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Detection of *Bonamia ostreae* and *B. exitiosa* (Haplosporidia) in *Ostrea edulis* from the Adriatic Sea (Italy)

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Abstract:

The flat oyster *Ostrea edulis* L. is widespread along the Italian coasts. In particular, the Manfredonia Gulf (Adriatic Sea) represents an important site where natural beds subsist. Previous monitoring conducted in 1990 by light microscopy and ultrastructural studies revealed the presence of *Bonamia*-like microcell parasites in some flat oysters: following this observation, a new sampling of *O. edulis* was carried out at this location in 2007. Of 750 oysters collected, 3 showed the presence of uninucleated microcells (2 to 3 µm diameter) free or inside the haemocyte cytoplasm by cytology and histopathology. Molecular analysis confirmed that the microcells in 2 oysters were *B. exitiosa,* whereas in the third oyster the microcells were *B. ostreae.* Moreover, molecular studies were carried out to confirm the existence of *Bonamia* sp. in archived samples, confirming the presence of *B. ostreae* in the Manfredonia Gulf since 1990.

Keywords: *Bonamia* spp. · *Ostrea edulis* · Molecular analysis · Electron microscopy

INTRODUCTION

Ostrea edulis L. is the native European oyster species and in the past represented an economically important oyster production in several European countries including France, Ireland, the UK, the Netherlands and Spain (Abollo et al. 2008; Culloty & Mulcahy 1996; Grizel et al. 1985).

In Italy, natural beds of *Ostrea edulis* are present along Adriatic coasts where they are mainly fished, directly sent to trade or, in some cases, left as stocks waiting for better trade periods. Moreover, in the last years experimental farming of *Crassostrea gigas* has been developed.

Considering the importance of the movements of live mollusc stocks around the world and the associated risk of disease spread from affected places to free areas, zoosanitary control of transfers is essential. It is thus necessary to establish status of oyster production areas and to characterize pathogen species.

The genus *Bonamia* encompasses protistan parasites causing a disease of oyster haemocytes known as Bonamiosis, "microcell disease" or "haemocyte disease of dredge oysters". This disease is responsible for extensive oyster mortality and has caused declines in oyster production in Europe in the late 1970s and early 1980s (Comps et al. 1980; Tigé et al. 1980).

The presence of haplosporosomes (Hine et al. 2001, Pichot et al. 1979) and the molecular analysis of the SSU rRNA gene (Carnegie & Cochennec 2004, Reece et al. 2004, Cochennec-Laureau et al. 2003, Carnegie et al. 2000; Cochennec et al. 2000) placed, phylogenetically, the genus *Bonamia* in the phylum Haplosporidia.

Four characterized species are included in this genus: *Bonamia ostreae*, *B. exitiosa*, *B. roughleyi* (O.I.E. 2006) and *B. perspora*. *B. ostreae* (Pichot et al. 1979) occurs naturally in *Ostrea edulis* L. (Grizel et al. 1983), and may infect also *O. puelchana* (Pascual et al. 1991), *O. angasi* (Bougrier et al. 1986) and *O. chilensis* (=*Tiostrea chilensis* = *Tiostrea lutaria*) when moved to endemic zones (Grizel et al. 1983). *B. exitiosa* infects *O. chilensis* in New Zealand (Hine et al. 2001) and *O. angasi* in Australia (Corbeil et al. 2006) and has been recently reported in *O. edulis* in Spain (Abollo et al. 2008). *B. roughleyi*, previously called *Mikrocytos roughleyi*, infects *Saccostrea glomerata* in Southeast Australia (Farley et al. 1988). *B. perspora* is a newly described protozoan

55 species found in *Ostreola equestris* (North Carolina, USA) and represents the first *Bonamia*
56 species producing a typical haplosporidian spore (Carnegie et al. 2006). Other *Bonamia*-like
57 organisms have also been described in *O. chilensis* from Chile (Kern 1993; Campalans et al.
58 2000), *O. puelchana* from Argentina (Kroeck & Montes 2005) and *Crassostrea ariakensis* from
59 North Carolina (Burreson et al. 2004).
60 Previous works have reported the occurrence in Italy of small parasites, similar to *Bonamia* sp., in
61 flat oysters collected from Southern (Tiscar et al. 1991) and Northern Adriatic Sea (Tiscar et al.
62 2002). Few individual molluscs appeared infected by the parasites, respectively 1/161 and 8/600,
63 nevertheless all the infected oysters showed high infection level of the haemocytes with presence
64 of numerous protozoans per cell (Tiscar et al. 2002, Tiscar et al. 1991).
65 Taking into account these previous reports, the aim of the present study was to investigate by
66 cytology and histology the occurrence of microcell parasites in flat oysters (*Ostrea edulis*) newly
67 collected from natural beds in the Southern Adriatic Sea and to characterize these parasites by
68 molecular and ultrastructural analyses as well as to confirm and characterize the presence of
69 *Bonamia* sp. in one archived sample collected in the past.

MATERIAL AND METHODS

Sample collection

A total number of 750 flat oyster specimens (*Ostrea edulis)* were collected in February, May, July, September and November 2007 (n=150/month) (Fig.I) from dispatch centre. The oysters were fished from natural beds widespread in the Manfredonia Gulf. Manfredonia Gulf is a large bay in the Southern Adriatic Sea situated on the Apulian coast, south of the Gargano Promontory (Fig.I).

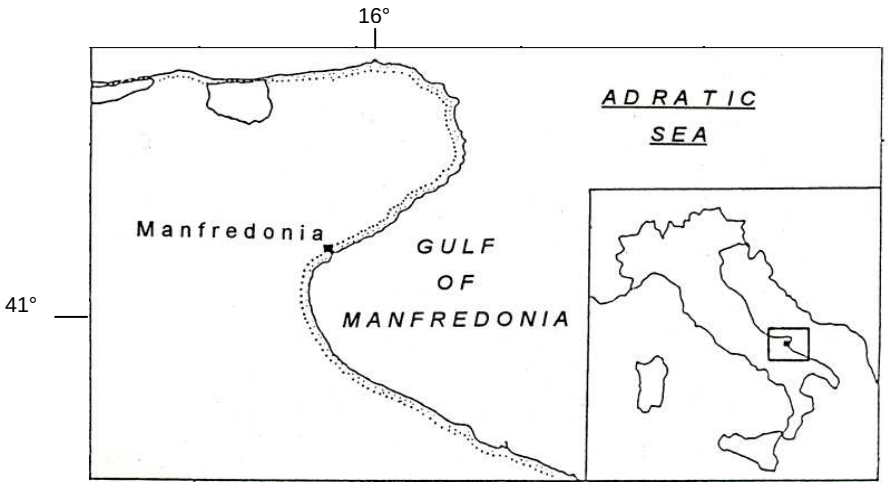

Fig.I: Manfredonia Gulf, site of natural beds of *Ostrea edulis.*

Moreover, one archived sample (coded as n.1640) embedded in paraffin block, obtained through a sampling carried out in the Manfredonia Gulf in 1991 (Tiscar et al. 1991), was included in the molecular study and some pictures acquired by transmission electron microscopy were analysed.

Microscopical examination

Each oyster was opened and several heart imprints were made on a glass slide. Slides were air-dried, fixed in methanol and stained using a commercially kit (Hemacolor®, Merk). Then, all slides were observed at 1000X magnification.

Sections of tissue including gills, digestive gland, mantle and gonad were fixed in phosphate-buffered formalin 10%. Cytology positive samples were processed for paraffin histology. Thin sections (5 μm) were cut from paraffin blocks and stained with haematoxylin and eosin for standard histopathological evaluation.

Molecular procedure

Gill fragments (25 mg), from each oyster were fixed in 95% ethanol. Genomic DNA was extracted from histological positive samples using a QIAamp® DNA Mini Kit (QIAGEN) following the manufacturer's instructions.

DNA was resuspended in sterile deionised water in order to produce a final DNA concentration of 100 ng/µl. Extracts were stored at 4°C until PCR analyses were performed.

DNA extracts were subjected to the PCR amplification of the partial small subunit ribosomal DNA (SSU rDNA) using the *Bonamia* specific primer Bo-Boas (Table 1) (Cochennec et al. 2000). PCR was performed in 50 µl volume in RedTaq® Mix (Sigma) containing 0.5 µl of each primer (100 mM) and 100 ng of DNA. PCR reactions were carried out in a GenAmp PCR System 2700 thermocycler (Applied Biosystems). The cycling protocol was 94° C for 7 min; 30 cycles: 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; final elongation at 72°C for 10 min.

Additionally, ITS-1 region was amplified using ITS For-8 and ITS Rev-8 primers (Table 1) (Corbeil et al. 2006); PCR cycling conditions were as follows: 7 min at 95°C for denaturation and 40 cycles: 30s at 94°C, 45s at 50°C and 45s at 72°C. Final elongation at 72° C was extended for 10 min. After amplification, 10 µl of the PCR products were separated by electrophoresis in 1.5% agarose gel (in Tris Acetate EDTA buffer 1X). Gels were stained with ethidium bromide.

Primer	*Sequence*	*Reference*
BO	5′-CAT TTA ATT GGT CGG GCC GC-3′	Cochennec et al. 2000
Boas	5′-CTG ATC GTC TTC GAT CCC CC -3′	Cochennec et al. 2000
ITS For-8	5′-CGT AAC AAG GTT TCC GTA GGT-3′	Corbeil et al. 2006
ITS Rev-8	5′-TGC TTT TTG CGT TTG TGT AGT-3′	Corbeil et al. 2006

Tab. I: Sequences of primers used in this study.

Sequence determination and comparison

PCR products obtained from infected samples were further purified using Ultrafree-DA columns (Millipore, Billerica, MA) and directly sequenced by MWG Biotech/M-Medical (Milan, Italy).

PCR products obtained from one individual (n. 29/07) were cloned using the TA cloning kit (Invitrogen, U.S.A.) and three clones were bidirectionally sequenced using the Big Dye V3 sequencing kit (Applied Biosystem, U.S.A.), specific and plasmidic primers (TopoF: 5'GAC CAT GAT TAC GCC AAG C 3' and TopoR: 5' CCC AGT CAC GAC GTT G3').

Sequences were searched for similarity with public databases lodget in GenBank (Benson et al. 2008) using BLAST (Basic Local Alignment Search Tool).

Ultrastructural studies

Two samples found positive by PCR (n. 29/07 and n. 77/09) were processed for transmission electron microscopy (TEM). Four % glutaraldehyde fixed gill tissue from infected *Ostrea edulis* were post-fixed for 1h in 1% osmium tetroxide (OsO_4) in 0,2M cacodylate buffer, cleared in propylene oxide and embedded in epon resin. Ultrathin sections were made using copper grids and double-stained with 5% uranyl acetate and 5% lead citrate and then examined at 120 kV on a JEOL 1110 transmission electron microscope equiped with a Morada digital camera and the analySIS imaging software.

RESULTS

Microscopic studies

Three oyster specimens (n. 29/07, n. 77/09, n. 78/11) – collected in July, September and November respectively – were considered infected by cytology and histopathology since they presented small spherical organisms within the cytoplasm of haemocytes or free in connective tissue and gills (Fig. II-III).

Examination of heart imprints showed intracellular or free organisms with basophilic cytoplasm and eosinophilic nucleus inside heart haemocytes (Fig. II)

Histopathology showed few parasites in each oyster, associated with focal haemocyte infiltrations in the connective tissue of the gill and mantle, and in the vascular sinuses around the stomach and the intestine. Parasitized haemocytes had an eccentric nucleus, and one or two parasites were

observed inside the cytoplasm (Fig. III). The parasites were basophilic, spherical or ovoid, 2-3 µm in diameter. Some parasites were observed free in the connective tissue.

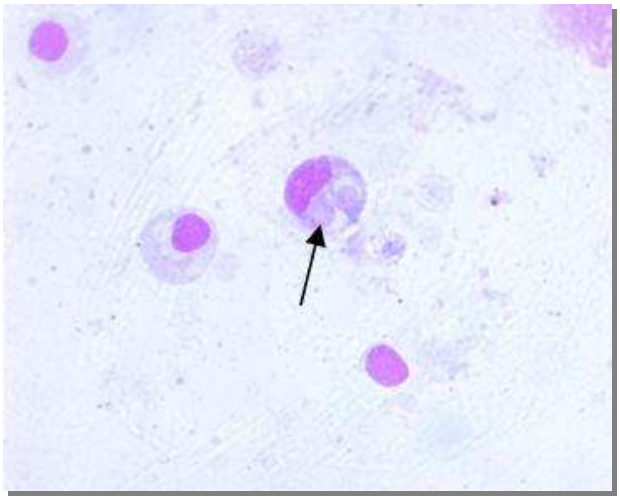

Fig. II: Heart imprint from *Ostrea edulis* (n.29/07). Some uninucleated cells, 2-5µm wide, can be observed within the cytoplasm of an haemocyte. Hemacolor® staining. (1000X).

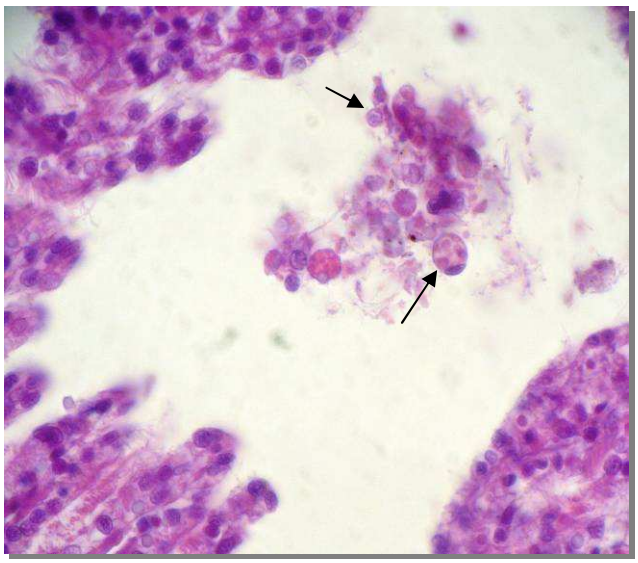

Fig. III: Histological section from *Ostrea edulis* (n.29/07). Some uninucleated cells can be observed within haemocytes and extracellularly in water lacuna in gills. Haematoxylin and eosin staining. (1000X).

Ultrastructural studies

The samples (n. 29/07 and n. 77/09) selected for ultrastructural studies showed dense and intermediate forms of Adriatic *Bonamia* sp.. In dense forms, the nucleus appeared central and nearly circular in section, consisting of a finely granular and electron-dense nucleoplasm and

containing a dense nucleolus located peripherally often seen against the nuclear membrane. The cytoplasm presented mitochondria (Fig. IV), Golgi bodies composed of stacked saccules and small vesicles (Fig. IV), lipid bodies (Fig. IV), haplosporosomes with the characteristic double membrane (Fig. V) and many ribosomes spread in the cytoplasm (Fig. IV & V). Spheroid inclusions (Fig. VI) were also present, these elements could correspond with different haplosoporogenesis stages. Mitochondrial size and shape were variable, even in the same section, with sparse vesicular inclusions or membranous features (Fig. VI). A stage containing large vacuoles derived from enlargement of one or more mitochondria has been observed in some sections (Fig. VI). Quantitative data from electron micrographs of cells are given in Table 2. The comparison of Adriatic *Bonamia* sp. with *B. ostreae* showed some differences; this last is smaller and has larger haplosporosomes, fewer lipoid bodies and mitochondrial profiles (Table 2). The Adriatic *Bonamia* sp. presented similarity with *B. exitiosa* group regarding diameter and mitochondria number.

	***Bonamia* sp.** (*O. edulis* Adriatic Sea)	*B. ostreae*[1]	*B. exitiosa*[1]
Number examined	62	64	61
Mean diameter (μm)	3±0.3	2.4±0.5	3±0.3
Mean no. Mitochondria	3.2	2±1	3±1
Mean no. Haplosporosomes	7.2±4.3	7±5	14±6
Mean haplosporosomes diameter (nm)	132±21	153±18	148±11
Range of haplosporosomes diameters (nm)	73-257	127-187	128-184
Lipid bodies present (%)	30	7	49
Mean no. lipid bodies	0.48	0.3±0.6	0.8±0.9

Table 2. A comparison of main features of dense forms of *Bonamia* spp. [1] Hine et al. 2001

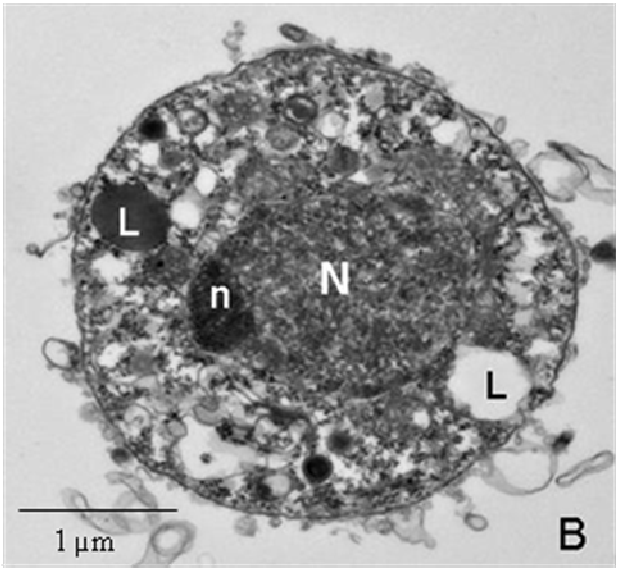

Fig. IV: **A** Dense form showing haplosporosomes (H) , mithocondria (M), Golgi-like profile (G) (x 25000); **B** Dense form with a central nucleus (N) an eccentric nucleolus (n), lipid bodies (L) (x 25000).

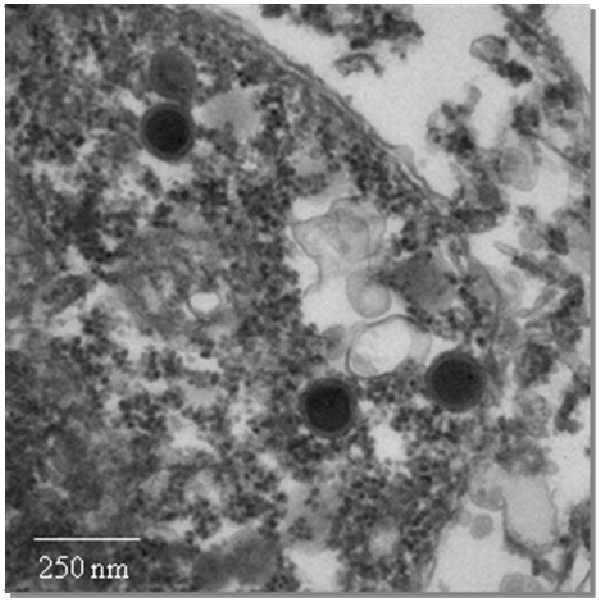

Fig. V: Haplosporosomes exhibiting double membrane within cytoplasm of *Bonamia* sp. dense form (x60000).

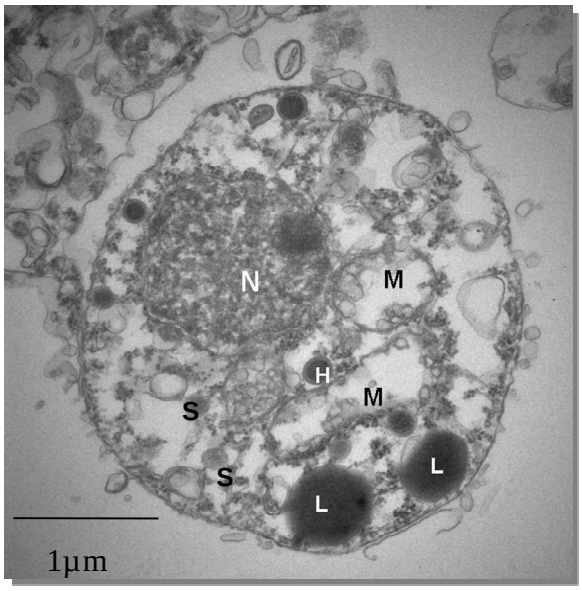

Fig. VI: Vacuolated form showing enlarged mithocondria (M), eccentric nucleus (N), lipid bodies (L), haplosporosomes (H) and spheroid inclusions (S) (x 25000).

Ultrastructural examination of the infected oyster collected in June 1990 revealed *Bonamia ostreae* like clear, intermediate and dense forms in the cytoplasm of haemocytes. Furthermore, a form surrounded by a dense layer included between two membranes was observed (Fig. VII) (Tiscar et al. 1991).

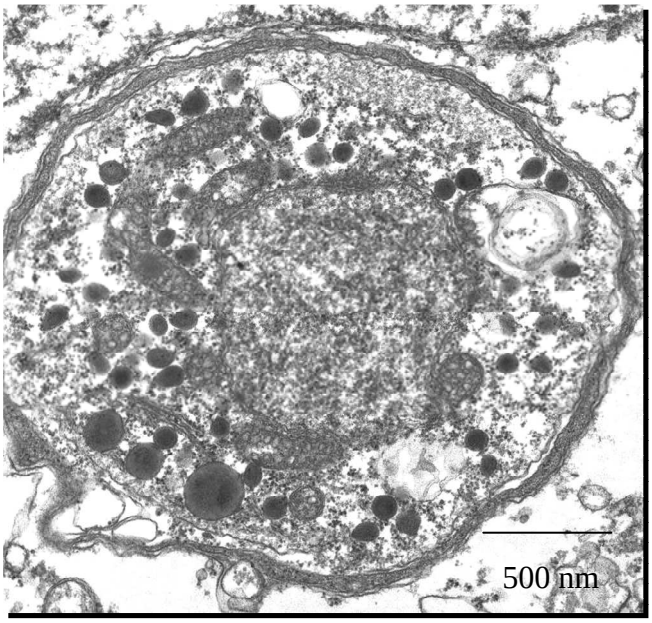

Fig. VII: *Bonamia* like cell surrounded by a dense layer included between two membranes (Tiscar et al. 1991) observed in sample n.1640 (x 40000)

PCR amplification and sequence analysis

When PCR was performed using primers Bo and Boas, amplicons of 300 bp were obtained for three samples collected in 2007 (29/07; 77/09; 78/11) and for DNA extracted from paraffin block n. 1640.

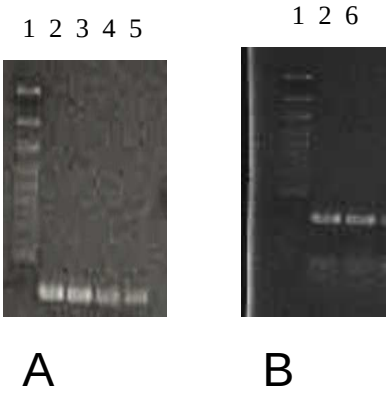

Fig. VIII: Agarose gel electrophoresis of PCR products with primers Bo and Boas. Lane 1 molecular weight marker; lane 2 DNA from purified *B. ostreae*; lane 3 DNA from sample 29/07; lane 4 DNA from sample 77/09; lane 5 DNA from sample 78/11; lane 6 DNA from sample 1640

These PCR products were directly sequenced. In addition, PCR products obtained from individual n. 29/07 were cloned and 3 clones were sequenced in order to evaluate intra-oyster polymorphism of the fragment Bo-Boas. The sequence obtained from the DNA extracted from paraffin block of the sample n.1640 and the sequence obtained from one sample (n.78/11) confirmed the presence of *Bonamia ostreae* with 100% nucleotide homology.

The sequences obtained directly from PCR products from samples 29/07 and 77/09 and sequences obtained from the 3 clones from individual n.29/07 were identical (GenBank Accession No. EU598800). The alignment of the sequences with the SSU rDNA genes of different *Bonamia* sp. showed 100% homology with parasites of the *B. exitiosa* groups, such as those from Australia (DQ312295), *Bonamia* sp. NAS-2004 (AY542903), *Bonamia* sp. Ca 1-16 (AY923857), *Bonamia* sp. (AF337563) in both samples.

The PCR using primers ITS For-8 and Rev-8 from samples 29/07 and 77/09 produced an amplicon of about 100 pb (GenBank Accession No. EU672891). Alignment of the obtained ITS

fragments presents further evidence of the genetic identity of the Adriatic isolate with Australian *B. exitiosa* (DQ312295).

DISCUSSION

The present study demonstrated the presence of protozoa belonging to the genus *Bonamia* in flat oysters *Ostrea edulis* coming from the Adriatic Sea and, particularly, from the Manfredonia Gulf. On 750 oysters collected during 2007, only three oysters were found infected by *Bonamia* sp. Moreover, the past researches carried out in Italy, showed one infected oyster on 161 subjects examined from April 1990 to January 1991 (Tiscar et al. 1991). A further Italian study highlighted 8 positive oysters on 600 individuals collected from 1997 to 2000 (Tiscar et al. 2002).

The oysters, detected infected during 2007 by cytology and histology, have been selected for molecular tests and two samples for TEM. In addition, the sample archived in paraffin block found infected by *Bonamia* like cells in 1990 was selected for molecular characterization.

Two *Bonamia* species were detected. Molecular analyses showed identities with *B. ostreae* in one sample as well as in the older oyster sample preserved in paraffin block, thus confirming the existence of this species in the Manfredonia Gulf since 1991 (Tiscar et al. 1991). Moreover, the ultrastructural study and the DNA sequence analysis supported, in two samples, the occurrence of microcells belonging to the *B. exitiosa* group, thus corroborating the recent report of this species-group in European flat oysters (Abollo et al. 2008). *B. exitiosa* is known to infect *Ostrea chilensis* in New Zealand (Dinamani et al. 1987) and *O. angasi* in Australia (Corbeil et al. 2006, Hine 1996, Hine & Jones 1994) while some close related –but not fully characterized - parasites have been reported in *O. chilensis* in Chile (Campalans et al. 2000), in *O. puelchana* in Argentina (Kroeck et al. 2005) and in *Crassostrea ariakensis* in North Carolina, U.S.A. (Burreson et al. 2004).

An ultrastructural study was carried out to morphologically characterize the microcell belonging to *Bonamia exitiosa* group occurring in Italy. Parasites observed presented similarity with *Bonamia exitiosa* group including diameter and mitochondria number. A dominant cell type of parasite was observed, corresponding to dense form with presence of lipoid bodies and

haplosporosomes. The number of haplosporosomes appeared equivalent to *B. ostreae* (Tab.I). The dense forms observed in Adriatic samples showed similarities with previous described forms of *B. exitiosa* (Hine et al. 2001) except that ribosomes were widely diffused through the cytoplasm including around the cell margin. Diplokaryotic cells were occasionally observed with dense material in opposite faces as showed in previous described form (Hine et al. 2001).

An earliest study reported the presence of haemocytic parasites in oysters collected from the same site (Tiscar et al. 1991). On the basis of the morphological characteristics of the parasite, the authors suggested that it was *Bonamia* sp. Unfortunately, only some pictures are now available and, at the moment, it is not possible to carry out further morphological studies. One of these pictures (Fig. VII) showed electrondense material included between two membranes around the parasite, but the exact significance of this structure was unclear. Generally, *Bonamia* species do not show resistance forms except the last characterized one, *B. perspora*. The presence of spores in *B. perspora* suggests that other *Bonamia* spp. may also produce spores (Carnegie et al. 2006). Molecular study carried out on this sample, during the present study, confirmed that the protozoan parasite is *B. ostreae* and is not a *Haplosporidium* species.

In order to evaluate the relatedness of the new recorded Adriatic microcells to other protozoans, part of the SSU rRNA gene and ITS-1 region were sequenced. The comparative molecular analysis with sequences available in GenBank showed that Adriatic *Bonamia* sp. from samples n. 29/07 and 77/09 was identical to *B. exitiosa* group, suggesting that Adriatic microcells belong to the same group. The third sample (n. 78/11) studied by PCR showed 100% homology with *B. ostreae*, thus confirming the presence of two *Bonamia* species in Manfredonia Gulf as reported in Spain (Abollo et al. 2008).

Bonamia sp. was previously reported in *Ostrea edulis* collected in the Northern Adriatic Sea in 1997, 1998 and 2000. In 1997, eight oysters out of the 300 analysed showed the presence of round and nucleated elements of 2-3 µm (Tiscar et al. 2002). The molecular analysis conducted on these samples by Bo-Boas primers, revealed identity with *B. ostreae* (data not show). Therefore, *B.*

ostreae was present in Adriatic Sea over the past years, but its real impact is difficult to evaluate since data on the population density of *O. edulis* natural beds are lacking.

Moreover, substantial differences of environmental conditions exist between the Adriatic Sea and the Atlantic Ocean, thus possibly inducing a different development of the parasite. In Manfredonia Gulf the sea water average temperature is 16.80°C with higher temperature in August (about 27°C) whereas the salinity is 37.34 g/L (Si.Di.Mar. 2007). These data are comparable with information about Quiberon bay in Atlantic Ocean where bonamiosis is endemic: the yearly temperature mean is 14.56°C, with maximum in August (about 23°C) and the yearly salinity mean is 33.47g/L and maximum in August is 38g/L; *B. ostreae* is present in Quiberon Bay since 1980 with prevalence usually lower than 0.15 (Arzul et al. 2005). A recent study on the influence of environmental factors showed a better survival of purified *B. ostreae* in hyper-saline (>35g/L) than in hyposaline media (<20g/L); moreover, high temperature (>25°) seems to have a negative impact on the parasite survival (Arzul et al. 2007).

The lack of previous data does not allow evaluating whether *Bonamia exitiosa* was present in Adriatic flat oyster in the past years or if it was recently introduced and it raises the following question: how *B. exitiosa* arrived in Italy? Oyster trade might exist with other European countries including Spain where *B. exitiosa* was recently reported in *O. edulis* (Abollo et al. 2008) and might have contributed to spread the parasite. However, epidemiological study is now necessary to answer this question.

The presence of *Bonamia ostreae* and *B. exitiosa* in natural beds of Manfredonia Gulf does not seem to cause mass mortality. The long time presence of these pathogens in flat oysters living in natural beds and subjected to continuous fishing activities might have contributed to generate oysters exhibiting low susceptibility to infection. In a number of other shellfish diseases, the only potential long-term method of control has been the development of resistance in the host species. "Resistance" in bivalve generally refers to relatively greater survival, thus implying a reduced susceptibility to the presence of the parasite and allowing the oysters to be grown to market size before disease-induced mortalities occur (Ford 1986).

In fact, some studies suggested that differences might exist between European populations of *Ostrea edulis* in regard to their susceptibility to infection with *Bonamia ostreae* (Culloty et al. 1999): e.g. the Rossmore oysters (Cork Harbour, Ireland) which were exposed to the parasite throughout life, showed lower prevalence of infection and percentage mortality than a number of other Irish strains, when exposed to the parasite in the laboratory and in the field (Culloty et al. 2004). In conclusion, two listed parasites, *B. ostreae* and *B. exitiosa* are present in flat oysters originating from Adriatic Sea at low prevalence. New epidemiological data are required in order to determine the distribution of *Bonamia* spp in the European waters and to avoid the spread of these pathogens. Moreover, further studies need to be carried out on the Adriatic *Ostrea edulis* in order to elucidate the parasite-host interactions.

Nucleotide sequence data relative at this paper are available in the GenBank (Benson et al. 2008) *under the Accession No. EU598800 and EU672891*.

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