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Cloning of Interferon in Sea Bream (*Sparus aurata*): An Important Marker for Immune Response after Viral Infection

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ABSTRACT

The interferons (IFNs) are soluble cytokines involved in the immune response against viral pathogens. In mammals, three families of IFNs have been identified (types I, II and III) and, recently, homologues of type I have been found in different fish species. In this paper we report the cloning of IFN genes from sea bream (*Sparus aurata*), expression analysis and gene structure. The sea bream IFN cDNA consists of 914 bp that translate in one reading frame to give the entire molecule containing 186 amino acids. The analysis of the sequences revealed the presence of a putative 20 amino acid signal peptide, two cysteine residues and two potential N-glycosylation sites and, finally, a high percentage of identity with type I IFN from mammals compared to type III IFN. Real time PCR was performed after poly I:C stimulation of head kidney leukocytes from sea bream to investigate the expression of sea bream IFN and Mx and an induction was observed for both genes at different time points and with different intensities. The sea bream IFN genes contains four introns, as type III IFN from mammals. The presented results will give the opportunity to investigate more in detail antiviral immune responses, vaccination and immunostimulation effects in sea bream, an economical important species for the South Mediterranean aquaculture.

Keywords: antiviral, IFN, Mx, real time PCR, teleost fish

INTRODUCTION

Interferons (IFNs) are potent cytokines capable of inducing an antiviral state in vertebrate cells after pathogen infections. In mammals, they have been divided in three families (types I, II and III) depending on their structural characteristics, the receptor used and their biological activities (Meager 2006). Type I IFNs can be further chategorised into homologous subgroups (α, β, ω, ε, κ in humans) and all play a crucial role in innate immune responses against virus infections with host cells secreting these IFNs after recognition of viral nucleic acids (Muller *et al.* 1994; Takeuchi and Akira 2007). In birds and mammals, type I IFN genes do not contain introns, and in mammals it has been shown they share a common heterodimeric receptor consisting of the IFNAR-1 and IFNAR-2 subunits (Coulombel *et al.* 1991; Sheppard *et al.* 2003). In addition, the size of these molecules is variable as along with the number of disulphide bonds but they all share a common 5 α-helices 3D structure (Radhakrishnan *et al.* 1996; Karpusas *et al.* 1997). Type II IFN (IFN-γ) is produced by Th1 cells in response to mitogens and antigens and regulates both innate and cell-mediated immune responses (Boehm *et al.* 1997). In mammals, IFN γ is encoded by genes with three introns and binds to a receptor complex containing IFN-γ receptor R1 and R2. Type III IFN (IFN-λ) shows biological activities comparable with that of type I IFNs but is encoded by genes with four introns and bind to a distinct receptor, composed of CRF2-12 and CRF2-4 subunits, also named IFN-λR1 and IL-10R2 (Kotenko *et al.* 2003).

In recent years, genes homologues to type I and II IFNs have been identified in teleost fish (Zou *et al.* 2005; Robertsen *at al.* 2006; Zou *et al.* 2007) and their functional activities have been studied and compared to mammalian IFNs (Robertesen *et al.* 2003; Martin *et al.* 2007). Fish type I IFNs show antiviral activities and are capable of inducing the expression of anti-viral proteins, which includes the Mx protein. They share limited sequence homology with their counterparts in birds and mammals and have a gene structure composed of 5 exons and 4 introns. Different subgroups of type I IFNs, with two or four cysteines in their primary structure, have also been identified in rainbow trout (*Oncorhynchus mykiss*) (Zou *et al.* 2007; Chang *et al.* 2009; Purcell *et al.* 2009) and Atlantic salmon (*Salmo salar*) (Sun *et al.* 2009), demonstrating that some fish possess more IFN genes than previously thought. In addition, an IFN receptor has been characterised in zebrafish (*Danio rerio*) (Levraud *et al.* 2007).

In this paper we report the identification of cDNAs encoding type I IFN homologues from sea bream (*Sparus aurata*), a fish of high economic impact for South Mediterranean countries, and analyse its gene structure and expression after poly I:C stimulation by real time PCR.

MATERIALS AND METHODS

Sea bream cDNA IFN cloning and sequencing

Leukocytes were obtained from a juvenile sea bream (150 g of weight) head kidney (HK) as described by Scapigliati *et al.* (2001) and were stimulated for 6 h with 20 μg/ml of polyinosinic polycytidylic acid (poly I:C, Sigma) in L-15 medium (Gibco). Total RNA was extracted with Tripure (Roche) and the primers IFNFR and IFNRV (**Table 1**) corresponding to highly conserved regions of known IFN genes were used in RT-PCR performed with Ready-To-Go RT-PCR Beads (GE Healthcare). The cycling protocol was one cycle of 94°C for 5 min, 35 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 45 s, followed by one cycle of 72°C for 10 min. PCR products (10 μl) were visualised on 1% (w/v) agarose gels containing 10 ng/ml GelRed™ (Biotium) using hyperladder IV (Bioline) as size marker. Controls for the presence of genomic DNA contamination were performed using the RNA samples as template. Plasmid DNA from at least six independent clones was sequenced (MWG) and generated sequences were analysed for similarity with other known sequences using the FASTA (Pearson and Lipman 1988) and BLAST (Altschul *et al.* 1990) programs.

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Table 1 Primers used for cDNA and genomic DNA cloning, and expression analyses of sea bass IFN and Mx.

Primer name	Sequence (5′-3′)	Use
IFNFR	ACAGCTCTGCATCATGGG	cDNA cloning for IFN
IFNRV	CTCCCAGGCT T/G CAGC A/G CT	cDNA cloning for IFN
IFNFWB	GCCACGTCCTAAAGAAAATGGGCC	3′ RACE-PCR for IFN
Oligo-dT adaptor	CTCGAGATCGATGCGGCCGCT$_{15}$	3′ RACE-PCR for IFN
Oligo-dG	GGGGGGIGGGIIGGGIIG	5′ RACE-PCR for IFN
IFNRW1	CTCCCATGATGCAGAGCTGTG	5′ RACE-PCR for IFN
ORAIFNFW	GCTCCTCGCTGAGCTGC	Genomic DNA cloning for IFN
ORAIFNRW1	GGCTTCAGCACTGTGGC	Genomic DNA cloning for IFN
RQIFNF2	GCTGAGGATCAACTTGCC	Q-PCR for IFN
RQIFNR1	GTGGCCCATTCTCTTGAG	Q-PCR for IFN
RQMxFW	GTCTGGAGATCGCCTCT	Q-PCR for Mx
RQMxRW	TCTCCATCAGGATCCAC	Q-PCR for Mx
RQRIBFR	CCAACGAGCTGCTGACC	Q-PCR for 18S rRNA
RQRIBRV	CCGTTACCCGTGGTCC	Q-PCR for 18S rRNA

Further primers were designed based on the initial sea bream IFN sequence for 5′- and 3′- rapid amplification of cDNA ends (RACE-PCR): IFNRW1 and IFNFWB (**Table 1**), respectively. cDNA was synthesised from head kidney leukocytes RNA stimulated with poly I:C with a First-strand cDNA Synthesis kit (GE Healthcare) following the manufacturer's instructions. For 3′ RACE-PCR, cDNA was transcribed using an oligo-dT adaptor primer (**Table 1**), and the PCR was performed with the IFNFWB primer and the oligo-dT adaptor primer. For 5′ RACE-PCR, cDNA was transcribed from total head kidney leukocytes RNA using the oligo-dT primer and successively treated with *E. coli* RNase H (Promega), purified using a PCR Purification Kit (QIAgen) and tailed with poly(C) at the 5′ end with terminal deoxynucleotidyl transferase (TdT, Promega). PCR was performed with IFNRW1 primer and an oligo-dG primer (**Table 1**). Cloning, sequencing and similarity searches related to the obtained products were as described above. The obtained cDNA sequence was analysed for the presence of a signal peptide, using SignalP (Nielsen *et al.* 1997) and for N- and O-linked glycosylation sites (NetNGlyc 1.0 Server, http://www.cbs.dtu.dk/services/NetNGlyc/; Julenius *et al.* 2005). Comparison of the sea bream IFN nucleotide and amino acid sequence to its counterparts from other species was carried out using the DIALIGN program (Brudno *et al.* 2003). Alignment of the sea bream IFN amino acid sequences to their counterparts from other species was carried out using MEGA 3.1 Software (Kumar *et al.* 2004). A phylogenetic tree was constructed by the "neighbour-joining" method using MEGA 3.1 Software (Kumar *et al.* 2004) on full-length amino acid sequences and bootstrap values calculated.

Basal Mx expression analysis

To study the Mx basal expression, six sea bream juveniles were sampled and leucocytes from different tissues (muscle, thymus, spleen, liver, gills, head kidney, gut) obtained as described in Scapigliati *et al.* (2001). Total RNA was isolated from each tissue separately with Tripure (Roche) following the manufacturer's instructions, resuspended in DEPC treated water and used for real-time quantitative PCR without pooling the tissue samples coming from the different fishes. Controls for the presence of genomic DNA contamination were performed using β-actin primers that bracket an intron. For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used with the protocol described in Buonocore *et al.* (2007). The expression level of Mx transcript was determined with a Mx3000P^{TM} real time PCR system (Stratagene) equipped with version 2.02 software and using the Brilliant SYBR Green Q-PCR Master Mix (Stratagene) following the manufacturer's instructions, with ROX as an internal passive reference dye. Specific PCR primers were designed for the amplification of about 200 bp products from Mx and the 18S ribosomal RNA, which was used as the house-keeping gene (**Table 1**). Approximately 10 ng of cDNA template was used in each PCR reaction. The PCR conditions were 95°C for 10 min, followed by 35 cycles of 95°C for 45 s, 52°C for 45 s and 72°C for 45 s. Triplicate reactions were performed for each template cDNA and the template was replaced with water in all blank control reactions. The analysis was carried out using the endpoints method option that causes the collection of the fluorescence data at the end of each extension stage of amplification. A relative quantitation has been performed, comparing the levels of the target transcript (Mx) to a reference transcript (calibrator, the tissue with the lowest Mx expression, in this case the muscle). A normalizer target (18S ribosomal RNA) is included to correct for differences in total cDNA input between samples. The results are expressed as the mean ± SD of the results obtained from the six considered fishes. The real-time PCR products from the different tissues were examined successively by agarose gel electrophoresis to investigate their specificity, size and sequence.

IFN and Mx expression analysis after *in vitro* stimulation

The *in vitro* IFN and Mx expression was studied using leukocytes isolated from six juveniles sea bream (150 g of weight) head kidney (HK) cells cultured in L-15 medium (Gibco) containing 10% FCS, adjusted to 1×10^5 cells/ml and incubated at 18°C for 6 h and 24 h with 50 μg/ml of poly I:C (Sigma). The cell control samples were stimulated with L-15 alone and analysed at the same time points. Total RNA was isolated with Tripure (Roche) following the manufacturer's instructions, resuspended in DEPC treated water and used for real-time quantitative PCR without pooling the samples coming from the different fishes. The primers are shown in **Table 1** and the real time PCR conditions were the same as described above, except that the calibrator for these experiments was the muscle. The results were expressed as the mean ± SD of the results obtained from six fishes and the differences from the control at the same time point were considered significant if $p < 0.05$ using the two-way ANOVA analysis following by Bonferroni's test.

Sea bream genomic DNA isolation and IFN gene cloning and sequencing

Genomic DNA was isolated from whole blood of a juvenile sea bream (150 g of weight) with the Wizard Genomic DNA purification kit (Promega) as described in Buonocore *et al.* (2003). The obtained DNA was resuspended in 30–100 μl of sterilized water at a final concentration of 10 ng μl^{-1}. The quality of the DNA was checked by running 1 μl of DNA on an GelRed™ stained agarose gel (0.5%, w/v). Based on the sea bream IFN cDNA sequence and on the genomic organisation of trout IFN (Zou *et al.* 2007) and of sea bass (Casani *et al.* 2009), two primers (ORAIFNFW and ORAIFNRW1, **Table 1**) were constructed to obtain the entire IFN gene. The cycling protocol was one cycle of 94°C for 5 min, 35 cycles of 94°C for 45 s, 55 °C for 45 s, 72°C for 45 s, followed by one cycle of 72°C for 10 min using the *Taq* PCR Master Mix Kit (QIAgen). 10 ng of genomic DNA were used for each PCR reaction. PCR products were visualised, sequenced and analysed as described above.

A

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                1                                                   60                                                  120
Sparusaurata1   MLNRIFFVCLSLSLYSAGSSLSCRWVMDHKFRQHSKNSLALLDTMANNSTNTTEDAEVEDTVAFPNLLYRQASKASAEDQLAFTVQILDETAALFEEDHSSASWEENTVENFVNVVTQQA
              121                                                 180   186
Sparusaurata1   DELRSCIGSHGHKKKNKKLHMYFQRLSSHFLQRMGHSAEAWELIRGEVKVHLMRANQLVTSATRTN
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B

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→ Oligo-dG
ACCAGTATAGCAATCAGTATAGCAATCAATACAGCAATCAGTATAGCAATCAGTGCAGCAA
CCAACACAACACCAGCACTCATCTGAACTTACTCTATACAAAGTCAATCTCTACCTTTTTG
AAA                                                  → ORAIFNFW
atgctcaacaggatttttctttgtgtgcctgtctctcagtctgtacagtgcaggctcctcg
 M  L  N  R  I  F  F  V  C  L  S  L  S  L  Y  S  A  G  S  S
ctgagctgcagatgggtcatggatcataaattcagacagcacagtaaaaactctttggct
 L  S  C  R  W  V  M  D  H  K  F  R  Q  H  S  K  N  S  L  A
ctactggatacgatggctaataactccactaacaccactgaggatgccgaagtggaggac
 L  L  D  T  M  A  N  N  S  T  N  T  T  E  D  A  E  V  E  D
                                                  → RQIFNF2
actgtggccttccctaatcttctgtacagacaggcgtccaaagcatcagctgaggatcaa
 T  V  A  F  P  N  L  L  Y  R  Q  A  S  K  A  S  A  E  D  Q
                                                     → IFNFR
cttgccttcacagttcagattcttgatgagacggctgccctgtttgaggaggatcacagc
 L  A  F  T  V  Q  I  L  D  E  T  A  A  L  F  E  E  D  H  S
   IFNRW1 ←
tctgcatcatgggaggagaacacagtggagaactttgtcaacgttgtgacccagcaggcc
 S  A  S  W  E  E  N  T  V  E  N  F  V  N  V  V  T  Q  Q  A
gacgagcttcgctcctgtatcgggagccacggacacaagaagaagaacaagaagctgcac
 D  E  L  R  S  C  I  G  S  H  G  H  K  K  K  N  K  K  L  H
           →        IFNFWB  RQIFNR1 ←  ORAIFNRW1 ←
atgtacttccagagactgtccagccacttcctccagagaatgggccacagtgctgaagcc
 M  Y  F  Q  R  L  S  S  H  F  L  Q  R  M  G  H  S  A  E  A
   IFNRV ←
tgggagctgatcaggggggaagtcaaagtccatctgatgagagcaaaccagctggttaca
 W  E  L  I  R  G  E  V  K  V  H  L  M  R  A  N  Q  L  V  T
tctgcaaccagaaccaactga
 S  A  T  R  T  N
GACCTGTCACATTAACCATCTGTTCAACTATTTATTTATCTATTTATTTTTTTTATTT
ATTATTGCACTTATTTATCTTCTCATTGAGTTGTTTGTCTATTTATTGAGTGATTGTTACT
TTTATACATGAAAATGTTGTTGATCAGATTATTTATTCAACTTTTCTATATTTATACCCGT
AAAAGACAATAAAAATAAGACTTTCTACAGCAAAAAAAAAAAAAAAAA
                    Oligo dT Adaptor ←
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Fig. 1 cDNA sequence of sea bream IFN. (**A**) The cDNA sequence of one characterised sea bream IFN molecule (*Sparus aurata* 1 = accession number AM937107). (**B**) The cDNA and putative encoded amino acid sequence of one characterised sea bream IFN molecule (accession number AM937107). The primers used for the cloning are indicated (see **Table 1**). The start and stop nucleotide sequences are in bold, the putative signal peptide is in italics, the two putative N-glycosylation sites are underlined, the putative O-glycosylation site is in bold and underlined, the polyadenylation signal is in bold and italics.

RESULTS

Sea bream cDNAs IFN sequence analysis

HK purified leukocytes were stimulated *in vitro* with poly I:C prior to RNA extraction to obtain high levels of sea bream IFN mRNA, as this compound is usually utilised for induction of mammalian and fish type I IFNs (Miley-Milovanovic *et al.* 2009). PCR with primers IFNFR and IFNRV gave a product of the expected size (191 bp) that when sequenced showed the presence of two slightly different clones (**Fig. 1A**) with good similarity versus other known IFN molecules (data not shown). 3′-RACE-PCR performed with IFNFWB (based on the initial 191 bp sequence) and the Oligo-dT adaptor primer gave a product of 368 bp (**Fig. 1B**). 5′-RACE-PCR performed with IFNRW1 (based on the initial 191 bp sequence) and oligo-dG gave a product of 490 bp (**Fig. 1B**). The full-length cDNAs (EMBL accession numbers AM937107 and AM937108) are comprised of 914 bp from the three overlapping products and were confirmed by PCR using primers that amplify the complete coding sequence (data not shown). The 3′-UTRs contained a polyadenylation signal (AATAAA) 18 bp upstream of the poly(A) tail (**Fig. 1B**).

An analysis of the sea bream IFN cDNA sequence (accession number AM937107) revealed the presence of a putative 20 amino acid signal peptide (most likely cleavage site between Ser^{20} and Lys^{21}), two potential N-glycosylation sites and one putative O-glycosylation site (**Fig. 1B**). Comparison of the sea bream IFN nucleotide and amino acid sequence (accession number AM937107) to its counterparts in other species is shown in **Table 2**. The highest nucleotide and amino acid identity (91.8 and 88.2%, respectively) was with sea bass (*Dicentrarchus labrax*), followed (73.0 and 65.1%, respectively) by stickleback (*Gasterosteus aculeatus*) and (69.1 and 56.7%, respectively) medaka (*Oryzias latipes*), whilst the lowest identity (41.8 and 17.9%, respectively) was to Western clawed frog (*Xenopus tropicalis*, accession number BN001169) followed by (45.5 and 20.7%, respectively) turkey (*Meleagris gallopavo*). The percentages of nucleotide and amino acid identity were higher (51.2 and 31.4%, respectively) to rainbow trout 2 (*Oncorhynchus mykiss*, accession number AJ582754), compared to rainbow trout 1 (accession number AJ580911) and 3 (accession number AM235738) and quite similar for zebrafish (*Danio rerio*) 1 (accession number AJ544820) and 2 (accession number NP_001104552). Moreover, the percentages of nucleotide and amino acid identity were lower (46.2 and 16.5%, respectively) with human (*Homo sapiens*) IFN-λ (type III IFN) compared to human IFN-α (type I IFN).

A multiple alignment of the sea bream IFN amino acid sequence with other known IFN sequences was assembled (**Fig. 2**) to investigate the conservation of characteristic amino acid residues involved in structural domains. In the sea bream IFN sequence two cysteines are present (Cys^{23} and Cys^{126}) and are conserved in all considered sequences. In human type I IFN-α two additional cysteine residues that form two disulphide bridges (Klaus *et al.* 1997) are present and they are conserved in rainbow trout 3, in zebrafish 2, in Western clawed frog 1 and 2 (accession number BN001170), and in bird IFN sequences (mallard and turkey).

Phylogenetic analysis (**Fig. 3**) performed using the amino acid sequences showed that sea bream IFNs grouped with the other known teleost sequences, very close to sea bass IFN and in the same cluster of the Tetraodontiformes. The rainbow trout 3 and zebrafish 2 sequences grouped in the clade of human IFN-α sequence.

Table 2 Percentage of nucleotide identity and amino acid identity and similarity of sea bream IFN with other known IFN sequences. The highest values are evidenced in bold.

Scientific name	Accession numbers	Common name	% Nucleotide identity	% Amino acid identity	% Amino acid similarity
Dicentrarchus labrax	AM765846	Sea bass	**91.8**	**88.2**	**94.1**
Gasterosteus aculeatus	CAM31707	Three-spined stickleback	73.0	65.1	78.5
Oryzias latipes	CAM32419	Japanese medaka	69.1	56.7	71.7
Takifugu rubripes	CAM82750	Fugu	68.0	54.8	70.4
Tetraodon nigroviridis	CAD67762	Spotted green pufferfish	68.0	54.8	72.0
Salmo salar	AAP51035	Atlantic salmon	52.6	32.1	51.9
Oncorhynchus mykiss	AJ580911	Rainbow trout 1	49.5	27.4	45.2
Oncorhynchus mykiss	AJ582754	Rainbow trout 2	51.2	31.4	47.6
Oncorhynchus mykiss	AM235738	Rainbow trout 3	46.3	24.4	42.1
Danio rerio	AJ544820	Zebrafish 1	49.3	31.8	51.0
Danio rerio	NP_001104552	Zebrafish 2	48.1	20.8	38.2
Carassius auratus	AAR20886	Goldfish	49.0	31.3	49.2
Ictalurus punctatus	AY847296	Channel catfish	49.3	27.6	46.7
Xenopus tropicalis	BN001169	Western clawed frog 1	41.8	17.9	38.6
Xenopus tropicalis	BN001170	Western clawed frog 2	48.0	23.4	39.3
Anas platyrhynchos	P51526	Mallard	46.7	21.5	34.0
Meleagris gallopavo	P51527	Turkey	45.5	20.7	36.6
Homo sapiens	NM_000605	Human α	49.4	24.9	42.0
Homo sapiens	NM_172138	Human λ	46.2	16.5	32.5

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                                                                    36                                                  86
Sparusaurata1*          ------------------------MLNRIFFVCLSLSLYSAGSSLSCRWVMDHKFRQHSKNSLALLDTMANNSTNTTE-DAEVEDTVAFPNLLYRQ---------ASKASAEDQLAFTVQ
Sparusaurata2*          ------------------------MLNRIFFVCLSLSLYSAGSSLSCRWVMDHKFRQHSKNSLALLDTMANNSTNTTE-DAEVEDTVAFPNLLYRQ---------ASKASAEDQLAFTVQ
Dicentrarchuslabrax     ------------------------MLNRIFFVCLSLSLYSAGSSLSCRWIMDHKFRQHSKNSLALLDTMANNSTNTTE-DAEVEDTVAFPNLLYRQ---------ASKASAEDQLAFTVQ
Gasterosteusaculeatus   ------------------------MLCRMFLVFVCLSLYSSASSLSCRWV-DHKFSHLSRTSMDLLDTLAHNSTNSTE-DAENN----FPNNLYSQ---------ASKASAEDKLRFSVQ
Oryziaslatipes          ------------------------MLHRLVFACALVSLAGAGFSLRCRWL-DHKFKQFSDTSLDLLEKMVNNATNSTEGDATEDIEVDFPHHLYRQ---------ASKESAENQVAFTVQ
Takifugurubripes           ---------------------MLP--LLVCLSLCVYSQGSPLGCRWL-DDKFRQYSHKSLELLDTMVNNSTNSSV---EPEEMVIFPQELYRQ---------TFNASAEDKLALAAQ
Tetraodonnigroviridis           -----------------MLT--VLLCLSLCVCSQGSPLGCRWL-EEKFTQYSSLSLSLLDNMKSNSTNSSL---EAEDTAIFPEVLYRQ---------TFNASAEDRLAFAAQ
Salmosalar               ----------------------MYTVQSWTCICLIICSMQSVCHCCDWI-RHHYGHLSSEYLSLLDQMGGD-------ITKQDAPVFFPTSLYRH---------IDDAEVEDQVRFLKE
Oncorhynchusmykiss1*    ---------------------------------------MQSVCHCCDWI-RHHYGHLSAEYLPLLDQMGGD-------ITKQNAPVLFPTSLYRH---------IDDAEFEDKVIFLKE
Oncorhynchusmykiss2*      ---------------------MCTMQSWTCLFLILCSMQSVCHCCDWI-QHHFGHLSAEYLSQLEQMGGD-------ITKQNAPVLFPTSLYRH---------IYDAEFEDKVRFLNE
Oncorhynchusmykiss3*    -----------------------MAVLKWLSICLTLFCQGTAASKPCRWT-QFRLGKLNDVSIDLLSDMGGIFP------LMCAEENVEQMFPEDLYKN------TEG---EDVSVVALE
Daniorerio1*             -------------------------MWTYIFVIYVILQSQSSASTCEWL-G-RYRIITTESLNLLKNMGGK-------YAD--LETPFPSRLYTL---------MDKSKVEDQVKFLVL
Daniorerio2*          ---------------------MEFWQFVAFLCPALFFAHITSK--PTNCFMRRKHVKT--AYSLLESMGGLFP---------RECLKENVRITFPKYALQS------NNSNQKTGVAKAVYK
Carassiusauratus        -----------------------MKTQMWTYMFVMFLTLQGQCSA-CEWLG-R-YRMISNESLSLLKEMGGK-------YPEG-TKVSFPGRLYNM---------IDNAKVEDQVKFLVL
Ictaluruspunctatus      ---------------------MDIKQSWICLYFLLFFIVQERSEA-CNWMI-SQYRAKNNFCVSLLKEMGGE-------IVQ--VNRPFPHKAYSE---------IDKAKAEDQVRFLAQ
Xenopustropicalis1*     MFKRNTFGSYCCINYAIYVSITVTTLHMASIQTILLLVLIPIVQSQNCKWLQ-PKQEYLNRQTLKTFEEMNPPE----DYDESCQYDSIELPNIDEI---------YSISQMEEMVLAVRG
Xenopustropicalis2*     -----------------------MLPMGQWSVLLLLSLTSIVHSQSCKWLH-PKQEYLNTQILKAFNEMMPLK----ETEEICEEHPTDLPNTEST---------YSVSQVEAGALAVRE
Anasplatyrhynchos       ----------------MPGPSAPPPPAIYSALALLLLLTPPANAFSCSPLR-LHDSAFAWDSLQLLRNMAPSP------TQPCPQQHAPCSFPDTL---------LDTNDTQQAAHTALH
Meleagrisgallopavo      ----------------MAVPASPQHPRGYGILLLTLLMKALAAAAACNHLR-PQDATFSRDSLQLLRDMAPSP------PQPCPQHNAPCSFNDTV---------LDTNNTQQADKTTHN
Homosapiensα            -----------------------MALTFALLVALLVLSCKSSCSVGCDLPQT--HSLGSRRTLMLLAQMRRISLFS------CLKDRHDFGFPQEEFG--------NQFQKAETIPVLHE
Homosapiensλ            --------------------MKLDMTGDCTPVLVLMAAVLTVTGAVPVARLHGALPDARGCHIAQFKSLSPQELQAFKRAKDALEESLLLKDCRCHSRLFPRTWDLRQLQVRERPMALEA

                        87                                                         135                                                  186
Sparusaurata1*          ILDETAALFEED----HSSASWEENTVENFVNVVT-QQADELRSCIGSHGHKKK--------NKKLHMYFQRLSSHFLQRMGHSAEAWELIRGEVKVHLMRANQLVTSATRTN-------
Sparusaurata2*          ILDETAALFEED----HSSASWEENTVENFVNVVT-QQADELRSCIGSHGHKKK--------NKKLHMYFQRLSSHVLKRMGHSAEAWELIRGEVKVHLMRANQLVTSATRTN-------
Dicentrarchuslabrax     ILNETAALFEED----HSSASWEENTVEDFVNVVT-RQADNLRSCIGSHGHKT---------NKKLQMYFMKLSSHVIKKMGHSAEAWELIRKEIKTHLMRADQLVSSLLTTN-------
Gasterosteusaculeatus   ILEEMAALFVED----HSNASWDEQTVDHFLFVVT-KQADSLHSCS--HGHKRK--------NKKLQMYFKRLSHHVLEQMGHSAESWELIRKEMKAHLMKTDQLVLSLLSN--------
Oryziaslatipes          VLKEVSALFEED----SSSASWQQITVEKFLGVVN-RQADELHSCVSESLVHKK--------NRKLRMYFKRLLDHILKKQGYSAEAWETIRKETKAHLLRAQRLLSPLISSK-------
Takifugurubripes        IMNETVALLMED----HSGASWDEKQVENLVNVLT-QQADNLQACMVSPGHKR---------SEEVERYFNRLSNHILKKMDYSAAAWELIREEIETLLMQTHLLVSTLLSTP-------
Tetraodonnigroviridis   ILNETAALFEED----YSGASWEEKSVENFVNILT-QQADNLGSCVASPGQSR---------SKELHKYFTRISTHILRKTDHSAGAWELVREKIRSLLMRAHLLLTTH-----------
Salmosalar              TIYQITKLFDG----NMKSVTWDKKKLDDFLNILE-RQLENLKSCVSPAMKPEK----------RLKRYFKKLNKNVLRKMNYSAQAWELIRKETKRHLQRLDILAAQMY----------
Oncorhynchusmykiss1*    TIYQITKLFDG----NMKSATWDKKNLDDFLNILE-RQLENLNSCVSPAMKPER----------RLKRYFKKLNSKVLRKMNYSAQAWELIGKETKRNLQRLDILAAQMY----------
Oncorhynchusmykiss2*    TIYQIIKLFDGNRNRNRKSVTWDKKNLDDFLNILD-RQFENLSSCVSPVVKPEK----------RLKRYFRNLNRKVLRKMNYSAEAWELIRKETKRHLQRLDILVGQMY----------
Oncorhynchusmykiss3*    AMRYVEQLYNNS----LTSVTWNKIKLNMFQNVIY-RQVQNLELCVVGGVWESSGDGG----SVTLKTYFNKL-NTVLKEKEHSACAWEIVRKEIRENLVQFKKFIDSRVKP--------
Daniorerio1*            TLDHIIHLMDAR--EHMNSVNWDQNTVEDFLNILH-RKSSDLKECVARYAKPAHKESY----EIRIKRHFRTL-KKILKKKQYSAEAWEQIRRVVKSHLQRMDIIASNARVNP-------
Daniorerio2*          --IMDHIDFLFAND----SYPEAWNKRKVDNFQNIVY--RLTKENQCIMRMRAQGTVDDFP-ARDDALKSYFNKL-ATLLRNKDNSFCAWEVVRHELLGVLS--DIIQPKL-----------
Carassiusauratus        TLDHIIRLMDAR--EHMNSVQWNLQTVEHFLTVLN-RQSSDLKECVARY-QPSHKESY----EKKINRHFKIL-KKNLKKKEYSAQAWEQIRRAVKHHLQRMDIIASIANRR--------
Ictaluruspunctatus      ATEQIISVFNV---SHVDEVKWDRSALDEFLNILNTRQLTELTKCTSTYAERAGHSPT----ERKLRKHFKKL-KKFLNEANYSADSLERIRNVVQHHLWRMDIIAANVKQKLLKRTN--
Xenopustropicalis1*     VLNETMRFYMKH----HESMGCKQQAWERFQQLLY-YQINQLEACIPETAENPVFN-------QTISDQYQALEQILQEK--NTACTRDIIQSEIRGNLQLVGQLASRARRQRLLQRTA--
Xenopustropicalis2*     VLNETMRFYMKH----HESMGCKQQAWERFQQLLY-YQIHQLEACVSQTEENDLLK-------ESISEEFNLLETMVLEKD-NSACVWDFIHLETRRNLQQVLQLSSRLRRQRLLQRPQ-
Anasplatyrhynchos       LLQHLFDTLSSP----STPAHWLHTARHDLLNQLQ-HHIHHLERCFPADAARLHRRGPR-NLHLSINKYFGCI-QHFLQNHTYSPCAWDHVRLEAHACFQRIHRLTRTMR----------
Meleagrisgallopavo      ILQHLFKILSGP----TTPAHWIDSQRQSLLNQIQ-RYAQHLEQCLADSHTRSRTRWPH-NPHLTINKHFSCL-HAFLHDNDYSACAWDHVRLRARAWLLHIHDLVRNTRT---------
Homosapiensα            MIQQIFNLFSTK----DSSAAWDETLLDKFYTELY-QQLNDLEACVIQGVGVTETPLMKEDSILAVRKYFQRI-TLYLKEKKYSPCAWEVVRAEIMRSFSLSTNLQESLRSKE-------
Homosapiensλ            ELALTLKVLEAT----ADTDPALVDVLDQPLHTLH-HILSQFRACIQPQPTAGP--------RTRGRLHHWLYRLQEAPKKESPGCLEASVTFNLFRLLTRDLNCVASGDLCV-------
```

Fig. 2 Alignment of the predicted sea bream IFN amino acid sequences with other known IFN molecules. Conserved cysteines are evidenced in bold, conserved leucine and phenylalanine residues are in bold and underlined. Accession numbers: sea bream 1 (*Sparus aurata*) AM937107; sea bream 2 (*Sparus aurata*) AM937108; sea bass (*Dicentrarchus labrax*) AM765846; three-spined stickleback (*Gasterosteus aculeatus*) CAM31707; Japanese medaka (*Oryzias latipes*) CAM32419; Fugu rubripes (*Takifugu rubripes*) CAM82750; spotted green pufferfish (*Tetraodon nigroviridis*) CAD67762; Atlantic salmon (*Salmo salar*) AAP51035; rainbow trout 1 (*Oncorhynchus mykiss*) AJ580911; rainbow trout 2 (*Oncorhynchus mykiss*) AJ582754; rainbow trout 3 (*Oncorhynchus mykiss*) AM235738; zebrafish 1 (*Danio rerio*) AJ544820; zebrafish 2 (*Danio rerio*) NP_001104552; goldfish (*Carassius auratus*) AAR20886; channel catfish (*Ictalurus punctatus*) AY847296; Western clawed frog 1 (*Xenopus tropicalis*) BN001169; Western clawed frog 2 (*Xenopus tropicalis*) BN001170; mallard (*Anas platyrhynchos*) P51526; turkey (*Meleagris gallopavo*) P51527; human α (*Homo sapiens*) NM_000605; human λ (*Homo sapiens*) NM_172138. *The different sequences related to the same species are due to the presence of more clones.

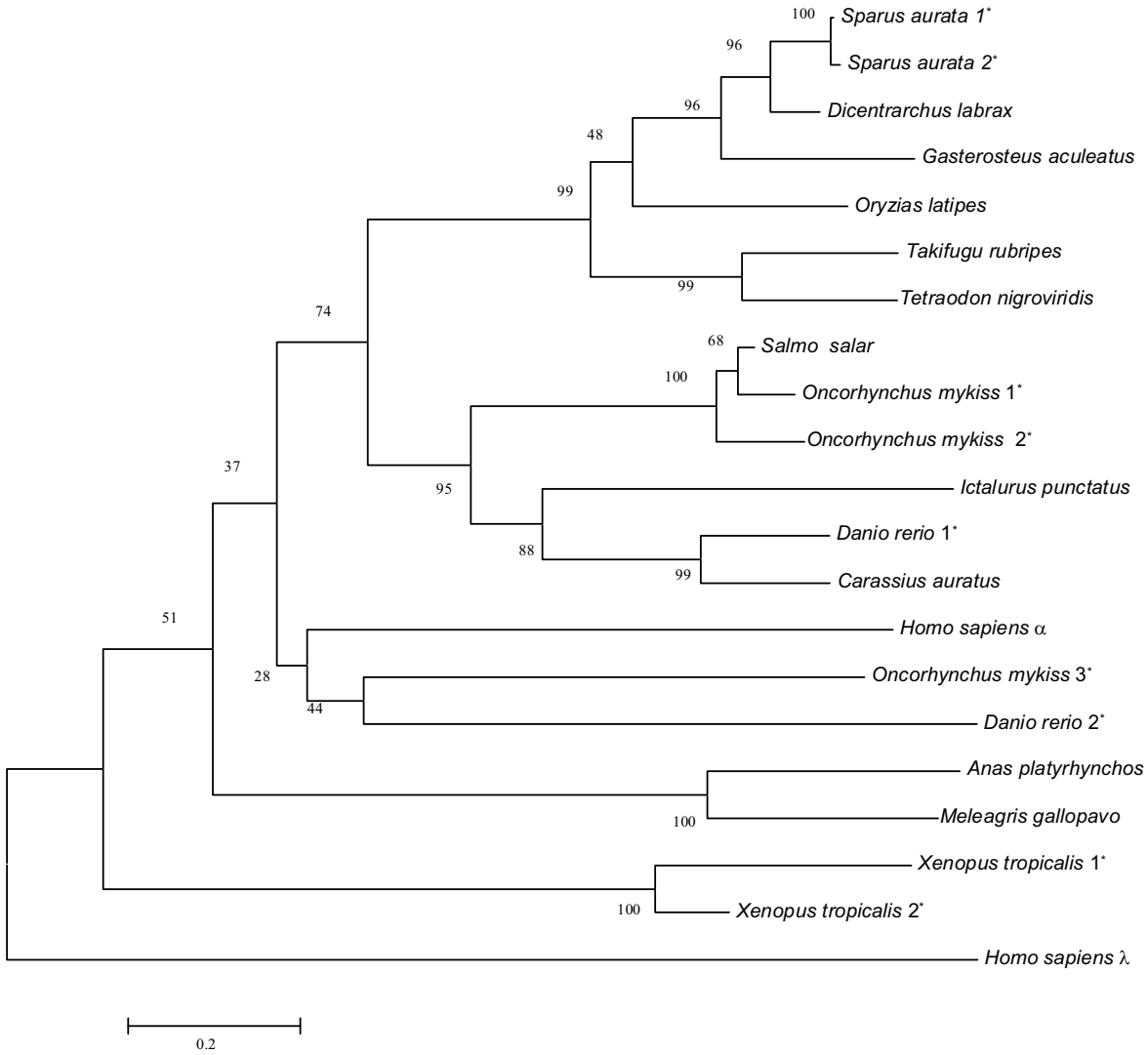

Fig. 3 Phylogenetic tree showing the relationship between sea bream IFN sequences with other known IFN molecules. The rooted tree was constructed by the "neighbour-joining" method and was bootstrapped 10000 times. 0.2 indicates the genetic distance. *The different sequences related to the same species are due to the presence of more clones.

Basal Mx expression analysis

The basal expression analysis of Mx in tissues of un-stimulated sea bream is shown in **Fig. 4**. Real-time PCR products were loaded on agarose gels to exclude the formation of non-specific amplicons and single bands of the expected sizes were obtained. Moreover, to take into consideration the individual genetic variability six different fishes were sampled and analysed separately. The highest Mx expression was detected in liver, followed by spleen, thymus and head kidney. The gills and muscle showed the lowest expression levels.

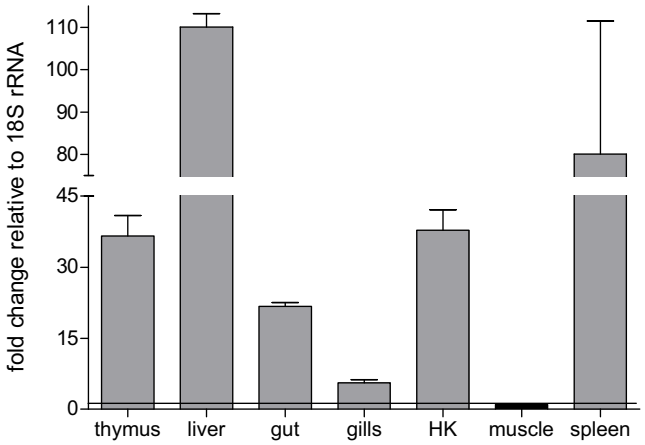

Fig. 4 Basal Mx expression analyses. Sea bream Mx basal expression in different tissues. Mx mRNA levels were expressed as a ratio relative to 18S rRNA levels in the same samples after real-time PCR analysis using the tissue with the lowest expression (muscle) as calibrator.

IFN and Mx expression analysis after *in vitro* stimulation

To investigate whether IFN and Mx expression levels could be modulated with poly I:C, *in vitro* stimulation of HK leucocytes for a short (6 h) and a longer (24 h) time was studied; RNA was extracted from the HK leucocytes of six individuals analysed separately. Real-time PCR products were loaded on agarose gels and single bands of the expected sizes were obtained. The results are shown in **Fig. 5**. Controls for 6 h and 24 h of incubation adding PBS only have been performed to show the differences from the time 0 control. A significant increase ($P < 0.05$) of IFN expression was detected after 6 h, whereas a decrease was evidenced after 24 h stimulation showing no significant difference from the 24 h control. With regard to Mx, after 6 h stimulation a significant increase ($P < 0.05$) of expression was detected, a further slight increase was observed after 24 h stimulation but showing no significant difference from the 24 h control.

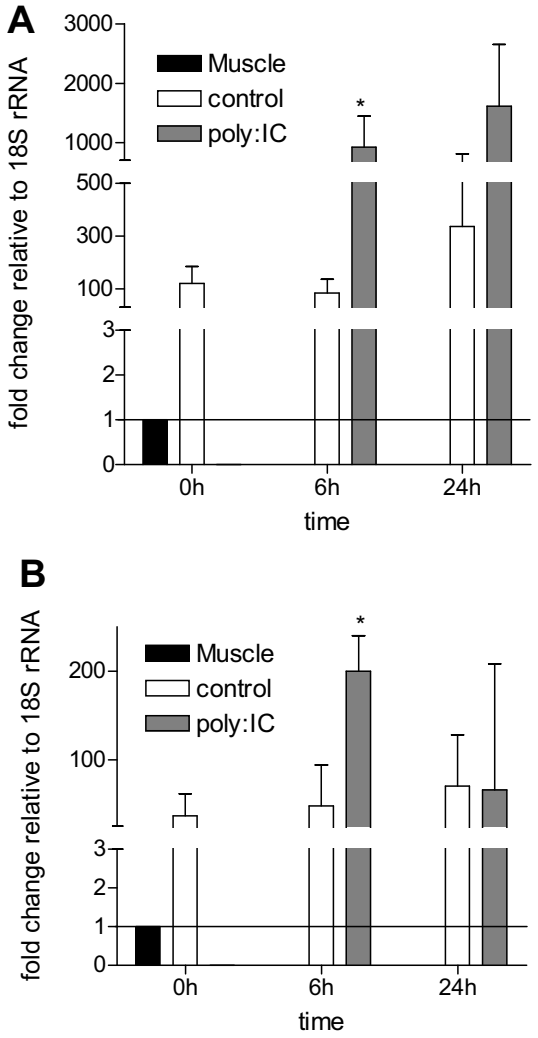

Fig. 5 ***In vitro*** **sea bream Mx (A) and IFN (B) expression analysis.** (**A**) Mx after stimulation with poly I:C. (**B**) IFN after stimulation with poly I:C. IFN and Mx mRNA levels expressed as a ratio relative to 18 S ribosomal RNA levels in the same samples after real-time PCR analysis of HK leukocytes stimulated with PBS (control) and with 20 μg/ml poly I:C for 6 and 24 h (the calibrator was the muscle). Data were expressed as the mean ± SEM; * indicates that $P < 0.05$.

Sea bream genomic DNA isolation and IFN gene cloning and sequencing

PCR amplification of genomic DNA, using the primers deduced from the sea bass and rainbow trout sequence, revealed the complete sea bream IFN gene organisation. Four different sequences have been evidenced (EMBL accession numbers FM882241, FM882242, FM882243, FM882244) with slight differences in the coding sequence (**Fig. 6**) and in the intron size (**Table 3**), but all showing the typical organisation of most fish and mammals type I IFNs, with five exons and four introns. All the introns started with the typical "GT" nucleotide sequence and ended with the usual "AG" sequence and were in phase between all exons.

DISCUSSION

In this report, IFN cDNA homologues from sea bream (*Sparus aurata*) were isolated and the IFN and Mx expression after *in vitro* stimulation with poly I:C has been analysed. Moreover, the sea bream IFN gene organisation has been studied and compared to counterparts in teleosts and mammals to reveal new insight with regard to the evolution of this fundamental antiviral component of the immune system.

The sea bream cDNA sequences translate in one reading frame to give a putative protein of 186 amino acids with two cysteine residues. The number of cysteines is the same of human type I IFN-β (Karpusas *et al*. 1997), where two cysteines form a single disulphide bond (in sea bream the cysteines are in different positions compared to human), but different from human type I IFN-α (4 cysteine residues) and human type III IFN-λ (6 cysteine residues). Most of the teleost fish IFN sequences show the presence of two conserved cysteines except for rainbow trout 3 and zebrafish 2 that have two additional cysteines. An analysis of the sequences shows the presence of two potential N-glycosylation sites and one O-glycosylation site. N-glycosylation seems not fundamental for antiviral activities of human IFN-α, but is believed to play a role in regulating protein solubility and stability of human IFN-β (Karpusas *et al.* 1998). Murine IFN-β contains three predicted N-glycosylation sites, one of which is in a region predicted to interact with type I IFN receptor. Mutation of individual sites had a weak negative influence of IFN antiviral activity, but the complete loss of glycosylation dramatically decreased antiviral activity (Sommereyns and Michiels 2006). The "CAWE" motif, that is usually present at the C-terminal region of the higher vertebrate type I IFNs (Zou *et al.* 2007), is not found in sea bream, but is present in most of the other teleost sequences isolated until now. Some residues are conserved between almost all fish sequences, in particular Leu[22] and Phe[44]. The substitution of these residues in human IFN-α led to a decrease in the IFN recombinant antiviral activity and showed their importance for IFN biological activity (Waine *et al.* 1992).

Table 3 Intron size (in bp) of the four characterised sea bream IFN gene sequences.

IFN gene sequences	Intron 1	Intron 2	Intron 3	Intron 4
Sparus aurata 1 (FM882241)	106	297	107	80
Sparus aurata 2 (FM882242)	106	297	106	80
Sparus aurata 3 (FM882243)	106	246	107	80
Sparus aurata 4 (FM882244)	140	333	107	90

In vitro IFN and Mx expression was studied using sea bream HK leukocytes stimulated with poly I:C to simulate a virus infection. The IFN level of expression increased after 6 h and decreased after 24 h and this is in agreement with previous findings in other teleosts like zebrafish, Atlantic salmon (*Salmo salar*) and rainbow trout (Altmann *et al.* 2003; Robertsen *et al.* 2003; Zou *et al.* 2007) stimulated with poly I:C as well. Moreover, the increase of Mx expression after 6 h and slightly more intensely after 24 h is a good indication that this gene is induced by the presence of IFN transcripts like it happens in mammals. The sea bream Mx basal levels were quite high in liver, spleen, head kidney and thymus, in agreement with the observations that these organs and tissues are intensively involved in the immune system responses. Similar experiments have been already performed in a previous work (Tafalla *et al.* 2004) that showed the constitutive presence of Mx transcript with higher expression in liver and spleen and Mx up-regulation after *in vitro* poly I:C stimulation in head-kidney macrophages and blood leukocytes.

Both from pairwise comparison of nucleotide and amino acid sequences and from phyologenetic analysis sea bream IFNs seems more related to human IFN-α than to human IFN-λ as expected from previous analysis of fish IFN genes (Zou *et al.* 2007). An analysis of the genome structure showed a feature common to sea bream and other teleost IFNs compared to mammalian type I homologues, which is the presence of four introns (**Fig. 7**) potentially the ancestral state for all IFNs and still seen in IFN-λ in mammals. The only exception to this is medaka that contains three introns (Casani *et al.* 2009). From the genomic analyses we have identified other IFN genes in sea bream and work is in progress to show if these genes are expressed and not pseudogenes and to study their expression in response to stimulation.

The identification of different type I IFN subgroups in rainbow trout (Zou *et al.* 2007; Chang *et al.* 2009) allowed

	1 … 60 … 120
	< EXON 1 >< EXON 2 >< EXON 3
Sparusaurata1*	MLNRIFFVCLSLSLYSAGSSLSCRW**ITAG**KF**KK**H**NERY**L**E**LLDTMANNSTN**S**TE**DD**EV**K**T**--**FP**DHL**Y**S**QAS**K**ASAED**K**LAFT**V**Q**V**L**N**ET**SV**LFEEDHSSASWEENTVENFVNVVTQQA**N**
Sparusaurata2*	MLNRIFFVCLSLSLYSAGSSLSCRW**ITAG**KF**KK**H**NERY**L**E**LLDTMANNSTN**S**TE**KT**EV**N**T**--**FP**RDP**Y**K**QAF**N**ASAED**K**LAFT**V**Q**V**L**N**ET**SV**LFEEDHSSASWEENTVENFVNVVTQQA**D**
Spruaaurata3*	MLNRIFFVCLSLSLYSAGSSLSCRW**ITDG**KF**RQ**H**SENY**L**E**LLDTMANNSTN**S**TE**DD**EV**K**T**--**FP**DHL**Y**S**QAS**K**ASAED**K**LAFT**A**Q**V**L**N**ET**SV**LFEEDHSSASWEENTVENFVNVVTQQA**N**
Sparusaurata4*	MLNRIFFVCLSLSLYSAGSSLSCRW**VMDH**KF**RQ**H**SKNS**L**A**LLDTMANNSTN**T**TE**DA**EV**E**T**VA**FP**NLL**Y**R**QAS**K**ASAED**Q**LAFT**V**Q**I**L**D**ET**AA**LFEEDHSSASWEENTVENFVNVVTQQA**D**

	121 … 180 186
	>< EXON 4 >< EXON 5 >
Sparusaurata1*	DELRSCIGSHGHKKKNKK**L**HMYFQRLSSHVLKRMGHSAEAWELIRGEVKVHLMRANQLVTSATRTN
Sparusaurata2*	DELRSCIGSHGHKKKNKK**L**HMYFQRLSSHVLKRMGHSAEAWELIRGEVKVHLMRANQLVTSATRTN
Spruaaurata3*	DELRSCIGSHGHKKKNKK**P**HMYFQRLSSHVLKRMGHSAEAWELIRGEVKVHLMRANQLVTSATRTN
Sparusaurata4*	DELRSCIGSHGHKKKNKK**L**HMYFQRLSSHVLKRMGHSAEAWELIRGEVKVHLMRANQLVTSATRTN

Fig. 6 The four sequenced sea bream IFN genes. Putative codifying region of the four characterised sea bream IFN gene sequences (*Sparus aurata* 1 = accession number FM882241; *Sparus aurata* 2 = accession number FM882242; *Sparus aurata* 3 = accession number FM882243; *Sparus aurata* 4 = accession number FM882244) with exon boundaries showed above the alignment; the different amino acids have been evidenced in bold. *The different sequences related to the same specie are due to the presence of more clones.

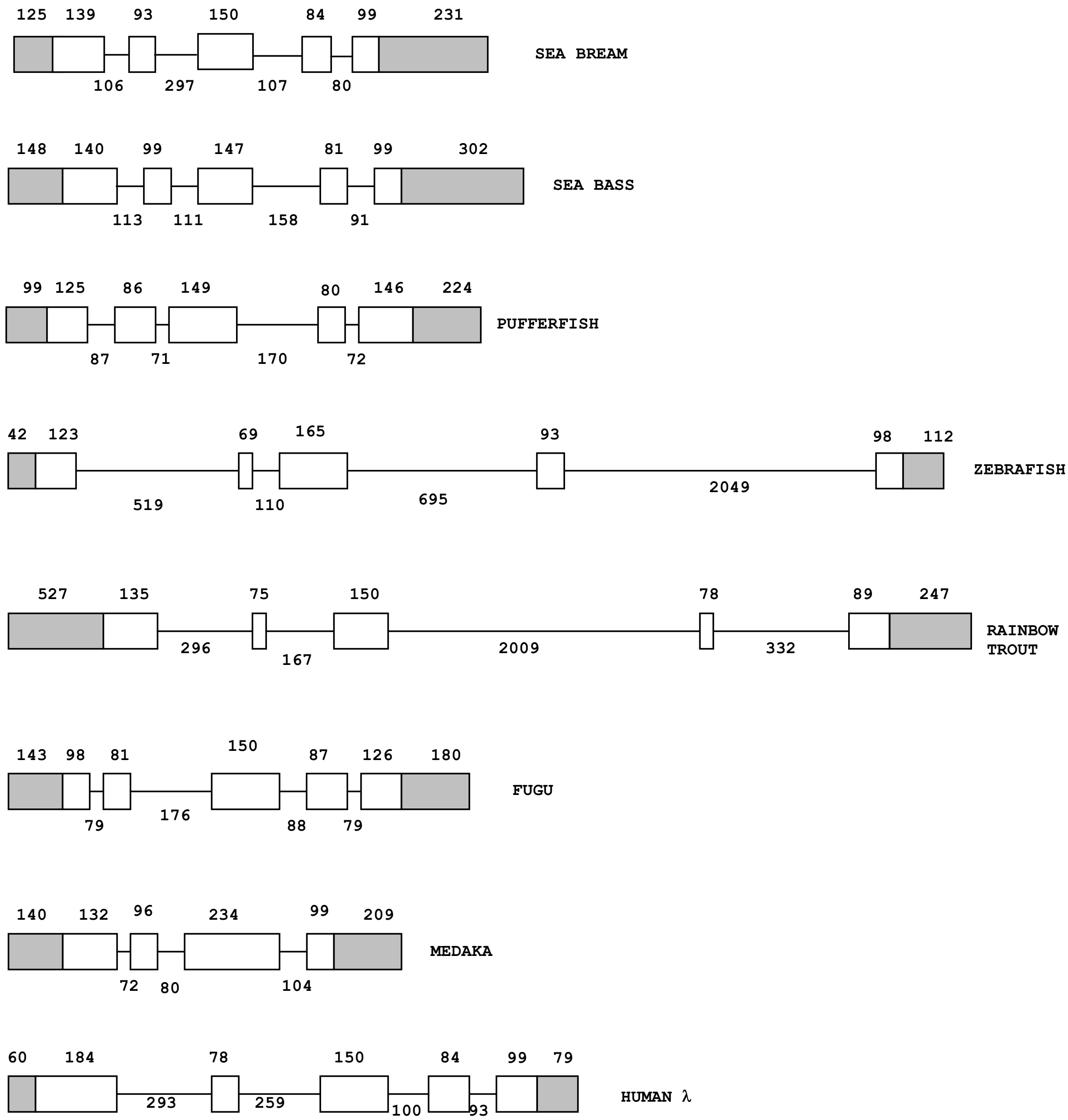

Fig. 7 A schematic representation of the IFN gene organisation and intron/exon sizes in sea bream (accession number FM882241) and other known IFN genes. The open boxes are the coding sequences, whether the grey boxes represent the untranslated regions of the mRNA, the lines show the intron sizes. Accession numbers: sea bass (*Dicentrarchus labrax*) AM946399; spotted green pufferfish (*Tetraodon nigroviridis*) AJ544889; Fugu rubripes (*Takifugu rubripes*) AJ583023; Japanese medaka (*Oryzias latipes*) BN001095; human λ (*Homo sapiens*) NT_011109; zebrafish (*Danio rerio*) AJ544820; rainbow trout (*Oncorhynchus mykiss*) AM489415.

the identification of two 2 cysteines IFN lineages in fish and phylogenetic analyses reveals that two major gene duplication events appear to have happened within teleosts (Chang *et al.* 2009). The IFN genes in amphibians were shown for the first time to contain introns and to conserve the four cysteine structure found in all type I IFN, except in IFN-β and most fish IFN (Zou *et al.* 2007). It seems that different vertebrate groups have independently formed their IFN subtypes with deletion of introns and cysteines during evolution.

In conclusion, the availability of these sea bream type I IFN sequences gives the opportunity to investigate more in detail antiviral immune responses in this species and will help in studying the effects of vaccination and immunostimulation trials against sea bream viral pathogens. Whether other types of type I IFNs with different numbers of cysteines are present in sea bream it remains to be determined although it could be anticipated that at least type II IFN should also be present as seen in other fish species.

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