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Molecular detection of parasites (Trematoda, Digenea: Bucephalidae and Monorchiidae) in the European flat oyster Ostrea edulis (Mollusca: Bivalvia)

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Abstract
Members of the globally distributed bivalve family Ostreidae (oysters) have a significant role in marine ecosystems and include species of high economic importance. In this work, we report the occurrence of digenean parasites of the families Bucephalidae (Prosorhynchoides sp.) and Monorchiidae (Postmonorchis sp.) in Mediterranean native populations of Ostrea edulis (but not in the introduced Magallana gigas). Molecular detection was based on DNA sequencing of the ribosomal intergenic spacer 2 (ITS2) marker. The importance of detecting the presence of overlooked digenean parasites in Mediterranean oysters is discussed.

Keywords: Ostrea edulis, Magallana gigas, Trematoda parasites, Mediterranean Sea, ITS2

Introduction
Oysters (family Ostreidae) are cosmopolitan sessile filter-feeder bivalves that play a remarkable ecological role in estuarine, intertidal and shallow-water ecosystems, through the mitigation of the excess of nutrients, algae and sediment. Among the c. 75 currently known species, many are of economic importance, being voluminously produced by the aquaculture industry. In Europe, oyster fisheries were historically based entirely on the autochthonous flat oyster Ostrea edulis Linnaeus, 1758, which occurs on hard substrata in estuarine and shallow coastal waters of the eastern Atlantic, from Scandinavia to North Africa, and into the Mediterranean Sea as far as the Black Sea (Yonge 1960). Oyster cultivation, based on the management of natural stocks, dates to the 17th century in Japan and earlier in China, and to Roman times in Europe, over 2000 years ago (Yonge 1960, 1970). A widespread decline occurred at the end of the 19th century (Gosling 2003) because of overfishing, habitat destruction and pollution (Orton 1937; Gosling 2003; Kirby 2004). Furthermore, the spread of a parasitic disease due to the haplosporidian protozoan Bonamia ostreae (Pichot et al. 1980), included in the International Aquatic Animal Health Code by the World Organisation for Animal Health (http://www.oie.int), led to massive mortality of European flat oysters in the last century (Renault et al. 1995). This has caused a shift to the rearing in Europe of the Pacific cupped oyster Magallana gigas Thunberg, 1793 (formely Crassostrea gigas; nomenclature after Salvi and Mariottini 2017; see also Bouchet and Marshall 2016), native to the Pacific coast of Asia, but introduced into North America, Australia, Europe and...
New Zealand. In 2004 *M. gigas* represented the bulk of farmed oyster world production (96.2%), whereas the production of the European flat oyster represented less than 0.11% of the total global production of all farmed oyster species (Svåsand et al. 2007). Today *M. gigas* is still among the most cultivated oysters, with a global annual production estimated at 583,464 tons, as compared to 2872 tons of *O. edulis* in 2015 (http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en). Monitoring of *O. edulis* and *M. gigas* populations for the presence (and putative overlapping) of parasite communities is necessary to set optimal aquaculture rearing practices and, thus, prevent or limit risks of cross-contamination (Mineur et al. 2014), as also specified by the World Organization for Animal Health – International Aquatic Animal Health Code (Alday-Sanz 2009). It is noteworthy that the co-presence in both oyster species of parasites, especially flatworms, is known in the natural environment (Aguirre-Macedo & Kennedy 1999). During laboratory experiments for a molecular phylogenetic study of Ostreidae (Salvi et al. 2014), the co-occurrence of Ostreidae-specific and non-target Polymerase Chain Reaction (PCR) products of the rRNA intergenic-spacer 2 (ITS2) was occasionally assessed as multiple bands in gel electrophoresis. These results suggested that the slightly degenerated ITS2 universal primers employed, originally designed by Oliverio and Mariottini (2001) and named ITS2-3d* (5ʹ-\text{GCATCGRTGAAGARCGCAG}-3ʹ) and ITS2-4r* (5ʹ-\text{AGTTTTYTTYTCCTCCGCTTA}-3ʹ), targeting the entire ITS2 region with the following thermal conditions: 3 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 50–55°C, 90 sec at 72°C, 6 min at 72°C for final extension. Oyster-specific ITS2 products were isolated, purified with the “Sure Clean” kit (Bioline) and sequenced by Macrogen Europe (Amsterdam, The Netherlands) (Genbank accession no. MF374290-MF374317). Co-amplified ITS2 extra bands (Figure 2(a,b)) having different molecular weights from those expected for *O. edulis* or *M. gigas* (i.e. ~600 and ~550 bp,

**Materials and methods**

Genomic DNA was extracted using the “DNeasy Blood and Tissue Kit” (Qiagen, Hilden, Germany) from mantle tissues of each alcohol-preserved specimen of *O. edulis* and *M. gigas*. Before PCR amplification, quality and quantity of the extracted DNA were verified by 1% agarose gel electrophoresis runs. ITS2 amplifications were performed on all collected specimens of *O. edulis* (*n = 18*) and *M. gigas* (*n = 18*) using slightly degenerated ITS2 universal primers derived from the ones described by Oliverio and Mariottini (2001) and named ITS2-3d* (5ʹ-\text{GCATCGRTGAAGARCGCAG}-3ʹ) and ITS2-4r* (5ʹ-\text{AGTTTTYTTYTCCTCCGCTTA}-3ʹ), targeting the entire ITS2 region with the following thermal conditions: 3 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 50–55°C, 90 sec at 72°C, 6 min at 72°C for final extension. Oyster-specific ITS2 products were isolated, purified with the “Sure Clean” kit (Bioline) and sequenced by Macrogen Europe (Amsterdam, The Netherlands) (Genbank accession no. MF374290-MF374317). Co-amplified ITS2 extra bands (Figure 2(a,b)) having different molecular weights from those expected for *O. edulis* or *M. gigas* (i.e. ~600 and ~550 bp,

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**Figure 1.** Map of the sampling localities of *Ostrea edulis* (small red circles) and *Magallana gigas* (blue squares). The presence of *Prosorhynchoides* sp. (grey circles) and *Postmonorchis* sp. (empty circles) is reported.
Table I. Geographic information of collection sites for all oyster specimens analysed in this study. Habitat/date of collection sites is reported (* indicates specimens collected in the Atlantic; all other samples were collected in the Mediterranean). The presence/absence of digenean parasites was confirmed by screening samples with the ITS2-DIG1/DIG2 PCR diagnostic tool. Voucher and ITS2 sequence accession numbers for both oysters and parasites are provided (for each locality one parasite specimen was vouchered and its sequence submitted to GenBank).

| Species | Specimen voucher | Oyster ITS2 (accession no.) | Sampling locality | Geographic coordinates | Habitat | Parasite taxon | Parasite voucher | Parasite ITS2 (accession no.) |
|---------|------------------|-----------------------------|-------------------|-----------------------|---------|---------------|----------------|--------------------------|
| Ostrea edulis | BAU01714 | LM993872 | Cape Circeo/April 2012 | 41°13'30"N, 13°05'44"E | 12 m depth, rocky substrate/June 2015 | Absent | – | – |
| Ostrea edulis | BAU01715 | LM993873 | Cape Circeo/April 2012 | 41°13'30"N, 13°05'44"E | 12 m depth, rocky substrate | Absent | – | – |
| Ostrea edulis | BAU01716 | LM993874 | Ginosa, Italy/August 2013 | 40°25'34"N, 16°53'41"E | 3 m depth, epibiont on hermit-crabbed shells of *Tritia mutabilis* | Absent | Postmonorchis sp. | BAU03025 MF374321 |
| Ostrea edulis | BAU01717 | LM993875 | Ginosa, Italy/August 2013 | 40°25'34"N, 16°53'41"E | 3 m depth, epibiont on hermit-crabbed shells of *Tritia mutabilis* | Absent | Postmonorchis sp. | – | – |
| Ostrea edulis | BAU02996 | MF374290 | Secche di Tor Paterno, Italy/September 2012 | 41°36'18"N, 12°20'20"E | 6 m depth, cemented on a buoy | Proserohiphidones sp. | BAU03022 MF374318 |
| Ostrea edulis | BAU02997 | MF374291 | Secche di Tor Paterno, Italy/September 2012 | 41°36'18"N, 12°20'20"E | 6 m depth, cemented on a buoy | Proserohiphidones sp. | – | – |
| Ostrea edulis | BAU02998 | MF374292 | Cape Mount Argentario, Italy/July 2015 | 42°26'42"N, 11°06'51"E | 5 m depth, rocky substrate | Absent | – | – |
| Ostrea edulis | BAU02999 | MF374293 | Cape Mount Argentario, Italy/July 2015 | 42°26'42"N, 11°06'51"E | 5 m depth, rocky substrate | Absent | – | – |
| Ostrea edulis | BAU03000 | MF374294 | San Nicola, Italy/February 2012 | 41°55'57"N, 12°06'27"E | 2 m depth, rocky substrate | Proserohiphidones sp. | BAU03023 MF374319 |
| Ostrea edulis | BAU03001 | MF374295 | San Nicola, Italy/February 2012 | 41°55'57"N, 12°06'27"E | 2 m depth, rocky substrate | Proserohiphidones sp. | – | – |
| Ostrea edulis | BAU03002 | MF374296 | Sliema Harbor, Malta/ March 2014 | 35°54'04"N, 14°30'20"E | 5 m depth, rocky substrate | Proserohiphidones sp. | BAU03024 MF374320 |
| Ostrea edulis | BAU03003 | MF374297 | Sliema Harbor, Malta/ March 2014 | 35°54'04"N, 14°30'20"E | 5 m depth, rocky substrate | Proserohiphidones sp. | – | – |
| Ostrea edulis | BAU03004 | MF374298 | Torvaianica, Italy/ February 2015 | 41°38'11"N, 12°26'31"E | Shored, epibiont on a buoy rope | Absent | – | – |
| Ostrea edulis | BAU03005 | MF374299 | Torvaianica, Italy/ February 2015 | 41°38'11"N, 12°26'31"E | Shored, epibiont on a buoy rope | Absent | – | – |
| Ostrea edulis | BAU03006 | MF374300 | Rab Island, Croatia/March 2016 | 44°47'29"N, 14°42'19"E | Cemented on the marina rocky berth, intertidal | Postmonorchis sp. | BAU03029 MF374322 |
| Ostrea edulis | BAU03007 | MF374301 | Rab Island, Croatia/March 2016 | 44°47'29"N, 14°42'19"E | Cemented on the marina rocky berth, intertidal | Postmonorchis sp. | – | – |
| Ostrea edulis | BAU03027 | MF374302 | Lido del Sole-Obia, Santinia/December 2015 | 40°54'54"N, 09°34'06"E | Epibiont on *Pomma nobilis*, 2 m depth | Postmonorchis sp. | BAU03030 MF374323 |
| Ostrea edulis | BAU03028 | MF374303 | Lido del Sole-Obia, Santinia/December 2015 | 40°54'54"N, 09°34'06"E | Epibiont on *Pomma nobilis*, 2 m depth | Postmonorchis sp. | – | – |

(Continued)
| Species       | Specimen voucher | Oyster ITS2 (accession no.) | Sampling locality                                      | Geographic coordinates       | Habitat                        | Parasite taxon | Parasite voucher | Parasite ITS2 (accession no.) |
|--------------|-----------------|-----------------------------|-------------------------------------------------------|-----------------------------|-------------------------------|----------------|-----------------|--------------------------|
| *Magallana gigas* | BAU01706         | LM993864                    | Touristic Harbour San Felice Circeo, Italy/February 2012 | 41°13′34″N, 13°05′38″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU01707         | LM993865                    | Touristic Harbour San Felice Circeo, Italy/February 2012 | 41°13′34″N, 13°05′38″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU01708         | LM993866                    | Rimini, Italy/August 2015                              | 44°04′43″N, 12°34′17″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU01709         | LM993867                    | Rimini, Italy/August 2015                              | 44°04′43″N, 12°34′17″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU03008         | MF374304                    | San Nicola, Italy/January 2015                         | 41°55′57″N, 12°06′29″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU03009         | MF374305                    | San Nicola, Italy/January 2015                         | 41°55′57″N, 12°06′29″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU03010         | MF374306                    | Canale dei Pescatori-Ostia, Italy/June 2013            | 41°43′09″N, 12°18′11″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU03011         | MF374307                    | Canale dei Pescatori-Ostia, Italy/June 2013            | 41°43′09″N, 12°18′11″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU03012         | MF374308                    | Goro, Italy/August 2013                                | 44°84′07″N, 12°30′26″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU03013         | MF374309                    | Goro, Italy/August 2013                                | 44°84′07″N, 12°30′26″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU03014         | MF374310                    | Cancale, France/August 2013                            | 48°04′04″N, 01°51′25″E       | Intertidal rocky substrate*   | Absent         | –               | –                        |
| *Magallana gigas* | BAU03015         | MF374311                    | Cancale, France/August 2013                            | 48°04′04″N, 01°51′25″E       | Intertidal rocky substrate*   | Absent         | –               | –                        |
| *Magallana gigas* | BAU03016         | MF374312                    | Cadiz, Spain/April 2014                               | 36°28′05″N, 06°15′10″E       | Intertidal rocky substrate*   | Absent         | –               | –                        |
| *Magallana gigas* | BAU03017         | MF374313                    | Cadiz, Spain/April 2014                               | 36°28′05″N, 06°15′10″E       | Intertidal rocky substrate*   | Absent         | –               | –                        |
| *Magallana gigas* | BAU03018         | MF374314                    | Touristic Harbour Riva di Traiano, Italy/February 2016  | 42°03′45″N, 11°48′49″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU03019         | MF374315                    | Touristic Harbour Riva di Traiano, Italy/February 2016  | 42°03′45″N, 11°48′49″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU03020         | MF374316                    | Harbour of Trieste, Italy/April 2017                   | 45°38′54″N, 13°49′47″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
| *Magallana gigas* | BAU03021         | MF374317                    | Harbour of Trieste, Italy/April 2017                   | 45°38′54″N, 13°49′47″E       | Intertidal rocky substrate    | Absent         | –               | –                        |
respectively; see Salvi et al. 2014) were observed in some cases. These extra bands were isolated, purified with the “PCR Clean up Gel Extraction” kit (Macherey-Nagel) and sequenced by Macrogen Europe. Non-oyster organisms whose DNA was co-amplified in ITS2 PCRs were taxonomically identified as belonging to two different digenean genera (Postmonorchis Hopkins, 1841 and Prosorhynchoides Dollfus, 1929; see Results) through the National Center for Biotechnology Information (NCBI) BLASTn tool using default search parameter settings. GenBank matching sequences returning the highest values of “Max Score” (= highest alignment score or bit-score between the query sequence and the database sequence segment), “Query Coverage” (= percentage of the query length included in the aligned segments calculated over all segments) and “Identity” (percentage of similarity between the query and subject sequences over the length of the coverage area) served to outline the most plausible genera parasitising the collected oysters. The ITS2 sequences of these digeneans were used to design a novel primer pair to target more specifically both parasitic ITS2 sources: ITS2-DIG1 (5’-AATGTGAACTGCGTACTG-3’) and ITS2-DIG2 (5’-AAGTTCCAGCGGTATTCA-3’) (Figure 3). These novel primers were used as a diagnostic PCR tool to screen the whole oyster sample (n = 36) of O. edulis and M. gigas to assess (or confirm) the presence of DNA from the two identified digeneans in the oysters’ mantle tissues used in DNA extractions [O. edulis ITS2 was then specifically amplified using ITS2-OED1 (5’-AATGTGAATTGCGAGGACA-3’) and ITS2-OED2 (5’-AAGTTCAGGCGGTAGTCT-3’).
Restriction enzyme digestion with BglII was carried on PCR products amplified with ITS2-3d*/4r*; ITS2-DIG1/DIG2; and ITS2-OED1/OED2 to confirm the presence of two distinct (but slightly overlapping) electrophoretic bands in the case of O. edulis parasitised by Prosorhynchoides sp. (Figure 2(a,b)). A PCR-amplified sample with ITS2-3d/4r was cut with BglII restriction enzyme and produced three bands, as shown in Figure 4 (lane 1), due to the restriction site occurring exclusively in the Prosorhynchoides sp. DNA sequence (see Results and Discussion). The Prosorhynchoides sp. PCR product amplified with the specific primers ITS2-DIG1/DIG2 was also cut with the BglII restriction enzyme to produce the two corresponding gel bands (Figure 4, lane 2). Digenean PCR products were obtained using the same thermal cycling protocol as reported above, then purified (using the “SureClean” Bioline Kit), sequenced and deposited in GenBank (Genbank Accession no. MF374318-MF374323).

**Results and Discussion**

We detected the presence of DNA from two distinct species of Digenea in mantle tissues of O. edulis, but not in those of M. gigas. In fact, electrophoresis of ITS2 products obtained with the primers ITS2-3d*/4r* (Figure 2(a)) revealed the co-amplification in O. edulis of a 480-bp extra band in six specimens and of a 658-bp extra band in six other individuals (Table I). BLASTn results indicated that the best match with the 480-bp extra-PCR product was the ITS2 sequence of Prosorhynchoides paralichthydis (Digenea: Buchephalidae; Accession no. KT273398; max score = 1068, query cover = 99%, identity 96%). Flatworm infection in the analysed oysters was then assessed by screening the whole sample with the novel primer pair (Figure 3) designed to target more specifically the ITS2 from the two identified digeneans.

Parasitism by digeneans has been already recorded in different oyster species (Millar 1963; Lee et al. 1996; Príncep et al. 1996; Aguirre-Macedo & Kennedy 1999). Bucephalid trematodes are known to be common parasites of commercially important molluscs (Cheng 1967) and cause parasitic castration in several bivalves (Lauckner 1983), including oysters (Cheng & Burton 1965; Feng & Canzonier 1970; Tripp 1973; Chun 1974; Mohan 1978). Infection by Bucephalus haimeanus was formerly reported in Mediterranean O. edulis (from the delta of the Ebro River in Spain), in which sporocysts were observed to invade the interstitial conjunctive tissues and to obstruct digestive gland tubes, affecting food absorption and ultimately causing host death (Príncep et al. 1996). Since the bucephaline genera Prosorhynchoides, Rhipidocotyle and Bucephalus as currently conceived are polyphyletic – as revealed by morphological and molecular data from ITS2 (D1–D3 region) and 28S rDNA markers (Nolan et al. 2015) – no conclusion can be drawn about the actual systematics of the trematode species associated with O. edulis in our study. However, our data revealed a bucephalid infection in O. edulis in the Mediterranean basin, as this occurred in the Italian and Maltese stocks (Figure 1). Bucephalidae are a large cosmopolitan family with a life cycle including sporocysts and cercariae developing in bivalves (intermediate host), metacercariae encysting within the tissues of fishes (second intermediate host), and sexual adult stages inhabiting the digestive tract, and rarely other sites, in piscivorous teleosts (definitive
host) (Overstreet & Curran 2002). The closest bucephalid to our targets retrieved in Genbank, i.e. Proserohynchoides paralichthydis, parasitises the southern flounder Paralichthys lethostigma. It would be interesting to identify the Mediterranean definitive fish host – arguably a benthic species such as flat fishes – parasitised by the Proserohynchoides species found in O. edulis, to improve our knowledge of the parasite life cycle and its potential impact on the health of oysters and fishes.

Larval stages of digeneans of the family Monorchidiidae have been described mostly in bivalves of the Atlantic and Pacific oceans (Carella et al. 2013). Only recently, metacercariae of Postmonorchis were detected in tissues of the Mediterranean wedge clam Donax trunculus Linnaeus, 1758 collected along the Italian Tyrrenian coast (Carella et al. 2013). This pathogen invades several molluscan tissues such as gills, labial palps, mantle, gut, kidney epithelium, and foot, triggering a strong inflammatory response in the host (Carella et al. 2013). The adult stages of Postmonorchis may occur in Sciaenidae (presumably Umbrina sp. in Mediterranean waters), but further data are required to identify the definitive host species (Carella et al. 2013). In our study, a DNA source of a parasite plausibly referred to Postmonorchis sp. was detected for the first time in oysters (Figure 1; Table I). This result would suggest the co-occurrence of this trematode in both Ostrea and Donax, consistent with previous observations indicating that digeneans parasitising oysters are commonly shared also with distantly related bivalve families (Lauckner 1983). On the other hand, no parasites were detected in any of our 18 specimens of M. gigas collected over a wide geographic range (Figure 1; Table I), although a further sampling effort would be needed to confirm the absence of bucephalids and monorchids in non-autochthonous populations of M. gigas. In this regard, it is relevant to remark that digeneans are known to parasitise the native Indo-Pacific M. gigas, and particularly the human intestinal trematode Gymnophalloides seoi (Digenea: Gymnostomida) is commonly transmitted by this oyster species in Korea (Lee et al. 1996; Pyo et al. 2013). It is possible that the successful thriving of the invasive M. gigas is related to a lack of parasites in the invaded areas, as expected under the enemy release hypothesis (ERH: Torchin et al. 2001; Keane & Crawley 2002; Mitchell & Power 2003). However, it is reasonable to assume that with time host-shifts by bucephalids and Postmonorchis sp. trematodes can potentially occur, so a risk of cross-infection between O. edulis and M. gigas cannot be excluded in the Mediterranean waters.

Conclusions

The growth of aquaculture – including oyster farming – has been accompanied by the emergence of new and transboundary diseases, stimulating epidemiological studies of aquatic animal pathogens. However, studies evaluating the occurrence and the impact of pathogens in wild aquatic animal populations are still sparse compared to those considering farmed species (Peeler & Taylor 2011). As a by-product of a molecular phylogenetic study on Ostreidae through a universal barcode marker (ITS2) (Salvi et al. 2014), it was possible to detect overlooked digenean parasites in wild Mediterranean populations of O. edulis. Notably, putative co-amplification of host/parasite bands were also occasionally observed during molecular phylogenetic studies on Mactridae and Donacidae (e.g. Mactra and Donax Salvi D., personal observation), suggesting that an ITS2 host/parasite DNA barcode approach may contribute in uncovering a still-hidden digenean diversity in benthic communities.

The identification of Bucephalidae and Monorchidiidae in the Mediterranean populations of flat oyster encourages future efforts in exploring their epidemiological consequences on such economically important molluscan species. Histopathological, taxonomical and ecological analyses on a larger collection of samples are certainly required to better characterise these digenean infections and to link the infection prevalence with environmental parameters and seasonality. These will allow evaluating the impact of these parasites on oyster health and fitness, the potential risks of cross-contamination to other oyster species (e.g. M. gigas) and, ultimately, the level of biosecurity and surveillance necessary to avoid the putative emergence of food-borne diseases.

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**Conflict of interest statement**

The authors declare that there are no competing interests.

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