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Short sequence report

Cloning and regulation of the major histocompatibility class I alpha gene in the teleost fish gilthead seabream

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Major histocompatibility complex (MHC) genes encode key molecules in the immune response. Antigens properly processed are expressed in the membrane bound to either class I or class II MHC proteins and then recognized by lymphocytes through the cell-surface T-lymphocyte receptors (TCR). However, contact with the CD8 (in cytotoxic T lymphocytes) or CD4 (in helper T lymphocytes) co-receptors are necessary for triggering the adaptive immune response leading to pathogen elimination [1]. MHC genes have been cloned in several species, representing all the vertebrate groups. From an evolutionary point of view, the adaptive immune response appears in fish, and the presence of MHC and MHC-related genes has been evidenced in all species studied. In teleost or bony fish MHC genes are not clustered, unlike in cartilaginous fish and tetrapods, indicating differences in evolution [2,3]. Therefore, a study of the teleost fish MHC genes is essential for understanding the importance and significance of this genetic divergence; at the same time, the knowledge will add to the recent advances made in fish immunology. Since the early 1990s when fish MHC genes were first cloned, numerous sequences have been published in many bony fish species but mainly for evolutionary purposes [3,4]. Interestingly, little information exists regarding their regulation and function. Thus, further characterization of the fish MHC genes at different levels will be of great help in understanding the innate–adaptive immune system interactions.

Class I MHC is a non-covalently bound heterodimer composed of an MHC class Iα chain (about 45 kDa) and β2-microglobulin (about 12 kDa). MHC class Iα is expressed on all the nucleated cells and generally presents endogenous antigens to cytotoxic T lymphocytes. The heavy chain, Iα, is encoded by a polymorphic gene containing a leader peptide, 3 extracellular domains (α1, α2 and α3) and transmembrane and cytoplasmic regions. However, recent findings point to the presence of soluble MHC class Iα proteins without a transmembrane region [5]. Genomic studies have demonstrated the variable copy numbers in the genome of teleost fish (from 3 to 17) as well as polymorphism in the peptide-binding region (PBR) within the α1 and α2 domains [4,6,7]. However, little information exists about MHC functioning in fish. At gene level, the expression of MHC class I (either Iα chain or β2-microglobulin) may be affected by physical factors (e.g. temperature), particulated antigens (viruses, bacteria, etc.) or endogenous soluble proteins (cytokines, growth factors, etc.) [8–10]. Moreover, the use of anti-fish MHC class Iα antibodies has revealed that its expression pattern is similar to that in mammals, including the immune system cells, and that it may be

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regulated in different fish species [6,11–13]. However, fish MHC class I needs to be studied in greater depth before we can fully understand its role in the fish immune response. Therefore, we aimed herein to clone the MHC class I alpha chain in the Mediterranean teleost gilthead seabream (*Sparus aurata* L.) and study its regulation.

Specimens (125 g bw) of the hermaphroditic protandrous seawater teleost gilthead seabream (*S. aurata* L.) (CULMAREX S.A., Murcia, Spain) were kept in running seawater aquaria at 20 ± 2 °C and fed daily (1% biomass). This study was approved by the Bioethical Committee of the University of Murcia. Brain, liver, gut, gills, head-kidney (HK), spleen, thymus and testis were obtained by dissection and immediately frozen in TRIzol Reagent (Gibco). Leucocytes from peripheral blood (PBLs), peritoneal exudate (PE) and HK were isolated and adjusted in sRPMI [RPMI-1640 (Gibco) culture medium containing 2% FCS (Gibco) and 0.35% NaCl]. Isolated leucocytes and cultured tumor cells (seabream fin, SAF-1, ECACC-00122301; mouse lymphoma, L-1210, ATCC CCL-219) were pelleted and also resuspended in TRIzol reagent. HK leucocytes from 3 different fish (10^7 per fish) were treated *in vitro* with medium (controls), heat-killed *Vibrio anguillarum* R-82 (5 bacterial cells per leucocyte) (pathogenic for seabream), heat-killed *Saccharomyces cerevisiae* S288C (2 yeast cells per leucocyte), concanavalin A (ConA; 5 μg/ml; Sigma), ConA + LPS (lipopolysaccharide; 5 μg/ml and 10 μg/ml, respectively; Sigma), phytohemagglutinin (PHA; 10 μg/ml; Sigma), sonicated and RNase treated SAF-1 or L-1210 tumor cells (10 leucocytes per tumor cell), CpG ODN (5′-TCCATGACGTTCCTGATGCT-3′; 50 μg/ml; Eurogentec) or poly I:C (25 μg/ml; Sigma). The mixed leucocyte reaction (MLR) was carried out by co-incubation of leucocytes from 3 specimens (10^7 leucocytes of each fish) in triplicate. After 4 h of incubation, leucocytes from the replicas were washed, pooled and resuspended in TRIzol reagent.

Total RNA was isolated from TRIzol reagent frozen samples following the manufacturer's instructions. First strand cDNA was synthesized by reverse transcription of 1–5 μg of total RNA using the ThermoScript™ RNAse H^- Reverse Transcriptase (Invitrogen) with an oligo-dT_{18} primer (Invitrogen). Spleen cDNA was used in a first PCR amplification with degenerate primers (Table 1) designed against known fish MHC class I alpha sequences. PCR reactions were carried out using *Taq* polymerase (Invitrogen) and the amplification was performed in a MasterCycler Gradient PCR: 95 °C for 5 min, 35 cycles of 95 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s, and followed by 72 °C for 10 min. PCR products were separated on a 1% agarose gel containing 0.5 μg/ml ethidium bromide and visualised under UV light. PCR fragments were purified from the gel and ligated into the pGEM-T Easy vector (Promega). Following transfection into competent *Escherichia coli* DH-5α cells, recombinants were identified through blue-white color selection in ampicillin Luria Broth Base plates, containing 40 mg/ml of X-Gal. Isolated plasmid DNA was sequenced using an ABI PRISM 377 sequencer. Based on the first partial sequence of seabream MHC class I alpha, specific primers were then designed (Table 1) and used to obtain the 3′ and 5′ ends by rapid amplification of cDNA ends (RACE). For the 5′RACE, an oligo-dT-synthesized spleen cDNA was treated with *E. coli* RNase H (Gibco), purified with the Concert Rapid PCR Purification System (Gibco) and finally tailed with poly(A) at the 5′ end using terminal deoxynucleotidyl transferase (TdT; Gibco). To obtain both ends, this cDNA was used as template in RT-PCR

Table 1
Oligonucleotide primers used in the study

Name	Nucleotide sequence (5′ → 3′)	Position (bp)
MHC-F1	ACTCBHTGAAGTWTTTCTDCAC	64
MHC-F2	GWGGATGWAYGGMTGTGAGTGG	341
MHC-R1	TCTGGAGGAGAGACACYGAKGG	632
MHC-R2	TBABACCWGAGAGMTGAAACAC	856
SpauCIAF5	TGGACCACGGAGAGATCCTCC	730
SpauCIAF7	ACGGAGGATATTCAGACTGTCTGTG	−75
SpauCIAF9	TGGAGGAAAGATGGAGAGGAGC	696
SpauCIF11	ATGAGGTTCTTGGTGTTTCTGG	1
SpauCIAR5	TGGAGCGATCCATGTCTCTGC	462
SpauCIAR8	ACAGCATCCATTGTTCCAGTGC	1169
SpauCIAR9	TCCAGTTTGGTTGAAGCGTGG	324
β-ActinF	ATCGTGGGGCGCCCCAGGCACC	
β-ActinR	CTCCTTAATGTCACGCACGATTTC	
RACET	GGCCACGCGTCGACTAGTAC$(T)_{16}$	
RACE	GGCCACGCGTCGACTAGTAC	

Degeneracy: B = C, G or T; H = A, C or T; W = A or T; D = A, C or T; Y = C or T; M = A or C; K = G or T.