lucent layers with a regular periodicity of 24.64 nm ± 2.45 (22.17 to 29.75 nm) for *Marteilia maurini* and 23.31 nm ± 3.02 (21.96 to 28.04 nm) for *M. refringens*.

DISCUSSION

The present study challenges current established criteria (Perkins 1976, Perkins & Wolf 1976, Comps et al. 1982, Auffret & Poder 1985) for the identification of species of *Marteilia* sp. Our measurements of haplosporosomes from *Marteilia* spp. in the different host species have shown considerable variation and overlap in size ranges as can be seen when comparing the results to previous studies. Consequently, haplosporosome morphology should be considered to be highly pleomorphic and therefore not a reliable means of defining species. It is believed that the use of haplosporosome measurements as a diagnostic tool to discriminate between species is problematic. Variations which may result from use of different processing, fixation and viewing methods may be expected to be as great or greater the size difference recorded by various authors. Similarly, the use of striated plate-like inclusions can equally be considered to be of no value in separating *Marteilia* sp. obtained from the 3 host species, as no differences were detected in their shape, pattern of striations or periodicity.

Finally, the use of spore wall ultrastructure was also considered as a possible taxonomic criterion. Perkins & Wolf (1976) stated that the spore wall of *Marteilia sydneyi* was surrounded by a heavy layer of concentric membranes, which was 'occasionally' present in *M. refringens*. *M. maurini* was also separated from *M. refringens* on the basis that these concentric membranes were absent in *M. maurini* (Comps et al. 1982). In the course of this study, it was found that in mature *M. refringens* specimens, i.e. those with noticeable breakdown of the sporangiosoral cytoplasm or mature haplosporosomes (those clearly showing medulla and cortex), there was a build-up of concentric membranes around the spore wall. This was also true for *M. maurini*. Spore wall thickness can therefore only be of taxonomic value when measured on a mature spore. The available information is of limited use because of the lack of details, in particular about maturity, provided in several previous studies. It is suggested that for speciation to be determined at the ultrastructural level, all life stages should be examined, particularly the later spore stages in the host.

Higher level structural criteria, discernable by light microscopy, were used by Perkins & Wolf (1976) to separate *Marteilia sydneyi* from *M. refringens*, namely that sporangiosori contained 8 to 16 sporangia, compared with 8 for *M. refringens*, and sporangia contain 2 to 3 rather than 4 spores (Perkins & Wolf 1976) (see Table 2). Using these criteria, it would be impossible to separate *M. maurini* from *M. refringens* as both 'species' of the parasite have 8 sporangia which contain 4 spores.

Based on the material examined in this study and the observation of pleomorphic haplosporosomes and variability of spore wall morphology, it is considered that *Marteilia maurini* from *Mytilus* spp. cannot be reliably separated from *Marteilia refringens* from *Ostrea edulis* using ultrastructural criteria. Spore wall morphology, and therefore wall thickness, appears to be dependent on the degree of maturation of the spores and sporan-

Table 2. Number of secondary and tertiary cells and sporoplasms in tertiary cells of paramyxean genera and species. ND = no data

Species	No. of secondary cells	No. of tertiary cells	No. of sporoplasm cells	Selected reference
Marteilia refringens	8	3–4	3	Perkins (1976)
Marteilia sydneyi	8–16	2(3)	3	Perkins & Wolf (1976)
Marteilia lengehi	8	?	ND	Comps (1976)
Marteilia maurini	8	3–4	3	Comps et al. (1982)
Marteilia christenseni	8	4	3	Comps (1985)
Marteilioides chungmuensis	2–3	1	3	Comps et al. (1986)
Marteilioides branchialis	2–6 (12)	1	2	Anderson & Lester (1992)
Paramarteilia orchestiae	1–12	2	2	Ginsberger-Vogel & Desportes (1979a,b)
Paramyxa paradoxa	4	4	4	Chatton (1911)

giosorus and can therefore not be used as a criterion for the discrimination of species. Haplosporosome size and shape of both species of *Marteilia* are highly variable. Taking into account the data obtained from previous studies, it is clear that haplosporosome morphology is too inconsistent and with too much overlap in size range between *Marteilia* spp. in different hosts to provide a robust diagnostic feature. *M. maurini* was described from *Mytilus galloprovincialis* collected in Venice Lagoon and imported into France (Comps et al. 1982).

On the basis that there are no reliable features at the light microscope level or at the ultrastructure level, and taking into account an apparent lack of host specificity, we propose that *Marteilia maurini* be relegated to a junior synonym of *M. refringens*.

Developments in DNA based methods can offer, in some cases, a more objective method of discriminating between putative protistan species compared with the ultrastructural morphological approach (Philippe et al. 1995, Adoutte et al. 1996). In the present case, the attempt to define parasite species based primarily on occurrence in different host species has resulted in attempts to correlate fine morphological differences to support this distinction. As reported here, detailed analysis of these differences do not support this. Two haplosporidians (*Haplosporidium nelsoni* and *H. costale*) that inhabit the same area and host and have a similar morphology in the plasmodial stage, but different spore stages, were found to be clearly different when their 18S rDNA sequence were compared (Fong et al. 1993, Ko et al. 1995, Stokes et al. 1995). *Perkinsus olseni* from Australia and *P. atlanticus* from Portugal are morphologically similar and were found to belong to a single species, but *P. marinus* (found in the USA) was well discriminated from these 2 species (Goggin 1994). It is possible, however, that different analyses of the DNA of *P. olseni* and *P. atlanticus* could show a difference. Although *Marteilia refringens* and *M. maurini* are here regarded as conspecific based on morphological features, it will be desirable to confirm this using DNA sequence data. In addition, the use of DNA sequences, DNA probes and techniques such as *in situ* hybridisation in tracking infections in putative alternate hosts and examining parasite maturation will prove invaluable in future studies of this parasite.

Acknowledgements. This work was supported by European Union FAIR contract 94C 85/13.

LITERATURE CITED

Adoutte A, Germot A, Le Guyader H, Philippe H (1996) Que savons-nous de l'historie évolutive des eucaryotyes? 2. De la diversification des protistes à la radiation des multicellulaires. Méd Sci 12(2):I–XVIII

Anderson TJ, Lester RJG (1992) Sporulation of *Marteilioides branchialis* n. sp. (Paramyxea) in the Sydney rock oyster, *Saccostrea commercialis*: an electron microscope study. J Protozool 39(4):502–508

Auffret M, Poder M (1985) Recherches sur *Marteilia maurini* parasite de *Mytilus edulis* sur les cotes de Bretagne Nord. Rev Trav Inst Peches Marit 47(1-2):105–109

Berthe FJ, Pernas M, Zerabib M, Haffner P, Thebault A, Figueras AJ (1998) Experimental transmission of *Marteilia refringens* with special consideration of its life cycle. Dis Aquat Org 43(2):135–144

Chatton E (1911) Sur une cnidospiridie sans cnidoblaste (*Paramyxa paradoxa*, n.g., n.sp.). C R Acad Sci Paris 152: 631–633

Comps (1976) *Marteilia lengehi* n.sp., parasite de l'huître *Crassostrea cucullata* Born. Rev Trav Inst Pêches Marit 40(2):347–349

Comps (1985) Etude morphologique de *Marteilia christenseni* sp.n., parasite du lavignon *Scrobicularia piperata* P. (Mollusque pélécypode). Rev Trav Inst Pêches Marit 47(1-2): 99–104

Comps M, Pichot Y, Papagianni P (1982) Recherche sur *Marteilia maurini* n. sp. parasite de la moule *Mytilus galloprovincialis* Lmk. Rev Trav Inst Peches Marit 45(3):211–214

Comps M, Park MS, Desportes I (1986) Etude ultrastructurale de *Marteilioides chungmuensis* n.g., n. sp. parasite des ovocytes de l'huitre *Crassostrea gigas* Th. Protistologica 22(3):279–285

Figueras A, Robledo JAF (1993) Does the *Marteilia* present in mussels (*Mytilus galloprovincialis*) infect flat oysters (*Ostrea edulis*)? Bull Eur Assoc Fish Pathol 13(3):97–99

Fong D, Chan MM, Rodriguez R, Chen C, Liang Y, Littlewood T, Ford S (1993) Small subunit ribosomal RNA gene sequence of the parasitic protozoan *Haplosporidium nelsoni* provides a molecular probe for the oyster MSX disease. Mol Biochem Parasitol 62:139–142

Ginsburger-Vogel T, Desportes I (1979a) Structure and biology of *Marteilia* sp. in the amphipod, *Orchestia gammarellus*. Mar Fish Rev 41(1-2):3–7

Ginsburger-Vogel T, Desportes I (1979b) Etude ultrastructurale de la sporulation de *Paramarteilia orchestiae* gen. n. sp. n. parasite de l'amphipode *Orchestia gammarellus* Pallas. Protistologica 26(3):390–403

Goggin CL (1994) Variation in the two internal transcribed spacers and 5.8S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). Mol Biochem Parasitol 65:179–182

Grizel H, Comps M, Bonami JR, Cousserans F, Duthoit JL, Le Pennec MA (1974) Recherche sur l'agent de la maladie de la glande digestive de *Ostrea edulis* Linne. Sci Peche, Bull Inst Peches Marit 240:7–30

Ko YT, Ford SE, Fong D (1995) Characterisation of the small subunit ribosomal RNA gene of the oyster parasite *Haplosporidium costale*. Mol Mar Biol Biotech 4(3):236–240

Lewis PR, Knight DP (1977) Staining methods for sectioned material. North-Holland Publishing Company, Amsterdam

Perkins FO (1976) Ultrastructure of sporulation in the European flat oyster pathogen *Marteilia refringens*—taxonomic implications. J Protozool 23(1):64–74

Perkins FO (1979) Cell structure of shellfish pathogens and hyperparasites in the genera *Minchinia*, *Urosporidium*, *Haplosporidium* and *Marteilia*—taxonomic implications. Mar Fish Rev 41(1–2):25–37

Perkins FO, Wolf PH (1976) Fine structure of *Marteilia sydneyi* sp. n.—Haplosporidian pathogen of Australian oysters. J Parasitol 62(4):528–538

Philippe H, Adouette A (1995) How reliable is our current view of eukaryotic phylogeny? In: Brugerolle G, Mignot JP (eds) Protistological actualities. Proceedings of the Second European Congress of Protistology, Clermont-Ferrand 1995. Université de Clermont-Ferrand, p 17–33

Robledo JAF, Figueras A (1995) The effects of culture-site, depth, season and stock-source on the prevalence of *Marteilia refringens* in cultured mussels (*Mytilus galloprovincialis* Lmk.) from Galicia, Spain. J Parasit 81(3): 354–363

Robledo JAF, Miahle E, Figueras A (1995) Purification of several phases of the paraite *Marteilia* (Protozoa: Ascetospora) from mussels (*Mytilus galloprovincialis*). In: Stolen JS, Fletcher TC, Smith SA, Zelikoff JT, Kaattari SL, Anderson RS, Söderhäll K, Weeks-Perkins BA (eds) Techniques in fish immunology, 4. Immunology and pathology of aquatic invertebrates. SOS Publications, Fair Haven, NJ, p 117–121

Stokes N, Burreson E (1995) A sensitive and specific DNA probe for the oyster pathogen *Haplosporidium nelsoni*. J Eukaryot Microbiol 42(4):350–357

Villalba A, Mourelle SG, Lopez MC, Carballal MJ, Azevedo C (1993) Marteiliasis affecting cultured mussels *Mytilus galloprovincialis* of Galicia (NW Spain). I. Etiology, phases of the infection, and temporal and spatial variability in prevalence. Dis Aquat Org 16:61–72

Editorial responsibility: Albert Sparks, Seattle, Washington, USA

Submitted: February 21, 2000; Accepted: November 6, 2000
Proofs received from author(s): February 12, 2001