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Molecular epizootiology of the European *Marteilia* spp., infecting mussels (*Mytilus galloprovincialis* and *M. edulis*) and oysters (*Ostrea edulis*): an update

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Abstract

The Internal Transcribed Spacer 1 (ITS-1) region from *Marteilia* spp. parasitizing Mediterranean mussels (*Mytilus galloprovincialis*) from Italy and flat oysters (*Ostrea edulis*) from Greece were characterized by RFLP with endonuclease *HhaI* and sequenced. Profiles "O" (*Marteilia refringens*), previously associated with heavy mortalities, and "M" (*Marteilia maurini*) were found in both mussels and oysters and coinfection with the two types was common. In addition, all the available molecular information on *Marteilia* spp. ITS-1 region in Europe was compared and summarized. All these molecular data, in addition to molecular data from other regions and ultrastructural evidence, support the synonymy of *M. maurini*, the parasite originally described from mussels, with *M. refringens*, which was originally described from oysters and has taxonomic priority. This emphasises the need for further molecular and biological studies to reach scientifically based conclusions regarding control of Marteiliosis.

Introduction

Marteiliosis is a mollusc disease with devastating consequences in European *Ostrea edulis* aquaculture. It is characterized by the rapid wasting of the digestive gland and the consequent death of the animal. Marteiliosis was first described in oysters from Arcachon Basin (SW France), naming the causative agent as *Marteilia refringens* (Grizel et al., 1974). *Marteilia maurini* was first described from mussels (*Mytilus galloprovincialis*) imported into France from the Venice Lagoon, Italy (Comps et al., 1982). Differences from previous species were based on ultrastructural characters and host specificity (Comps et al., 1982; Auffret & Poder, 1985). However, *M. refringens* was also detected in *M. galloprovincialis*, but there were no data about ultrastructure (Tigé & Rabouin, 1976; Gutiérrez, 1977; Comps & Joly, 1980).

Ultrastructural characters employed to distinguish *Marteilia* species (length and

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shape of haplosporosomes and thickness of the spore wall) could be caused by intraspecific variation rather than inter-specific variation or simply Transmission Electron Microscopy (TEM) processing artefacts (Longshaw et al., 2001). Since the detection of *M. refringens* in mussels (Villalba et al., 1993; Robledo & Figueras, 1995), host specificity seems to be an invalid argument. Moreover, the use of TEM, needed for ultrastructural characterization, is not feasible for surveillance studies and is not easily accessible to laboratories conducting routine surveillance of mollusc diseases.

Parasitological molecular taxonomy is mainly based on the Small Subunit Ribosomal RNA (SSU rRNA) gene sequence, due to the availability of sequences in public databases and the existence of regions showing certain degree of variability intercalated with regions highly conserved among organisms (Medlin et al., 1988; Hillis & Dixon, 1991; Page & Holmes, 1998). The SSU rRNA gene sequence from *M. refringens* and *M. maurini* is identical (Berthe et al., 2000) and not useful for *Marteilia* spp. discrimination.

Internal Transcribed Spacers (ITS) are regions belonging to the RNA cluster which have a lower evolution constraint than SSU rRNA gene. For this reason they are more suitable for discriminating among closely related groups, including strains (Page & Holmes, 1998). ITS-1 RFLP was employed for distinguishing between *M. refringens* from *O. edulis* and *M. maurini* from *Mytilus* spp., resulting in two different restriction profiles which matched almost perfectly with the host of the parasite (Le Roux et al., 2001). However, subsequent analysis from *Marteilia* spp. samples from different origins showed that this matching was not as clear as Le Roux and co-workers suggested and both types could be found in both hosts (López-Flores et al., 2004; Novoa et al., 2005). Intergenic Spacer (IGS) is another region belonging to the RNA cluster with a low evolutionary constraint which could not distinguish perfectly among sequences from parasites infecting oyster and mussel (López-Flores et al., 2004).

In the present study, we update available molecular data with more DNA sequences corresponding to the ITS-1 fragment and RFLP information from *Marteilia* sp. present in flat oysters (*O. edulis*) and Mediterranean mussels (*M. galloprovincialis*) from Greece and Italy in order to improve the understanding of Marteiliosis in Europe.

Materials and methods

Samples

Mediterranean mussels, *M. galloprovincialis*, (n=18) were obtained from La Spezia (Italy) and flat oysters, *O. edulis*, (n=30) were collected from Thessaloniki (n=2) and Kavála (n=28) (Greece). All samples were collected in Spring 2003. Mussels were cultured in La Spezia under *pergolari* system, while wild oysters were collected in Greece. In addition, mussels in Kavála were cultivated under *pergolari* system in the vicinity of the oyster beds. Whole animals were fixed in ethanol 70% and the digestive gland was dissected out for subsequent DNA extraction.

	Location	Samples n/N	Profile		
			Type O	Type M	Coinfected
M. galloprovincialis	La Spezia	14/18	5	3	6
O. edulis	Kavála	9/28	4	1	4
	Thessaloniki	0/2	0	0	0

Table 1. Number of individuals showing each profile as found by RFLP of PCR products obtained directly from the host. *M. galloprovincialis* from La Spezia (Italy) and flat oysters (*O. edulis*) from Kavála and Thessaloniki (Greece). n: number of infected mollusks. N: number of analyzed mollusks.

DNA extraction and RFLP

DNA from animals was isolated with DNAzol (Invitrogen) following manufacturer's instructions and PCR for the detection of *Marteilia* spp. was carried out as previously reported (Le Roux et al., 2001; Novoa et al., 2005). PCR reactions were performed in 25 µl using standard conditions with a final concentration of 2.5 mM $MgCl_2$ in a Gene Amp PCRsystem 2700 thermocycler (Applied Biosystems, Foster City, CA, USA). Reactions not containing DNA were also carried out as negative controls. Following an initial denaturation at 94°C reactions were subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 55 °C for 1 min and elongation at 72°C for 1 min. The final extension lasted 10 min at 72°C. PCR products were subjected to restriction fragment length polymorphism with *HhaI* (Promega). Since coinfected molluscs were found (Table 1), PCR products from coinfected animals and from at least three different representative profiles "O" and "M" were cloned using the TOPO TA cloning kit (Invitrogen Life Technologies) following the manufacturer's instructions. TOP 10 F′ competent bacteria (Invitrogen) were transformed and clones were screened for the presence of the inserted band and subjected to RFLP as described above.

DNA sequencing

Different profiles from different species and geographical origins were selected for sequencing and corroboration of the RFLP. PCR products were purified by digestion with the enzymes Exonuclease I (*Exo I*) and Shrimp Alkalyne Phosphatase (*SAP*) (Amershan Pharmacia Biotech) for 1 hour at 37°C. The enzymes were then denatured for 15 minutes at 80°C. Sequencing was carried out in an ABI Prism 310 or ABI Prism 377 automated sequencers using Big Dye Terminator Cycle Sequencing Ready Reaction Kit v 3.1 (Applied Biosystems) according to manufacturer directions.

Results and discussion

Table 1 shows the presence of Marteiliosis in both hosts. Even though the results obtained herein indicated that *Marteilia* infection was more common for mussels than for oysters (Table 1), the low number of samples does not support a reliable comparison between hosts. Although *Marteilia* sp. was not recorded in oysters from Thessaloniki gulf in the present work, the low sample numbers and the presence

Host	Clones	Type M	Type O	Other	Coinfection	References
France (La Trinité)						
M. edulis	16	15	1	0	1	Le Roux et al., 2001
O. edulis	21	1	20	0	1	"
France (Marennes)						
O. edulis	15	0	15	0	0	"
France (Leucate)						
O. edulis	16	0	16	0	0	"
Spain (Rías Baixas)						
M. galloprovincialis	4	4	0	0	0	"
M. galloprovincialis	3	0	3	0	0	López-Flores et al., 2004
M. galloprovincialis	38	30	8	0	0	Novoa et al., 2005
Spain (Huelva)						
M. galloprovincialis	4	2	2	0	1	López-Flores et al., 2004
O. edulis	4	0	4	0	0	"
O. edulis	26	16	7	3(Type X)	1*	Novoa et al., 2005
Spain (Delta of Ebro)						
O. edulis	16	2	14	0	2	"
Italy (Trieste)						
M. galloprovincialis	4	0	4	0	0	López-Flores et al., 2004
Italy (Venice)						
M. galloprovincialis	2	0	2	0	0	López-Flores et al., 2004
Italy (La Spezia)						
M. galloprovincialis	60	28	32	0	6	Present paper
Croatia (Istria)						
M. galloprovincialis	7	7	0	0	0	Le Roux et al., 2001
Greece (Kavála)						
O. edulis	40	10	30	0	4	Present paper

Table 2. Comparative data of RFLP profiling of *Marteilia* spp. from infected mussels and oysters in European waters. * Coinfection with "M" profile and a third profile termed type "X".

of *Marteilia* sp. in wild *O. edulis* and *M. galloprovincialis* in the past (Virvilis et al., 2003), indicates that this zone should be maintained as *Marteilia* non-free area.

Ultrastructural characters employed to differentiate *Marteilia* spp. are length and shape of haplosporosomes, an organelle of unknown function shared by organisms of phylum Haplosporidia, Myxozoa and Paramyxea, and thickness of the wall of the sporont. Measures reported from haplosporosomes both in oyster and mussel showed high variability in length and shape: 230 x 140 (Grizel et al., 1974); 175-203 x 71-158 (Perkins, 1976); 92-312 x 40-150 (Villalba et al., 1993); 130-400 x 130-200 (Robledo et al., 1994); 106-191 x 71-117 (oyster), 107-296 x 41-86 (mussel) (Longshaw et al., 2001); 230-320 x 70-90 (Comps et al., 1982), 260 x 120 (Auffret & Poder, 1985), being often overlapped in

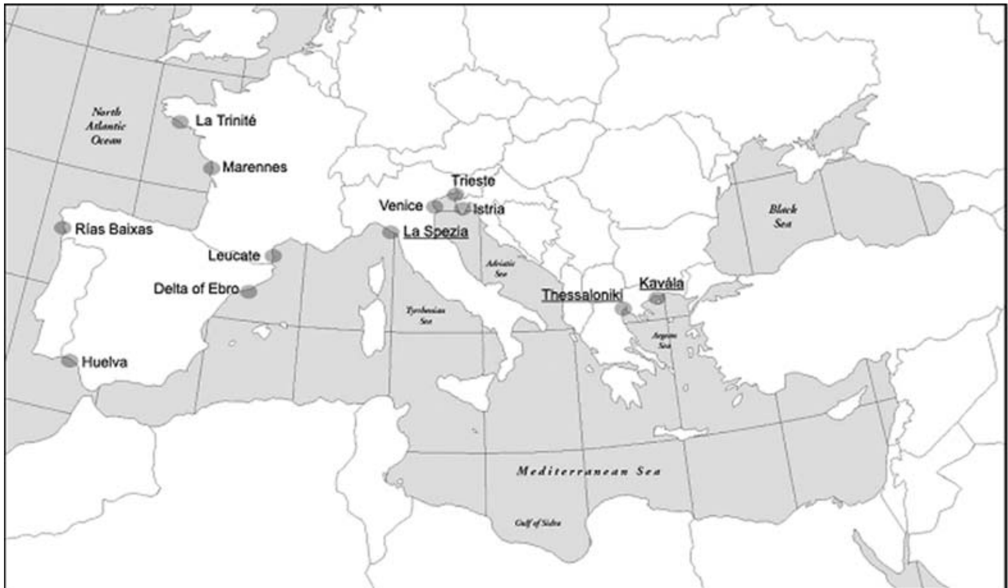

Figure 1. Map summarizing sample locations for molecular profiling of ITS-1 region of *Marteilia* spp. in the literature and in the present study (underlined locations).

Marteilia spp. from oyster and from mussel. Differences were only based on the presence of a multimembranaceous envelope surrounding the spore wall in *M. maurini*. However, this could be a product from intraspecific variation rather than interspecific variation, different morphologies of the parasite in different environmental conditions or simply TEM processing artefacts (Longshaw et al., 2001).

Small Subunit Ribosomal (SSU rRNA) gene was used to clarify the taxonomic position of genus *Marteilia* in the "tree of life", but failed in the discrimination between the putative different species *M. maurini* and *M. refringens* (Berthe et al., 2000; Cavalier-Smith & Chao, 2003a; 2003b) since this gene is highly constrained evolutionarily. However, although this gene is widely employed in molecular parasitology for distinguishing even among species of the same group (Kotob et al., 1999a, Stokes & Burreson, 2001; Reece et al., 2004), it is not useful for discriminating between *M. refringens* and *M. maurini*.

The Internal Transcribed Spacers (ITS) sequences are more suitable for characterization of closely related groups, even strains, because the evolutionary constraint is lower in these regions than in other ribosomal RNA subunit genes (Hillis & Dixon, 1991; Goggin, 1994; Page & Holmes, 1998; Kotob et al., 1999b; Cremonte et al., 2005). For this reason and because of the failure of SSU rRNA gene, ITS1 sequences are more appropriate for distinguishing between the taxa infecting mussels and oysters in Europe.

Results summarized in Tables 1 and 2 show that both "O" profile, which corresponds to

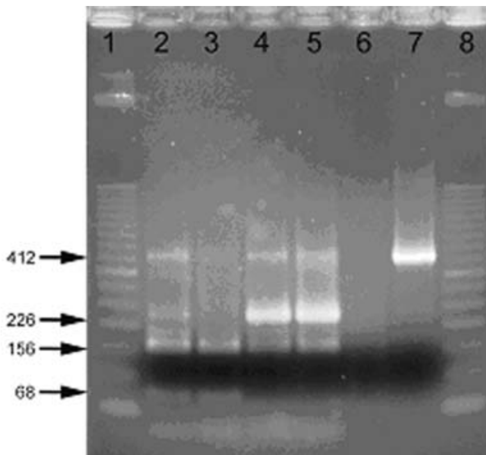

Figure 2. Restriction Length Fragment Polymorphism in the ITS region of *Marteilia* spp. Lanes 1 and 8: 50 bp DNA ladder. Lane 2: O and M profiles (coinfection). Lane 3: "M" profile. Lane 4 and 5: "O" Profile. Lane 6: Negative control without DNA. Lane 7: Negative control without *Hha I* enzyme. Sizes of the different fragments in base pairs are indicated.

M. refringens from oysters (Le Roux et al. 2001) and "M" profile, which would correspond to *M. maurini* are present in mussels and oysters from different locations. Coinfection with both RFLP profiles is also a common event. Obtained sequences were in agreement with RFLP profiles and deposited in the GenBank database (accession numbers DQ426545 to DQ426644).

Previously reported profiles of ITS-1 sequence of *Marteilia* spp. are summarized in table 2. In contrast with Le Roux et al., (2001), the "O" type is the predominant RFLP profile in mussels from Rías Baixas, Trieste, Venice (López-Flores et al., 2004) and La Spezia (present results) and the "M" type is the predominant profile in oyster from Huelva (Novoa et al., 2005). As previously mentioned, coinfection is a common event, which thus refutes the theory of host specificity of *M. maurini* for mussels, and *M. refringens* for flat oysters.

Furthermore, the profiles detected could not be linked to their geographical origin, which might have suggested an allopatric speciation between populations from the Mediterranean Sea and the Atlantic Ocean. High detection frequencies of the "M" and "O" profiles were detected in both the Mediterranean and Atlantic samples (Table 1, Figure 1).

Differences between profiles "O" and "M" are reduced only to presence or absence of one restriction locus (Figure 2). This character, in such a variable region, could easily switch more than once in the course of evolution which would invalidate the ITS-1 RFLP as a tool for distinguishing among *M. refringens* and *M. maurini*. Furthermore, the presence of an identical restriction site close to the diagnostic locus causes an "O" type to look like an "M" type when they are loaded on a gel (Novoa et al., 2005). In addition, even if there was only one evolution event which gave rise to such restriction locus, the variation on ITS-1 sequence among oysters and mussels from different locations are of similar magnitude and they could correspond to intraspecific rather than interspecific variations (López-Flores et al., 2004, Novoa et al., 2005). RFLP gives biased data since identical profiles belong to different sequences.

In summary, characterisation of *M. refringens* and *M. maurini* based an ultrastructural morphology, host specifity or molecular taxonomy, both with SSU rRNA gene and

ITS or IGS regions, shows that these parasites can be regarded as synonymous with *M. refringens.* This name has priority because of its earliest description**.** In order to definitely confirm this, more research is needed: molecular investigation in new genes, especially coding genes; studies on relationships between parasites and its different hosts and also ecological studies to complete knowledge about the life cycle of the parasite. This issue has great consequence for international trade of live molluscs. *Marteilia refringens* is an important risk to *O. edulis* stocks (O.I.E., 2006), and if mussels can really act as vectors of the parasite, an update on mussel management is suggested: *M. galloprovincialis* and *M. edulis* from *M. refringens* infected areas should not be placed in a *M. refringens* free area unless an appropriate analysis is performed to estimate the risk of introduction of the parasite including cultural practices aspects, and partial knowledge concerning the parasite life cycle.

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