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Severe glomerular disease in juvenile grey snapper *Lutjanus griseus* L. in the Gulf of Mexico caused by the myxozoan *Sphaerospora motemarini* n. sp.

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ABSTRACT

In the eastern Gulf of Mexico, off the coast of Florida, grey snapper, *Lutjanus griseus* was found to be infected with the myxozoan parasite *Sphaerospora motemarini* n. sp., with high prevalence (83%) and intensity of infection occuring in age-0 fish, and with parasite levels decreasing with age (age-1 snapper 40%; age-2 snapper 0%). The morphological, molecular and phylogenetic characterisation of the myxozoan showed that it is a member of the typically marine, polysporoplasmid *Sphaerospora* spp. which form a subclade within the *Sphaerospora sensu stricto* clade of myxozoans, which is characterised by large expansion segments in their SSU rDNA sequences. Presporogonic stages of *S. motemarini* n. sp. were detected in the blood, using PCR. Pseudoplasmodia and spores were found to develop in the renal corpuscles of the host, causing their massive expansion. Macroscopic and histopathological changes were observed in age-0 fish and show that *S. motemarini* n. sp. causes severe glomerulonephritis in *L. griseus* leading to a compromised host condition, which makes it more susceptible to stress (catch-and-release, predators, water quality) and can result in mortalities. These results are discussed in relation to the exploitation of grey snapper populations by commercial and recreational fisheries and with the observed increased mortalities with temperature along the coast of Florida. In the future, we would like to determine prevalence and intensity of infection with *S. motemarini* n. sp. in juvenile *L. griseus* in different areas of the Gulf of Mexico in order to be able to estimate the temperature dependence of *S. motemarini* n. sp. proliferation and to be able to predict its distribution and severity during climatic changes in the Gulf.

1. Introduction

The grey snapper *Lutjanus griseus* (L.), commonly also known as mangrove snapper, is a medium-sized snapper species which is restricted to the Western Atlantic, with its centre of distribution in the Gulf of Mexico and the Caribbean Sea (Robins et al., 1986; Allman and Grimes, 2002). *L. griseus* is a target species for commercial and recreational fisheries and the largest number of animals is being caught along the Gulf coast of Florida, with approx. 2 million lbs of grey snapper landed between 2005 and 2009 (NMFS, 2012). Ault et al. (2002, 2005) reported that the spawning potential ratio (SPR) of mangrove snapper in the Florida Keys coral-reef system was below that of a healthy stock, indicating overfishing. Increasing mortality and decreasing size-at-age along a temperature gradient from northwest to southwest Florida has also been reported (Allman, cited by Gold et al., 2009). During fishing efforts conducted by Mote Marine Laboratory and aiming at fish population studies along the mangrove shores of the Eastern Gulf of Mexico, it was noted that juvenile mangrove snapper caught by seine nets and subsequently released, sometimes failed to recover from the stress of capture. During a parasitological study of moribund mangrove snapper after their transport to the laboratory we detected macroscopic changes in their kidneys and large numbers of myxozoan spores in squash preparations. Myxozoans are microparasites belonging to the Cnidaria (Holland et al., 2011), and several species have been related to pathological changes and mortalities in wild and cultured fish species around the globe. In order to better understand the influence this specific myxozoan has on its host *L. griseus*, we aimed to characterise this species morphologically and molecularly, determine its prevalence and intensity of infection in different age classes of fish, describe the pathological changes it causes and determine whether it can be related to mortalities.

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2. Materials and methods

2.1. Study site

L. griseus were obtained within the framework of fishery-independent surveys of fish communities occupying mangrove shorelines and creeks in the Gulf of Mexico. In November and December 2012 fish were caught at the Southeast coast of the Gulf, in Florida, between Anna Maria Island in the north (27°26′36.15″N 82°41′36.09″W) and New Pass in the south (27°19′37.59″N 82°35′14.06″W). Fishing was conducted in shallow areas between the keys and the mainland, and in the canals connecting this area with the Gulf. The capture areas were characterised by sandy or muddy sediment, sometimes covered with sea grass, and a depth of 1–10 m. Seine nets were used and some fish were caught by line fishing from the shore. A total of 27 *L. griseus* were captured for parasitological study. On thin sections of otoliths, annual growth zones (annuli) were examined and the fish were ascribed to three year-based age classes: age-0 ($n = 12$; total length (TL) 7.5–10.5 cm), age-1 ($n = 10$; TL 20.6–25.2 cm) and age-2 ($n = 5$; TL 23.2–34.0 cm). Thereby, the relation between age and TL was in accordance with the results from other studies (Burton, 2001; Fisher et al., 2005; Jones et al., 2010).

2.2. Parasitological study – sampling procedures and morphological details

Fish were transported live to the aquaculture facilities of Mote Aquaculture Research Park (MAP) in large, aerated containers and were transferred to an isolated holding tank on site. All specimens were maintained in the facilities for a maximum of 48 h before they were euthanised by an overdosis of MS222. Thereafter, blood was taken from the caudal vein, using a sterile syringe, and 4 μl of blood were collected in 100% ethanol. Fish were weighed, measured and necropsied. Fresh kidney smears were observed under the light microscope at ×400 to ×1000 magnification. Spores were measured on digital images using ImageJ v.1·44p (Wayne Rasband, http://imagej.nih.gov/ij) calibrated against a digital image of a graticule. Measurements ($n = 15$ spores per fish, 3 *L. griseus*) were obtained following the guidelines of Arthur and Lom (1989) and Sitjà-Bobadilla and Álvarez-Pellitero (1994). Kidney tissue from one heavily infected fish was minced up in an Eppendorf tube using a peston and incubated with sterile sea water in order to disrupt the tissues and release spores from it. After 3 days the solution was pelleted at 2000 g, the supernatant was discarded and the pellet washed several times. Finally, a pellet consisting predominantly of spores was collected and left to settle for 30 min onto poly-L-lysine coated slides and coverslips. Some spore preparations were fixed in methanol and incubated with DAPI overnight to be able to count the number of sporoplasms in the spores. Lysine-coated coverslips with spores were fixed in 2.5% glutaraldehyde and processed for scanning electron microscopy (SEM) as described in Alama-Bermejo et al. (2009). For molecular analyses, blood obtained from the caudal vein and small kidney pieces of each fish were fixed in 100% ethanol. Furthermore, kidney pieces of all age-0 fish were fixed in 10% neutral buffered formalin for histological analyses.

2.3. Molecular analyses and phylogeny of parasite isolates

Ethanol–fixed blood was pelleted and the ethanol left to evaporate before adding TNES urea buffer (Asahida et al., 1996) to the tubes. Ethanol-fixed kidneys were briefly dried on filter paper and transferred to TNES urea buffer. All samples were stored in buffer for several days, until DNA extraction. DNA was extracted using a simplified phenol-chloroform extraction protocol (Holzer et al., 2004) and, after drying, DNA was redissolved in RNAse/DNAse-free water. Myxozoan small subunit ribosomal DNA (SSU rDNA) was amplified in three overlapping segments using universal and semi-specific primers designed in previous studies. Primers ERIB1 (5′-ACCTGGTTGATCCTGCCAG-3′) and ERIB10 (5′-CTTCCGCAGGTTCACCTACGG-3′) (both Barta et al., 1997) were used in the first round PCR at 62 °C annealing temperature (AT). This PCR product was used as a template in three different nested PCR reactions: (1) MyxGP2F (5′-TGGATAACCGTGGGAAA-3′ Kent et al., 1998) and Act1R (5′-AATTTCACCTCTCGCTGCCA-3′ Hallett et al., 2002) at 58 °CAT, (2) ERIB1 and BasalSphCladeSSU1850R (5′-AACCRATACCCRTACAYRGRRRGTGC-3′ Bartošová et al., in press) at 53 °C AT, and (3) Myxgen4F (5′-GTGCCTTGAATAAATCAGAG-3′ Diamant et al., 2004) and ERIB10 at 60 °C AT. PCRs were conducted in 20 μl reactions with 0.025U μl^{-1} Titanium Taq DNA polymerase and 10× buffer which contained 1.5 mM $MgCl_2$ (BD Biosciences Clontech), with 0.2 mM of each dNTP, 0.5 mM of each primer, and 50–150 ng of template DNA. Denaturation of DNA (95 °C for 2 min) was followed by 30 cycles of amplification (95 °C for 40 s, AT (see above) for 40 s, and 68 °C for 2 min) and terminated by a 4 min extension (68 °C). Overlapping fragments of the kidney myxozoan of three *L. griseus* individuals were sequenced in the sequencing facility of the Faculty of Science at the Biological Centre of the Academy of Sciences of the Czech Republic. The three partial SSU fragments were self-assembled and aligned in SeqMan II (DNASTAR, Inc.). The obtained consensus sequence was submitted to the BLAST tool on GenBank™ to check for the closest relatives. Based on this, the sequence was aligned to a subset of SSU rDNA sequences from a previous alignment (Bartošová et al., in press) which were used as a profile in ClustalX version 1.18 (Thompson et al., 1997). The alignment was manually edited and the ambiguous regions were excluded in BioEdit (v7.0.5.2; Hall, 1999). To estimate phylogenetic relationships to other myxozoans, we performed Maximum Parsimony (MP) analysis in PAUP* (v4.b10; Swofford, 2001), using a heuristic search with random taxa addition, the ACCTRAN option, TBR swapping algorithm, all characters treated as unordered and gaps treated as missing data. The Maximum Likelihood (ML) analysis was calculated in RAxML (Stamatakis, 2006) using GTR + Γ model. Clade support values were calculated from 1000 bootstrap replicates with random sequence additions in both MP and ML analyses. The Bayesian Inference (BI) analysis was performed in MrBayes (v. 3.0; Ronquist and Huelsenbeck, 2003) using the GTR + Γ + I model of evolution. Posterior probabilities were estimated from 1,000,000 trees via two independent runs of four simultaneous Markov Chain Monte Carlo chains with every 100th tree saved. The burn-in period (50,000 generations) was determined in Tracer (v. 1.4.1; Rambaut and Drummond, 2007).

Based on differences in the variable regions of the SSU rDNA gene region, specific primers were designed for the polysporoplasmid parasite from *L. griseus*. SmoteF (5′-TCGTGTGGCTAGACTGGCATC-3′) and SmoteR (5′-AGTGAGAGAGGGAGAAGGCAC-3′) were then applied in PCR to determine whether the parasite occurred in the blood and whether the prevalence of infection observed by microscopy was the same as determined by PCR. PCR conditions were as above with 66 °C AT and a shorter elongation step (40 s). To test the specificity of the newly designed PCR primers, DNA extractions of twelve species belonging to *Sphaerospora sensu stricto* were checked for cross-reactivity. These included *Sphaerospora sparis* (Sitjà-Bobadilla and Álvarez-Pellitero, 1995), *Sphaerospora elegans* Thélohan (1892), *Sphaerospora hankai* Lom et al. (1989), *Sphaerospora molnari* Lom et al. (1983), *Sphaerospora angulata* Fujita (1912), *Sphaerospora dykovae* (Lom and Dyková, 1982) and four unidentified/undescribed *Sphaerospora* spp. (GenBank accession numbers JX286621-22, JX286625-26).

2.4. Intensity of infection and pathological changes

On fish dissection, the macroscopic appearance of the kidney was reported for each individual. Formalin-fixed tissues were dehydrated through an ethanol series, transferred into xylene