New pathological condition in cultured mullet *Argyrosomus japonicus*: histopathological, ultrastructural and molecular studies

Dina Zilberg¹, J. Brian Jones², Mieke A. A. Burger², Philip K. Nicholls³,
Druime Nolan², Melanie Crockford², Fran Stephens^{2,*}

¹French Associates Institute for Agriculture and Biotechnology of Drylands, Blaustein Institutes for Desert Research,
Ben-Gurion University of the Negev, Midreshet Ben Gurion 84990, Israel

²Fish Health Laboratory, Department of Fisheries, Government of, North Beach, Western Australia 6020, Australia

³School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia

ABSTRACT: Mullet *Argyrosomus japonicus* is a native fish species in Western Australia, for which aquaculture production has recently been developed. A single cohort was stocked in a cage offshore at Geraldton, Western Australia, at a water depth of 6 m. Fish appeared healthy before stocking. Routine histological analysis was carried out from 10 mo post stocking and until completion of harvest (about 2.5 yr post stocking). No gross pathology was evident. Microscopically, however, granulomatous lesions were present in the kidneys of almost 100% of the fish examined. Enclosed in the granuloma was an aggregate of organisms, 4.2 to 5.4 µm in diameter. Kidney granulomas appeared as multi-focal aggregates. Granulomas at different stages of formation and finally fibrosing granulomas were observed. Granulomas also appeared infrequently in other organs: a few granulomas were found in the liver and spleen and a single granuloma in the heart of one fish. Transmission electron microscopy (TEM) revealed that the organism was composed of 2 cells, an outer cell enclosing an inner cell. The inner cell was surrounded by a double membrane and the outer cell by a single membrane. Cellular material, presumably of parasitic nature, surrounded the outer cell. The organism contained primitive mitochondria and abundant free ribosomes. Small subunit ribosomal DNA (SSU rDNA) sequence obtained by PCR revealed an 84% sequence identity with the myxosporean *Latyspora scomberomori*. Based on TEM and preliminary molecular results, we suggest that the organism is the extrasporogonic developmental stage of a myxozoan parasite, which failed to form spores in the mullet host.

KEY WORDS: Granuloma · Parasite · Mullet · Liver · Fish

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INTRODUCTION

Mullet *Argyrosomus japonicus*, order Perciformes, is an estuarine and coastal water fish distributed through the Indian and Western Pacific Oceans, occurring around Australia, Africa, India, Pakistan, China, Korea and Japan (Silberschneider & Gray 2008). It provides important high value fisheries and

is also a popular recreational angling fish. Research suggests that overfishing around southeastern Australia is leading to depletion of spawning stock (Silberschneider et al. 2009). Mullet has been cultured for about 8 yr at a few locations in Australia, including cage culture offshore in the Indian Ocean at Geraldton (Western Australia), and offshore and inshore in the Southern Ocean at Port Lincoln (South

*Corresponding author. Email: fstephens@agric.wa.gov.au

Australia). Mulloway has also been cultured in inland saline ponds in New South Wales. Western Australian production, thus far, has been limited to a single cohort of 50 000 fish, investigated in the current study.

The main health problems described in mulloway include infection with ectoparasitic protozoans and monogenean trematodes that have been reported to cause mortalities in juvenile and adult mulloway (PIRSA 2001). The monogenean (Monopisthocotylea, Capsalidae) *Benedenia sciaenae* was diagnosed in caged mulloway (Whittington 1996). Farmed mulloway from Port Lincoln had gills infested with Monogenea, *Sciaenacotyle sciaenicola* (Polyopisthocotylea) and *Calceostoma glandulosum* (Monopisthocotylea), and sea lice *Caligus* cf. *elongatus* (Copepoda: Caligidae) (Hayward et al. 2007).

In this case study of sea-caged mulloway in Western Australia, extensive kidney pathology was diagnosed. Based on a literature search, to our knowledge the condition has not been previously reported either in mulloway or other fish species. Although no evident adverse effect was noted, such as associated change in behavior, morbidity or mortality, estimated infection rate was close to 100 %. The aim of the present study was to characterize the pathology and describe the infecting organism.

MATERIALS AND METHODS

Fish

The fish studied were mulloway from a commercial aquaculture farm, Indian Ocean Fresh Australia, located at Geraldton, Western Australia (28° 45' S, 114° 35' E). The fish were stocked in cages offshore in the Indian Ocean, at a water depth of about 6 m. The farm was established in February 2008 with a single cohort of 50 000 mulloway fingerlings obtained from the Challenger TAFE hatchery (Fremantle), that were stocked in a sea cage at the age of 50 d. Health examination of 150 fingerlings was carried out before shipment to the sea cage. On arrival the fish were stocked into two 600 m² D-nets within a single 60 m circumference circular cage. Post transport mortality of 924 out of 50 000 fish was documented, but no parasite, bacterial infection or lesions were found in a sample of examined survivors. This was the only mulloway culture at Western Australia at the time and therefore all examinations were carried out on this stock.

Fish sampling and histological analysis

All health examinations were carried out in Perth at the Fish Health Laboratory, Department of Fisheries, Western Australia. From December 2008 to September 2010 (when the harvest was completed), 5 fish were sampled monthly for routine health check, i.e. a total of 105 fish. The fish were anaesthetized using AQUI-S. Organs were fixed in 10% neutral buffered formalin (NBF) and sent for histological processing and diagnosis at the Fish Health Laboratory. Organs sampled included liver, spleen, heart, gut, muscle, brain and kidney.

Samples were processed for histological analysis, embedded in paraffin blocks and sectioned at 5 µm. Sections were stained with haematoxylin and eosin (H&E) using standard techniques. Additional stains applied included Giemsa, which is used to stain protozoa and produces a pronounced staining of microsporidian polar capsule, as well as Grocott, a silver stain, and Periodic Acid Schiff (PAS), which both stain carbohydrates and are used to demonstrate fungal organisms. Positive controls were used to validate the stains as per the laboratory's ISO 17205 accreditation. Slides were examined under a light microscope (Olympus BX51) and documented with an attached camera (Olympus DP70).

Transmission electron microscopy (TEM)

Kidneys were dissected from deeply anaesthetized fish, immediately trimmed to a size of 1 mm or less and fixed in 2.5% cold glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Samples were post fixed in 1% osmium tetroxide, embedded in Epon, sectioned and stained with uranyl acetate lead citrate and examined with a Philips CM10 Transmission Electron Microscope.

DNA analysis

Samples included for DNA analysis are listed in Table 1. DNA was extracted from frozen kidney tissue and flesh samples using DNAzol (Invitrogen) solution as described in Crockford et al. (2005). DNA was extracted from paraffin blocks using Quick-Extract FFPE DNA Extraction Kit (Epicentre Biotechnologies) as per the manufacturer's specifications. Granulomas from infected kidneys were also cut by laser micro-dissection on a Leica LMD 6500 microscope and DNA was successfully extracted from this

Table 1. Samples used to identify the kidney pathogen in mulloway and results for each sample using the nested PCR for Myxosporea as described in Fiala (2006). Samples are listed as case numbers from frozen kidney, paraffin embedded tissues (FFPE), laser microdissected tissues (LMD) and flesh preserved in 100% ethanol. The primary PCR, using primers ERIB1 and ERIB10, amplifies any universal eukaryote (~1400 bp expected size) and the nested PCR, using primers myxospec F and myxospec R, targets specific Myxosporea (900 bp expected size). Amplicons that showed a product of 900 bp following nested PCR were sequenced and matched best with *Latyspora scomberomori*

Sample description	Primary PCR	Nested PCR
FH09-128 frozen kidney 2	+/-	-
FH09-128 frozen kidney 3	+	-
FH09-128 frozen kidney 4	+	+
FH09-128 frozen kidney 5	+	+
FH09-128 FFPE tissues 2	-	-
FH09-128 FFPE tissues 3	-	-
FH09-128 LMD 1	-	-
FH09-128 LMD 2	-	+
FH09-128 LMD 3	-	-
FH10-101 frozen kidney 2	-	-
FH10-101 frozen kidney 3	+	+
FH10-101 frozen kidney 4	+/-	-
FH10-101 frozen kidney 5	-	-
FH10-101 K frozen kidney 2	+	-
FH10-101 K frozen kidney 3	-	-
FH10-101 K frozen kidney 4	+++	-
FH10-101 K frozen kidney 5	+	+
FH12-12 Flesh 4	+	+
FH12-12 Flesh 5	+	+
FH12-12 Flesh 6	+	+

tissue using the QIAamp DNA FFPE Tissue Kit (QIAGEN). PCR tests were carried out using generic primers on the DNA for various groups of organisms that the parasite was suspected to belong to, including Fungi, Myxozoa, and Protozoa. The primers are shown in Table 2.

Testing also included a nested PCR. A primary PCR was set up to amplify the Small subunit ribosomal DNA (SSU rDNA) gene using universal eukaryotic primers ERIB1 and ERIB10 (Barta et al. 1997); 1 µl of product from the first round PCR was used as template for the nested PCR using myxosporean specific SSU rDNA primers MyxospecF and MyxospecR (Fiala 2006). PCR reactions were made up with ~300 ng of DNA template, 12.5 µl of 2X BioMix™ Red (Bioline), and 1 µl of 10 µM primer made up to 25 µl reactions. Cycling of PCR reactions was carried out in a cp2-01 thermocycler (Corbett Research) as per conditions shown in Table 2. All PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by UV transillumination for a band of the estimated size.

For the fungal and lower eukaryote PCR tests *Saccharomyces cerevisiae* (bakers yeast) was used as a

positive control. The positive control used for the myxosporean and universal eukaryote PCR tests was an uncharacterized muscle-dwelling *Unicapsula* sp. (Myxosporea: Multivalvulida) ex *Seriola lalandi*; *Unicapsula* sp. spore DNA was extracted by tissue preparation and DNA extraction from frozen fish tissue as per Burger & Adlard (2010). The identity of the myxosporean as coming from the genus *Unicapsula* was confirmed by visualization of spores using light microscopy and sequencing of the SSU as per Burger & Adlard (2010). NCBI BLAST results showed 97% sequence identity to *Unicapsula* sp. 18S SSU ribosomal RNA, partial sequence (Genbank accession number AY302725).

PCR products of the expected size were extracted using a freeze-squeeze method as described in Crockford et al. (2005). Purified PCR products were sent to the Australia Genome Research Facility (Perth) for sequencing using their standard protocol.

The resulting consensus sequence was aligned with a selected group of other myxozoan SSU sequences from the marine urinary clade and representative of species from each of the other clades, selecting particularly species that infect the kidney (from Barošová et al. 2011), plus *Kudoa neurophila* ex *Seriola lalandi* (collected from a similar geographic locality to the mulloway parasite). Alignment was carried out using ClustalW within Geneious Pro™ (Drummond et al. 2011) with gap open cost 10 and gap extend cost 2. A distance neighbor-joining tree with bootstrap supports based on 1000 replicates and pairwise distance matrix was generated (also in Geneious Pro™) from a trimmed alignment (to the full length of the mulloway parasite sequence) to demonstrate the basic relationship of this organism with other myxozoans using the Geneious tree builder with genetic distance model Tamura-Nei. The malacosporan *Boddenbrockia plumatellae* (Genbank accession number FJ981824) was used as an outgroup.

RESULTS

A total of 105 fish were histologically examined from December 2008 to September 2010 (5 fish mo⁻¹). Other infections that were diagnosed in these sea-caged fish included: systemic bacterial infections by *Vibrio harveyi* and *Photobacterium damsela*;

Table 2. PCR primers used for identifying an unknown kidney pathogen of mulloway. The majority of the PCRs attempted were unsuccessful. The PCR for the specific detection of *Myxosporeia* described by Fiala (2006) produced amplicons of the expected size

Target group	Gene	Forward primer	Reverse primer	Amplicon size	Initial cycle	Amplification	Elongation	Reference
Universal fungi	Large subunit rDNA	C1 5'ACC CGC TGA TTT AAG CAT3'	D2 5'TCC GTG TTT CAA GAC GG3'	620+	4 min, 94°C 2 min, 54°C 2 min, 72°C	1 min, 94°C 1 min, 54°C 1 min, 72°C 30 cycles	4 min, 72°C	Leclerc et al. (2000) (primer and cycle conditions)
	ITS + 5.8S	ITS1 5'TCC GTA GGT GAA CCT GCG G3'	ITS4 5'TCC TCC GCT TAT TGA TAT GC3'	600+	– 4 min, 94°C 2 min, 54°C 2 min, 72°C	3 min, 94°C 30 s, 90°C 30 s, 50°C 1.5 min, 72°C 35 cycles	7 min, 72°C	Hulvey et al. (2007) (cycle conditions)
	ITS + 5.8S	ITS1 5'TCC GTA GGT GAA CCT GCG G3'	ITS4 5'TCC TCC GCT TAT TGA TAT GC3'	600+	– 4 min, 94°C 2 min, 54°C 2 min, 72°C	3 min, 94°C 30 s, 90°C 30 s, 50°C 1.5 min, 72°C 35 cycles	7 min, 72°C	White et al. (1990) (primers), Leclerc et al. (2000) (cycle conditions)
Mesomycetozoea	Small subunit rDNA	MesoF 5'TAA GCC ATG CAT GTC AAG TAT AA3'	MesoR 5'ACT AGG AAT TCC TCG TBA AGA TS3'	1600	90 s, 95°C	45 s, 95°C 60 s, 55°C 140 s, 72°C 35 cycles	5 min, 72°C	Feldman et al. (2005)
	Small subunit rDNA	V1f 5'CAC CAG GTT GAT TCT GCC3'	1492R 5'GGT TAC CTT GTT ACG ACT T3'	1400	–	1 min, 94°C 1 min, 50°C 2 min, 72°C 35 cycles	10 min, 72°C	Nilsen (2000)
Myxosporea	Small subunit rDNA	MyxospecF 5'TTC TGC CCT ATC AAC TWG TTG3'	18R 5'CTA CGG AAA CCT TGT TAC G3'	1500	2.5 min, 94°C	20 s, 94°C 30 s, 46°C 60 s, 72°C 30 cycles	10 min, 72°C	Fiala (2006) (MyxospecF), Whipps et al. (2003) (18R), Heiniger et al. (2008) (cycle conditions)
Universal eukaryote	Small subunit rDNA	GO1 5'CAC CTG GTT GAT CCT GCC AG3'	GO7 5'AGC TTG ATC CTT CTG CAG GTT CAC CTA C3'	1850	–	30 s, 96°C 30 s, 65°C 60 s, 72°C 30 cycles	–	Rand et al. (2000), Saunders & Kraft (1994)
Universal eukaryote	Small subunit rDNA	ERIB10 5'CTT CCG CAG GTT CAC CTA CGG3'	ERIB1 5'ACC TGG TTG ATC CTG CCA G3'	2028	–	1 min, 95°C 1 min, 48°C 1 min, 72°C 30 cycles	10 min, 72°C	Barta et al. (1997), Fiala (2006) (cycle conditions)
Myxosporea	Small subunit rDNA	MyxospecR 5'GGT TTC NCD GRG GGM CCA AC3'	Myxospec F 5'TTC TGC CCT ATC AAC TWG TTG3'	800+	–	1 min, 95°C 1 min, 48°C 1 min, 72°C 30 cycles	10 min, 72°C	Fiala (2006), Barta et al. (1997) ERBI primary primers

parasitic infections by monogenean parasites, most commonly gill flukes, including a blood-feeding polyopisthocotylean morphologically similar to *Sciaenacotyle sciaenicola*; a monopisthocotylean resembling *Calceostoma glandulosom*; and the capsalid skin fluke *Benedinia scianiae* (monopisthocotylean). The latter was the most significant in terms of morbidity and mortality.

Histopathology

Macroscopically, no lesions were evident and all internal organs appeared normal. Histological analysis of fixed tissues revealed granulomatous lesions, mainly in the kidney, where they were present in all the examined fish. Focal aggregates as well as focal single granulomas were seen in the kidney. Granulomas were typically characterized by a thick wall composed mainly of layered macrophages/epithelioid cells and an outer layer of fibroblasts with little or no fibrous material (Fig. 1). Inside the granuloma was an aggregate of round shaped organisms with a diameter of 4.2 to 5.4 μm (average 4.7 μm , $n = 25$). Granulomas appeared individually or in clusters (Fig. 1).

Occasionally an aggregate of organisms surrounded by macrophages but no well-developed granuloma could be seen (Fig. 2). These were few compared to the granuloma-surrounded aggregates and were presumed to be a developing granuloma following a recent invasion of the organism. The few granulomas found in the spleen were thin-walled and surrounded by melanomacrophages (Fig. 3).

The organism was characterized by a central round light basophilic circle with darker basophilic staining at the periphery, with an average diameter of 2.33 μm ($n = 25$), which was assumed to be the nucleus with marginated chromatin. This organelle was surrounded by an eosinophilic, slightly foamy cytoplasm. An elliptical basophilic organelle was present at the edge of the cytoplasm, adjacent to the cell membrane (Fig. 1); this was later revealed to be the nucleus of the outer cell, as observed under TEM (see Fig. 11b). In aggregates that were not surrounded by a fully developed granuloma, the organism appeared less circular and the cytoplasm appeared more foamy (Fig. 2).

Granulomas were mainly present in the kidney, but sporadic granulomas (1 to 2 per section) were identified in the liver and spleen of about 50% of the fish. In the liver, granulomas were usually, but not always, associated with the hepatopancreas and blood vessels (Fig. 4). In one case, an aggregate of the organism

surrounded by macrophages was lodged on or adjacent to and distorting a hepatic blood vessel wall (Fig. 5). It is possible that the organism had entered the circulation and attached itself to the wall of the hepatic blood vessel on its way to invade the liver (Fig. 5). In the spleen, most of the granulomas were thin walled and surrounded by macrophages containing brownish pigment (Fig. 3). In one fish a single granuloma was found in the heart (Fig. 6). Fibrosing granulomas contained eosinophilic stained amorphous material and a thickened cellular capsule where the parasite appeared to have been destroyed (Figs. 4 & 7).

Throughout the sampling period, both fibrosing granulomas (with no live organism) and what appeared to be new granulomas were evident and infection was heavy throughout the study.

In an attempt to identify the nature of the organism several histological stains were applied. The parasite did not have the pronounced staining with Giemsa that is characteristic of polar capsules within myxozoan spores (Fig. 8). It was strongly argyrophilic, as it stained with Grocott (Fig. 9) and with PAS (not shown), both of which positively stain fungus.

TEM

TEM revealed that the organism enclosed in the granuloma was composed of 2 cells, i.e. a peripheral cell (average size 4.9 $\times$ 6.4 μm , $n = 5$) surrounding a central cell (Figs. 10 & 11). Each cell contained a nucleus (average size 1.9 μm , $n = 8$). The outer cell appeared to be surrounded by more cellular material. The nucleus of the central cell was centrally located (Fig. 10) and that of the peripheral cell was often flattened against the outer cell membrane (Fig. 11c). The central cell appeared to be surrounded by a double cell membrane whereas the membrane of the outer cell was single layered (Fig. 11). The organism contained a primitive mitochondria and abundant free ribosomes (Fig. 11). Epithelioid cells surrounded organism aggregates and cellular material of unidentified nature. Tight junctions were evident between the epithelioid cells. There were no evident host cells within the granuloma.

DNA analysis

All PCR methods attempted are shown in Table 2. PCR analysis using primers for Mesomycetozoa and Microsporidia produced no product. However, some

Figs. 1 to 6. *Argyrosomus japonicus*. Granulomas in the kidney and other organs of mulloway. **Fig. 1.** Granulomas in the kidney containing the infecting organism (arrowhead), enclosed by layers of epithelioid cells (long arrow) and a second layer of fibroblasts (short arrow). SG: split granuloma. **Fig. 2.** Aggregate of the infecting organism (arrowhead) in the kidney (presumably a newly developing granuloma), surrounded by haematopoietic tissue and/or mononuclear inflammatory cells and few macrophages/epithelioid cells (arrow). Parasite cells at the center of the granuloma display foamy eosinophilic cytoplasm. **Fig. 3.** Granuloma in the spleen, enclosing the infecting organisms (arrowhead) surrounded by a thin layer of macrophages containing brown pigment (arrows). BV: blood vessel; MMC: melanomacrophage center. **Fig. 4.** Hepatic granulomas, containing viable infecting organism (V) and a parasite-free granuloma with a necrotic core (N) in the hepatopancreas. H&E staining. **Fig. 5.** Aggregate of the infecting organism (arrowhead) surrounded by macrophages (arrow), located in or adjacent to a hepatic blood vessel (BV). **Fig. 6.** Granuloma in the heart containing the infectious organism surrounded by fibroblasts (long arrow) and inflammatory cells (short arrow)

Figs. 7 to 9. *Argyrosomus japonicus*. Fig. 7. Fibrosing granuloma in the kidney. Organisms were unidentifiable in the core. Fig. 8. Kidney granulomas stained with Giemsa. Fig. 9. Silver staining (Grocott), showing intense staining of the infecting organism in kidney granulomas

DNA amplification was achieved using the DNAzol extracted samples with primer combinations C1 and D2 for the large subunit rDNA (Leclerc et al. 2000) and ITS1 and ITS4 for the internal transcriber gene region (White et al. 1990), under cycling conditions as per Hulvey et al. (2007). None of the 4 forward and 4 reverse ITS samples sequenced well. Of the 12 large subunit sequencing reactions carried out (6 each with primers C1 and D2), one C1 sequence produced a quality sequence for 400 bp of the 721 bp fragment. Because only one forward primer was successful in amplifying any useful DNA and the sequence produced was sub-optimal, a consensus sequence could not be obtained. The TEM suggested that the organism was a myxosporidian, so this sequence was aligned with known myxosporidian sequences from Freeman et al. (2008). The partial sequence obtained had 85 % sequence identity to *Kudoa ovivora* 28S large subunit ribosomal RNA gene, partial sequence (GenBank accession number AY302731.1) using NCBI BLAST. Therefore further PCR tests using more myxosporidian specific primers were carried out as follows.

The template from a primary reaction using ERIB1 and ERIB10 primers for SSU rDNA (Table 2) was used in a nested reaction with MyxopsecF and MyxospecR primers, with a target PCR product of approximately 900 bp (Table 2, Fig. 12). Out of 20 samples tested, 8 positive products (including one from a Laser microdissected granuloma) were obtained with the nested PCR. The bands obtained from 4 positive samples are presented in Fig. 12.

This product was sequenced and submitted to GenBank (accession number JQ868766). Using NCBI BLAST, the consensus sequence revealed 84 % sequence identity with *Latyspora scomberomori* 18S SSU rDNA gene (GenBank accession number HM230826) with 100 % coverage of the 863 nucleotide query.

The trimmed alignment generated with other myxozoan SSU sequences consisted of 1106 characters which spanned the entire mulloway parasite sequence. The overall distance relationships can be seen in the neighbor-joining tree in Fig. 13. The percentage similarities between the sequences included in the analysis are shown in Table S1 in the supplement at www.int-res.com/articles/suppl/d100p219_supp.pdf. The mulloway parasite sequence shows most similarity to *Latyspora scomberomori* (Bartošová et al. 2011) from the family Sinuolineidae, but at a low level (81.9 % identity from the alignment), and secondly to *Zchokkella lophii* (Freeman et al. 2008) from the family Myxidiidae (74.7 % identity).

Figs. 10 & 11. *Argyrosomus japonicus*. TEM images of a granuloma. Fig. 10. Edge of a granuloma showing host epithelioid cells (Ep) and 6 of the encysted organisms (eO). Scale bar = 11.26 μ m. Fig. 11a–c. Encysted organisms within a granuloma. Scale bars = 2.56 μ m. rER: rough endoplasmic reticulum; G: Golgi; J: host cell junctions; L: lysosome; hL: host lipid vacuole; hM: host cell membrane; iMe: inner cell membrane; oMe: outer organism's membrane; Mi: mitochondria; cN: central nucleus; hN: host nucleus; pN: peripheral nucleus

DISCUSSION

The reported study suggests that the lesions identified mainly in the kidney but also in other organs are granulomatous in nature and the cells present in these granulomas are of parasitic origin. This parasite appears to be the causative agent of the described pathology.

The parasites stained positive with Grocott staining (a silver stain) and PAS. In the Grocott stain, chromic acid oxidation forms aldehydes from polysaccharide components, for example in the fungal cell wall, which are subsequently demonstrated by reduction of an alkaline hexamine-silver complex that precipitates and blackens the tissue. Thus, the positive staining suggests the presence of polysaccharide.

Fig. 12. PCR products amplified by nested PCR using primers MyxospecF and MyxospecR (Fiala 2006). M: 100 bp DNA ladder marker; Lanes 1 to 8: PCR products in duplicate from DNA extractions FH09-128 #4 (Lanes 1+2), FH09-128 #5 (Lanes 3+4), FH09-128 LMD Cs (Lanes 5+6), and FH10-101 # 3 (Lanes 7+8); N: no template control (negative control); P: positive control (undescribed muscle-dwelling microsporidian, *Uncapsula* sp. ex *Seriola lalandi*)

According to the TEM analysis, the organism does not have the cell wall characteristic of fungi. However, some myxosporidians do stain with silver stains (Wolf & Markiw 1979)

Fish initially stocked from the hatchery appeared healthy with no evident pathology (by histology). Granulomas were present when routine histological assessment began, 10 mo post stocking. Possibly the fish encountered the pathogen shortly after arrival to the culture site, and within 10 mo the infection was well established. Granulomas appeared as individual or split. The latter could have been formed by macrophages infiltrating between the enclosed organisms or on exit of the organism and the formation of coalescing granulomas.

TEM analysis revealed that the organism was multicellular. The innermost cell was surrounded by a double membrane and enclosed within the cytoplasm of a larger cell (enclosing cell). Enclosing cells appeared to be surrounded by cytoplasm of a third larger cell (Fig. 10). Assuming that the third outer cell is parasitic in nature, this can be interpreted as being a primary cell that contains a large number of secondary cells, most of which enclose a tertiary cell, thus presenting a polysporic pseudoplasmodia (Lom & Dykova 1992). Alternatively the parasite may be in

Fig. 13. Neighbor-joining distance tree of partial small subunit rDNA including the novel mulloay kidney parasite with other myxozoan organisms from an alignment of 1106 characters. Bootstrap values based on 1000 replicates

the form of pseudoplasmodia aggregates (large number of primary cells containing a single secondary cell each). Myxosporean cells contain most of the cytoplasmic organelles, constituents and reserve materials (fat, glycogen) of an animal cell (Lom & Dykova 1992). The abundant free ribosomes observed in the mulloway parasite are characteristic of myxozoans, especially in extrasporogonic stages (Lom & Dykova 1992).

Myxosporeans are known to have a proliferative stage in their life cycle (Lom & Dykova 1992). This stage is localized in sites or organs different from the sporogonic phase. At this stage, development starts with growth and nuclear division, producing an inner secondary cell within the primary cell, within a tightly fitting membrane bound vacuole, a significant characteristic in myxosporeans (Lom 1987, Lom & Dykova 1992). A primary cell containing a secondary cell was demonstrated for *Myxidium lieberkuhni* (see Lom 1987). Primary cells of an unknown myxosporidian infecting coho salmon *Onchorhynchus kisutch* kidneys, CKX, contained one to several secondary cells (Jones et al. 2004). CKX is different from the described infection as it occurs intracellularly and primary cells contain one or more secondary cells, as opposed to a single secondary cell in the mulloway parasite. *Sphaerospora renicola*, a myxozoan parasite infecting carp *Cyprinus carpio*, has extrasporogonic development occurring in the bloodstream where a primary cell containing a single secondary cell is formed, growing into 8 secondary cells, each containing a tertiary cell (Lom & Dykova 1992). The primary cells then fall apart and the released secondary cells restart the cycle either in the bloodstream, the swim bladder (where inflammatory response is associated) or reach the kidney to initiate sporogonic development (Lom & Dykova 1992). Such extrasporogonic proliferation may have occurred in the mulloway, producing the extensive infection described. A similar form of extrasporogonic proliferation and formation of a monosporic plasmodia was reported for *Tetracapsuloides bryosalmonae*, a malacosporean species (a different myxozoan class to the Myxosporea) identified as the causative agent of proliferative kidney disease (Feist & Longshaw 2006).

Lack of mature spores made the classification of the mulloway parasite much more complicated, as characteristic histological stains for myxozoans failed to demonstrate the nature of this organism. Similarly, the extrasporogonic stages of *Tetracapsuloides bryosalmonae* do not stain positively with Giemsa (Ferguson 2006). Infection with *T. bryosalmonae* results in a host response characterized by granuloma formation

in the kidney, similar to the pathology observed in the mulloway. However, significant tissue damage is associated with *T. bryosalmonae* infection in salmonid hosts (Feist & Longshaw 2006). In contrast, the granulomatous response in mulloway was of a chronic nature, with no obvious implication on fish health. *T. bryosalmonae* infection in Arctic char results in a large number of spores without an extensive host reaction (Feist & Longshaw 2006), suggesting host specificity for *T. bryosalmonae* with Arctic char.

From the many primer sets used in this study (Table 2) only the nested PCR using template from the universal eukaryotic primers ERIB1 and ERIB10 and then the myxosporean specific MyxospecF/MyxospecR PCR primer combination (Fiala 2006) proved able to amplify an appropriately sized SSU rDNA fragment from the mulloway kidney. The fragment had an 81.9% pairwise identity based on neighbor-joining analysis with the myxosporean *Latyspora scomberomori* (family Sinuolineidae); this species also infects kidney tissue of fish (*Scomberomorus guttatus*) but as mature spores in the kidney tubules (Bartošová et al. 2011). Without mature spores, it is still difficult to assign the mulloway parasite even to family level using the DNA data. For example, the family Sinuolineidae is not monophyletic and there is as low as 48% sequence identity between 2 species from this family, *Biptera formosa* (FJ790309) and *Latyspora scomberomori* (HM230826), over this region of the SSU (see Fig. 13). Perhaps the association between these 2 sequences is derived more from their tissue tropism, which is known to be a considerable correlating factor in sequence similarities in the Myxosporea (Burger et al. 2007). Overall, this DNA sequence similarity, together with the TEM evidence, indicates that the mulloway parasite is a novel myxosporean.

The importance of myxosporean parasite infections in cultured fish is increasing (Bartošová et al. 2011). Diagnosis of pathogens is not always straightforward. There have been other reported pathogen-associated pathological conditions in fish which were not identified, or that were only fully or partially identification after a considerable time. For example the X-cell parasite, originally described in 1969 (Brooks et al. 1969), was only years later identified as a protozoan, related to Haplosporidia, using molecular techniques (Miwa et al. 2004). A parasitic amoeba-like organism was described in granulomatous lesions of goldfish, the taxonomy of which is yet to be finalized (Dykova et al. 1996). Recently Con-

stenla & Padrós (2010) have reported a new pathological condition in sole *Solea senegalensis*, associated with a unicellular parasite of unknown nature.

Infection occurred within the sea cage, since no infection was apparent in fish prior to them being stocked in the sea cage. From our observation the host response appeared to eventually destroy the enclosed organism and there was no evidence of progression to spore formation in the mullocky.

Further research is needed to determine the route of transmission of this parasite, including identification of potential alternate hosts (presumably an annelid worm or a bryozoan, if this is indeed a myxozoan parasite) at the culture site and transmission experiments to determine whether the life cycle can be completed in the mullocky.

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