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Short communication

Genetic characterization and transcription analyses of the European sea bass (*Dicentrarchus labrax*) *isg15* gene

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ABSTRACT

Fish interferons are cytokines involved in its resistance to viral infections by inducing the transcription of several interferon-induced genes, such as *isg15*. The aim of the present study was the genetic characterization of the European sea bass *isg15* gene, describing the regulatory motifs found in its sequence. In addition, an in vivo analysis of transcription in response to betanodavirus (RGNNV genotype) and poly I:C has been performed. The analysis of the resulting sequences showed that sea bass isg15 gene is composed of two exons and a single 276-bp intron located at the 5′-UTR region. The full length cDNA is 1143-bp, including a 102-bp 5′-UTR region, a 474-bp ORF, and a 291-bp 3′-UTR region. Several mRNA-regulatory elements, including three unusual ATTTA instability motifs in the intron, and four ATTTA motifs along with a cytoplasmic polyadenylation element in the 3′-UTR region, have been found in this sequence. The *in vivo* analyses revealed a similar kinetics and level of transcription in fish brain and head kidney after poly I:C inoculation; however, the induction caused by RGNNV started earlier in brain, where the upregulation of *isg15* gene transcription was high. The present study contributes to further characterize the European sea bass IFN I response against RGNNV infections.

Type I interferon (IFN I) is one of the main factors involved in the fish anti-viral innate response, inducing the transcription of numerous interferon-stimulated genes (ISG). One of the earliest and most highly-expressed ISGs upon viral infections is the interferon-stimulated gene 15 (*isg15*), coding for ISG15, which are 15-kDa effector proteins with a proven antiviral role [1]. ISG15 activity occurs through the covalent conjugation (ISGylation) of the LRGG motif, sited in the C-terminal end of all ISG15 proteins, to cellular or viral proteins. ISGylation is controlled by IFN-induced enzymes [2,3], and occurs through pathways similar to those of ubiquitination, resulting in the modification of some characteristics of the conjugated proteins, such as location, stability and activity [3,4]. In addition, unconjugated ISG15 may also play an important role in host response [5], since they can be secreted, acting as cytokines [6].

One of the main threats to the culture of European sea bass (*Dicentrarchus labrax*) is the appearance of viral nervous necrosis (VNN) outbreaks. This disease is characterized by damages in nervous tissues, which are responsible for the typical external symptoms, such as altered swimming and floatability, anorexia and visual dysfunction [7]. VNN is caused by Nervous Necrosis Virus (NNV, *Betanodavirus* genus, *Nodaviridae* family), with a single-stranded, positive-sense RNA bipartite genome [8]. RNA1 and RNA2 viral segments encode the viral polymerase and the capsid protein (CP), respectively. Fish nodaviruses have been grouped into four genotypes [9], being red-spotted grouper nervous necrosis virus (RGNNV) the only one associated with mass mortality episodes in sea bass to date. Although some aspects of the immune response of this fish species against NNV infections have been characterized, no information about the role of sea bass *isg15* is available. The present study contributes to further characterize the European sea bass IFN I response against RGNNV infections, addressing the *isg15* gene structure and transcription after polyinosinic:polycytidylic acid (poly I:C) and RGNNV inoculation.

For *isg15* gene characterization, sea bass cDNA and DNA were obtained and sequenced. Primers were designed according to the sea bass genome database (GenBank accession no. HG916840) by alignment with the expressed sequence tag (EST) of sea bass *isg15* (accession no. CV186275) [10] following the diagram displayed in Fig. 1. Those primers used to sequence and characterize the untranslated regions (UTR) were designed considering the polyadenylation signal and the transcription start site predicted with

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1050-4648/

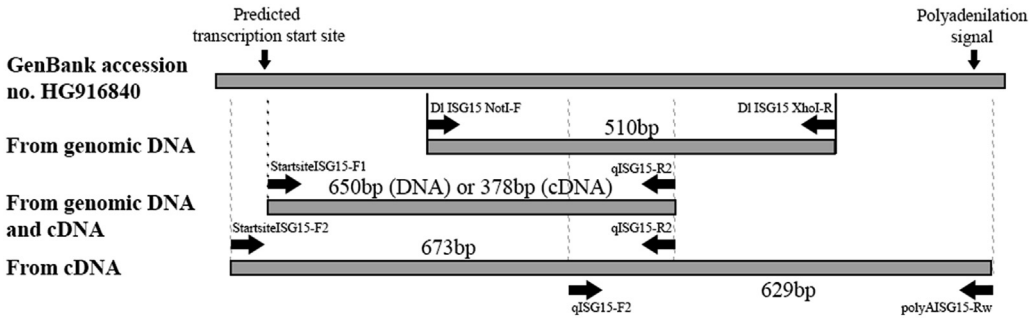

Fig. 1. Diagram showing fragments amplified and primers used for elucidating the genomic structure of the European sea bass *isg15*.

the PROSCAN v1.7 (BIMAS) software.

To obtain the *isg15* cDNA, juvenile European sea bass were intraperitoneally injected with poly I:C (25 mg/kg, CalBiochem). Animals were killed by anaesthetic overdose (MS-222, Sigma) at 8 h post-injection, and head kidneys were immediately collected and homogenized (10%, w/v) in Leibovitz medium (L15, Gibco) with 2% foetal bovine serum (FBS, Gibco), 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Lonza). Homogenates were clarified, and total RNA extraction and cDNA synthesis were conducted using TRI Reagent solution (Sigma) and the Transcriptor First Strand cDNA Synthesis Kit (Roche), respectively, following manufacturer's recommendations. After the DNase I treatment (Roche), cDNA was amplified in 50-μl PCR mixtures containing 400 ng cDNA, 10 μM each primer (Table 1, Fig. 1, S1), 10 mM dNTPs, 2x Universe Buffer (Biotool.com), and 1 U Universe High-Fidelity Hot Start DNA polymerase (Biotool.com). Amplification conditions were: 95 °C for 3 min, 35 cycles at 95 °C for 15 s, 48–65 °C (depending on the primers used, Table 1) for 15 s and 72 °C for 15 s, and a final extension at 72 °C for 5 min.

Genomic DNA was isolated from caudal fin by saline precipitation [11], and the non-coding regions were sequenced and characterized by PCR amplification using the primers shown in Table 1 (Fig. 1, S1), and the above described conditions and mixture reactions.

Sequencing was conducted with the ABI 3730 (STABVIDA) system. Nucleotide sequence was translated into aminoacid sequence using the EditSeq (DNASTAR Lasergene 7) software. Intron screening was performed by alignment of the resulting cDNA and genomic DNA sequences.

The analysis of the resulting sequences showed the typical structure for fish *isg15* genes (Fig. 2). European sea bass *isg15* gene is composed of two exons and a single 276-bp intron, flanking by the canonical splicing GT/AG motif (S1). The intron region is located within the 5′-UTR, as it happens in other fish *isg15* genes (Fig. 2).

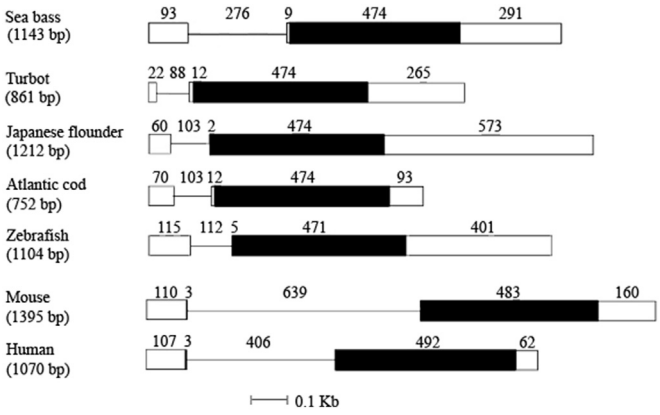

Fig. 2. Comparison between the European sea bass *isg15* genomic structure and other known *isg15* genes. Lines represent introns, and boxes are exons. Coding regions are in black, and the untranslated regions in white. Numbers above exons and introns indicate the size (bp) of each element.

This location suggests that the intron may be involved in repressing the silencing, and, therefore, in regulating ISG15 protein level [10]. Surprisingly, the sea bass *isg15* intron contains three instability motifs (ATTTA) (S1), which has only been described in a few genes, such as Atlantic cod (*Gadus morhua*) *isg15* (with a single ATTTA motif in the intron) or rainbow trout (*Onchorynchus mykiss*) interlaeukin-1β gen [12]. The ATTTA motifs in introns seem to have a role in regulating the splicing of heterogeneous nuclear RNA or the mRNA expression [12].

Based on the putative start and stop codons, the estimated complete ORF is 474-bp long. The 5′-UTR starts 102 bp upstream the start codon (Fig. 2, S1), whereas the 3′-UTR region is 291-bp long, containing one AATAAA polyadenylation signal and four instability motifs (S1). These motifs are frequently found in 3′-UTR

Table 1
Primers used in this study.

Name	Sequence	Annealing temperature (°C)	Usage
DllSG15 *Not*I-F	5′-GATCACAAGGGCGGCCGCATGATGGATATAACC-3′	65	*isg15* ORF
DllSG15 *Xho*I-R	5′-TATGAGCCTCGAGGGTGCTCAGCCTCCTCTCAG-3′	65	*isg15* ORF
PolyAISG15-Rw	5′-TTTTTTTTTTTTTACTTTAAATAGTTTC-3′	48	*isg15* cDNA sequencing
StartsiteISG15-F1	5′-CACAGCTGTTCCTAACAATCCTC-3′	57	*isg15* cDNA, DNA sequencing
StartsiteISG15-F2	5′-CTGTGGGGAGCTGAAACCTCT-3′	58	*isg15* cDNA, DNA sequencing
qISG15-F2	5′-CGACTCAAAGCCTCTCTGCTACT-3′	60	*isg15* qPCR, cDNA sequencing
qISG15-R2	5′-CGTTTCTGACGAACACCTGGAT-3′	60	*isg15* qPCR, cDNA, DNA sequencing
RG_965_RNA2-F4	5′-ACCGTCCGCTGTCTATTGACTA-3′	60	RGNNV qPCR
RG_965_RNA2-R1	5′-CAGATGCCCCAGCGAAACC-3′	60	RGNNV qPCR
18S rRNA–Fw[a]	5′-CCAACGAGCTGCTGACC-3′	60	Housekeeping gene qPCR
18S rRNA-Rw[a]	5′-CCGTTACCCGTGGTCC-3′	60	Housekeeping gene qPCR

[a] [24].