Manuscript Details

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Title	Report and genetic identification of Amyloodinium ocellatum in a sea bass (Dicentrarchus labrax) broodstock in Portugal
Short title	Amyloodiniosis in a seabass broodstock
Article type	Case Report

Abstract

In this paper we report a case of amyloodiniosis in a sea bass (Dicentrarchus labrax) broodstock in Portugal. Microscopic examination of gill filaments showed the presence of trophonts while histological observation revealed gills epithelial hyperplasia, hypertrophy and lamellar fusion of secondary lamellae. The amplification and sequencing of the small subunit ribosomal RNA gene allowed the identification of the parasite as Amyloodinium ocellatum. It was also possible to amplify a partial sequence of ribosomal RNA from a Colpodellid, a predator of protists.

Keywords	Amyloodinium ocellatum; Dicentrarchus labrax; Fish parasites; Colpodellids; broodstock;
Manuscript category	Genetics, Pathology and Health
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Suggested reviewers	Isabel Bandin, Carmen Sarasquete, Ana Roque

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Figure 1.png [Figure]

Figure 2.png [Figure]

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Dear Editors,

We would like to submit the attached research article, entitled “Report and genetic identification of Amyloodinium ocellatum in a sea bass (Dicentrarchus labrax) broodstock in Portugal” for consideration in the Aquaculture reports journal.

In this case report we describe the occurrence of an infection by the dinoflagelate *Amyloodinium ocellatum* in a seabass broodstoock in Portugal and we present the molecular identification of the parasite. Besides, it was also possible to genetically identify the presence of a protist´s parasite belonging to the group of colpodellids that lead us to the hypothesis of these small predatory flagelates could be acting as potential controllers for *A. ocellatum* infestations and if this is confirmed that they can be used as a preventive agents of *Amyloodiniosis*.

We confirm that this work is original and it has not been published elsewhere nor is under consideration for publication elsewhere. Furthermore we assure that all authors have contributed significantly for the manuscript and are in agreement with its content. Additionally, authors declare no financial support or relationship that may pose conflict of interest.

We are thrilled to read the comments made by the editor and referees and we are at your disposal for any further clarification.

Thank you for your consideration of this manuscript.

Sincerely,

Cátia Marques

Dear editor,

You can find below the answers to reviewers' comments.

Reviewer 1:

The report addresses an important disease from an important aquacultured teleost species which justifies submission.

The manuscript is well written with no major linguistic problems found.

Comment #1

The description of the PCR mentions a primer set applied but it is not mentioned if the authors developed the primer set themselves or if the primers had been published previously. The authors are advised to give a reference for the primers.

Answer #1

The primers used in this study were designed by us with exception for the AOce_Fw2, used in the nested PCR, which is the same used by Levy and co-workers (2007). We realized that the primer Rv used in the above mentioned study was not presented in the correct orientation (5' – 3'), thus we decided to design new primers for *Amyloodinium ocellatum*. Besides, in order to improve specificity we decided to perform a second PCR (nested) using the amplification product of a first PCR and for that new primers have been developed (AOce_Fw2 and AOce_Rv1. Reference for the primer has been included in the manuscript text.

Comment #2

In addition no information is provided about the target region for the primers in the parasite genome. This should be added and further details from their aligment studies on the recovered sequence should be provided.

Answer #2

The *Amyloodinium ocellatum* sequence used to design the primers of this study was recovered from GenBank (http://www.ncbi.nlm.nih.gov) and accession number is provided in line 68 of the manuscript (GenBank accession number: DQ490256).

Comment #3

The authors have performed a histopathological investigation but the presented photos are not of good quality. It is suggested to omit these photos.

The authors are requested to present 1) a good photo (LM, TEM or SEM) of the parasite in order to support their molecular analyses and 2) a written description on morphometric details of the parasite.

Answer #3

A better quality image of the parasite as well as the gills lesions' has been provided. A morphometric description with the details of the parasite has also been included in the manuscript text.

Reviewer 2

The present manuscript by Marques et al. [AQREP_2019_8] titled "Report and genetic identification of Amyloodinium ocellatum in a sea bass (Dicentrarchus labrax) broodstock in Portugal." Marques et al. provided interesting observations of AO in broodstock of ESB. AO is a major parasitic disease in ESB farming. AO causes fast outbreaks in ESB with high mortality. It is a major threat for aquaculture species worldwide.

In this study, Marques et al. conducted microscopical, histological, and genetic analysis in order to understand the causative agent of infection in ESB broodstock. The present study data on ESB are compelling, although lacking novelty somewhat. However, there are several weaknesses in the study (methodology section) and manuscript (results presentation) that should be addressed before publication.

The following are the comments and suggestions:

Comment #1

The abstract is confusing, requires reorganization of the material and presentation. Also, no clear description of the results.

Answer #1

Abstract has been modified in order to include more details on the procedure and results and make it clear.

Comment #2

Spell check *Amyloodinium ocellatum* throughout the manuscript.

Answer #2

Spell of *Amyloodinium ocellatum* has been carefully checked throughout the manuscript and misspellings have been corrected.

Comment #3

Line 50-51: the details on broodstock length and weight should be mentioned.

Answer #3

Length and weight of the broodstock has been included in the manuscript text.

Comment #4

Line 84: mention how did the author identify AO infestation in broodstock at first.

Answer #4

At the beginning of the infestation fish started to present suspicious behavioral changes such as scratching their skin against the bottom of the tank and feeble movements, stopped eating and started positioning close to water entrances. After, infestation with *A. ocellatum* was confirmed by microscopical observation of the gills and further by PCR and sequencing.

This information has been included in the manuscript text.

Comment #5

Line 56-59: mention all the equipment's used for histological procedures. I think this information is necessary for the benefit of the readers.

Answer #5

Information on equipment used for histological procedures has been included in the manuscript text.

Comment #6

Line 60 and 61: It is difficult to understand the protocol for detaching trophonts from the gills. Did author used freshwater or marine water to immerse infected broodstock? How did author eliminated mucus and other debris during trophonts detach? Did author performed gradient purification? Technically, these details are required to obtain the purified trophonts from the infected gills.

Answer #6

Moribund fish were euthanized by cutting the spinal cord immediately posterior to the head. Gills were extracted, washed twice in distilled water to remove the attached trophonts. The water from the washes, containing the trophonts, was centrifuged to collect the parasite. Several washes, with destilled water, were performed to remove as much mucus and other impurities as possible. Trophonts were then preserved in ethanol 70% for DNA extraction.

Text has been changed in order to include this important information.

Comment #7

Authors have used the primers to amplify the AO 18s SSU from the literature. Citation is required for the primers.

Answer #7

See answer to comment #1 of reviewer #1.

Comment #8

Line 79. Sequencing platform for the product should be mentioned. Also, the methodology to verify the amplified product (sequence) should be mentioned.

Answer #8

Information about the methodology to verify the amplified product and on the sequencing platform has been included in the manuscript text.

Comment #9

Line 82: it is always fish not fishes.

Answer #9

Word has been corrected.

Comment #10

Line 89-95- Fig 1 has a, b, c, d and author should specify this details according to the results mentioned in the section, for ex. Fig 1a.

Answer #10

Text has been modified accordingly.

Comment #11

The results section has be to be improved a lot with presentation on the gel image (single band or multiple product amplification), similarity of small SSU after BLAST analysis with other sequences in the database. It is difficult to understand how the primer specific to AO could amplify Colpodellid. The author should emphasize this in the discussion.

Answer #11

A figure with the gel image has been added (Figure 2) and information about similarity of SSU with other sequences in the database have been included in the manuscript text. The primers used in this study were designed according to the information available in the GenBank (accession number DQ490256) and hence are specific to *Amyloodinium ocellatum*. However,

and due to the high conservation of the small subunit ribosomal RNA gene across species, we believe that a Colpodellid sequence was amplified because of an unspecific hybridization of the primers. This information has been included into the manuscript text.

Comment #12

Concerning highlights: it is the molecular identification of both AO and Colpodellid based on the results obtained from PCR. Author concluded that Colpodellid may be used as a potential agent to control A. ocellatum infestations; this is presented without any further evidence, so this conclusion must be eliminated.

Answer #12

The molecular identification of *A. ocellatum* and a Colpodellid sequence are based on the sequence of the PCR fragments obtained from parasites collected from sea bass branchial arches. Since Colpodellids are described as parasites of protists and algae we hypothesized that they could potentially act as potential controllers of *A. ocellatum* infestations. Conclusions on this have been toned down.

Comment #13

From the molecular analysis, it is evident that the author have amplified both AO and Colpodellid however, it is interesting to note that the Colpodellid is not observed from the histological analysis. Therefore, could be possible that Colpodellid is present in the water rather then the gills of the ESB broodstock. Hence the protocol for detaching trophonts should be very specific and detail. This observation must be discussed in the discussion section.

Answer #13

In fact from the histological preparation it was not possible to observe Colpodellids, however this fact may be due to its small size (approximately 10 times smaller than *Amyloodinium ocellatum* trophonts). This information has been included in the discussion. Trophonts have been detached by washing the gills with distilled water. After, parasites were collected by centrifugation and washed with distilled water to remove any impurities. The information on the procedure to collect trophonts from the gills has been included in the manuscript text.

Comment #14

Overall, the discussion is too short without much detail. Even though author have not described the conclusion, an elaborate conclusion is needed discussing the data in light of other data in the field and the implications of their findings with emphasize on future studies.

Answer #14

A small conclusion resuming the principal findings has been included in the manuscript.

Highlights

- Report of an amyloodiniosis outbreak in a sea bass broodstock in Portugal;
- Molecular identification of *A. ocellatum*;
- Gills epithelial hyperplasia, hypertrophy and lamellar fusion were associated with the presence of the parasite;
- Colpodellids may be used as a potential agents to control A. ocellatum infestations;

Report and genetic identification of *Amyloodinium ocellatum* in a sea bass (*Dicentrarchus labrax*) broodstock in Portugal

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Abstract

In this paper we report a case of amyloodiniosis in a sea bass (*Dicentrarchus labrax*) broodstock in Portugal. Microscopic examination of gill filaments showed the presence of trophonts while histological observation revealed gills epithelial hyperplasia, hypertrophy and lamellar fusion of secondary lamellae. The amplification and sequencing of the small subunit ribosomal RNA gene allowed the identification of the parasite as *Amyloodinium ocellatum*. It was also possible to amplify a partial sequence of ribosomal RNA from a Colpodellid, a predator of protists.

Key words

Amyloodinium ocellatum; *Dicentrarchus labrax*; *Fish parasites*; Colpodellids; broodstock;

Introduction

A basic requirement of intensive farming for any fish species is a constant supply of good quality eggs. Therefore, it is essential to maintain captive broodfish under controlled conditions in order to produce eggs and larvae with the highest quality and, consequently, that can reach a higher market value (Bromage, 1995). However, sometimes, several diseases caused by parasites can occur and compromise the expected output. Among them is a disease provoked by the dinoflagellate *A. ocellatum* (Paperna 1980), that can be an important factor limiting aquaculture productivity (Soares et al., 2012), particularly in estuaries and semi-intensive aquaculture systems, where outbreaks can rapidly occur, resulting in massive mortalities (Alvarez-Pellitero et al., 1993).

A. ocellatum is a non-specific extremely prolific and devastating dinoflagellate fish parasite and the disease caused by this organism is commonly referred as amyloodiniosis or marine velvet disease (Kumar et al., 2015). It mainly infect gills, and less frequently the skin (fins and body) and buccal cavity of the host fish (Kumar et al., 2015). Paperna (1980) described, in the region of Eliat, in the Red Sea, outbreaks of *A. ocellatum* in reared breeders of sea bass. This parasite has also been reported in sea bass breeders in Italy, but their presence did not cause serious mortalities in any broodstock (Giavenni, 1988). In the Mediterranean area and the Red Sea, the parasite also produced a massive mortality in sea bass juveniles (Alvarez-Pellitero et al., 1993). *D. labrax* broodstock outbreaks infested with amyloodiniosis have never been reported in Portugal, and were only reported in cultured juveniles of seabass and in natural population of sea bass juveniles from the Óbidos coastal lagoon and the Sado estuary (Menezes, 2000).

The present communication describes a case of *amyloodiniosis* in sea bass broodstock maintained at the Olhão Aquaculture Research Station of the Portuguese Institute of Sea

and Atmosphere (IPMA-EPPO) and reports the molecular identification of the parasite causing this infestation as being the dinoflagelate *A. ocellatum*.

Material and methods

The sea bass broodstock was composed by 24 individuals, with an average weight of 5165,17 g ± 920,27 g and an average length of 76,41 cm ± 3,84 cm, that were kept in a 18m^3 tank under natural conditions of light and temperature, with continuous sand filtered water inflow (5 m^3h^{-1}) and aeration. At the time of epizootic, stocking density was 7.6 $kg.m^{-3}$, salinity was 38 psu and mean water temperature was 24ºC.

After identification of *A. ocellatum*, infestation was contained by repeated treatments of 1.5 $g.m^{-3}$ copper sulfate, applied for 11 days. Gill filaments from dead fish were preserved in 10% neutral buffered formalin for 24 hours, and then transferred to ethanol 70% for histology purposes. Dehydration, clearing, infiltration and embedding were performed according to standard procedures using a tissue processor (Leica TP1020) and a paraffin dispenser (Leica EG 1140 H) , followed by thin sectioning (5 μm thick) with a micrometer (Leica RM-2155) and staining using haematoxylin eosin (Martoja, 1967). Slides were observed on a Nikon H550S microscope using bright-field illumination. Selected gill tissues were then scanned in a Hamamatsu Nano Zoomer Digital Pathology, and representative images were taken and processed using NDP View 2 software.

Moribund fish were euthanized by cutting the spinal cord immediately posterior to the head. Gills were extracted and trophonts of *A. ocellatum* were stripped from the gills, with two washes of distilled water. The water from the washes was then centrifuged to collect the parasite. Trophonts were washed three times with distilled water to remove mucus and other debris and preserved in ethanol 70%, until further use. DNA extraction

was performed using two different commercial kits available, the DNeasy Blood and tissue kit, from Qiagen and the FastDNA spin kit for soil, from MP Biomedicals, following manufacturer's instructions, with this last one being the one that provided the best results. DNA quality and quantity was assessed using a NanoDrop spectrophotometer (Thermo Scientific). A total of 100 ng of the extracted DNA was used to amplify a fragment of the *Amyloodinium ocellatum* small subunit ribosomal RNA gene (GenBank accession number: DQ490256). For the first PCR amplification we used the combination of AOce_Fw1 – 5' TAGATGTTCTGGGCTGCACG 3', AOce_Rv2 – 5' CCTACGGAAACCTTGTTACGAC 3' and Taq DNA polymerase (Thermo Fisher Scientific) with the following conditions: 3 minutes at 94ºC, 35 cycles of amplification (45 seconds at 94ºC, 30 seconds at 55ºC and 30 seconds at 72ºC) and a final step of amplification during 10 minutes at 72ºC. The product of the first amplification (5 µl) was used as a template for a second amplification using the primer's combination AOce_Fw2 - 5' GACCTTGCCCGAGAGGG 3' (Levy et al., 2007) and AOce_Rv1 – 5' CCGCCACAGTTTTCAGAAGC 3' and the conditions previously described. The result of the second PCR was loaded in a 1.5% agarose gel and the fragment of approximately 220 bp was excised, purified using the Gene Jet Gel extraction kit (Thermo Fisher Scientific), cloned and sequenced at CCMAR's Sequencing Platform, with an Applied Biosystems 3130xl Genetic Analyzer, BigDye®Terminatorv3.1 chemistry and POP7 polymer.

Results and discussion

At the beginning of the epizootic infestation, fish started to develop behavioral changes (e.g. scratching their skin against the bottom of the tank and feeble movements, characteristic of hypoxic fish, stopped feeding and started positioning close to the water

entries). No uncommon external features in sea bass infested by *A. ocellatum* were found except excessive mucus production and slight discoloration of the gills.

Under microscope observations, gill filaments showed spherical to oval, dark brown trophonts dispersed between and inside them. On the histology slides, we can easily confirm the presence of the three life stages of *Amyloodinium ocellatum* in the gills (Figure 1A): dinospores (Din), a free living state with 11.6 µm length and 11.7 µm width (Landsberg et al., 1994); trophonts (Tr), a parasitic state that has a nonpigmented pyriform shape with starch granules, vacuoles, large nucleus, a stomopode and attachment rhizoids, with approximately 100-350 µm, and that attaches to gills and skin; and tomonts (Tm), a 150-350 µm cyst that develops after the trophont leaves the fish (Lawler, 1980), with the ability to produce up to 256 dinospores in three days at 25 ºC, each one capable to infect a new host and produce a trophont (Brown and Hovasse, 1946).

Histopathological examination of the gills showed large parasites attached to the filaments between the lamellae and varying degrees of epithelial lesions (Figures 1B and 1C). Epithelial hyperplasia and hypertrophy of the primary and secondary lamellae was observed, with fusion of secondary lamellae, vacuolization and lifting of the lamellar epithelium were extensive throughout the length of the gill filament and resulted in destruction and necrosis of the lamellar structure of the gill (Figure 1).

The injuries observed, depending on the intensity, may be reversible if the parasite is detected and adequately treated on time. Still, it is highly important to keep the broodfish under controlled conditions and these should be optimized by appropriated management and husbandry practices.

Concerning the molecular characterization of the parasite, we were able to amplify a fragment of 225 bp (Figure 2; GenBank accession number MG768977 and MG768978),

that shared 99% identity with the small subunit ribosomal RNA (SSU) gene from *A. ocellatum* (GenBank accession number KU761581). It was also possible to amplify another sequence, possibly because of the high conservation of the SSU ribosomal RNA across species or to a unspecific binding of the primers, that was identified as a partial 18S ribosomal RNA sequence (98%) from a Colpodellid (GenBank accession number MG770590), a parasite of protists and algae which is described to have an apical complex and a complex life cycle (Brugerolle, 2002). Colpodellids live mostly in freshwater and marine habitats, however not much is known about their predatory behavior, in particular in marine parasites (Mylnikov and Mylnikova, 2008). Although it was not evident in the histological observation of the gills, most likely due to its small size (ten times smaller than *A. ocellatum* trophonts), it was possible to co-amplify *A. ocellatum* and Colpodellid sequences. Thus, it would be interesting to evaluate how these two parasites interact, especially if we consider that Colpodellids can potentially parasitize *A. ocellatum*. In this sense it would be important to verify in a future work whether these small predatory flagellates can act as potential controllers for *A. ocellatum* infestations.

Data presented in this report evidenced for the first time an infestation of *A. ocellatum* in a sea bass broodstock in Portugal, confirmed by molecular tools. Amyloodiniosis can severely impact on aquaculture production, thus we intend to create awareness for the need to implement hygiene and disinfection measures (e.g. filtration of the water) that, in most cases, can prevent the infection provoked by this parasite. Also the presence of a Colpodellid in an *Amyloodinium ocellatum* infection is a novelty, and the interaction of these parasites may be worth to evaluate in future studies to understand if Colpodellids may be used as preventive agents against Amyloodiniosis.

Funding

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Figure's caption

Figure 1 – Histological section of H&E stained gills from European seabass (Dicentrarchus labrax) during an Amyloodinium ocellatum infestation. A (20x) represent the different life stages of Amyloodinium ocellatum observed in the gill: dinospores (Din), trophonts (Tr) and a tomont (Tm). B (40x) and C (20x) represent gills with parasite trophonts (8) and several histopathological alterations: lifting of the lamellar epithelium (1), hypertrophy and hyperplasia of the lamellar epithelium (2), primary (4) and secondary lamellae (5), with vacuolization (3, 6), and fusion of secondary lamellae (7).

Figure 2 – Gel electrophoresis of *Amyloodinium ocellatum* fragments amplified by PCR. *Lane 1*, 100 bp plus DNA ladder, *lane 2*, first reaction amplification product showing a fragment of 336 base pairs (bp); *lane 3*, second reaction amplification product showing a fragment of 225 bp.