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Title: MOLECULAR CLONING, DIFFERENTIAL EXPRESSION AND 3D STRUCTURAL ANALYSIS OF THE MHC CLASS-II beta CHAIN FROM SEA BASS (Dicentrarchus labrax L.)

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Keywords: major histocompatibility complex class (MHC) II beta chain; sea bass; Dicentrarchus labrax; cloning; polymorphism; expression analysis; quantitative PCR; 3D structure.

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Abstract: The major histocompatibility complex class I and II molecules (MHC-I and MHC-II) plays a pivotal role in vertebrate immune response to antigenic peptides. In this paper we report the cloning and sequencing of the MHC class II b chain from sea bass (Dicentrarchus labrax L.). The six obtained cDNA sequences (designated as Dila-DAB) code for 250 amino acids, with a predicted 21 amino acid signal peptide and contain a 28 bp 5'-UTR and a 478 bp 3'-UTR. A multiple alignment of the predicted translation of the Dila-DAB sequences was assembled together with other fish and mammalian sequences and it showed the conservation of most amino acid residues characteristic of the MHC class II b chain structure. The highest basal Dila-DAB expression was found in gills, followed by gut and thymus, lower mRNA levels

were evidenced in spleen, peripheral blood leucocytes (PBL) and liver. Stimulation of head kidney leukocytes with LPS for 4 h showed very little difference in the Dila-DAB expression, but after 24 h the Dila-DAB level decreased to a large extent and the difference was statistically significant. Stimulation of head kidney leukocytes with different concentrations of rIL-1b (ranging from 0 to 100 ng/ml) resulted in a dose-dependent reduction of the Dila-DAB expression. Moreover, two 3D Dila-DAB*0101 homology models were obtained based on crystallographic mouse MHC-II structures complexed with D10 T-cell antigen receptor or human CD4: features and differences between the models were evaluated and discussed. Taken together these results are of interest as MHC-II structure and function, molecular polymorphism and differential gene expression are in correlation with disease resistance to virus and bacteria in teleost fish.

Dear Prof. Tony Ellis,

I am sending you a revised version of the paper entitled: "MOLECULAR CLONING, DIFFERENTIAL EXPRESSION AND 3D STRUCTURAL ANALYSIS OF THE MHC CLASS-II beta CHAIN FROM SEA BASS (Dicentrarchus labrax L.)" (Ref. n. FSIM-D-07-00013), author team: Francesco Buonocore, Elisa Randelli, Daniela Casani, Susan Costantini, Angelo Facchiano, Giuseppe Scapigliati, Renè J.M. Stet.

I have taken into account most of the suggestions from the referees and I have attached a list of all the changes made in the paper (in the text you will find **in bold** the phrases that differ form the first version).

I hope that now the paper will be accepted for the publication, but let me know if there is any problem.

Best regards

Dr. Francesco Buonocore

*** Response to Reviewers**

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Best regards

Dr. Francesco Buonocore

Reviewer #1:

This manuscript describes the cloning of 6 cDNA sequences encoding MHC class II beta from sea bass. The sequences have been aligned and analyzed in a phylogenetic tree, the expression of the gene in head kidney leukocytes in response to stimulation with LPS and Il-beta is examined by real time PCR and some homology models have been derived.

General Comments

This paper is generally well written, although the English need polishing. The data provided are interesting, but much of it is in silico derived from the sequences and the addition of a few more "wet lab" experiments would greatly enhance the story the authors are trying to tell. For example, sequencing cDNAs does not really give gene copy number or even number of expressed genes due to primer bias/fit among other problems. The addition of a Southern blot, an analysis of the expression levels in different tissues or the examination of the real degree of polymorphism by sequencing the putative PBR regions from several other individuals (up to 50 sequences) would provide more evidence that these are the functional genes of Sea Bass and enhance the publish ability of this manuscript.

I have added an analysis of the MHC expression levels in different tissues. The Southern blot has not been added as an attempt to evidence the number of class II *B* loci in sea bass is already present in the paper (see the beginning of "Results" section). The examination of the real degree of polymorphism was not in the aim of the authors, but the idea was to focus on the analysis of functional evidences related to the MHC gene.

The discussion is very weak and fails to discuss important questions such as why the expression of genes that are key to the immune response is down-regulated after stimulation - this seems to mean that the immune response is being shut down. The rest of the discussion needs more explanation of the data rather than simply saying it agrees or disagrees with the literature.

I have improved the Discussion taking into account the reviewer suggestions.

Specific comments:

line 63: Redundant language

I have changed the phrase.

line 65: in terms of linkage? Polymorphism? Classical/non-classical? Clarify
I have clarified the sentence.

line 229 "they encode"

I have corrected the mistake.

line 244; 11% identity - no "of"

I have corrected the mistake.

line 257 ; The trout glycosylation site is more than just "potential" the protein is definitely glycosylated - see: Immunogenetics (2006) 58: 443-453

"Potential" was referred to sea bass and not to the other species, I have added the reference.

Line 258: Figure 2 - why were amino acid sequences used? DNA has more usable data and avoid convergence more. Why are some names capitalized and others not?

Yes, usually DNA has more usable data and avoid convergence more, but we got similar results with DNA or amino acid, so we decide to use the latter as it was more straightforward after the alignment. I have used only capitalized names in the tree.

Line 265, figure 3 Did you measure IL-1b expression after LPS stimulation to ensure you actually got stimulation? (oh I see you did at line 392-394 - you should mention this earlier). Sometimes LPS doesn't work so well on fish, especially from E coli. This concentration seems very low - other papers have used as much as 100ug/ml. Why was this concentration chosen - was a preliminary experiment using a range of concentrations done to assess how much is needed to elicit a response? Why did the expt only go to 24 hours? It seems that MHC expression should be affected for a long time after that.

I have added a phrase in the "Results" section related to the studying of IL-1β expression to ensure LPS stimulation. The LPS concentration worked well in a lot of different experiments we performed in the past (for a reference see: G. Scapigliati, F. Buonocore, S. Bird, J. Zou, P. Pelegrin, C. Falasca, D. Prugnoli and C.J. Secombes. Phylogeny of cytokines: molecular cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1β. *Fish and Shellfish Immunology* (2001), 11(8):711-726; F. Buonocore, E. Randelli, D. Casani, M. Mazzini, I. Cappuccio, C.J. Secombes, G. Scapigliati. cDNA cloning and expression analysis of a cyclooxygenase-2 from sea bass (*Dicentrarchus labrax* L.) after vaccination. *Aquaculture* (2005), 245(1-4): 301-310; F. Buonocore, M. Forlenza, E. Randelli, S. Benedetti, P. Bossù, S. Meloni, C.J. Secombes, M. Mazzini, G. Scapigliati. Biological activity of sea bass (*Dicentrarchus labrax*) recombinant interleukin-1β. *Marine Biotechnology* (2005), 7(6): 609-617), so we used the same. We did not look at the MHC expression after 24 h both because LPS elicit inflammatory responses quickly (few hours post-stimulation) and as the viability of

fish leukocyte cultured cells is quite low, so to study the MHC expression for a long time we should perform "in vivo" experiments.

Line 290 is 34% identity enough to do this?

Yes, it is enough with the used computational methods (for a reference see: Facchiano AM, Stiuso P, Chiusano ML, Caraglia M, Giuberti G, Marra M, et al. Homology modelling of the human eukaryotic initiation factor 5A (eIF-5A). Protein Engineering 2001; 14: 881-90; Marabotti A, D'Auria S, Rossi M, Facchiano AM. Theoretical model of the three-dimensional structure of a sugar binding protein from *Pyrococcus horikoshii*: structural analysis and sugar binding simulations. Biochem J 2004; 280:677-84). I have added a phrase in the "Results" section.

Line 369-370: there is variability - you can't say it is "high" based on 6 sequences

I have changed the phrase.

Lines 393-403: but why does MHC expression go down - this is decreasing the immune response in response to a simulated infection - isn't this bad?? You need to discuss the implications of this? Does the MHC expression increase later?????

MHC down-regulation in our samples could be very likely linked to the already reported mechanisms for the control of inflammation responses in mammals that, uncontrolled, may have dangerous effects. For example, during early inflammation, various cytokines are produced and they down-regulate the expression of inflammation-related molecules.

Reviewer #2

General comments:

The manuscript outlines the isolation and structural analysis of six full length MHC class II B sequences cDNA sequences in Sea Bass, a topic which should be of relevance and interest to the readership of Fish and Shellfish Immunology. While I can't speak authoritatively about the structural analysis, the science appears sound and the methods clear. There are a few instances where the English wording could use improvement (see below), but generally the manuscript is well organised and written.

I would have liked further elaboration about the potential impact of the modeling exercise on our understanding of the function of this gene.

I have improved the Discussion taking into account the reviewer suggestions.

Given that the two models differed in perhaps the most important site-the ligand binding region, how can one differentiate which is correct?

The two models represent two conformations of the same protein under different conditions, i.e. the interaction with two different ligands, and this may explain why the main structural differences involve the binding sites, as observed by the referee. It is not possible to indicate if one model is better than the other. Each model predicts the structural organization of the protein when it interacts with CD4 or TCR, so both models can be useful for further experimental studies to simulate the interaction with the respective ligand and confirm the structural prediction.

How does the size of the binding site relate to the size of the potential ligand?

The binding sites for the antigen peptide and CD4 resulted larger in the MHC conformation which interacts with these ligands, and the binding site for TCR resulted larger in the MHC conformation which interacts with TCR. We wrote that this observation confirms the reliability of the two models. In fact, this results is in agreement with the ability of any protein to adopt a conformation more suitable to the interaction. The larger surface implies a better ability to interact with the ligand. As an example, it is well known that enzymes expose better the catalytic site in the presence of the substrate. Therefore, the measures of the surfaces for the binding sites confirm that our models are suitable to simulate the different conformations of MHC when it interacts with the two different ligands.

The authors should consider revising their final concluding sentence, as it is too long and cumbersome. As a note, they have not demonstrated that the genes they identified are actually maintained by pathogen-driven selection.

The final sentence has been revised.

English could be better in places, some of which are outlined below:

Line 194: significativity -- what is that? Should be significance

I have changed the word.

Line 232: in order to verify the number of class II B locus (should be loci)

I have changed the word.

Line 233: perfomed should be performed

I have changed the word.

Line 239: seems to express up to three class II b loci at least (up to and at least are contradictory terms. Should read at least three class IIb loci)

I have changed the phrase.

Line 325: where the strands include less amino acids (should read fewer amino acids)

I have changed the word.

Line 361: growing evidences suggest should read growing evidence suggests

I have changed the phrase.

Line 397: I am not sure what is meant by similar results…after infection with IHNV on MHC class I-what? That expression of class I or class II was downregulated?

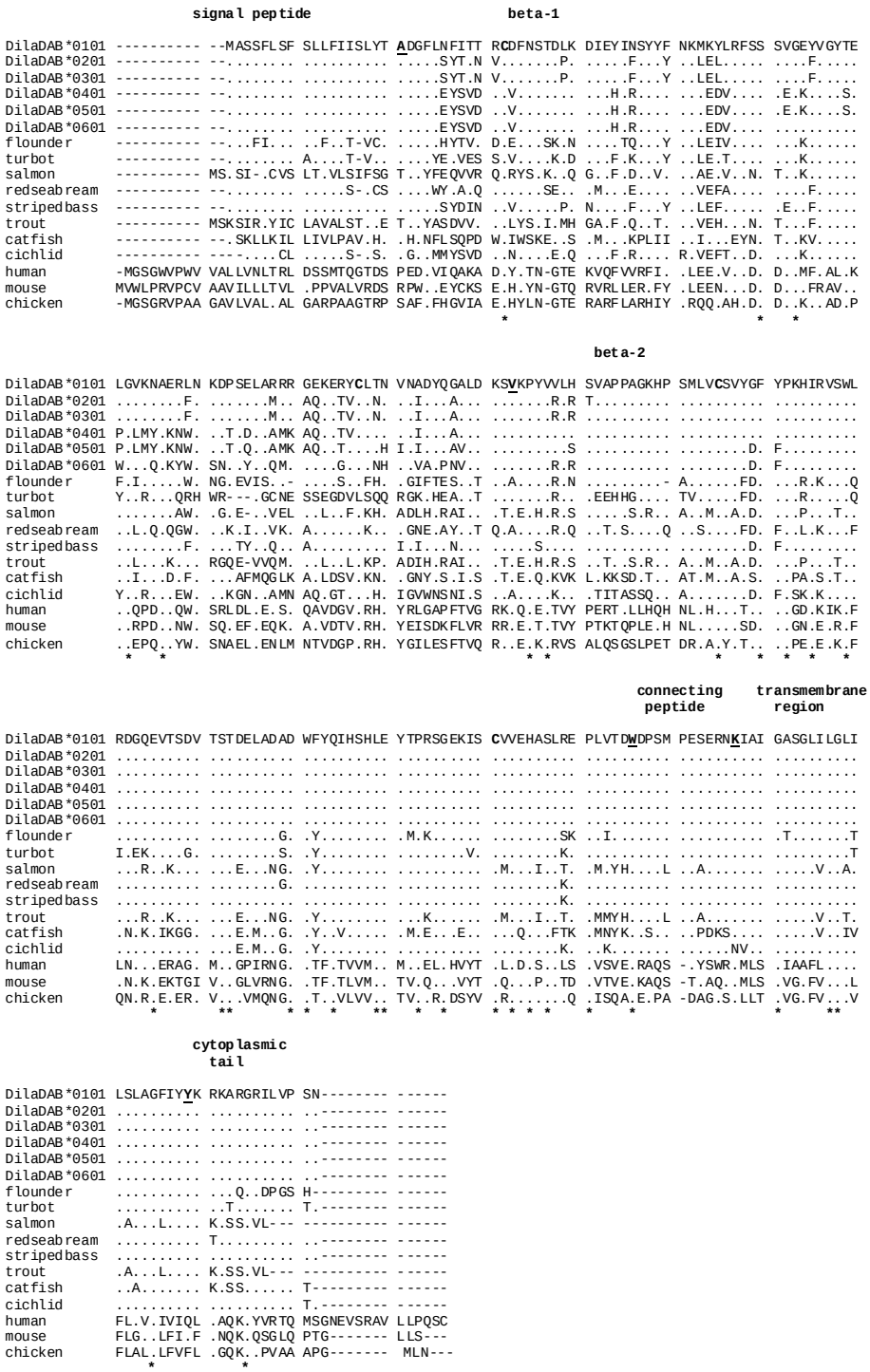
I have changed the phrase.

Line 404: the identity percentage should read percentage identity

I have changed the phrase.

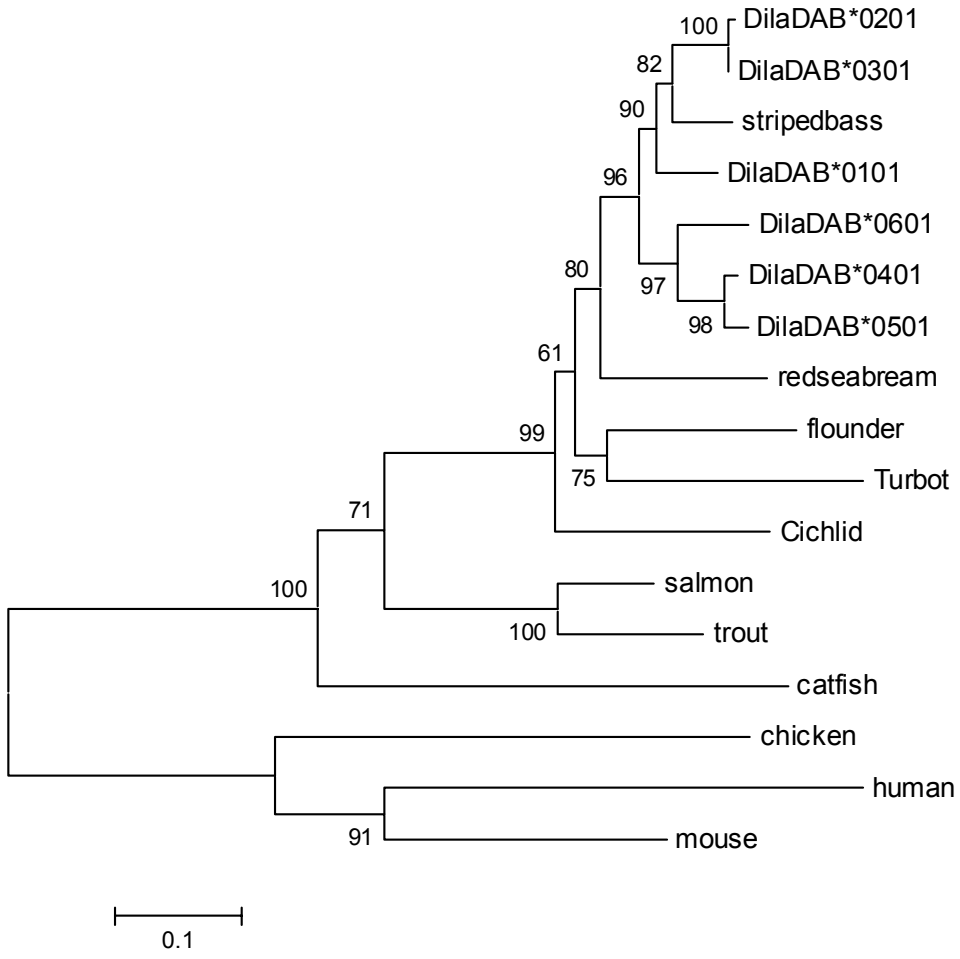
Figure(s)

Figure 1



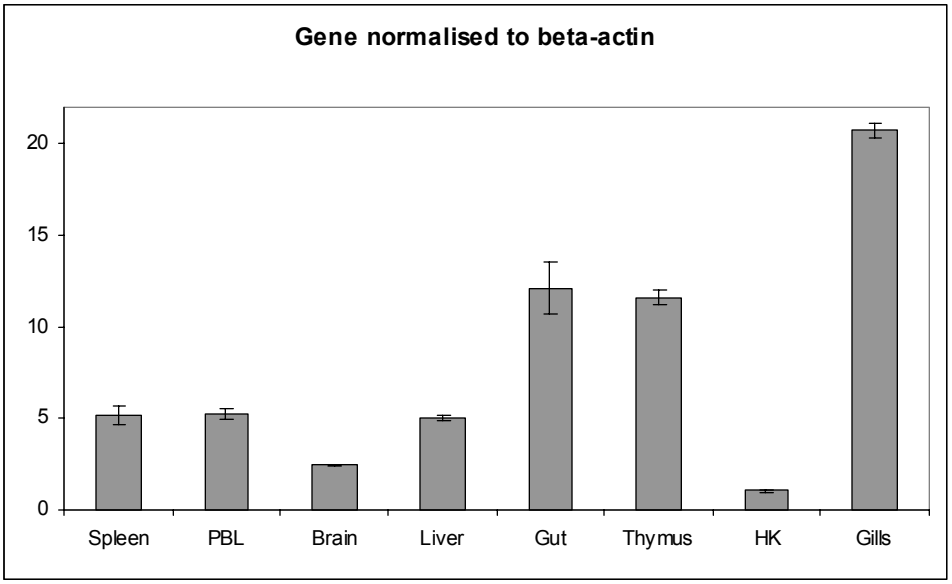
Figure(s)

Figure 2

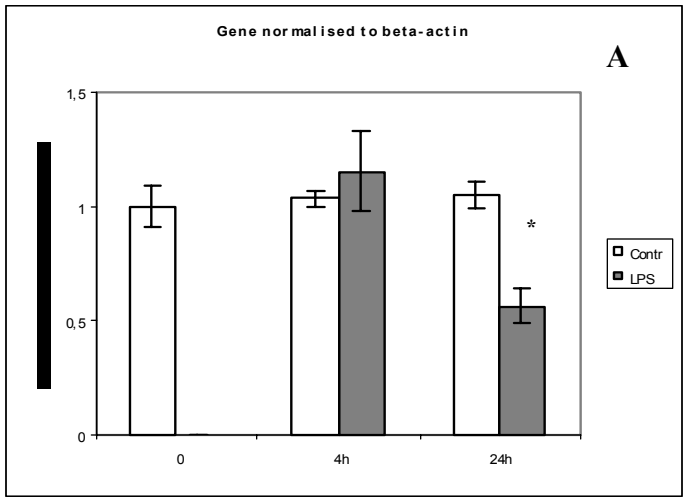


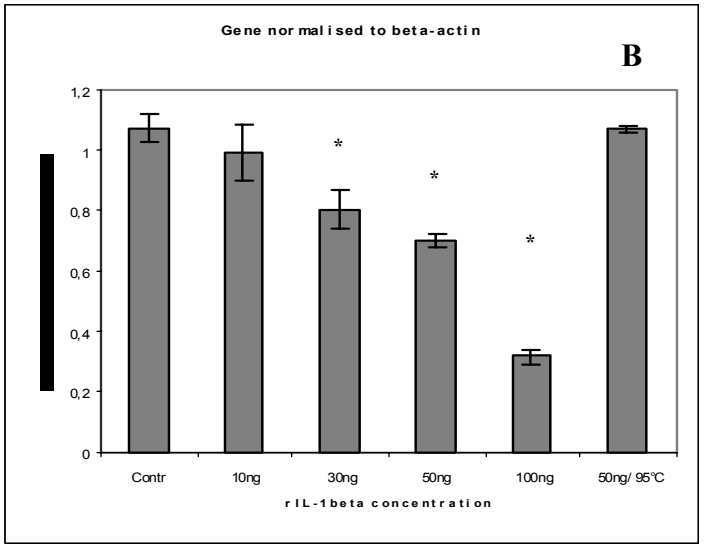
Figure(s)

Figure 3



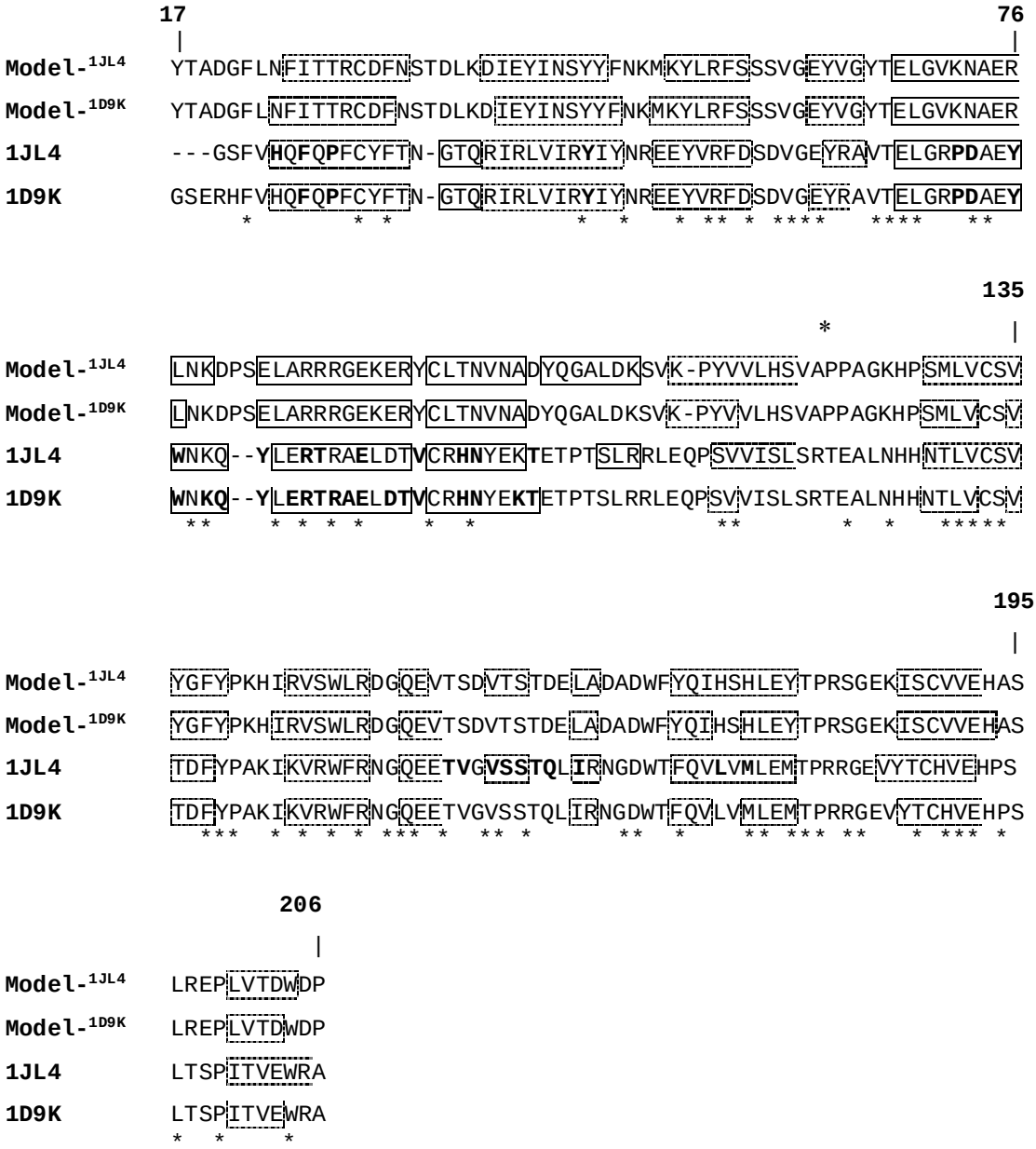
Figure(s)


Gene normalised to beta-actin
A
1,5
1
0,5
0
*
Contr
LPS
0
4h
24h


Gene normalised to beta-actin
B
1,2
1
0,8
0,6
0,4
0,2
0
*
*
*
Contr
10ng
30ng
50ng
100ng
50ng/ 95°C
rIL-1beta concentration

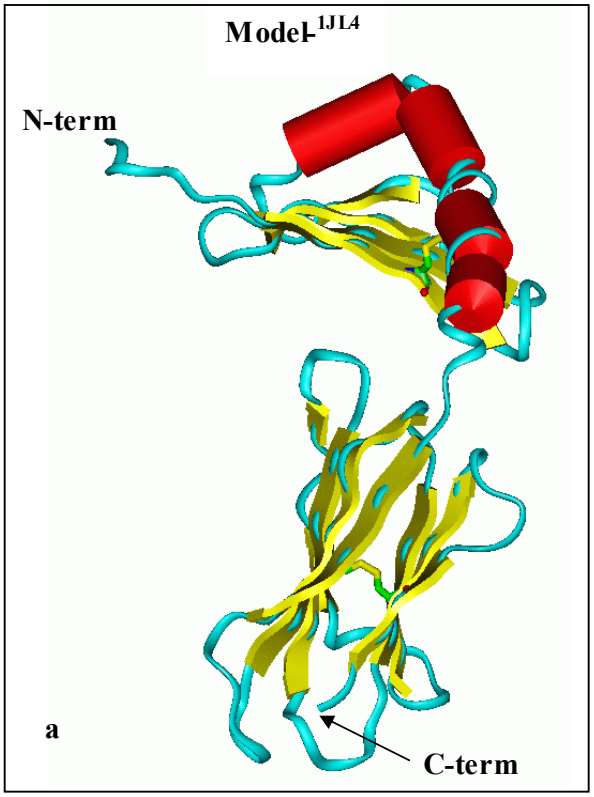
Figure(s)

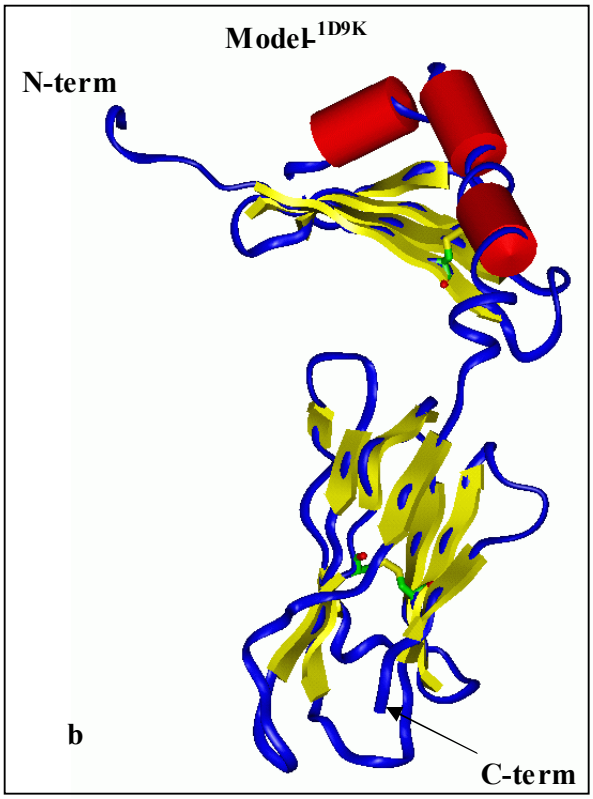
Figure 5



Figure(s)

Figure 6





MOLECULAR CLONING, DIFFERENTIAL EXPRESSION AND 3D STRUCTURAL ANALYSIS OF THE MHC CLASS-II β CHAIN FROM SEA BASS (*Dicentrarchus labrax* L.)

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ABSTRACT

The major histocompatibility complex class I and II molecules (MHC-I and MHC-II) plays a pivotal role in vertebrate immune response to antigenic peptides. In this paper we report the cloning and sequencing of the MHC class II β chain from sea bass (*Dicentrarchus labrax* L.). The six obtained cDNA sequences (designated as Dila-DAB) code for 250 amino acids, with a predicted 21 amino acid signal peptide and contain a 28 bp 5'-UTR and a 478 bp 3'-UTR. A multiple alignment of the predicted translation of the Dila-DAB sequences was assembled together with other fish and mammalian sequences and it showed the conservation of most amino acid residues characteristic of the MHC class II β chain structure. **The highest basal Dila-DAB expression was found in gills, followed by gut and thymus, lower mRNA levels were found in spleen, peripheral blood leucocytes (PBL) and liver.** Stimulation **of head kidney leukocytes** with LPS for 4 h showed very little difference in the Dila-DAB expression, but after 24 h the Dila-DAB level decreased to a large extent and the difference was statistically significant. Stimulation **of head kidney leukocytes** with different concentrations of rIL-1β (ranging from 0 to 100 ng/ml) resulted in a dose-dependent reduction of the Dila-DAB expression. Moreover, two 3D Dila-DAB*0101 homology models were obtained based on crystallographic mouse MHC-II structures complexed with D10 T-cell antigen receptor or human CD4: features and differences between the models were evaluated and discussed. Taken together these results are of interest as MHC-II structure and function, molecular polymorphism and differential gene expression are in correlation with disease resistance to virus and bacteria in teleost fish.

Keywords: major histocompatibility complex class (MHC) II β chain; sea bass; *Dicentrarchus labrax*; cloning; polymorphism; expression analysis; quantitative PCR; 3D structure.

1. INTRODUCTION

The major histocompatibility complex class I and II molecules (MHC-I and MHC-II) are fundamental components of the immune response to foreign protein antigens. They have been extensively studied in mammals, especially in humans [1], mouse and rat. The MHC molecules are heterodimers formed by α and β membrane glycoproteins that bind self and non-self peptides for presentation on the cell surface to T-cells. MHC-II binds peptides for presentation to the $CD4^+$ T helper cells [2-3] and it is encoded by two genes, *A* and *B* [4], in mammals. MHC-II genes are constitutively expressed in antigen-presenting cells such as macrophages, B cells, monocytes and dendritic cells. They are highly polymorphic with multiple loci and alleles and this polymorphism gives the possibility to bind a large number of peptide ligands. Crystal structures of different MHC II proteins in mammals have shown that bound peptides are deeply integrated into the MHC structure using two main classes of interactions: 1) conserved hydrogen bonds to the peptide backbone; 2) at least four prominent pockets that accept peptide side chains [5-7].

In teleosts, MHC class I and II genes have been identified in various species and it has been established that class I and II loci **reside in different linkage groups** [8]. Fish MHC class II molecules are comprised of α and β subunits, like in mammals, and **class II *B* loci are in separate linkage groups in all Euteleostei** [9]. MHC class II genes have been isolated from numerous fish species such as striped sea bass (*Morone saxatilis*) [10], red sea bream (*Chrysophrys major*) [11], various cichlids [12], salmonids [13-15] and different cyprinids [16-20].

MHC II genes in teleosts are also polymorphic and various studies have tried to establish the association of the MHC diversity [21-22] or differential gene expression [22-27] with disease resistance to virus and bacteria, as such associations were identified in humans [28] and chicken [29].

In this study we report the cloning and sequencing of the MHC class II β chain from sea bass (*Dicentrarchus labrax* L.), one of the most important species in aquaculture in the South Mediterranean, and studied **its basal expression levels** and under different "in vitro" conditions by

real-time PCR. These results will add a new tool for studying the effects of vaccination and immuno-stimulation on the sea bass immune system. Moreover, we predicted, for the first time, the 3D structure of a MHC class II β chain from a teleost fish by homology modelling, a starting point for successive structural-functional investigations on this fundamental immune molecule.

2. MATERIALS AND METHODS

2.1 Sea bass MHC class II β chain cloning and sequencing

Two degenerate primers (MHCFR1 5'- TGCWGYGYRTAYGRSTTCTACCC – 3' and MHCRV1 5' AGGCTKGKRTGCTCCACCWRRCA –3' where Y = C/T, K = G/T, R = G/A, S = G/C, W = A/T) corresponding to highly conserved regions of known MHC class II β genes [30] were used for RT-PCR on total RNA extracted with Tripure (Roche) solution from one juvenile sea bass (150 g of weight) head kidney. The leukocyte head kidney cells were obtained after Percoll purification as a single fraction following the procedures described in [31]. RT-PCR was performed using Ready-To-Go RT-PCR Beads (Amersham Pharmacia). For cDNA synthesis, 1 µg of total RNA and 0.5 µg of random primers [$pd(N)_6$] were used in each reverse transcription reaction in a total volume of 50 µl. Reactions were conducted using the Mastercycler personal (Eppendorf). The cycling protocol was one cycle of 94°C for 5 min, 35 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 45 s, followed by one cycle of 72 °C for 10 min. PCR products (15 µl) were visualised on 1% (w/v) agarose gels containing ethidium bromide (10 ng/ml) using hyperladder IV (Bioline) as size marker. Controls for the presence of DNA contamination were performed using the RNA samples as template. DNA amplified by PCR was purified using the QIAquick Gel Extraction Kit (QIAgen), inserted into the pGEM-T Easy vector (Promega) and transfected into competent JM109 *Escherichia coli* cells. Plasmid DNA from at least ten independent clones was purified using the Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced using MWG DNA Sequencing Services. Sequences generated were analysed for similarity with other known MHC class II β sequences using the FASTA [32] and BLAST [33] programs and multiple alignments were made with MEGA 3.1 Software [34].

Further primers were designed based on the initial sea bass MHC class II β sequences for 5'- and 3'- rapid amplification of cDNA ends (RACE)-PCR (MHC-F1 5'-TCAGAGTGAGCTGGCTCAGA-3' and MHC-F2 5'-GGTCTGGAGAGAAGATCTCC-3'; MHC-

141 R1 5'-GGAACCAGAATCCTTCCTCG-3' and MHC-R2 5'- TGTGTTTGGGGTAGAAGCCG-
142 3'). cDNA was synthesised from the same total head kidney RNA with the First-strand cDNA
143 Synthesis kit (Amersham Pharmacia) following the manufacturers instructions. For 3' RACE-PCR,
144 cDNA was transcribed from the same total head kidney RNA using an oligo-dT adaptor primer (5'-
145 CTCGAGATCGATGCGGCCGCT$_{15}$-3'). PCR was performed initially with the MHC-F1 primer
146 and the oligo-dT adaptor primer, followed by a semi-nested PCR using MHC-F2 primer and the
147 adaptor primer (5'-CTCGAGATCGATGCGGCCGC-3'). For 5' RACE-PCR, cDNA was
148 transcribed from total RNA using the oligo-dT primer, treated with *E. coli* RNase H (Promega),
149 purified using a PCR Purification Kit (QIAgen), and tailed with poly(C) at the 5' end with terminal
150 deoxynucleotidyl transferase (TdT, Promega). PCR was performed initially with MHC-R1 primer
151 and an Oligo-dG primer (5'-GGGGGGIGGGIIGGGIIG-3'), and then semi-nested with MHC-R2
152 and the oligo-dG primers. Sequencing and similarity searches were as described above.
153 The obtained cDNA sequences were analysed for the presence of a signal peptide, using SignalP
154 software [35], and of N- (with the NetNGlyc 1.0 Server) and O-linked glycosylation sites [36].
155 Comparison of the sea bass MHC class II β amino acid sequences to their counterparts from other
156 fish and mammalian species was carried out using the DIALIGN program [37]. A phylogenetic tree
157 was constructed by the "neighbour-joining" method using MEGA 3.1 Software [34] on full-length
158 amino acid sequences and bootstrap values calculated.
159 ***2.2 Basal MHC class II β chain expression***
160 **To study the IL-10 basal expression, 5 sea bass juveniles (150 g of weight) were sampled**
161 **and leucocytes from different tissues and organs [spleen, peripheral blood leukocytes (PBL),**
162 **brain, liver, gut, thymus, head kidney (HK), gills] obtained as described in [31].**
163 **Total RNA was extracted with Tripure (Roche). For the reverse transcription the**
164 **BioScript RNase H minus (Bioline) enzyme was used with the following protocol: 2 μg of total**
165 **RNA was mixed with 1 μl of random hexamer (0.2 μg/μl; Amersham Pharmacia) and nuclease**
166 **free water was added to a final volume of 12 μl. This mixture was incubated at 70° C for 5**

min and then cooled on ice. Successively, 0.4 µl of a reaction mix containing 100 mM dNTPs (25mM each; Promega), 4µl of 5X Reaction buffer , nuclease free water to a final volume of 19.75 µl and 0.25 µl of BioScript at 200 u/µl were added and the solution incubated first at 25 °C for 10 min and than at 37 °C for 60 min. Finally, the reaction was stopped by heating at 70 °C for 10 min.

The expression level of MHC class II β chain was determined with a Mx3000P™ real time PCR system (Stratagene) equipped with version 2.02 software and using the Brilliant SYBR Green Q-PCR Master Mix (Stratagene) following manufacturer's instructions, with ROX as internal reference dye. Specific PCR primers were designed for the amplification of about 200 bp products from both MHC class II β chain and β-actin (used as housekeeping gene) transcripts. The primers were: RTMHCFR 5'-CAGAGACGGACAGGAAG–3' and RTMHCRV2 5'- CAAGATCAGACCCAGGA-3', RTACTFR2: 5'-ATGTACGTTGCCATCC-3' and RTACTRV2: 5'-GAGATGCCACGCTCTC-3', respectively. Approximately 50 ng of cDNA template was used in each PCR reaction. The PCR cycle 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. Each run was terminated with a melting curve analysis (all points method) which resulted in a melting peak profile specific for the amplified target DNA and the PCR products were examined by agarose gel electrophoresis. Fluorescence data were collected during the extension stage of amplification. Analysis of the data was carried out using the endpoints method option of the Mx3000P™ software.

Data were expressed as the mean ± SE and the tissue with the lowest MHC class II β chain expression was used as calibrator and the β-actin as the normaliser.

2.3 In vitro sea bass MHC class II β chain expression after stimulation

192 The “in vitro” expression of MHC class II β chain was studied by stimulating leukocytes
193 isolated by Percoll gradients [31] from the head kidney of 5 sea bass juveniles (150 g of weight).
194 In one stimulation, the head kidney leucocytes from the single fishes after Percoll purification,
195 were adjusted to 1 X 10^5 cells/ml and incubated at 18 °C for 4 h and 24 h with 5 μg/ml of
196 lipopolysaccharide (LPS from *E. coli* 0127:B8, Sigma).
197 In another stimulation, the head kidney leucocytes from the single fishes after Percoll
198 purification, were adjusted to 1 X 10^5 cells/ml and incubated at 18 °C for 24 h in the presence of 10,
199 30, 50 or 100 ng/ml of sea bass recombinant IL-1β (rIL-1β) [38-39], with 50 ng/ml of rIL-1β heated
200 at 95 °C for 20 min or with no recombinant protein, in 5 ml of L15 medium (Gibco).
201 Total RNA was extracted from experimental cultures after the stimulations with Tripure
202 (Roche) and real-time PCR conditions were as described above for the basal expression.
203 Data were expressed as the mean ± SE and the differences from the control at the same time
204 have been considered significant if $p < 0.05$ using the standard student *t* test to analyse the
205 **significance**.
206 ***2.4 Sea bass MHC class II β chain protein modelling***
207 Three-dimensional models of sea bass MHC class II β chain were created following the
208 homology modelling procedure described in previous papers [40-44], also in agreement with the
209 rules recently reviewed [45] to improve the quality of the modelling results. The BLAST program
210 [33] was used to find homologous proteins in databases. Structure predictions of sea bass MHC
211 class II β chain were based on the availability of the three-dimensional models of the homologous
212 mouse MHC I-A^K chain β protein complexed with antigen peptide and D10 T-cell antigen receptor
213 [46] (PDB code: 1D9K) and with antigen peptide and human CD4 [47] (PDB code: 1JL4). The
214 alignment of the protein sequences was made with CLUSTALW program [48] and a few manual
215 refinements were added to account for the position of secondary structures. Full-atom models of sea
216 bass MHC were created with the MODELLER module [49] of Quanta (Accelrys, Inc., San Diego,

CA, USA) by using as template each of two crystallographic structures, by setting 4.0 Angstroms as RMS deviation among initial models and by full optimization of models, i.e. multiple cycles of refining with conjugate gradients minimization and molecular dynamics with simulated annealing. The best models were chosen by evaluating their stereochemical quality with the PROCHECK program [50] and a scoring function with ProsaII program [51]. Secondary structures were assigned by the DSSP program [52]. Search for structural classification was performed on CATH database [53]. The "Protein-Protein Interaction Server" (http://www.biochem.ucl.ac.uk/bsm/PP/server) [54] and the program NACCESS [55] were used to identify the amino acids at the protein-protein interface in the crystallographic complexes. Molecular superimposition, RMSD values and figures were obtained with the InsightII package (Accelrys, Inc., San Diego, CA, USA).

3. RESULTS

3.1 Sea bass MHC class II β chain cloning and sequencing

PCR with primers MHCFR1 and MHCRV1 resulted in products of the expected size (190 bp) with similarity to other known MHC class II β sequences (data not shown). 3'-RACE-PCR performed with MHC-F2 (based on the initial 190 bp sequence) and the adaptor primer to extend the sea bass MHC sequence gave a product of about 690 bp that contained the 3'-end of the gene. 5'-RACE-PCR was then performed with MHC-R2 (based on the initial 190 bp sequence) and oligo-dG, and gave a product of about 460 bp that contained the 5'-end of the gene, with some differences between the selected clones. The six full-length nucleotide sequences (EMBL accession numbers **AM113466**, **AM113467**, **AM113468**, **AM113469**, **AM113470**, **AM113471**) are comprised of 1259 and have been designated as Dila-DAB sequences (from *0101 to *0601). **They encode** for 250 amino acids, with a predicted 21 amino acid signal peptide, and a 28 bp 5'-UTR and a 478 bp 3'-UTR (Fig. 1). The 3'-UTR contained a polyadenylation signal (AATAAA) 12 bp upstream of the poly(A) tail.

In order to verify the number of class II *B* **loci**, PCR of the open reading frame was performed on the cDNA used for the MHC class II β chain cloning with specific primers (MHCTOTFR 5'-GGCTTCATCCTTTCTCAG-3' and MHCTOTRV 5'-TACTGGGAACCAGAATCC-3') and 10 clones were sequenced, confirming the presence of the six different sequences already obtained with the superimposition of the fragments coming from 3' and 5' RACE. In addition, 10 clones of the 3' UTR sequences from the same individual were sequenced and three different sequences were identified, which should demonstrate that sea bass seems to express **at least three class II *B* loci**.

A multiple alignment of the predicted translation of Dila-DAB sequences was assembled (Fig. 1) together with some fish, mammalian and avian species to investigate the conservation of characteristic amino acid residues. The β-1 domain comprises of 92 amino acids, starts with an alanine residue, which is conserved in all fish sequences except in salmon (*Salmo salar*) and trout (*Oncorhynchus mykiss*), and contains few conserved amino acids (**11% identity** between fish

284 species). The β-2 domain is 94 amino acids long, starts with a valine residue conserved in all
285 sequences except in Japanese flounder (*Paralichthys olivaceus*), red sea bream (*Pagrus major*) and
286 cichlid (*Cyphotilapia frontosa*), shows high identity (35 %) in the C-proximal region and ends with
287 a conserved tryptophan residue. The connecting peptide consists of 10 amino acids and the
288 transmembrane domain, that contains 22 residues, and has the highest identity percentage (55 %).
289 The cytoplasmic tail varies in length in the different species and starts with a conserved tyrosine
290 residue in fish species. The cytoplasmic region contains 13 residues in the Dila-DAB sequences.
291 The cysteine residues present in the β-1 and β-2 domains are well conserved in all sequences
292 except in the turbot sequence that lacks the cysteine at position 94 of the Dila-DAB sequences.
293 These residues, presumably forming two disulfide intra-chain bonds, are consistent with previous
294 findings in other species [56-57]. No potential O- or N-glycosylation sites were found in the Dila-
295 DAB sequences, in contrast to one present in the red sea bream [11], in the catfish [58] and in
296 rainbow trout [59-60].
297 Phylogenetic analysis (Fig. 2) conducted using amino acid sequences showed that all fish
298 sequences are in the same cluster and that the six Dila-DAB sequences are in two different groups:
299 one with sequences Dila-DAB*0101, *0201 and *0301 and the other with sequences Dila-
300 DAB*0401, *0501, *0601. The first group of sea bass sequences also contains the striped bass
301 Mosa-DAB sequence. The mammalian and avian sequences are in a different cluster with respect to
302 the fish one.
303 ***3.2 Basal MHC class II β chain expression***
304 **The expression analysis of IL-10 in organs and tissues of unstimulated sea bass is shown in**
305 **Figure 3. Real-time PCR products were loaded on agarose gels to exclude the formation of**
306 **non-specific amplicons and, to take into consideration the individual genetic variability, five**
307 **different fishes were sampled. MHC class II β chain levels were expressed as a ratio relative to**
308 **β-actin levels in the same samples after real-time PCR analysis using the tissue with the lowest**
309 **expression as calibrator. The highest MHC class II β chain expression was detected in gills,**

followed by gut and thymus. Lower IL-10 mRNA levels were observed in spleen, PBL, and liver; brain and HK showed the lowest expression levels.

3.3 In vitro sea bass MHC class II β chain expression after stimulation

To investigate the differential "in vitro" sea bass MHC class II β chain expression after stimulation with LPS at 4 and 24 hours and with various rIL-1β concentrations, we used RNA extracted from head kidney leucocytes. Total RNA from 5 different fishes was collected and real-time PCR primers were selected in the conserved region of all Dila-DAB sequences. After amplification and real-time analysis, PCR products for both MHC class II β and β-actin were loaded on agarose gels to exclude the formation of non-specific amplicons. Dila-DAB mRNA levels were compared to the housekeeping gene β-actin levels in the same samples and the values of the quantitative analysis were expressed as a ratio relative to β-actin.

Stimulation with LPS (Fig. 4A) resulted in very little differences in the MHC class II β expression after 4 h with respect to the control at the same time and the statistical analysis performed showed that it was not significant. After 24 h the MHC expression has decreased to a large extent and, in this case, the difference was statistically significant. **Moreover, IL-1β expression (data not shown) was studied in the same samples to be sure of the LPS stimulation and showed an increase of IL-1β levels, as was expected.**

Stimulation with different concentrations of rIL-1β (from 0 to 100 ng/ml) resulted in a dose-dependent reduction of the Dila-DAB expression (Fig. 4B), with the lowest value obtained from the sample treated with 100 ng/ml of rIL-1β. The differences were statistically significant for all the samples, except for the cell culture stimulated with 10 ng of rIL-1β and the cell culture stimulated with 50 ng/ml of rIL-1β pre-heated at 95 °C for 20 min. The latter observation is consistent with those reported by Hong et al., (2001) [61]. The Dila-DAB mRNA level in this case was almost equal to the control and significantly different with the sample stimulated with the same dose of rIL-1β but not heated.

335 ***3.4 Sea bass MHC class II β protein modelling***

336 The Dila-DAB sequences have been analysed with the BLAST program in order to find similar
337 sequences in databases and to perform the structural predictions. The crystallographic structures of
338 mouse MHC I-AK β chain complexed with D10 T-cell antigen receptor (PDB code: 1D9K) and
339 human CD4 (PDB code: 1JL4) were selected as template models and the mouse sequence showed
340 the maximum identity percentage (34%) with the Dila-DAB*0101 sequence (data not shown). **This**
341 **low level of sequence identity required an accurate procedure to build a 3D model of the**
342 **protein by comparative modeling, in agreement with rules recently reviewed [45], as already**
343 **applied in previous papers [40-44].**

344 We aligned the Dila-DAB*0101 and mouse MHC sequences using the CLUSTALW program
345 and we performed a few manual adjustments in order to remove gaps within α-helices or β-strands
346 (Figure 5). Starting from this alignment two sets of ten structural models were created, in two
347 distinct sessions, for the Dila-DAB 17-206 region using the templates above indicated. We selected
348 the best model created in each session, i.e. Model-^{1D9K} and Model-^{1JL4} by evaluating the stereo
349 chemical quality of the models with the PROCHECK package [50] and a scoring function with
350 ProsaII program [51]. The models have been deposited in the Protein Data Bank and accepted with
351 the PDB codes 2H37 and 2H38, respectively.

352 Figure 6 shows the two Dila-DAB homology models with their secondary structure elements.
353 These models have a classical organization in two distinct domains in agreement with the structural
354 classification reported by CATH database [53] for the model structures of mouse MHC class II β
355 chain. The N-terminal domains are classified as "alpha-beta" and consist of an alpha helical region
356 and a beta sheet of four strands in antiparallel orientation. The C-terminal domains have a "mainly
357 beta" fold and are characterized by an immunoglobulin-like beta-sandwich made of two antiparallel
358 sheets, each consisting of three main strands and few shorter strands, organized in greek-keys
359 motifs. In both models of Dila-DAB four cysteine residues are located in the same positions of the
360 mouse structures and they may form an S-S bond (30-95) in the N-terminal domains, and another S-

S bond (133-189) in the C-terminal domains between the two sheets of the sandwich architecture, as described in mammals [56-57].

The two Dila-DAB models were compared by structural superimposition, RMSD evaluation and secondary structures (Figure 5) to evidence structural changes due to the binding with different ligands (in one case T-cell receptor and in another CD4). The presence of gaps in the alignment made it difficult to perform a complete structural comparison of Dila-DAB models with the template structures. Superimposition of structurally conserved regions of Dila-DAB Model-^{1JL4} and Model-^{1D9K} with their respective templates gave RMSD values of 1 Angstrom and 0.96 Angstrom, respectively. In contrast, an RMSD value of 1.22 Angstrom was obtained by superimposition of the two Dila-DAB models, i.e. Model-^{1D9K} and Model-^{1JL4}, indicating that these two conformations present some structural differences.

The comparison of secondary structures, assigned by DSSP program in all four models, shows that the alpha helices and the beta-strands are quite conserved in the mouse and Dila-DAB models, although some differences are noted. The short 3_{10} helix (GTQ) observed in both crystallographic mouse structures is not present in the two Dila-DAB models. In Dila-DAB Model-^{1JL4}an alpha helix (Tyr^{103}-Lys^{110}) is observed where in mouse MHC I-A^K complexed with human CD4 (PDB code: 1JL4) a short 3_{10} helix (SLR) is present. By comparing the two Dila-DAB models, we note that Model-^{1D9K} has a lower content of beta-strand in the C-terminal domain, where the strands include **fewer** amino acids and are often broken in two shorter strands. In particular, two short beta-strands, spanning residues Tyr^{171}-Ile^{173} and His^{176}-Tyr^{179}, are present in Model-^{1D9K}, while only one long beta-strand (Tyr^{171}-Tyr^{179}) is present in Model-^{1JL4}. Moreover, Model-^{1JL4} shows one more short beta strand (Val^{158}-Ser^{160}), similar to its template.

Finally, we analyzed the mouse structures to identify the regions involved in the interactions with peptides, T-cell antigen receptor and CD4, by means of solvent accessibility and protein-protein interaction analyses (data not shown). By homology considerations, the aligned regions in the Dila-DAB sequence can be hypothesized to be similarly involved in ligand binding.

4. DISCUSSION

435 The MHC class I and class II molecules are involved in the presentation of antigens to the
436 adaptive immune system. High polymorphism in MHC molecules has been observed in vertebrates
437 and growing **evidence suggests** that MHC variants influence many important biological traits,
438 including immune recognition, susceptibility to infectious and autoimmune diseases, mating
439 preferences and pregnancy outcome [62]. For these reasons MHC genes are among the best
440 candidates for studies of mechanisms and significance of molecular adaptation in vertebrates.

441 In this study, we report the homology cloning of the MHC class II β chain from the teleost sea
442 bass (*Dicentrarchus labrax*). Six different sequences, named Dila-DAB, were obtained. The Dila-
443 DAB cDNAs were predicted to code for proteins of 250 amino acids and their size was in
444 accordance with other fish and mammalian MHC class II β chain molecules. The alignment of
445 Figure 1 showed that the β-1 domains present **a variability** indicative of a functional peptide
446 binding region and most of the differences between the six Dila-DAB sequences are in this region.
447 On the contrary, the β-2 domains are quite well conserved, especially in the membrane-proximal
448 region. Proposed sites for the interaction of the MHC class II β chain with the MHC class II α chain
449 in mammals [57-58] involve three amino acid residues: two histidines and a glutamic acid. Two of
450 these residues are conserved in all fish sequences (His^{127} and Glu^{178} in the Dila-DAB sequences),
451 while the second histidine residue is substituted in the majority of fish molecules by a proline
452 (Pro^{128} in the Dila-DAB sequences). Putative sites important for human MHC class II β chain
453 interaction with CD4 co-receptor are three residues: a valine, a threonine and a serine [57], and
454 these residues are conserved in all fish sequences (Val^{158}, Thr^{159}, Ser^{160} in the Dila-DAB
455 sequences). The transmembrane domain is highly conserved and has multiple hydrophobic residues
456 that are interspersed with uncharged glycines at position 219, 222, 226 and 233 in the Dila-DAB
457 sequences as seen in the sequences from other species [63].

458 Phylogenetic analysis, generated using amino acid sequences, showed a close relationship
459 between the Dila-DAB and Mosa-DAB sequences (Fig. 2) providing evidence for trans-species

evolution of the class II sequences. The bootstrapped tree was divided into two different branches with one containing all fish sequences and the other with mammalian and avian ones.

Moreover, our data suggest that MHC class II β is present in a ubiquitous manner in non-stimulated tissues and organs, although with different expression levels. The highest expression was found in gills that in fish are constantly exposed to a plethora of water born antigens. Moreover, some pathogens use the gills as a portal of entry into the host [64] while others use this organ as the site of infection [65]. Therefore, the ability to mount strong local immune reactions to pathogens is fundamental to avoid disease as already observed in Atlantic salmon [66]. It should be noted that expression of MHC class II β is notably elevated in the gut that is a mucosal tissue particularly rich in T-cells in sea bass [67].

The "in vitro" MHC class II β chain expression was studied using head kidney leukocytes, because this tissue in the red sea bream, a species related to sea bass, showed the highest basal MHC β chain expression [11]. The selected stimulants were LPS, to simulate a pathogen infection, and the sea bass rIL-1β. This cytokine was chosen as it should promote phagocytosis of foreign particles, which are subsequently presented to T-cells in conjunction with MHC molecules [68]. LPS stimulation resulted in a down-regulation of Dila-DAB, which was statistically significant after 24 h and in an increase of IL-1β gene expression (data not shown), that was used to test the efficacy of the activation. This is in accordance with the results found in a macrophage-like cell line (SHK-1) derived from Atlantic salmon [69-70]. **A similar down-regulation of MHC class II was also obtained after challenge with *Vibrio anguillarum* in red sea bream [11] and of MHC class I after infection with hematopoietic necrosis virus (IHNV) in rainbow trout [71].** Recombinant sea bass IL-1β produced a dose-dependent down-regulation in the concentrations used and this has also been observed in various human cell types [72]. It has been postulated that IL-1β plays a role in regulating immunoreactivity by inhibiting transcription of the CIITA gene, thereby reducing class II MHC expression in mammal cell lines [73]. The stimulation has also been performed with

heat inactivated rIL-1β to confirm that the observed down-regulation was due to the cytokine stimulation. **MHC down-regulation could be very likely linked to the already reported mechanisms for the control of inflammation responses in mammals that, uncontrolled, may have dangerous effects. During early inflammation, various cytokines are produced and they down-regulate the expression of inflammation-related molecules [74]. More experiments and tools will be required to demonstrate that something similar happens in fish.**

The **percentage identity** between the N-terminal region of the Dila-DAB*0101 sequence (amino acids 17-206) and mouse MHC sequence, for which crystallographic structures complexed with D10 T-cell antigen receptor and human CD4 are available, gave us the opportunity to apply homology modelling techniques and study the predicted 3D Dila-DAB structures. The two obtained models (Fig. 5) differ mainly in the ligand binding regions. In Model-^{1JL4}, we observed that both the antigen peptide binding site (1360 vs 1309 Angstrom^2) and the interaction surface with CD4 (775 vs 742 Angstrom^2) were larger than in the Model-^{1D9K}. On the contrary, the interaction surface with the T-cell antigen receptor was larger in Model-^{1D9K} (1144 vs 1095 Angstrom^2). **The fact that each model presents a larger interaction surface for the respective ligand confirms the reliability of the prediction. The experimental use of synthetic peptides designed on the basis of the amino acids present on the interaction surfaces with the two different ligands should block the non-covalent bond and will be useful to confirm the structural prediction and for functional investigations.**

In conclusion, the availability of these Dila-DAB sequences will add new insight into the MHC variability in vertebrates, that is linked to parasite resistance, and will give the possibility to analyse the sea bass MHC class II expression levels after vaccination and immuno-stimulation protocols.

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708 **FIGURE LEGENDS**

709 **Fig. 1**. Alignment of the predicted sea bass Dila-DAB amino acid sequences with other known
710 MHC class II β chain molecules. Regions corresponding to the putative signal peptide,
711 β-1 domain, β-2 domain, connecting peptide, transmembrane region and cytoplasmic tail
712 are shown above the sequences. Conserved cysteine residues are evidenced in bold,

conserved amino acid residues are indicated with an asterisk, while amino acid residues present at the starting point of the different regions considering the human crystal structure are in bold and underlined. Accession numbers: Dila-DAB *0101 (*Dicentrarchus labrax*) AM113466; Dila-DAB *0201 (*Dicentrarchus labrax*) AM113467; Dila-DAB *0301 (*Dicentrarchus labrax*) AM113468; Dila-DAB *0401 (*Dicentrarchus labrax*) AM113469; Dila-DAB *0501 (*Dicentrarchus labrax*) AM113470; Dila-DAB *0601 (*Dicentrarchus labrax*) AM113471; Japanese flounder (*Paralichthys olivaceus*) AY848955; turbot (*Scophthalmus maximus*) DQ001730; Atlantic salmon (*Salmo salar*) X70167; red sea bream (*Pagrus major*) AY190711; striped sea bass (*Morone saxatilis*) L33967; rainbow trout (*Oncorhynchus mykiss*) AF115529; catfish (*Ictalurus punctatus*) U77597; humphead cichlid (*Cyphotilapia frontosa*) L13231; human (*Homo sapiens*) CAA47028; mouse (*Mus musculus*) AAC05286); chicken (*Gallus gallus*) NP_001038144).

Fig. 2. Phylogenetic tree showing the relationship between Dila-DAB sequences with other known MHC class II β molecules. The tree was constructed by the "neighbour-joining" method and was bootstrapped 10000 times. 0.1 indicates the genetic distance.

Fig. 3. MHC class II β chain basal expression in different tissues [spleen, peripheral blood leukocytes (PBL), brain, liver, gut, thymus, head kidney (HK), gills]. MHC class II β mRNA levels were expressed as a ratio relative to β-actin levels in the same samples after real-time PCR analysis using the tissue with the lowest expression as calibrator.

Fig. 4. MHC class II β expression in different stimulating conditions. (A): MHC mRNA levels expressed as a ratio relative to β-actin levels in the same samples after real-time PCR analysis of the head kidney leukocytes stimulated with no LPS (control) and with LPS for 4 and 24 h. (B): MHC mRNA levels expressed as a ratio relative to β-actin levels in

the same samples after real-time PCR analysis of the head kidney leukocytes stimulated with various rIL-1β concentrations.

Data were expressed as the mean ± SE, * indicates $p < 0.05$ and therefore significantly lower than the respective control.

Fig. 5. Primary and secondary structures of mouse and Dila-DAB*0101 chains, aligned as for the modelling procedure. Stars (*) indicate identical amino acids. The interaction residues of mouse MHC-II with the antigen peptide, the D10 T-cell antigen receptors alpha and beta chains (N-terminal region) and human CD4 (C-terminal region) are reported in bold. Helices and beta strands are marked with continuous and dashed line boxes, respectively.

Fig. 6. Molecular models of sea bass MHC class II β obtained by homology modelling using as template the structure of mouse MHC I-A^K chain β complexed with human CD4, i.e. Models-^{1JL4} (a) and with D10 T-cell antigen receptor, i.e. Models-^{1D9K} (b). Backbone ribbon and the secondary structure topology are shown: yellow arrows represent beta strands, red cylinders represent alpha helices. Amino and carboxy terminal ends are indicated. Green and yellow sticks indicate the possible presence of two Cys-Cys bonds in each model.