

Manuscript Details

Manuscript number	AQREP_2019_8_R1
Title	Report and genetic identification of <i>Amyloodinium ocellatum</i> in a sea bass (<i>Dicentrarchus labrax</i>) 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	<i>Amyloodinium ocellatum</i> ; <i>Dicentrarchus labrax</i> ; Fish parasites; Colpodellids; broodstock;
Manuscript category	Genetics, Pathology and Health
Corresponding Author	Cátia Marques
Order of Authors	Cátia Marques, Ana Medeiros, Márcio Moreira, Hugo Quental-Ferreira, Ana Candeias-Mendes, Pedro Pousão-Ferreira, Florbela Soares
Suggested reviewers	Isabel Bandin, Carmen Sarasquete, Ana Roque

Submission Files Included in this PDF

File Name [File Type]

Cover letter.docx [Cover Letter]

Response to reviewers.docx [Response to Reviewers]

Highlights.docx [Highlights]

Manuscript_final.docx [Manuscript File]

Submission Files Not Included in this PDF

File Name [File Type]

Figure 1.png [Figure]

Figure 2.png [Figure]

To view all the submission files, including those not included in the PDF, click on the manuscript title on your EVISE Homepage, then click 'Download zip file'.

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 dinoflagellate *Amyloodinium ocellatum* in a seabass broodstock 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 alignment 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 distilled 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 than 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;

1
2
3
4 1 **Report and genetic identification of *Amyloodinium ocellatum* in a sea**
5
6 2 **bass (*Dicentrarchus labrax*) broodstock in Portugal**
7

8
9 3 Cátia L. Marques^{*a}, Ana Medeiros ^a, Márcio Moreira ^a, Hugo Quental-Ferreira ^a, Ana C.
10
11 4 Mendes ^a, Pedro Pousão-Ferreira ^a, Florbela Soares ^a
12

13
14 5 ^a IPMA - Portuguese Institute of the Sea and Atmosphere EPPO - Aquaculture Research
15
16 6 Station, Av. 5 de Outubro s/n, 8700-305 Olhão, Portugal
17

18 7 [*catia.marques@ipma.pt](mailto:catia.marques@ipma.pt)
19
20 8

21
22 9 **Abstract**
23

24 10 In this paper we report a case of amyloodiniosis in a sea bass (*Dicentrarchus labrax*)
25
26 11 broodstock in Portugal. Microscopic examination of gill filaments showed the presence
27
28 12 of trophonts while histological observation revealed gills epithelial hyperplasia,
29
30 13 hypertrophy and lamellar fusion of secondary lamellae. The amplification and
31
32 14 sequencing of the small subunit ribosomal RNA gene allowed the identification of the
33
34 15 parasite as *Amyloodinium ocellatum*. It was also possible to amplify a partial sequence
35
36 16 of ribosomal RNA from a Colpodellid, a predator of protists.
37
38 17

39
40
41
42 18 **Key words**
43

44 19 *Amyloodinium ocellatum*; *Dicentrarchus labrax*; *Fish parasites*; Colpodellids;
45
46 20 broodstock;
47
48 21

60
61
62 **23 Introduction**
63

64 24 A basic requirement of intensive farming for any fish species is a constant supply of
65
66 25 good quality eggs. Therefore, it is essential to maintain captive broodfish under
67
68 26 controlled conditions in order to produce eggs and larvae with the highest quality and,
69
70 27 consequently, that can reach a higher market value (Bromage, 1995). However,
71
72 28 sometimes, several diseases caused by parasites can occur and compromise the expected
73
74 29 output. Among them is a disease provoked by the dinoflagellate *A. ocellatum* (Paperna
75
76 30 1980), that can be an important factor limiting aquaculture productivity (Soares et al.,
77
78 31 2012), particularly in estuaries and semi-intensive aquaculture systems, where outbreaks
79
80 32 can rapidly occur, resulting in massive mortalities (Alvarez-Pellitero et al., 1993).
81
82

83 33 *A. ocellatum* is a non-specific extremely prolific and devastating dinoflagellate fish
84
85 34 parasite and the disease caused by this organism is commonly referred as
86
87 35 amyloodiniosis or marine velvet disease (Kumar et al., 2015). It mainly infect gills, and
88
89 36 less frequently the skin (fins and body) and buccal cavity of the host fish (Kumar et al.,
90
91 37 2015). Paperna (1980) described, in the region of Eliat, in the Red Sea, outbreaks of *A.*
92
93 38 *ocellatum* in reared breeders of sea bass. This parasite has also been reported in sea bass
94
95 39 breeders in Italy, but their presence did not cause serious mortalities in any broodstock
96
97 40 (Giavenni, 1988). In the Mediterranean area and the Red Sea, the parasite also produced
98
99 41 a massive mortality in sea bass juveniles (Alvarez-Pellitero et al., 1993). *D. labrax*
100
101 42 broodstock outbreaks infested with amyloodiniosis have never been reported in
102
103 43 Portugal, and were only reported in cultured juveniles of seabass and in natural
104
105 44 population of sea bass juveniles from the Óbidos coastal lagoon and the Sado estuary
106
107 45 (Menezes, 2000).

108
109 46 The present communication describes a case of *amyloodiniosis* in sea bass broodstock
110
111 47 maintained at the Olhão Aquaculture Research Station of the Portuguese Institute of Sea
112
113
114
115
116
117
118

119
120
121 and Atmosphere (IPMA-EPPO) and reports the molecular identification of the parasite
122
123 causing this infestation as being the dinoflagellate *A. ocellatum*.
124

125
126
127
128 **Material and methods**
129

130 The sea bass broodstock was composed by 24 individuals, with an average weight of
131
132 5165,17 g \pm 920,27 g and an average length of 76,41 cm \pm 3,84 cm, that were kept in a
133
134 18m³ tank under natural conditions of light and temperature, with continuous sand
135
136 filtered water inflow (5 m³h⁻¹) and aeration. At the time of epizootic, stocking density
137
138 was 7.6 kg.m⁻³, salinity was 38 psu and mean water temperature was 24°C.
139

140 After identification of *A. ocellatum*, infestation was contained by repeated treatments of
141
142 1.5 g.m⁻³ copper sulfate, applied for 11 days. Gill filaments from dead fish were
143
144 preserved in 10% neutral buffered formalin for 24 hours, and then transferred to ethanol
145
146 70% for histology purposes. Dehydration, clearing, infiltration and embedding were
147
148 performed according to standard procedures using a tissue processor (Leica TP1020)
149
150 and a paraffin dispenser (Leica EG 1140 H) , followed by thin sectioning (5 μ m thick)
151
152 with a micrometer (Leica RM-2155) and staining using haematoxylin eosin (Martoja,
153
154 1967). Slides were observed on a Nikon H550S microscope using bright-field
155
156 illumination. Selected gill tissues were then scanned in a Hamamatsu Nano Zoomer
157
158 Digital Pathology, and representative images were taken and processed using NDP
159
160 View 2 software.
161
162

163 Moribund fish were euthanized by cutting the spinal cord immediately posterior to the
164
165 head. Gills were extracted and trophonts of *A. ocellatum* were stripped from the gills,
166
167 with two washes of distilled water. The water from the washes was then centrifuged to
168
169 collect the parasite. Trophonts were washed three times with distilled water to remove
170
171 mucus and other debris and preserved in ethanol 70%, until further use. DNA extraction
172
173
174
175
176
177

178
179
180 73 was performed using two different commercial kits available, the DNeasy Blood and
181
182 74 tissue kit, from Qiagen and the FastDNA spin kit for soil, from MP Biomedicals,
183
184 75 following manufacturer's instructions, with this last one being the one that provided the
185
186 76 best results. DNA quality and quantity was assessed using a NanoDrop
187
188 77 spectrophotometer (Thermo Scientific). A total of 100 ng of the extracted DNA was
189
190 78 used to amplify a fragment of the *Amyloodinium ocellatum* small subunit ribosomal
191
192 79 RNA gene (GenBank accession number: DQ490256). For the first PCR amplification
193
194 80 we used the combination of AOce_Fw1 – 5' TAGATGTTCTGGGCTGCACG 3',
195
196 81 AOce_Rv2 – 5' CCTACGGAAACCTTGTTACGAC 3' and Taq DNA polymerase
197
198 82 (Thermo Fisher Scientific) with the following conditions: 3 minutes at 94°C, 35 cycles
199
200 83 of amplification (45 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C) and a
201
202 84 final step of amplification during 10 minutes at 72°C. The product of the first
203
204 85 amplification (5 µl) was used as a template for a second amplification using the
205
206 86 primer's combination AOce_Fw2 - 5' GACCTTGCCCGAGAGGG 3' (Levy et al.,
207
208 87 2007) and AOce_Rv1 – 5' CCGCCACAGTTTTTCAGAAGC 3' and the conditions
209
210 88 previously described. The result of the second PCR was loaded in a 1.5% agarose gel
211
212 89 and the fragment of approximately 220 bp was excised, purified using the Gene Jet Gel
213
214 90 extraction kit (Thermo Fisher Scientific), cloned and sequenced at CCMAR's
215
216 91 Sequencing Platform, with an Applied Biosystems 3130xl Genetic Analyzer,
217
218 92 BigDye®Terminatorv3.1 chemistry and POP7 polymer.
219
220
221
222
223
224

225 94 **Results and discussion**

226
227 95 At the beginning of the epizootic infestation, fish started to develop behavioral changes
228
229 96 (e.g. scratching their skin against the bottom of the tank and feeble movements,
230
231 97 characteristic of hypoxic fish, stopped feeding and started positioning close to the water
232
233
234
235
236

237
238
239 98 entries). No uncommon external features in sea bass infested by *A. ocellatum* were
240
241 99 found except excessive mucus production and slight discoloration of the gills.
242
243 100 Under microscope observations, gill filaments showed spherical to oval, dark brown
244
245 101 trophonts dispersed between and inside them. On the histology slides, we can easily
246
247 102 confirm the presence of the three life stages of *Amyloodinium ocellatum* in the gills
248
249 103 (Figure 1A): dinospores (Din), a free living state with 11.6 μm length and 11.7 μm
250
251 104 width (Landsberg et al., 1994); trophonts (Tr), a parasitic state that has a nonpigmented
252
253 105 pyriform shape with starch granules, vacuoles, large nucleus, a stomopode and
254
255 106 attachment rhizoids, with approximately 100-350 μm , and that attaches to gills and skin;
256
257 107 and tomites (Tm), a 150-350 μm cyst that develops after the trophont leaves the fish
258
259 108 (Lawler, 1980), with the ability to produce up to 256 dinospores in three days at 25 °C,
260
261 109 each one capable to infect a new host and produce a trophont (Brown and Hovasse,
262
263 110 1946).
264
265 111 Histopathological examination of the gills showed large parasites attached to the
266
267 112 filaments between the lamellae and varying degrees of epithelial lesions (Figures 1B
268
269 113 and 1C). Epithelial hyperplasia and hypertrophy of the primary and secondary lamellae
270
271 114 was observed, with fusion of secondary lamellae, vacuolization and lifting of the
272
273 115 lamellar epithelium were extensive throughout the length of the gill filament and
274
275 116 resulted in destruction and necrosis of the lamellar structure of the gill (Figure 1).
276
277 117 The injuries observed, depending on the intensity, may be reversible if the parasite is
278
279 118 detected and adequately treated on time. Still, it is highly important to keep the
280
281 119 broodfish under controlled conditions and these should be optimized by appropriated
282
283 120 management and husbandry practices.
284
285 121 Concerning the molecular characterization of the parasite, we were able to amplify a
286
287 122 fragment of 225 bp (Figure 2; GenBank accession number MG768977 and MG768978),
288
289
290
291
292
293
294
295

296
297
298 123 that shared 99% identity with the small subunit ribosomal RNA (SSU) gene from *A.*
299
300 124 *ocellatum* (GenBank accession number KU761581). It was also possible to amplify
301
302 125 another sequence, possibly because of the high conservation of the SSU ribosomal RNA
303
304 126 across species or to a unspecific binding of the primers, that was identified as a partial
305
306 127 18S ribosomal RNA sequence (98%) from a Colpodellid (GenBank accession number
307
308 128 MG770590), a parasite of protists and algae which is described to have an apical
309
310 129 complex and a complex life cycle (Brugerolle, 2002). Colpodellids live mostly in
311
312 130 freshwater and marine habitats, however not much is known about their predatory
313
314 131 behavior, in particular in marine parasites (Mylnikov and Mylnikova, 2008). Although
315
316 132 it was not evident in the histological observation of the gills, most likely due to its small
317
318 133 size (ten times smaller than *A. ocellatum* trophonts), it was possible to co-amplify *A.*
319
320 134 *ocellatum* and Colpodellid sequences. Thus, it would be interesting to evaluate how
321
322 135 these two parasites interact, especially if we consider that Colpodellids can potentially
323
324 136 parasitize *A. ocellatum*. In this sense it would be important to verify in a future work
325
326 137 whether these small predatory flagellates can act as potential controllers for *A.*
327
328 138 *ocellatum* infestations.
329
330
331
332 139 Data presented in this report evidenced for the first time an infestation of *A. ocellatum*
333
334 140 in a sea bass broodstock in Portugal, confirmed by molecular tools. Amyloodiniosis can
335
336 141 severely impact on aquaculture production, thus we intend to create awareness for the
337
338 142 need to implement hygiene and disinfection measures (e.g. filtration of the water) that,
339
340 143 in most cases, can prevent the infection provoked by this parasite. Also the presence of
341
342 144 a Colpodellid in an *Amyloodinium ocellatum* infection is a novelty, and the interaction
343
344 145 of these parasites may be worth to evaluate in future studies to understand if
345
346 146 Colpodellids may be used as preventive agents against Amyloodiniosis.
347
348
349 147
350
351
352
353
354

355
356
357 **148 Funding**
358

359 149 This work was supported by DIVERSIAQUA and SAUDE&AQUA projects
360
361 150 [MAR2020 16-02-01-FMP-0066 and Mar2020 02.05.01-FEAMP-0009, respectively].
362
363 151 Márcio Moreira has a PhD grant from FCT - Foundation for Science and Technology
364
365 152 (SFRH/BD/118601/2016). This study also received Portuguese national funds from
366
367 153 FCT - Foundation for Science and Technology through project UID/Multi/04326/2019.
368
369
370
371

372 **155 References**
373
374

- 375 156 Alvarez-Pellitero, P., Sitjà-Bobadilla, A., Franco-Sierra, A., 1993. Protozoan parasites of wild
376 and cultured sea bass, *Dicentrarchus labrax* (L.), from the Mediterranean area. *Aquac.*
377 *Fish. Manag.* 24, 101–108. doi:10.1111/j.1365-2109.1993.tb00832.x
378
- 379 159 Bromage, N., 1995. Broodstock Management and Seed Quality - General Considerations, in:
380 Bromage, N.R., Roberts, R.J. (Eds.), *Broodstock Management and Egg and Larval*
381 *Quality*. Wiley, pp. 1–24.
382
- 383 162 Brown, E.M., Hovasse, R., 1946. *Amyloodinium ocellatum*,. *Proc. Zool. Soc. London* 116, 33–
384 46. doi:10.1111/j.1096-3642.1946.tb00107.x
385
- 386 164 Brugerolle, G., 2002. *Colpodella vorax*: ultrastructure, predation, life-cycle, mitosis, and
387 phylogenetic relationships. *Eur. J. Protistol.* 38, 113–125. doi:10.1078/0932-4739-00864
388
- 389 166 Giavenni, R., 1988. Some Parasitic and other diseases occurring in Sea bass (*Dicentrarchus labrax*
390 L.) broodstocks in Italy. *Bull. Eur. Assoc. Fish Pathol.* 8, 45–46.
391
- 392 168 Kumar, P.R., Nazar, A.K.A., Jayakumar, R., Tamilmani, G., Sakthivel, M., Kalidas, C.,
393 Balamurugan, V., Sirajudeen, S., Thiagu, R., Gopakumar, G., 2015. *Amyloodinium*
394 *ocellatum* infestation in the broodstock of silver pompano *Trachinotus blochii* (Lacepede,
395 1801) and its therapeutic control. *Indian J. Fish.* 62, 131–134.
396
- 397 172 Landsberg, J.H., Steidinger, K.A., Blakesley, B.A., Zondervan, R.L., 1994. Scanning electron
398 microscope study of dinospores of *Amyloodinium cf. ocellatum*, a pathogenic
399 dinoflagellate parasite of marine fish, and comments on its relationship to the Peridiniales.
400 *Dis. Aquat. Organ.* 20, 23–32. doi:10.3354/dao020023
401
- 402 176 Lawler, A.R., 1980. Studies on *Amyloodinium ocellatum* (Dinoflagellata) in Mississippi sound:
403 experimental hosts. *Gulf Res. Reports* 6, 403–413. doi:10.18785/grr.0604.08
404
- 405 178 Levy, M.G., Poore, M.F., Colorni, A., Noga, E.J., Vandersea, M.W., Litaker, R.W., 2007. A
406 highly specific PCR assay for detecting the fish ectoparasite *Amyloodinium ocellatum*.
407 *Dis. Aquat. Organ.* 73, 219–26. doi:10.3354/dao073219
408
- 409 181 Martoja, R., 1967. *Initiation aux techniques de l'histologie animale*. Mason, Paris.
410
411
412
413

414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472

182 Menezes, J., 2000. Manual sobre doenças de peixes ósseos. Publicações Avulsas do IPIMAR.

183 Mylnikov, A.P., Mylnikova, Z.M., 2008. Feeding spectra and pseudoconoid structure in
184 predatory alveolate flagellates. *Inl. Water Biol.* 1, 210–216.
185 doi:10.1134/s1995082908030036

186 Paperna, I.H., 1980. *Amyloodinium ocellatum* (Brown, 1931) (Dinoflagellida) infestations in
187 cultured marine fish at Eilat, Red Sea: epizootiology and pathology. *J. Fish Dis.* 3, 363–
188 372. doi:10.1111/j.1365-2761.1980.tb00421.x

189 Soares, F., Quental-Ferreira, H., Moreira, M., Cunha, E., Ribeiro, L., Pousão-Ferreira, P., 2012.
190 First report of *Amyloodinium ocellatum* in farmed meagre (*Argyrosomus regius*). *Bull.*
191 *Eur. Assoc. Fish Pathol.* 32.

192

193

473
474
475 **194 Figure's caption**
476

477 **195 Figure 1** – Histological section of H&E stained gills from European seabass
478
479 (Dicentrarchus labrax) during an Amyloodinium ocellatum infestation. A (20x)
480
481
482 **197** represent the different life stages of Amyloodinium ocellatum observed in the gill:
483
484 **198** dinospores (Din), trophonts (Tr) and a tomont (Tm). B (40x) and C (20x) represent gills
485
486 **199** with parasite trophonts (8) and several histopathological alterations: lifting of the
487
488 **200** lamellar epithelium (1), hypertrophy and hyperplasia of the lamellar epithelium (2),
489
490 **201** primary (4) and secondary lamellae (5), with vacuolization (3, 6), and fusion of
491
492 **202** secondary lamellae (7).
493

494 **203**

495
496 **204 Figure 2** – Gel electrophoresis of *Amyloodinium ocellatum* fragments amplified by
497
498 **205** PCR. *Lane 1*, 100 bp plus DNA ladder, *lane 2*, first reaction amplification product
499
500 **206** showing a fragment of 336 base pairs (bp); *lane 3*, second reaction amplification
501
502 **207** product showing a fragment of 225 bp.
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531