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Amyloodiniosis in cultured *Dicentrarchus labrax*: parasitological and molecular diagnosis, and an improved treatment protocol

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ABSTRACT: *Amyloodinium ocellatum*, the causative agent of amyloodiniosis (marine velvet, velvet disease), affects marine and brackish fish in various warm and temperate habitats. We recorded disease outbreaks with high morbidity and mortality rates in marine-cultured European seabass *Dicentrarchus labrax* fry at 2 locations in northwest Egypt. The sudden outbreak, high morbidity and mortality rates, and skin lesions with a velvety appearance in affected fish all indicated *A. ocellatum* infection. This was further confirmed by microscopic findings of the parasitic stage (trophonts) in skin and gill smears. While ecological factors including water temperature and salinity were all amenable to parasite establishment and propagation, mortality rates differed between the 2 farms, with rates of mortality well correlated with prevalence and intensity of *A. ocellatum* infections. Characterization by PCR targeting rDNA gene fragments and subsequent DNA sequencing and phylogenetic analysis further confirmed the molecular identity of the *A. ocellatum* isolate, which was genetically similar to isolates from other geographical locations. Finally, an improved treatment method using dual hyposalination and copper sulfate exposure to increase the efficiency and decrease the toxicity of copper sulfate was tested. The gradual reduction in water salinity coupled with copper sulfate treatment was more efficient at controlling the disease than only applying copper sulfate. To our knowledge, this is the first parasitological and molecular characterization of *A. ocellatum* in marine cultures in Egypt. The high molecular identity and close phylogenetic relationship further confirmed the monophyletic nature of *A. ocellatum* isolates.

KEY WORDS: European seabass · *Dicentrarchus labrax* · Amyloodiniosis · PCR · Phylogenetic analysis · Copper sulfate treatment

INTRODUCTION

Dinoflagellates are an assemblage of autotrophic, myxotrophic, and heterotrophic species that play important roles in marine ecosystems. Most are free-living, but several are known to be symbionts of marine invertebrates or parasites of aquatic animals (Paperna 1984, Noga & Bower 1987). *Amyloodinium ocellatum* is an ectoparasitic dinoflagellate with a free-living phase first described by Brown (1931). The life cycle involves 3 developmental stages. In the first stage, trophonts feed on fish tissues, causing various disease lesions. After detaching from the host, parasites develop into the second stage, in which actively reproductive tomonts produce dinospores. In the last stage, dinospores are the free-floating spores that enter a new fish host to complete the life cycle (Noga & Bower 1987).

Amyloodiniosis is commonly known as velvet disease, or marine velvet, with affected fish showing a

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powdered (velvety) appearance, along with other symptoms including, but not limited to, loss of coordination and sporadic gasping. Parasites penetrate deep into host tissues using rhizoid-root like structures, causing severe damage at the attachment sites, particularly skin and gills (Francis-Floyd & Floyd 2011).

A. ocellatum infects a wide range of fish species in different habitats, including temperate and tropical geographical locations (Francis-Floyd & Floyd 2011). It is often considered the most consequential pathogen of marine fish and can cause serious morbidity and mortality at aquaculture facilities worldwide in brackish and marine fish (Paperna et al. 1981). It affects various fish species in the wild, as well as in culture and aquarium rearing systems. In severe disease epizootics, outbreaks can occur extremely rapidly, resulting in 100% mortality within a few days (Kuperman & Matey 1999).

Outbreaks of amyloodiniosis associated with considerable economic losses have been recorded in a broad range of fish species in various aquaculture settings, including hatcheries of milkfish *Chanos chanos* (Shinn et al. 2015), cultured gilthead seabream *Sparus aurata* and meagre *Argyrosomus regius* in Portugal (Soares et al. 2012), and seabass *Dicentrarchus labrax* reared in sea cages in the Adriatic Sea off the coast of Italy (Rigos & Troisi 2005, Abowei et al. 2011), with economic losses of hundreds of thousands US dollars in each outbreak.

Diagnosis of *A. ocellatum* infection depends on clinical signs and microscopic identification of trophonts (or recently divided tomonts) in skin and gill biopsies collected from infected fish (Bower et al. 1987, Noga & Bower 1987, Francis-Floyd & Floyd 2011). A more specific sensitive molecular assay has been developed based on specific amplification of the target rDNA region by PCR and subsequent phylogenetic analysis from a single parasite from the 3 developmental stages (trophont, tomonts, or dinospore) in samples of gill or water (Levy et al. 2007).

Different methods have been tested and applied in treating and controlling *A. ocellatum* infections in different aquaculture systems, including immunization, chemical and drug treatments, and optimizing aquatic water parameters such as salinity and temperature (Buchmann 2015). In general, treatment of amyloodiniosis involves breaking the parasite life cycle by killing the free-swimming dinospores before they can attach to a new fish host or by removing trophonts from the fish or killing them. The traditional treatment protocols include decreasing water salinity and applying chemical solutions such as formalin (Johnson 1990, Buchmann 2015).

Here we report natural infections of *A. ocellatum* as a cause of outbreaks associated with morbidity and mortalities in seabass *Dicentrarchus labrax* from 2 localities in northwest Egypt. In addition to the microscopic examination identifying the amyloodiniosis, molecular identification by PCR coupled with bioinformatics and phylogenetic analysis confirmed the presence of an *A. ocellatum* isolate that is genetically similar to isolates from other geographic locations. Moreover, an improved treatment regimen that included dual applications (hyposalination/freshwater bath in the morning coupled with copper sulfate at night) was successfully applied to stop or slow down infection and mortalities of affected fish.

MATERIALS AND METHODS

Study locations

Two aquaculture stations housing seabass *Dicentrarchus labrax* fry were affected by disease epidemics: one in Wadi El-Natroun district, northwestern Egypt (30° 26′ 19.5″ N, 30° 15′ 24.6″ E), and the second at El-Max station, Alexandria Governorate, in northern Egypt (31° 8′ 17.2″ N, 29° 49′ 52.4″ E). The study ran from April to September 2016. No histories of diseases or rearing problems were evident at the 2 sites prior to this study.

Fish and water sampling

A total of 720 fish were collected from each farm throughout the 6 mo study (approx. 120 fish mo^{-1}). Collected fry were apparently healthy, freshly dead, or moribund. Samples were examined as soon as possible after collection.

Concurrent to fish samples, water samples were also collected and were examined for temperature, salinity, dissolved oxygen, and ammonia levels by using electronic probes and an environmental testing photometer (Hanna Instruments), according to previously used methods (APHA 2005).

Clinical and macroscopic examination

Clinical and macroscopic examination was mainly performed according to Noga (2010). Observed clinical signs (e.g. 'piping' and gathering at the water surface) or other physical and behavioral changes were carefully recorded. Other clinical lesions visible to

the naked eye, such as hemorrhages, emaciation, deformities, and abrasions, on different body parts and fins were also noted. Skin surface, fins, and gills were examined by eye to detect any attached parasites, lesions, or external changes, as well as disease characteristics, e.g. skin lesions and noticeably cloudy or velvety appearance (Levy et al. 2007).

Microscopic examination

Microscopic examination was performed on skin and gill biopsies collected from affected fish for identification of parasite/trophonts following the routine clinical procedure used by Noga (2010), and as reviewed in detail by Francis-Floyd & Floyd (2011). Biopsies prepared by dissection under a dissecting microscope (Optika) were placed on a slide with a drop of aquarium water if necessary, covered with coverslips, and then examined fresh under a light microscope (Optika). Stained smears were obtained by staining thin biopsies according to a previously applied procedure (Bruno et al. 2006). Throughout the microscopic examination procedure, the most important consideration when examining or collecting biopsies was to avoid exposure of fish or biopsies to fresh water, which would most likely cause parasitic stages to fall off or disintegrate, leading to false negative results (Francis-Floyd & Floyd 2011).

Extraction of genomic DNA, PCR, and sequencing of rDNA-PCR products

Total genomic DNA was extracted from tissue specimens of fish suspected to be diseased (2 from each farm) using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, K0721) according to the manufacturer's instructions for extracting genomic DNA from tissue samples. Briefly, affected fry with disease lesions were preserved in 70% ethyl alcohol at −20°C, before an excision of 2 cm^2 spanning the skin and the underlying tissue was sampled into a microcentrifuge tube. The tissue specimen was then homogenized using a pellet pestle in 400 µl of lysis buffer. After complete homogenization, 40 µl of Proteinase K (20 mg ml^{-1}) were added, and the homogenate was incubated for 3 h. The non-digested parts were removed by centrifugation while the clear supernatant was transferred to the spin column of the purification kit. DNA genome purification was continued following the kit's instructions. Extracted genomic DNA was finally eluted with 200 µl of elution buffer, and the DNA quality and concentration were estimated using agarose gel electrophoresis and 100 bp DNA rulers as the ladder. To amplify a target-specific sequence of suspected *Amyloodinium ocellatum*, the primer pair of AO18SF (5′-GAC CTT GCC CGA GAG GG-3′) and AO18SR (5′-GAA AGT GTG GTG AAT CTT AAC-3′) was selected to amplify a 248 bp insert located in the small subunit (SSU) 3′ region of the rDNA gene as a specific assay to detect genomic DNA of *A. ocellatum* (Levy et al. 2007). PCR amplification was done using the DreamTaq™ master mix (Thermo Scientific) according to the manufacturer's protocol, and by using a GeneAmp thermal cycler (Creacon Thermocycler). Conditions for PCR amplification were as previously applied (Levy et al. 2007), with only the minor modification of using 55°C as the annealing temperature instead of 58°C.

For sequencing, the amplified PCR amplicon was purified, cloned, and sequenced in one direction using the forward primer (AO18SF), and by using the ABI PRISM® 3100 Genetic Analyzer (Micron) following the manufacturer's sequencing protocol.

To establish the molecular identity of the sequenced samples, complete nucleotide sequences after manual scanning and trimming junk 3′ and 5′ ends were subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Molecular identity and similarity to resultant top BLAST hits were further consolidated by aligning sequences using ClustalW in MEGA 7.0 software (Kumar et al. 2016). The molecular relationship with other *A. ocellatum* isolates was inferred using phylogenetic analysis based on the maximum likelihood (ML) method (Tamura & Nei 1993). Phylogenetic analysis and the resulting tree were validated by using the bootstrap test (1000 replicates) in MEGA 7.0 (Felsenstein 1985). The ML phylogenetic tree was obtained by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach (Nei & Kumar 2000), with evolutionary analyses conducted in MEGA 7.0 (Kumar et al. 2016).

Treatment of amyloodiniosis

Reported amyloodiniosis in this study was subjected to 3 simultaneous different treatment regimens following the methods described by Bower (1983) and Cardeilhac & Whitaker (1988) for the copper sulfate (Bluestone) treatment, and by Kingsford (1975) and Lawler (1977b) for the hyposalination treatment.

For the experimental design, 300 *D. labrax* (mean ±SD body mass: 23 ± 6.4 g) were distributed equally in 5 groups (60 fish each) in fiberglass tanks (1000 l capacity). All experimental groups were set up in triplicate. Fish were reared in a closed re-circulating unit supplied with sand filters and UV-treated, aerated sea water. Dissolved oxygen was maintained at 6 ± 2 mg l^{-1}, pH was 7.4–8.8, and salinity averaged 33 ± 1 ppt; a 12:12 h light:dark cycle was kept throughout. Water parameters were monitored following standard methods of water and waste water examination (APHA 2005) and maintained within the permissible limits during the trial. The fish were fed twice a day with a commercial pellet containing 40% crude protein at 3–5% body weight.

Experimental groups were subjected to different treatment regimens as follows. The first and second groups (T1 and T2) were apparently healthy (negative controls) and naturally infected fish (positive controls), respectively. The third (T3) group was naturally infected and treated with copper sulfate (Bluestone) applied at a concentration of 0.30 mg l^{-1} in a daily routine of 2 h baths for 14 d. The fourth group (T4) was subjected to a daily (morning) routine of hyposalination/freshwater bath for 2 wk. Finally, fish in the fifth group (T5) were subject to a dual routine (hyposalination/freshwater bath in the morning coupled with copper sulfate at night), achieved via a gradual desalination program followed by copper sulfate bath. This was accomplished by replacing seawater with dechlorinated freshwater (freshwater bath) to gradually lower the salinity (~5 ppt d^{-1}) until ~10 ppt salinity was reached (within 4–5 d); this hyposalination routine was then followed by copper sulfate bath treatment applied at a concentration of 0.10 mg l^{-1} in a daily routine of a 4 h bath for 14 d. Experimental steps and processing were performed following the universal directive on the protection of animals used for scientific purposes.

Toward the end of each treatment, prevalence rate, mortality rate, and the presence/absence of infection in tissue sample biopsies (skin/gill) were carefully observed and recorded, and the efficiencies of single treatments (copper sulfate or hyposalination) versus dual treatments (desalination/fresh water bath coupled with copper sulfate) were compared at the end of the treatment routines.

Throughout the experiment, water conditions (temperature, dissolved oxygen, pH, and salinity) were kept at the recommended ranges (www.fao.org/docrep/field/003/AB707E/AB707E09.htm) in the closed seawater system with artificial aeration, and ~25% of the water volume was changed daily. Data collected for the different experimental groups were analyzed with a chi-squared test using the SAS package (SAS 2004).

RESULTS

Clinical picture of amyloodiniosis

Observed clinical signs on fish from 2 localities included aggregation either at the water surface or oxygen source, poor appetite, and sluggish movement. Signs of respiratory distress were also noted, including piping behavior and rapid opercular movement ('gilling'). Macroscopic examination of moribund or dead fry showed debilitation and congested gills. The skin had a hazy, velvety appearance with dark/brown discoloration, which is more evident when using the indirect lighting of a flashlight (Fig. 1). Despite these obvious clinical and postmortem signs in moribund and dead fish, many fish were found dead with no obvious gross skin lesions.

Detection of *Amyloodinium ocellatum*

Microscopic examination of skin and gill tissue smears (both non-stained and Giemsa-stained) of moribund and freshly dead fish revealed a high prevalence of *A. ocellatum* infection (Figs. 2 & 3). Trophont stages of slightly variable size could be easily detected in the gill and skin tissue specimens from infected *Dicentrarchus labrax*, with sizes varying from as small as ~150 µM to large trophonts up to ~350 µM (Figs. 2 & 3). *A. ocellatum* infections in different tissue preparations were recorded with different intensities ranging from light (<10 trophonts per examination field) to moderate (10–20 trophonts) and severe infection (>20 trophonts) (Tables 1 & 2)

Occurrence of amyloodiniosis

The 2 localities (Wadi El-Natroun and El-Max) were compared with regard to disease (amyloodiniosis) parameters that included prevalence rate, infection intensity, and rate of mortality during the experimental period (Tables 1 & 2). Average prevalence rates were 84.86 and 39.58%, while average mortality rates were 42.78 and 9.86% in Wadi El-Natroun and El-Max, respectively. In Wadi El-Natroun, relatively high prevalence rates were constant throughout the 6 mo experiment (62.5–100%), with the highest rates

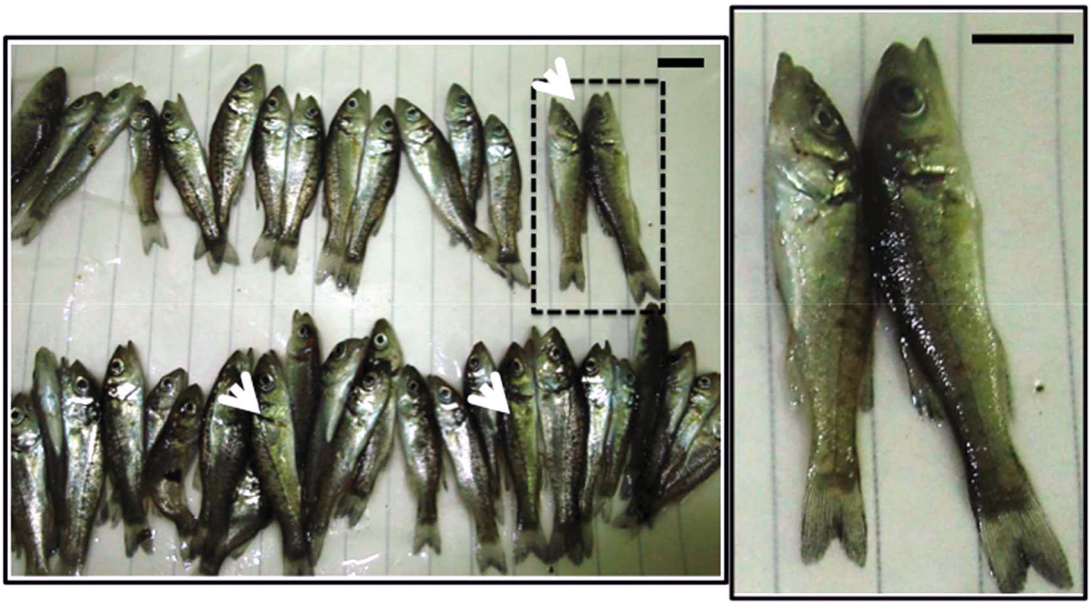

Fig. 1. European seabass infected by the dinoflagellate ectoparasite *Amyloodinium ocellatum*. The seabass fry show a velvet sheen (arrowhead) due to the heavy skin infection with parasites, enhanced by using indirect illumination. Scale bars = 1 cm

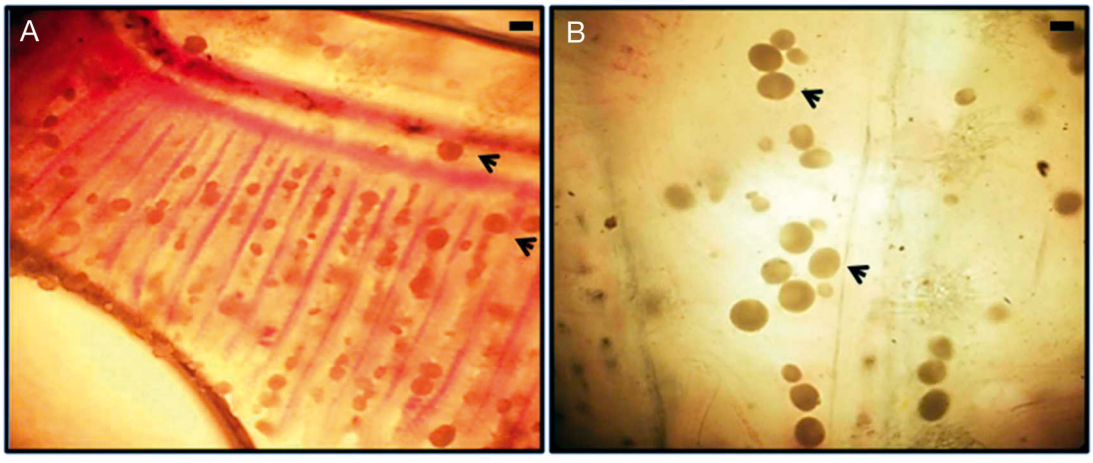

Fig. 2. Smears (non-stained) from European seabass (A) gill and (B) skin showing trophonts of *Amyloodinium ocellatum* (arrowheads). Scale bars = 300 µM

recorded in April and August. Mortality rates were in accordance with prevalence rates, which also peaked in April and August. On the other hand, fish at El-Max had relatively low prevalence rates with no evident mortalities throughout the first months, but with surprisingly high rates during the last 2 mo (Tables 1 & 2). In both cases, the intensities of infections correlated well with prevalence and mortality rates, with severe infection intensity (>20 trophonts per examination field) always associated with high mortality rates.

Different physico-chemical properties of water, including temperature, salinity, oxygen tension, oxygen saturation, and ammonia readings, were very similar between the 2 locations (Appendix 1).

Molecular identification

A. ocellatum-specific PCR applied to affected fish samples from 2 localities consistently produced a specific band when tested with agarose gel electrophoresis (Fig. 4). The produced amplicons were located between 200 and 300 bp, which is consistent with a 248 bp *A. ocellatum*-specific amplicon obtained in a previous study (Levy et al. 2007), using

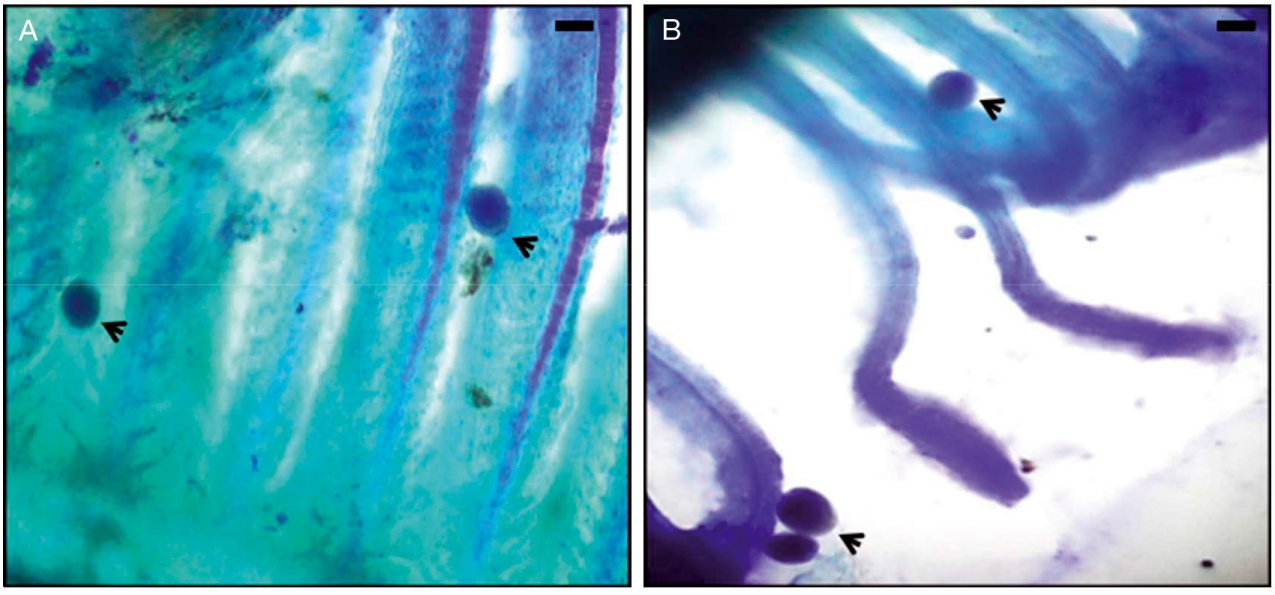

Fig. 3. Giemsa-stained European seabass (A) gill and (B) skin tissue preparations showing trophonts of *Amyloodinium ocellatum* (arrowheads). Scale bars = 300 µM

the same PCR primer combination. To corroborate the molecular identity of the amplified PCR product, DNA sequencing associated with manual scanning and trimming of junk and unidentified nucleotides from 3′ and 5′ ends identified a 237 bp sequence. When this sequence was applied in the nucleotide BLAST analysis, several hits were returned, with the top 15 hits being SSU rRNA gene sequences from different *A. ocellatum* strains from the Mediterranean Sea, Adriatic Sea (Italy), Rea Sea, Gulf of Mexico ('clone FL'), and DC1 isolates, with identities of 97–100%, query sequence coverage of >70%, and a minimum authentic E-value of 1×10^{-25} (data not

Table 1. Occurrence of *Amyloodinium ocellatum* in naturally infected European seabass from Wadi El-Natroun farm. The degree (intensity) of infection was categorized as severe (+++, >20 trophonts per examination field); moderate (++, 10–20 trophonts), or light (+, <10 trophonts). The prevalence rate was calculated based on the number of examined fish

Month	Infection intensity Skin	Gill	Prevalence rate (%)	Mortality rate (%)
April	++	+++	100	50.83
May	+	++	71.67	33.33
June	++	+++	81.67	37.5
July	++	+++	93.33	48.33
August	++	+++	100	55
September	+	++	62.5	31.67

Table 2. Occurrence of *Amyloodinium ocellatum* in naturally infected *Dicentrarchus labrax* in El-Max farm. Details as in Table 1

Month	Infection intensity Skin	Gill	Prevalence rate (%)	Mortality rate (%)
April	+	+	10	0
May	+	+	15	0
June	+	+	22.5	0
July	+	+	19.17	0
August	++	+++	80.83	22.5
September	++	+++	90	36.67

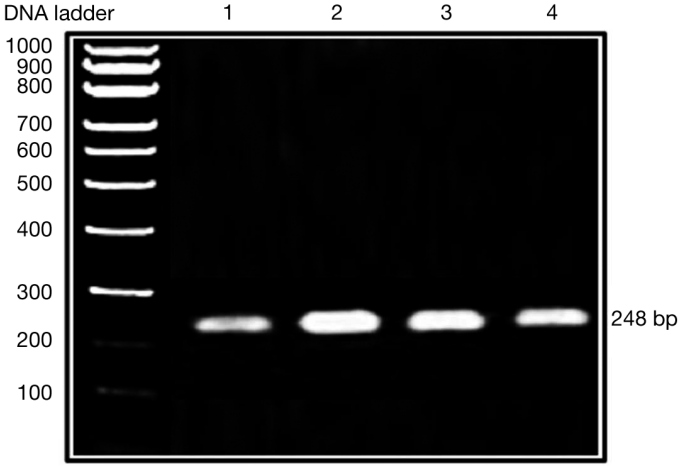

Fig. 4. PCR assay for the detection of *Amyloodinium ocellatum* infection in European seabass. The first lane is the 100 bp DNA ladder. Lanes 1–4 represent samples from 2 locations (1,2 = Wadi El-Natroun; 3,4 = El-Max). The PCR products shown correspond to the predicted molecular mass of 248 bp

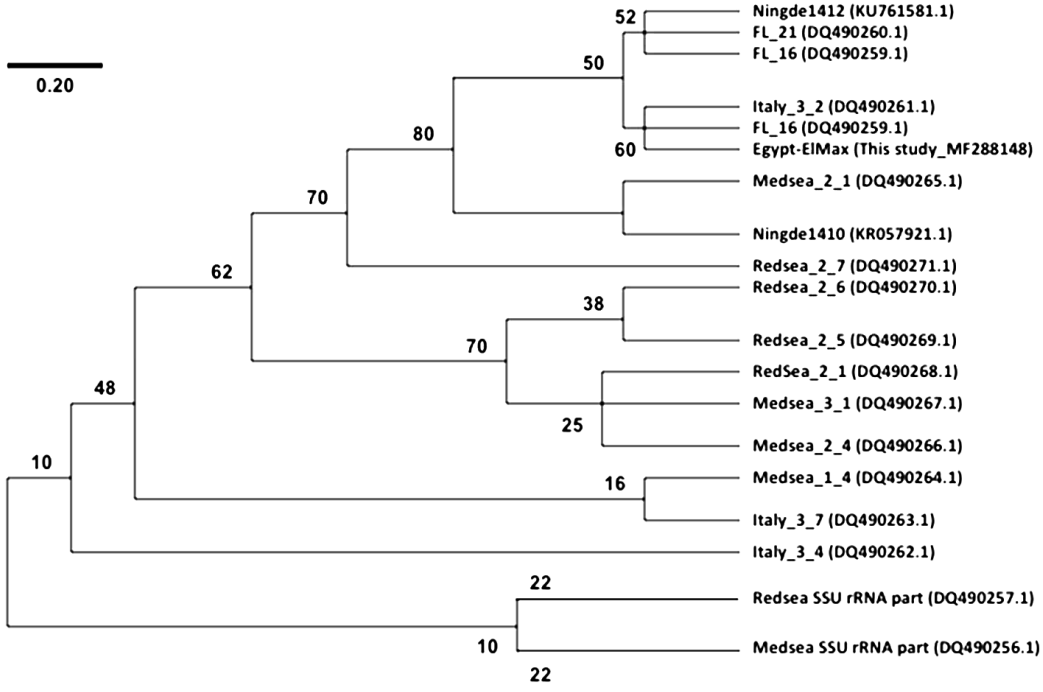

Fig. 5. Molecular phylogenetic analysis of *Amyloodinium ocellatum*. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (Tamura & Nei 1993). The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein 1985). Branches corresponding to partitions reproduced in <50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (as indicated by the scale bar). The analysis involved 19 nucleotide sequences. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). Medsea: Mediterranean Sea; Redsea: Red Sea; FL: Gulf of Mexico off Florida, USA; Italy: Adriatic Sea, Italy; Ningde: Fujian isolate, China

shown). We named the sequence in this study 'Egypt-ElMax' in reference to the isolate's geographic location, and it was deposited in GenBank under accession number MF288148. The full sequence is provided in Appendix 2.

Molecular identification and relation to other *A. ocellatum* isolates were further established when a phylogenetic tree placed Egypt-ElMax in very close proximity to *A. ocellatum* isolates from other geographical locations (Fig. 5). Again and similar to previously published data (Levy et al. 2007), no specific groupings or out-groupings to indicate different species or subspecies among tested isolates were evident after the phylogenetic analysis.

Treatment of amyloodiniosis

We achieved a marked improvement in the health status of fish following the 3 treatments, albeit with different efficiencies as revealed by relative differences in prevalence rates and cumulative mortalities at the end of each treatment (Table 3). In T3 (copper sulfate), ~12% cumulative mortality was registered. This was slightly better than in T4 (hyposalination/freshwater bath), for which a higher cumulative mortality rate of 15% was recorded. Nevertheless, and despite controlling mortalities in both cases, parasite stages could still be detected in tissue biopsies of some fish samples, though with much lower intensities when compared with pre-treatment samples (Table 3). The use of the dual hyposalination + copper sulfate (T5) was more efficient when compared to previous regimens as shown by both the low rate of cumulative mortality (12%), as well as the absence of parasite stages in the examined tissue biopsies of both skin and gill smears of fish (Table 3).

DISCUSSION

The current outbreak of *Amyloodinium ocellatum* occurred in cultured seabass *Dicentrarchus labrax*, which is one of the most popular marine fish species cultured in Egypt. Similar outbreaks were previously recorded in *D. labrax* in sea cages in the Adriatic Sea

Table 3. Amyloodiniosis treatment in European seabass; data were recorded at the end of the treatment routine and are cumulative. Experimental groups were set up in triplicate. Examined fish were recorded as either positive (+; see Table 1 for categories of infection intensity) when ≥1 parasitic trophont was detected, or negative (–) when no trophonts could be detected by microscopic examination. *p < 0.05

Experimental group (n = 60 in each)	Infection intensity Skin	Gill	Cumulative prevalence (%)	Cumulative mortality (%)
T1 (negative control)	–	–	1.67	1.67
T2 (positive control)	++	+++	76.67*	65
T3 (copper sulfate)	+	+	26.67*	11.67*
T4 (freshwater bath)	+	+	40*	15
T5 (freshwater bath + copper sulfate)	–	–	20*	11.67*

(Rigos et al. 1998, Rigos & Troisi 2005, Abowei et al. 2011). Moreover, *A. ocellatum* can infest both elasmobranchs and teleosts and has continued to be one of the most serious impediments to warmwater mariculture (Lawler 1980, Noga & Bower 1987, Kuperman & Matey 1999).

Our clinical diagnosis revealed that the affected seabass from the 2 investigated farms exhibited behavioral changes and clinical signs characteristic of *A. ocellatum* infection, especially signs of respiratory distress and dark/brown discoloration (velvety appearance) of the skin (Levy et al. 2007, Francis-Floyd & Floyd, 2011, Soares et al. 2012).

In addition to the clinical evidence, the amyloodiniosis outbreak was further confirmed by the microscopic detection of *A. ocellatum* trophonts, which has been the standard routine method of diagnosing the disease (Noga & Levy 1995). Under microscopy, it is normal to see different sizes of parasitic stages reflecting the developmental stages of *A. ocellatum* (Noga & Levy 1995). We did not further investigate other life cycle stages, e.g. tomonts and dinospores, as we assumed their propagation in the ecological environments of the 2 studied farms, and as we preferred to perform molecular diagnosis. The absence of any other types of parasites and/or protozoa in skin and gill tissue after microscopy further confirmed amyloodiniosis as the current disease outbreak.

The affected farms both showed disease morbidity and mortalities, albeit with varying rates. Clinical signs of amyloodiniosis and microscopic detection of *A. ocellatum* were evident at both sites. However, higher prevalence and mortality rates and more severe parasitic infections were observed at Wadi El-Natroun than at El-max. This is difficult to explain in light of the similarities of the rearing systems between the 2 sites, particularly because the physicochemical properties were very similar. The only difference was the more intensive system of rearing at Wadi El-Natroun, with subsequent heavier organic matter aggregation, which might provide less favorable ecological conditions for fish and a more hospitable environment for parasite propagation and infection. Adverse ecological conditions can alter both the physiological (Colorni et al. 2008, Noga et al. 2011) and immunological (Alvarez-Pellitero et al. 1993, Smith et al. 1994, Woo 2007, Woo & Ardelli 2014) responses of the fish host to *A. ocellatum*. Propagation and infection of the parasite are resistant to adverse ecological and aquatic conditions, and the parasite tolerates a broad range of salinity and temperature (Kuperman & Matey 1999). Taken together, this might explain the higher prevalence and mortality rates in Wadi El-Natroun when compared to El-Max. Nevertheless, the environmental factors of temperature, salinity, and ammonia levels in the 2 farms were favorable to the growth and propagation of the parasite. At an average temperature of 26°C and a salinity range of 28–37, the life cycle of *A. ocellatum*, from dinospore multiplication to attachment and infection, can be completed in as short as 3–6 d (Paperna 1984). High correlations between prevalence rates and mortalities were registered at both locations. Mortalities due to amyloodiniosis are most likely attributed to anoxia associated with serious gill hyperplasia, inflammation, hemorrhage, and necrosis (Lawler 1980) and/or osmoregulatory impairment and secondary microbial infections due to severe epithelial damage (Noga et al. 1991).

Molecular identification by PCR confirmed the parasitological findings and again proved to be a highly specific technique for the detection of *A. ocellatum* infestation (Levy et al. 2007). ITS-specific PCR has been successfully applied in detecting *A. ocellatum*, similar to other species of dinoflagellates (Litaker et al. 2003). The DNA sequence of the partial SSU rRNA gene fragment is highly identical to ITS sequences of *A. ocellatum* isolates from geographic regions as diverse as the Gulf of Mexico, Rea Sea, Adriatic Sea, and Mediterranean Sea (Levy et al. 2007). The phylogenetic assay also placed the current isolate in close association with other geographical isolates. Taken together, molecular data strongly point to the monophylogeny and that different isolates are indeed subspecies/strains of a single species of *A. ocellatum* (Litaker et al. 1999). Although the phylogenetic infer-

ence of a close relationship between the current isolate and other *A. ocellatum* isolates was obtained by aligning only a partial SSU rRNA sequence (237 bp), the results would likely be very similar if we applied full sequences in a similar analysis.

Based on PCR and subsequent phylogenetic analysis, it has been suggested that isolates of *A. ocellatum* from different fish species and from various geographic locations are all the same species (Levy et al. 2007). This contradicts previous morphological studies which suggested that several species of *Amyloodinium* might exist (Landsberg et al. 1994). This contradiction is particularly interesting in light of behavioral variations and differences in environmental tolerances between *A. ocellatum* isolates, especially salinity tolerance, which is greater in estuarine specimens than in all other isolates (Levy et al. 2007). This suggests that a single species of *A. ocellatum* exists which may have strains or subspecies that show variation in behavior and tolerance; however, more studies need to be performed on isolates obtained from various ecological and geographic regions to confirm this conclusion.

The efficiency of the gradual decrease in salinity through freshwater baths coupled with copper sulfate exposure as a treatment measure for amyloodiniosis was very evident toward the end of the treatment course and was evident in the nearly complete control of mortality and parasitic prevalence rates in the fifth experimental group. Thus, lowered salinity (~10 ppt) can play an important role in controlling outbreaks of amyloodiniosis in cultured European seabass, which in theory should be effective in similar marine cultured fish species. This is very important and is in agreement with a previous conclusion made by Hojgaard (1962) in which a lowered salinity value of 10 ppt proved effective in protecting marine aquarium fish against infestation with *Oodinium* (ex. *Amyloodinium*) *ocellatum*. Nevertheless, wild fish in estuarine waters of the Gulf of Mexico were found with *A. ocellatum* at 2 to 3 ppt salinity (Noga & Levy 1995). These differential tolerances may be explained in 2 ways: first, different fish species vary in their salinity tolerances, and second, different geographic isolates of *A. ocellatum* may have differential salinity tolerances (Francis-Floyd & Floyd 2011).

The near-complete control of amyloodiniosis after the freshwater bath and copper sulfate treatment is easily explained by zero parasite prevalence in all examined smears; this may also explain the ongoing fish mortality after applying the copper sulfate bath as the only treatment measure, when parasitic stages could still be detected in most of the samples examined. Copper sulfate as a toxic agent to parasitic stages of *A. ocellatum* has been the standard treatment of amyloodiniosis in many aquaculture systems in which the chemical agent has been applied at concentrations ranging from 0.15 to 0.20 mg l^{-1} (Cardeilhac & Whitaker 1988) since its first introduction as a treatment for amyloodiniosis in 1955 (Dempster 1955). However, in the current study, copper sulfate alone was not enough to fully control the disease despite its application at a slightly higher dose (0.30 mg l^{-1}). Another explanation is that only dinospores are the most susceptible to fish-safe copper sulfate therapy and that tomonts and trophonts lodged in fish hosts are more resistant and can still propagate (Paperna 1980, 1984), causing the observed prevalence and mortalities during copper sulfate treatment. On the other hand, the freshwater bath was more efficient at dislodging attached parasites (Kingsford 1975, Lawler 1977a), which then, at lowered salinity, became more susceptible to chemical eradication by copper sulfate.

Copper sulfate is applied as a control measure against several fish diseases and pathogens, and specifically is the drug of choice in the case of amyloodiniosis. Currently, copper sulfate is not approved by the US Food and Drug Administration (FDA) for the treatment of amyloodiniosis, despite its approval as an effective algicide by the US Environmental Protection Agency. Nevertheless, the chemical has been in use in the aquaculture of food fish for many years without a formal objection from the FDA authority (Francis-Floyd & Floyd 2011). We cannot ignore its possible adverse effects on fish and their environmental ecosystems, as the algicidal effect of copper sulfate might lead to oxygen shortages due to algal death and subsequent decay. Nevertheless, there is no current alternative to replace copper sulfate as an effective treatment of amyloodiniosis, and that is why we had tried to alleviate its negative effects by diluting the chemical with freshwater baths (hyposalination) in the dual treatment protocol. Also, the rearing system in the current study uses fully concrete ponds (including the pond floor) with continuous cleaning measures for walls and floors, which to a large extent inhibit the growth of algae and phytoplankton throughout these ponds.

Application of sufficient biosecurity measures is essential in controlling outbreaks of infectious agents, including *A. ocellatum*, in marine cultures and other aquarium fish industries. The historical background of the farms investigated in this study suggests that the disease outbreak started in one pond (El-Max station) and then spread quickly to other close ponds

at the same location. Better biosecurity measures should therefore always be maintained, including proper disinfection treatments of personnel and equipment, and introduction of infected fish to parasite-free aquatic cultures must be avoided.

In summary, the diagnosis of disease outbreaks due to *A. ocellatum* infestation clinically proved to be a straightforward process that requires outlining the farm history, observing clinical signs, and having access to light microscopy for parasitological examination. Moreover, molecular identification can be applied as a second, confirmatory, diagnostic step in addition to clinical and microscopic identification, and can be of further help when a differential diagnosis is required, e.g. for *Cryptocaryon irritans*, and also as a routine screening for subclinical infections and new aquatic habitats before introducing susceptible fish species. Finally, a combined treatment protocol consisting of bathing in fresh water free of disease agents and application of a chemical agent (copper sulfate) proved essential in this study to control high mortalities and morbidities associated with amyloodiniosis. Future studies should consider vaccine development and application of immunostimulants to boost fish resistance against parasitic infections as well as other microbial pathogens.

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Appendix 1. Physico-chemical water parameters at the 2 study locations

	Temperature (°C)	Salinity (ppt)	Oxygen tension (mg l^{-1})	Oxygen saturation (%)	Ammonia (ppm)
Location 1 (Wadi El-Natroun)	26.3	37	8.2	95	0.47
Location 2 (El-Max)	25.9	28	8.3	85	0.42

Appendix 2. Egypt-ElMax isolate sequence deposited in GenBank under accession number MF288148

TTTTCTCTCA CCTTTTCGTG CGGTGTGCTT GTGGAGTAGG AGCGGAGAAC AGCTATCCGA TATCCGAGCT TCCGAATTGG CCTACTCTGG GTAACGGAGA ATTAGGGTTT TCTTCGTTTC CTTCCGGTGG GGTGAAGACC GTGGCACTGA CCTTGATGCA TTGAAAGGCC GATTGCCTGA GGGCGACTGA TTGATCGAGC GTTTCGACTG GGGGGGCAGA GGTTCTTAGT CCAGTTCCCC CT

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