Fish & Shellfish Immunology xxx (2012) 1–11

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Characterization of RAG1 and IgM (mu chain) marking development of the immune system in red-spotted grouper (*Epinephelus akaara*)

Q4 Ming-Guang Mao [a], Ji-Lin Lei [a,b,*], Perálvarez-Marín Alex [c], Wan-Shu Hong [a], Ke-Jian Wang [a,**]

Q1 [a] State Key Laboratory of Marine Environmental Science, College of Oceanography and Environmental Science, Xiamen University, Xiamen 361005, Fujian, China
[b] Qingdao Key Laboratory for Marine Fish Breeding and Biology, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, Shandong, China
[c] Centre d'Estudis Biofísics, Universitat Autònoma de Barcelona, Barcelona 08193, Spain

ARTICLE INFO

Article history:
Received 1 February 2012
Received in revised form
18 May 2012
Accepted 15 June 2012
Available online xxx

Keywords:
RAG1
IgM mu chain
Epinephelus akaara
Thymus
Development

ABSTRACT

In vertebrates, lymphoid-specific recombinase protein encoded by recombination-activating genes (RAG1/2) plays a key role in V(D)J recombination of the T-cell receptor and B-cell receptor. In this study, both RAG1 and the immunoglobulin M (IgM) mu chain were cloned to characterize their potential role in the immune defense at developmental stages of red-spotted grouper, *Epinephelus akaara*. The open reading frame (ORF) of *E. akaara* RAG1 included 2778 nucleotide residues encoding a putative protein of 925 amino acids, while the ORF of the IgM mu chain had 1734 nucleotide residues encoding 578 amino acids including variable (VH) and constant (CH1–CH2–CH3–CH4) regions. *E. akaara* RAG1 was composed of a zinc-binding dimerization domain (ZDD) with a RING finger and zinc finger A (ZFA) in the non-core region and a nonamer-binding region (NBR), with a zinc finger B (ZFB), the central and C-terminal domains in the core region. Tridimensional models of the ZDD and NBR of *E. akaara* RAG1 were constructed for the first time in fishes, while a 3D model of the *E. akaara* IgM mu chain was also clarified. The RAG1 mRNA was only detected in the thymus and kidney of 4-month and 1.5-year old groupers using qPCR, and the RAG1 protein was confirmed using western blotting and immunohistochemistry. The IgM mu mRNA was examined in most tissues except the gonad. RAG1 and IgM mu gene expression were observed at 15 dph (days post-hatching) and 23 dph respectively, and increased to a higher level at 37 dph. In addition, this was the first time that the morphology of the *E. akaara* thymus was characterized. The oval-shaped thymus of 4-month old fish was clearly seen and there were amounts of T lymphocytes present. The results suggested that the immune action of *E. akaara* was likely to start to develop around 15 dph to 29 dph. The transcript level of the RAG1 gene and the number of lymphocytes in the thymus between 4-month and 1.5-year old groupers indicated that age-related thymic atrophy also occurs in fishes. The similar functional structures of RAG1 and IgM protein between fish and mammals indicated that teleost species share a similar mechanism of V(D)J recombination with higher vertebrates.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Red-spotted grouper, *Epinephelus akaara*, is considered a good model for studies of sex-inversion, development and genetic diversity [1]. Moreover, *E. akaara* (commonly named the Hong Kong grouper) is listed as threatened on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species based on the data available. Currently, *E. akaara* breeding has been conducted in some areas of China, but the aquaculture technology and the knowledge of *E. akaara* immunity are limited. Approximately 90% mortality during the larvae stages baffles the farmers in China [2], which attracted our interest to characterize how their immune system works during the developmental stages.

Recently, lymphoid organs and both T and B cells have been chosen to study the ontogeny of the fish immune system in several teleost species [3–6], and recombination-activating gene1 (RAG1) and immunoglobulin M (IgM) have been considered as very useful markers in the study of the physiological maturity of the immune system [7–14]. Previous studies show that the appearance of the immune system during early developmental stages is different in different fish species, such as 4 days post fertilization (dpf) in

* Corresponding author. State Key Laboratory of Marine Environmental Science, College of Oceanography and Environmental Science, Xiamen University, Xiamen 361005, Fujian, China. Fax: +86 592 2180655.
** Corresponding author.
E-mail addresses: leijilin@seacul.com (J.-L. Lei), wkjian@xmu.edu.cn (K.-J. Wang).

1050-4648/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2012.06.011

zebrafish [9] and common carp [11], 8 dpf in Japanese flounder [12] and 21 dpf in gadoid haddock [7]. On the other hand, maternal antibody is proved to protect the offspring during early developmental stages in teleost fishes [15,16]. Therefore, provided that the maternal antibodies run out before the time of endogenous Ig generation in *E. akaara*, the larvae are prone to be infected by pathogens in seawater. However, the knowledge of the development of immune system in *E. akaara* is limited and in demand now.

Recent developments in mammals have increased our understanding of how V(D)J recombination is regulated to ensure that antigen receptor gene assembly occurs in the appropriate cell lineage and in the proper developmental order [17,18]. The RAG proteins are expressed at high levels during the early stages of lymphocyte development with no occurrence in other tissue or cell types, and so it is considered to be a very useful marker for studies of immune system development [7]. The RAG proteins catalyze DNA cleavage in the first phase of the reaction using a recombination signal sequence (RSS) that flanks variable (V), diversity (D) and joining (J) gene segments. Each RSS of V, D, J gene segments is composed of well-conserved heptamer (consensus 5′-CACAGTG) and nonamer (consensus 5′-ACAAAAACC) sequences separated by a spacer of either 12 bp or 23 bp in a phenomenon known as the 12/23 rule [17]. RAG1 Core region mediates protein–protein and protein–DNA interactions that are essential for V(D)J recombination, and nonamer binding region (NBR) forms a tightly interwoven dimer that binds and synapses two nonamer elements [19]. Zinc-binding dimerization domain (ZDD) in the non-core region plays role in ubiquitin ligase activity and binding karyopherin alpha 1 (KPNA1) [20]. However, little knowledge of the functional regions of RAG1 has been reported in teleost species. The IgM class is the primary immunoglobulin in most teleost fish, and has been isolated from many species [21–26]. Since the IgM mu chain is one of the B cell specific proteins, it was chosen to study the ontogenesis of the immune system of *E. akaara* in our study.

Here we aimed to characterize the *E. akaara* RAG1 and IgM mu genes, and to construct 3D models of ZDD, NBR and IgM mu chain. Further studies on the development of the *E. akaara* immune system are to be summarized in our next study. In addition, the thymus, which is considered a key organ of the immune system in fish, is responsible for producing and educating T cells [27], but the histology of the thymus can vary considerably between different fish species, and so the general morphology of the *E. akaara* thymus will also be dealt with here.

2. Materials and methods

2.1. Fish

Eggs and larvae of *E. akaara* were collected from a hatchery in Zhangzhou, Fujian Province, China during the spawning season in 2010. While in the process of hatching, a pool of 100 eggs and larvae were collected 0, 1 and 4 dph (days post-hatching), a pool of 50 larvae were collected 6, 9 and 12 dph, and a pool of 30 larvae 15, 18, 23 and 29 dph. Larvae at 30 dph were maintained in the aquarium facilities of the College of Oceanography and Environmental Science, Xiamen University, in a recirculation unit supplied with saltwater at 27 ± 3 °C, and fish were sampled 37, 60 and 80 dph. Four to six 4-month and 1.5-year old groupers were raised in order to harvest tissues for RNA extraction.

2.2. Cloning and sequencing of RAG1 and IgM mu chain cDNA

Total RNA was extracted from the thymus and head kidney separately. The full-length sequence of RAG1 was determined using 3′-Rapid Amplification of cDNA Ends (3′-RACE) and 5′ RACE using commercial kits (Clontech) and following the procedures described by the manufacturer. Specific primers were designed based on the conserved regions of the known RAG1, IgM mu chain and β-actin sequences. The primer details are given in Table 1 and PCR conditions were as follows: 94 °C/4 min; 40 cycles of 94 °C/5 s, 55 °C/40 s, 72 °C/90 s; and 72 °C/10 min. PCR products were first detected using electrophoresis with 1.2% agarose gel prepared with TAE buffer (Tris/acetic acid/EDTA), then purified from the gel using an AxyPrep DNA gel extraction kit (Axygen) and subcloned into pMD18-T vectors (TaKaRa) for sequencing (Invitrogen) to verify the cDNA sequence of the *E. akaara* RAG1. The same procedure for cloning and sequencing of the mu chain cDNA was used.

2.3. Tissue distribution of RAG1 and IgM (mu chain) mRNA transcripts

Six groupers of 4-month and 1.5-year old were randomly chosen to harvest various tissues including muscle, kidney, heart, thymus, brain, intestine, gonad, spleen, liver and gill. Total RNAs were prepared from all tissues and then treated with DNase I (Tiangen Biotech) to remove residual genomic DNA. The relative quantification of RAG1 and mu gene mRNA transcripts was evaluated respectively using the comparative CT (Cycle threshold) method using a 7500 Real Time PCR System (AB Applied Biosystem) and Power SYBR Green PCR Master Mix (AB Applied Biosystem). The β-actin gene was employed as the endogenous control. The gene specific primers for qPCR are listed in Table 1. The cycling profile was as follows: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of denaturing at 95 °C for 15 s, followed by annealing and primer extension at 60 °C for 1 min. Expression of RAG1 and IgM mu gene was normalized to an endogenous reference β-actin and presented as subtraction of target CT values from β-actin CT values (ΔCT value). Comparison of gene expression between tissues and calibrator was derived from subtraction of the calibrator ΔCT values from the target ΔCT values to give a ΔΔCT value, and relative gene expression was calculated to determine fold difference ($2^{-\Delta\Delta CT}$). Results were expressed as mean ± standard deviation. Comparisons between groups were made by One-way Anova analysis, followed by a Tukey test for identification of the statistically distinct groups. Significant differences were accepted for $P < 0.05$.

Table 1
Primers used in this experiment. Q3

Primer name	Sequence of primer (5′–3′)	Target
Actin-F1	ATGGAAGATGAAATCGCCGC	ORF of β-actin
Actin-R1	TGATGCTGTTGTAGGTGGTCT	
Actin-F2	GTGGAATCCACGAGACCA	
Actin-R2	TAACCGTACCGAAGTCAATAA	
RAGF1	CAGYCAGTAYCAYAAGATGTACCGCAC	Partial sequence
RAGF1	GCATAGTTYCCATTCATCCTCATCACAG	of RAG1
3GSP1	GGCAATGCGGCGGAGTTCTAC	3′RACE PCR of
3GSP2	AAACTATGCCCGCAGGCTAATGAC	RAG1
3GSP3	ACACGGTCACTCTTGGTTGCC	3′RACE PCR of
3GSP4	GAGTTTGCTTCCTCGTCATC	IgM mu chain
5GSP1	CCAGTGCCACATCATAGCGAAACC	5′RACE PCR of
5GSP2	CACCGAGGAAGCCCATCCAGAG	RAG1
5GSP3	TGAGTAGGTCAGTGAGGAGGGTG	5′RACE PCR of
5GSP4	GGCAACCAAGAGTGACCGTGT	IgM mu chain
5′adaptor	$(T)_{25}$VN	Universal primer
3′adaptor	AAGCAGTGGTATATCAACGCAGA GTAC$(T)_{30}$VN	of RACE PCR
qRAGF	GCAATGCGGCGGAGTTCTAC	RAG1 (q-PCR)
qRAGR	AGCCTGCGGGCATAGTTTCC	
qIgMF	GCCTCAGCGTCCTTCAGTTT	IgM (q-PCR)
qIgMR	TGGCGTCCCAGTCCTGTTTGC	
qactinF	GTCCCTGTATGCCTCTGGTCG	β-actin (q-PCR)
qctinR	CTGTGGTGGTGAAGGAGTAGC	

F = forward sequence, R = reverse sequence.

Please cite this article in press as: Mao M-G, et al., Characterization of RAG1 and IgM (mu chain) marking development of the immune system in red-spotted grouper (*Epinephelus akaara*), Fish & Shellfish Immunology (2012), http://dx.doi.org/10.1016/j.fsi.2012.06.011

```
   1 ATGAACTGCAAGTTCCTCAGCTGGCCAGAGGTGATCCTCAAGGTCTTCAAAGTGGATGTGACAGAAGACACAGAATCCGTCCACCCTCTG   90
   1 M N C K F L S W P E V I L K V F K V D V T E D T E S V H P L   30
  91 TCCTTCTGCCATCGCTGCTGGGTGATTGCCATACGAGGAGGAGGCGTCTGCAGCTTCTCCAAAACGAATGTCCCCGAGTGGAATCCCCAC  180
  31 S F C H R C W V I A I R G G G V C S F S K T N V P E W N P H   60
 181 TCTTCCCCCTGCCACCTTTGCTCCCCCAAAAAACCTTCATTCAAGCGGACTGGGAGGAAGAGGAGGTTAGCCATTCCCAGAGCCCAGAGT  270
  61 S S P C H L C S P K K P S F K R T G R K R R L A I P R A Q S   90
 271 TTGGCAAAAAGAAGCAGGAGAGACCACGGAGACAGCACTGCGGGTGGTGAGAGGAGGGCTCTGAGACAATTCGCCGACCATTATCATGGT  360
  91 L A K R S R R D H G D S T A G G E R R A L R Q F A D H Y H G  120
 361 CCTGTGCTCAGGGGGTGGAGAAAACCTACCATCCAGAGAGAGCAATGGGTGAGGAACATCACCCACTGCCAGAAAGACCACCTGAATACC  450
 121 P V L R G W R K P T I Q R E Q W V R N I T H C Q K D H L N T  150
 451 AAGCTGATCTCTGAGAAGCTCCCTGTTGACTTCCTCTTCTCTTTCACCTGCCTGGTGTGTGACCACCTGCTCTCTGATCCAGTCCAGTCC  540
 151 K L I S E K L P V D F L F S F T C L V C D H L L S D P V Q S  180
 541 CCCTGTGGGCACCTCTTCTGCCGCAGCTGCATTATAAAATACATCCACGTCCTGGGACCTCACTGCCCGGCCTGCAACTTGTCCTGCACC  630
 181 P C G H L F C R S C I I K Y I H V L G P H C P A C N L S C T  210
 631 CCCGATGATCTCAGTCTGCCTGCCAGAGCCTTCTTATCAGCCCTACATTCCCTGCCTCTGCTCTGCCCCAAGAGCGGCTGTGGCAAGCAG  720
 211 P D D L S L P A R A F L S A L H S L P L L C P K S G C G K Q  240
 721 GTAAGGCTAGACTCATTTAAAACTCATTGTCTGGGCCATGAGCTGTGTGAGCAGGACACAAAGCAGCAGTCATCAGACCTTGACAACTAC  810
 241 V R L D S F K T H C L G H E L C E Q D T K Q Q S S D L D N Y  270
 811 CTGCTAGCCAATAAAGGGGGAAGACCCCGTCAGCACCTGCTATCGCTAACCCGCCGTGCCCAGAAGCATCGGCTCAAGGATATGAAGAAC  900
 271 L L A N K G G R P R Q H L L S L T R R A Q K H R L K D M K N  300
 901 CAGCTGAAGGCGTTTGCAGACAGAGAGGAAGGTGGCGACCTCAAGTCTGTGTGTCAGACTCTGTTTCTGCTCTCGCTGAGGTCTGCGAAT  990
 301 Q L K A F A D R E E G G D L K S V C Q T L F L L S L R S A N  330
 991 GAACACCGGCAGGCGGACGAGCTGGAGGCCTTGATGCAAGGCAGAGGCTTTGGGTTGCATCCTGCTGTGTGCTTGGCCATTCGGGTCAAC 1080
 331 E H R Q A D E L E A L M Q G R G F G L H P A V C L A I R V N  360
1081 ACTTTCCTGAGCTGCAGCCAGTATCACAAGATGTACCGGACTGTCAAAGCCACCAGCGGCCGCCAGATCTTTCAGCCCCTGCACACCCTG 1170
 361 T F L S C S Q Y H K M Y R T V K A T S G R Q I F Q P L H T L  390
1171 CGAGCCGCAGAGAAGGAGCTTCTCCCTGGCTTTCACCAGTTTGAATGGCAGCCGGCTCTCAAAAATGTGTCCACATCGTGCAATGTTGGC 1260
 391 R A A E K E L L P G F H Q F E W Q P A L K N V S T S C N V G  420
1261 ATTATTAATGGGCTCTCTGGATGGGCTTCCTCGGTGGATGACATCCCAGCTGACACCATCACTCGACGGTTTCGCTATGATGTGGCACTG 1350
 421 I I N G L S G W A S S V D D I P A D T I T R R F R Y D V A L  450
1351 GTGTCAGCATTAAAGGATCTGGAGGAGGACATCATGGACGGGCTGAGAGAGTGTGGGATGGAAGACAGTACATGCACCTCAGGCTTCAGT 1440
 451 V S A L K D L E E D I M D G L R E C G M E D S T C T S G F S  480
1441 GTCATGATCAAGGAATCCTGTGATGGCATGGGCGATGTCAGCGAGAAGCACGGTGGAGGACCAGTTGTTCCTGAGAAGGCTGTGCGTTTC 1530
 481 V M I K E S C D G M G D V S E K H G G G P V V P E K A V R F  510
1531 TCTTTCACTGTTATGTCTGTCTCTGTCCTGGCAGATGAGCAGGAGAAAGAGGTTACCATCTTCACTGAGGCAAAGCCAAACTCAGAGATG 1620
 511 S F T V M S V S V L A D E Q E K E V T I F T E A K P N S E M  540
1621 TCCTGTAAGCCCCTTTGCCTGATGTTTGTGGATGAGTCAGACCACGAGACACTAACAGCCGTCCTGGGGCCTATAGTTGCAGAGCGTAAT 1710
 541 S C K P L C L M F V D E S D H E T L T A V L G P I V A E R N  570
1711 GCAATGAAAGAGAGCAGGCTCATCCTATCCGTGGGCGGACTCCCTCGCGCTTTCCGCTTTCACTTCAGAGGCACGGGATATGATGAGAAG 1800
 571 A M K E S R L I L S V G G L P R A F R F H F R G T G Y D E K  600
1801 ATGGTGCGCGAGATGGAAGGCCTCGAAGCCTCAGGGTCCACCTATGTCTGCACTCTTTGTGACTCCACTCGGGCAGAGGCCTCCCAAAAC 1890
 601 M V R E M E G L E A S G S T Y V C T L C D S T R A E A S Q N  630
1891 ATGGTGCTCCACTCCATCACTCGCAGTCATGACGAGAACCTAGACCGTTATGAAATATGGAGAACCAATCCTTTTTCTGAGTCTGCAGAT 1980
 631 M V L H S I T R S H D E N L D R Y E I W R T N P F S E S A D  660
1981 GAGCTGCGAGACAGAGTCAAAGGAGTCTCTGCCAAGCCCTTCATGGAGACCCAGCCCACGCTGGATGCATTACACTGCGACATCGGCAAT 2070
 661 E L R D R V K G V S A K P F M E T Q P T L D A L H C D I G N  690
2071 GCGGCGGAGTTCTACAAAATCTTCCAGGACGAGATAGGGGAAGTGTACCAAAAGGTCAACCCCAGCCGGGAGGAGCGGCGCAGCTGGAGG 2160
 691 A A E F Y K I F Q D E I G E V Y Q K V N P S R E E R R S W R  720
2161 GCAGCCCTAGATAAACAGCTGAGGAAGAAGCTGAAGCTCAAACCTGTGATGAGGATGAATGGAAACTATGCCCGCAGGCTAATGACCCAG 2250
 721 A A L D K Q L R K K L K L K P V M R M N G N Y A R R L M T Q  750
2251 GAGGCTGTGGAGGTGGTGTGTGAGCTGGTGCCCTCGGAGGAGAGGAGGGAGGCCCTGAGGGAGCTCATGAGGATCTACCTCCAGATGAAG 2340
 751 E A V E V V C E L V P S E E R R E A L R E L M R I Y L Q M K  780
2341 CCCGTGTGGCGCGCCACCTGCCCTGCCAAGGAGTGCCCCGACCAGCTGTGCCGCTACAGTTTTAACTCCCAGCGCTTCGCCGACCTTCTC 2430
 781 P V W R A T C P A K E C P D Q L C R Y S F N S Q R F A D L L  810
2431 TCCTCTACCTTCAAATACAGGTACAACGGTAAAATAACCAATTACCTGCACAAGACCCTGGCCCATGTGCCTGAGATCATAGAGAGAGAA 2520
 811 S S T F K Y R Y N G K I T N Y L H K T L A H V P E I I E R E  840
2521 GGATCCATAGGAGCCTGGGCCAGCGAGGGGAACGAGTCGGCGAACAAACTGTTCAGACGTTTATGGAAGATGAATGCACGTCAGTCAAAG 2610
 841 G S I G A W A S E G N E S A N K L F R R L W K M N A R Q S K  870
2611 GCGTTTGAGCTTGAGGACGTGTTGAAACATCACTGGCTCTACACCTCAAAGTGCTTGCAGAAGTTTATGGAAGCCCACAAGGACTCTGCC 2700
 871 A F E L E D V L K H H W L Y T S K C L Q K F M E A H K D S A  900
2701 AAAGCTCTGCAAGCCACTATTGACCCGATAGAGAGCCAGGATTATGAGGACATGTCTCTAGAAGATAATGACTTTTGATTTTTCTCAAGA 2790
 901 K A L Q A T I D P I E S Q D Y E D M S L E D N D F *          925
2791 TAGTTGTGTAATATTTGGACTGCCTCCATCAGTGATATGGACTTGTTTACTAATATACCCATTCACTGATATTGAGAATAATGAGGGAGT 2880
2881 GGAGTTTATGGACTTGAAGCCAAGTTTTCTACATTTTTTTTGTATTTTGTTGTGATTAAATGAATTTTGGAAAAAAAAAAAAAAAAAAAA 2970
2971 AAAAAAAAAA                                                                                2980
```

Fig. 1. *E. akaara* RAG1 cDNA and putative amino acid sequence. The stop codon is indicated with an asterisk (*), and a typical polyadenylation signal (AATAA) in the 3′-UTR is boxed. The core region is underlined, and the nonamer-binding region (NBR) is in italics. The zinc-binding dimerization domain (ZDD) is shaded.

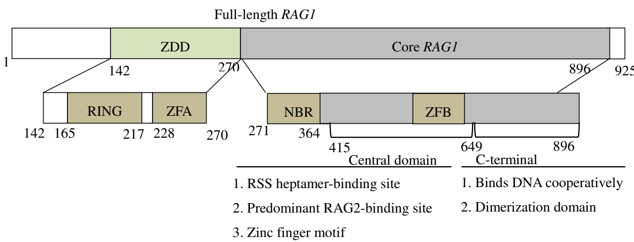

Fig. 2. Schematic representation of full-length *E. akaara* RAG1 protein. The RAG1 protein, with a core and a non-core region, is represented schematically as bars. NBR is the nonamer-binding region (within the core region) that binds specifically to the RSS nonamer. Boxes represent zinc-binding motifs including a RING finger and zinc finger A (ZFA) in the zinc-binding dimerization domain (ZDD) of the non-core region and a zinc finger B (ZFB) in the core region of RAG1.

2.4. Expression pattern of RAG1 and IgM mu gene during development

Total RNA in the pool of *E. akaara* larvae at 0, 1, 4, 6, 9, 12, 15, 18, 23 and 29 dph and the heads of larvae at 37, 60 and 80 dph were separately extracted using the RNAprep pure tissue kit according to the manufacturer's instructions (Tiangen Biotech). All the samples were tested using real time PCR. The procedure for the relative quantification of RAG1 and IgM mu chain mRNA was the same as in Section 2.3.

2.5. Western blotting

The thymus and head-kidney of 4-month old grouper were prepared using RIPA and SPMF reagents, containing protease and phosphatase inhibitors (Roche Applied Science). SDS-PAGE was carried out, and RAG1 protein was detected using western blot with the primary antibody, anti-RAG1 protein from goat IgG (sc-34270, Santa Cruz). The detection was performed using the Polink-2 plus® Polymer HRP Detection System (PV-9003, GBI). Visualization of western blot results was developed using an enhanced chemiluminescence (ECL) detection system.

2.6. Immunohistochemistry (IHC)

5-μ-thick paraffin sections of thymus from 4-month old grouper and head-kidney from of 1-month old grouper were cut and mounted on silanized slides. Heat-induced epitope retrieval was performed in a water bath and the primary antibody Anti-RAG1 goat IgG (sc-34270, Santa Cruz) was used. IHC was performed using the Polink-2 plus® Polymer HRP Detection System (PV-9003, GBI).

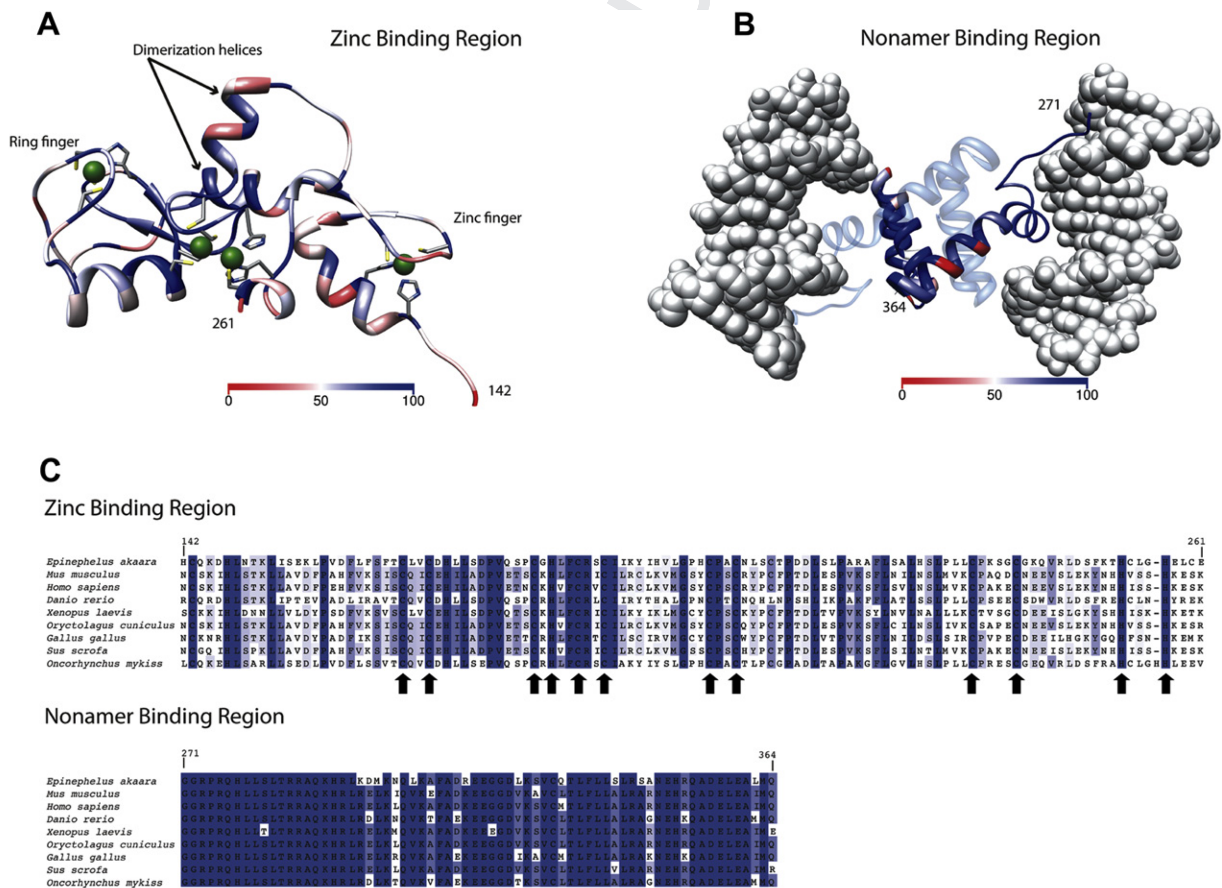

Fig. 3. Tridimensional models of the zinc and nonamer binding regions of *E. akaara*. The models were constructed using Modeller and visualized using Chimera. A. 3D structure for the zinc binding region of *E. akaara* modeled using the *M. musculus* crystal structure (pdb code 1RMD). The protein domain is represented as a ribbon colored using a conservation coding (color bar scale indicates the percentage of identity). The histidines and cysteines involved in zinc (green spheres) coordination are represented as sticks. B. 3D structure for the nonamer binding region modeled using the *M. musculus* crystal structure (pdb code 3GNA) in complex with DNA (gray spheres). The protein domain is represented as a ribbon colored using a conservation coding (color bar scale indicates the percentage of identity). For clarity purposes, the second subunit of the dimer is represented as a light blue transparent ribbon. C. Alignment of several RAG1 sequences of vertebrates used for the definition of the conservation coloring code. The alignments were carried out using T-coffee. The residues are shaded using the percentage of identity coloring mode of JalView. The consistent C3HC4 motif and C2H2 zinc finger are marked with arrows (⬆). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

```
   1 CGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAT   90
  91 TAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCC  180
 181 CAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGAT  270
 271 TACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGATTAAGCAGTGGTATCAACGCAGAGTACGCGGGGGTTCACAGCACGTCAGTTT  360
 361 CAACATAAACC                                                                                 371
 372 ATGTTCTCTGTAGCTCTGCTGCTGCTGTTGGCAGCTGGATGTGTGAAGTGTGAACAGTTGACACAGCCAGCCTCTGTGACTGTGCAGCCA  461
   1  M  F  S  V  A  L  L  L  L  L  A  A  G  C  V  K  C  E  Q  L  T  Q  P  A  S  V  T  V  Q  P   30
 462 GGTCAACGTCTGACCATCACCTGTCAGGTCTCTTATTCTGTTAGTGGCTACTACACAGCTTGGATCAGACAGCCTGCAGGGAAAGGACTG  551
  31  G  Q  R  L  T  I  T  C  Q  V  S  Y  S  V  S  G  Y  Y  T  A  W  I  R  Q  P  A  G  K  G  L   60
 552 GAGTGGATTGGACAAGCAGCTGGATCCACCACATACTACAAAGATTCACTGAAGAACAAGTTCAGTATCAGCTTAGACTCTTCCAGCAAC  641
  61  E  W  I  G  Q  A  A  G  S  T  T  Y  Y  K  D  S  L  K  N  K  F  S  I  S  L  D  S  S  S  N   90
 642 ACAGTGACTCTAAACGGACAGAATGTGCAGCCTGAAGACACTGCTGTGTATTACTATGCCAGAATACGGGACTACGGTTTTGACTACTGG  731
  91  T  V  T  L  N  G  Q  N  V  Q  P  E  D  T  A  V  Y  Y  Y  A  R  I  R  D  Y  G  F  D  Y  W  120
 732 GGAAAAGGCACCACGGTCACCGTCACCTCAGCCACTCCAAATGCACCAACTGTGTTCCCTCTGATGCAATGTGGTTCTGGGACTGGAAAC  821
 121  G  K  G  T  T  V  T  V  T  S  A  T  P  N  A  P  T  V  F  P  L  M  Q  C  G  S  G  T  G  N  150
 822 ACGGTCACTCTTGGTTGCCTTGCCACCGGCTTCACACCCTCCTCACTGACCTACTCATGGAGCGTGGTAAATGGGGCTGCCTTGACAGAC  911
 151  T  V  T  L  G  C  L  A  T  G  F  T  P  S  S  L  T  Y  S  W  S  V  V  N  G  A  A  L  T  D  180
 912 TTCATTCAGTACCCTCCAGTACTGAAAGACAACCTTTATACTGGAGTAAGTCAAGTCCAAGTGAGCAAACAGGACTGGGACGCCATGAAA 1001
 181  F  I  Q  Y  P  P  V  L  K  D  N  L  Y  T  G  V  S  Q  V  Q  V  S  K  Q  D  W  D  A  M  K  210
1002 TCCTTCAGATGTGATGTGACACATGCAGCGGGAACTCCACATGTTACTATCACAAAGCCAAGCGTGCATTATCAGTTGCCAACTCTTAAA 1091
 211  S  F  R  C  D  V  T  H  A  A  G  T  P  H  V  T  I  T  K  P  S  V  H  Y  Q  L  P  T  L  K  240
1092 GTAATGACCTGCTCTGATGAGGGCACCGAGACTACCTTCTCCTGCTTTGCCAAAGATTTTTCACCAAAAGATTTTGAGTTCAAATGGCTG 1181
 241  V  M  T  C  S  D  E  G  T  E  T  T  F  S  C  F  A  K  D  F  S  P  K  D  F  E  F  K  W  L  270
1182 AAAAATGGAGAAAAAATCACCACTGGAATAACCAACATCAAAACACCTTTTGATGAAAAAAAGACAGACAATGCAACACTGTACAGTGCA 1271
 271  K  N  G  E  K  I  T  T  G  I  T  N  I  K  T  P  F  D  E  K  K  T  D  N  A  T  L  Y  S  A  300
1272 GCAAGTTTTCTGACAGTGCAGCCAACTGATTGGGCTTCTGACATTAAATATACATGTGAGTTCACGGGAAAAGGTGAAAAAGGTCCAACA 1361
 301  A  S  F  L  T  V  Q  P  T  D  W  A  S  D  I  K  Y  T  C  E  F  T  G  K  G  E  K  G  P  T  330
1362 TATGTGAATTCATCTGCAACCCGCCAAATGGCAACTGAATGTATAGGATGCCTGCAAGCAGATGTGGAAGTAAAGATCATAGAACCAACA 1451
 331  Y  V  N  S  S  A  T  R  Q  M  A  T  E  C  I  G  C  L  Q  A  D  V  E  V  K  I  I  E  P  T  360
1452 ATGAGGGACTTTGTTGAAAACAGAAAGGGAGTTGTAAAATGTCAAGTCAAGATAAACAAACCATCTGTCATCAAGATTTTCTGGGAGACC 1541
 361  M  R  D  F  V  E  N  R  K  G  V  V  K  C  Q  V  K  I  N  K  P  S  V  I  K  I  F  W  E  T  390
1542 CATGATGGCAAAGAAATACCTGGTGCTGTGGAGCCCAAAAAAGAAGAAACAGGTGTAAAAACCGCTTTACTTCCCATCACATTTGAAGAA 1631
 391  H  D  G  K  E  I  P  G  A  V  E  P  K  K  E  E  T  G  V  K  T  A  L  L  P  I  T  F  E  E  420
1632 TGGAGACAGGGGGAAAAATTCATCTGCACCATTCAACATGACAACTGGCTAGAGCCACGTACGGAAGTCTACAAAAGGGCGATTGAACGA 1721
 421  W  R  Q  G  E  K  F  I  C  T  I  Q  H  D  N  W  L  E  P  R  T  E  V  Y  K  R  A  I  E  R  450
1722 CTGCCTCAGCGTCCTTCAGTTTTTATGCTACCTCCACTAGAACATATTAAAACAGAAACAGTGACCCTGACTTGCTATGTGAAAGACTTC 1811
 451  L  P  Q  R  P  S  V  F  M  L  P  P  L  E  H  I  K  T  E  T  V  T  L  T  C  Y  V  K  D  F  480
1812 TTCCCTCGGGACATTTATGTGTCTTGGCTTGTCGATGACGAGGAAGCAAACTCAAAACACAAGTTCCATACCACAACGCCTGTAGAAAAT 1901
 481  F  P  R  D  I  Y  V  S  W  L  V  D  D  E  E  A  N  S  K  H  K  F  H  T  T  T  P  V  E  N  510
1902 GATGGATCATACTCTGCCTATAGCCAGTTAACCCTCACCCTCGAGCAGTGGAAAAATGATGACATGGTGTACAGCTGTGCAGTTCACCAT 1991
 511  D  G  S  Y  S  A  Y  S  Q  L  T  L  T  L  E  Q  W  K  N  D  D  M  V  Y  S  C  A  V  H  H  540
1992 GAATCTGTGGTCAACACAACTAGAGCTATCGTCAGGTCCATCGGGCACAGAACATTTGAAAAAACCAACATGGTCAACCTCAACATGAAC 2081
 541  E  S  V  V  N  T  T  R  A  I  V  R  S  I  G  H  R  T  F  E  K  T  N  M  V  N  L  N  M  N  570
2082 ATCCCTGAAACGTGCAAGGCCCAGTAGATGTTGTTCTGTGTCACTGTGTCTTCTGCTGTTTGTTGTTTAATGTTTGCTGCTTGTGATATG 2171
 571  I  P  E  T  C  K  A  Q  *                                                                  578
2172 ACACTGTGTTTGTCTTTTTAATGCAGATTCAAAATCAAAATAAAAAAAAAGCACTTTGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA   2259
```

Fig. 4. *E. akaara* IgM (mu chain) cDNA and putative amino acid sequence. The deduced amino acid sequence is reported in one-letter code and the open reading frame of 1737 bp encodes a protein of 578 amino acid residues. The stop codon is indicated with an asterisk (*), and the predicted polyadenylation signal is boxed.

Please cite this article in press as: Mao M-G, et al., Characterization of RAG1 and IgM (mu chain) marking development of the immune system in red-spotted grouper (*Epinephelus akaara*), Fish & Shellfish Immunology (2012), http://dx.doi.org/10.1016/j.fsi.2012.06.011

2.7. General morphology of the thymus

The thymus of both 4-month and 1.5-year old groupers were sampled to investigate the morphological and histological structure of the thymus. Anatomical and histological techniques were used in the study. Hematoxylin and Eosin staining (H&E) was performed and the result was screened using a light microscope (Leica). An electron microscope (Phenom, FEI) was used to obtain electron micrographs of the thymus.

2.8. Gene analysis and protein structure assessment

cDNA sequences of the RAG1, IgM mu chain and β-actin gene were submitted to the National Center for Biotechnology Information (NCBI) in order to perform the multiple alignments (http://www.ncbi.nlm.nih.gov/). The deduced amino acid sequence was submitted to multiple alignments using ClustalW. A phylogenetic tree was constructed using the Neighbor-Joining Method with MEGA version 5 [28]. The models of both ZDD and NBR were constructed using Modeller [29] and visualized using Chimera [30]. Alignments of ZDD and NBR sequences of vertebrates were carried out using T-coffee [31]. A 3D model of the *E. akaara* IgM mu chain was predicted using SWISS-MODEL (http://swissmodel.expasy.org/) [32,33].

3. Results

3.1. cDNA sequence analysis of E. akaara RAG1

Blast analysis against nr database in NCBI GenBank of all the resultant sequences from the positive clones revealed that the open read frame (ORF) including 2778 bp was high similar to RAG1 of Tilapia, *Oreochromis niloticus* (GenBank no. XM003440495). The complete cDNA sequence of *E. akaara* RAG1 has been deposited in GenBank under accession no. HQ007253. The deduced RAG1 amino acid sequence was 925 amino acids and the deduced molecular

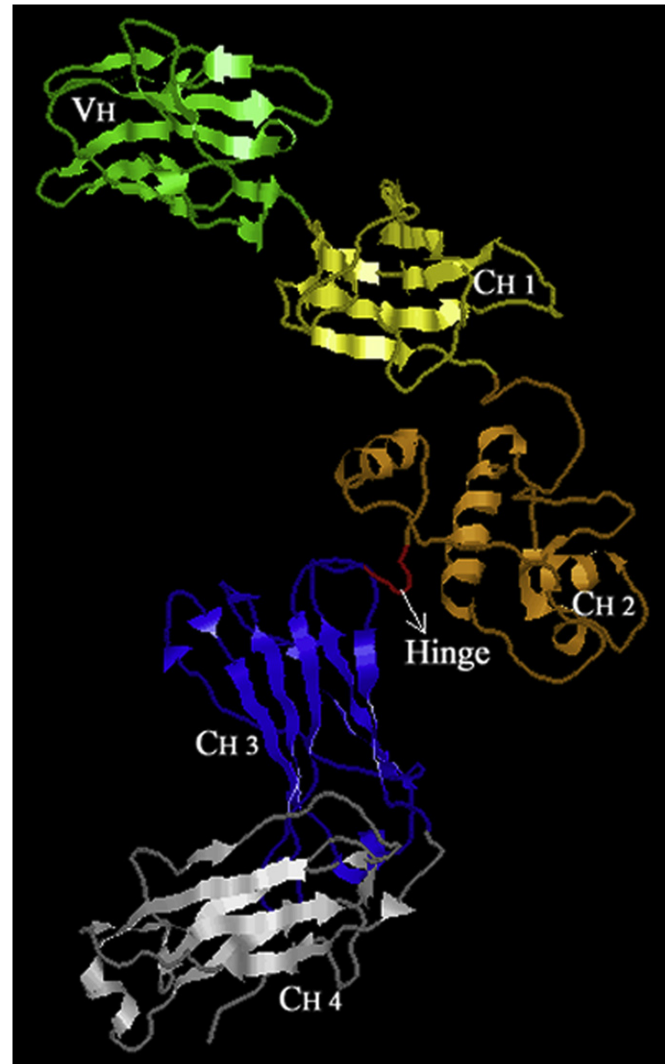

Fig. 5. A putative 3D model of the IgM mu chain. 'VH–CH1–CH2–Hinge–CH3–CH4' structures are shown in different colors. The model was predicted using SWISS-MODEL (http://swissmodel.expasy.org/).

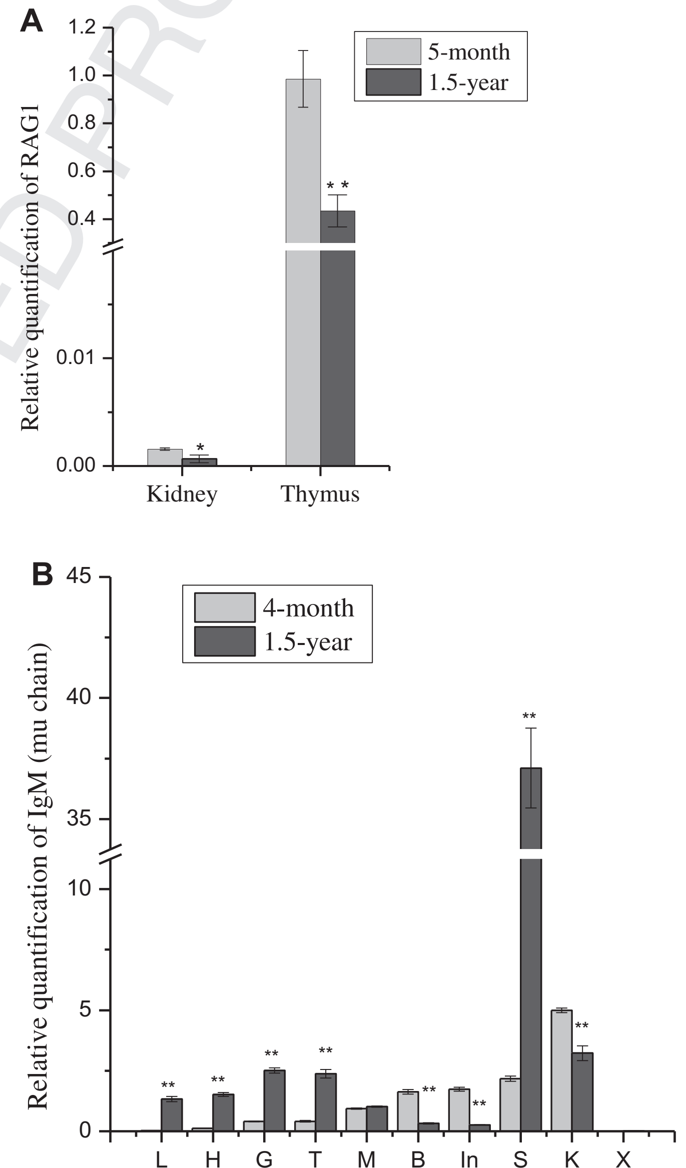

Fig. 6. Distribution of RAG1 (A) and IgM mu chain mRNA (B) in both 4-month and 1.5-year old *E. akaara* using real-time PCR. Tissues analyzed: muscle (M), kidney (K), heart (H), thymus (T), brain (B), intestine (I), gonad (X), spleen (S), liver (L) and gill (G). The β-actin gene was used as the endogenous control. Bars indicate mean ± S.E. ($n = 3$). The significant difference of gene expression between both 4-month and 1.5-year old groupers is indicated with asterisks (**: $P < 0.01$, *: $P < 0.5$).

Please cite this article in press as: Mao M-G, et al., Characterization of RAG1 and IgM (mu chain) marking development of the immune system in red-spotted grouper (*Epinephelus akaara*), Fish & Shellfish Immunology (2012), http://dx.doi.org/10.1016/j.fsi.2012.06.011

mass was approximately 105 kDa (Fig. 1). Multiple alignment of the deduced amino acid sequence indicated that *E. akaara* RAG1 shared high similarity in the functional region, which possesses a zinc Cys–Cys–Cys–His–Cys–Cys–Cys–Cys (C3HC4) RING finger, a Cys–Cys–His–His (C2H2) zinc finger A (ZFA) in the non-core region and an NBR, a zinc finger B (ZFB), the central and C-terminal domains in the core region (Fig. 2).

3.2. 3D structure of the ZDD and NBR

The predicted structure of RAG1 ZDD showed the expected zinc-binding subdomains, dimerization helices, a RING finger and zinc finger, with the four zinc ions bound to three distinct sites within the domain (Fig. 3A). A poor level of conservation was found in the 3D structure and the T-coffee alignment of the ZDD region (Fig. 3A, C), but the C3HC4 motif and C2H2 zinc finger structure were well consistent (Fig. 3C). The 3D structure for the NBR was a symmetrical homodimer, which interacted with two molecules of DNA, which were bound to the outside of the protein core in a near anti-parallel configuration offset (Fig. 3B).

3.3. cDNA sequence analysis of *E. akaara* IgM (mu chain)

The IgM mu chain was cloned using RACE-PCR with primers 3GSP3, 3GSP4, 5GSP3, 5GSP4 and the adaptor primers. The full-length cDNA sequence was 2259 bp, including 371 bp of 5′ untranslated region (UTR), 1737 bp of the mu chain ORF, 151 bp of 3′ UTR (excluding the poly(A) + tail). The complete sequence of the IgM mu chain has been deposited in GenBank under accession no. HQ007252. The deduced amino acid sequence is composed of 578 amino acids (Fig. 4) (GenBank no. AEK82140) and the deduced molecular mass is approximately 65 kDa.

Multiple alignment of the deduced amino acid sequence of mu chain with those of other known IgM family members indicated that the *E. akaara* IgM mu chain shared high similarity with other fish. The IgM mu chain is composed of a leader peptide (L), variable domain (VH), constant domain CH1, CH2, Hinge, CH3, CH4, and C-terminus, as shown in the 3D model (Fig. 5). Compared with the amino acid sequences of both the IgM secreted form (GenBank no. AAW66975) and the membrane-bound form (GenBank no. AAA56663) in rainbow trout, it was estimated that the *E. akaara*

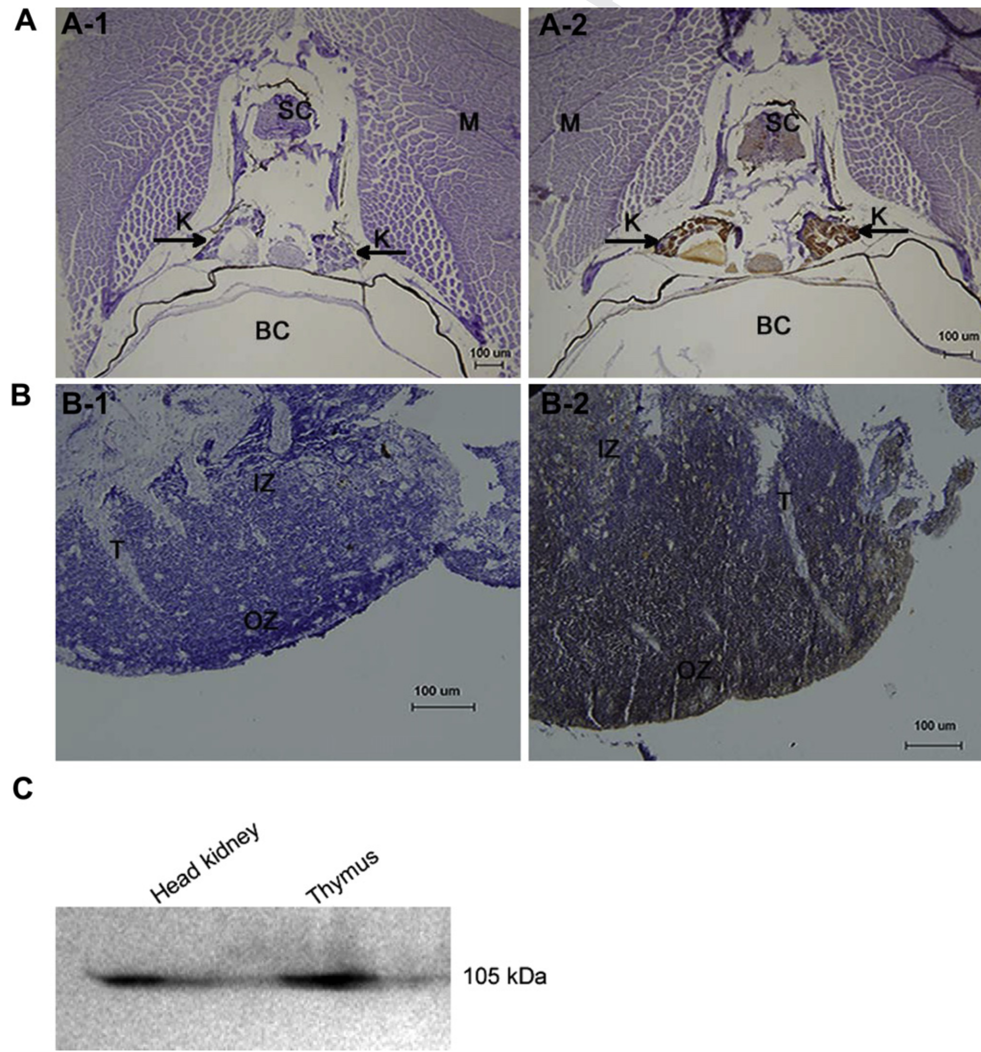

Fig. 7. Detection of RAG1 in the kidney and thymus of 4-month old grouper using immunohistochemistry and western blot. A. RAG1 detection in the kidney. Panoramic view of the kidney, exhibiting positive immunostaining for anti-RAG1 antibody in the kidney (K) (A-2) compared to control (A-1). M: muscle. SC: spinal cord. BC: body cavity. Bar = 100 μm. B. Immunohistochemistry for RAG1 detection in the thymus. Compared to the control (B-1), both the inner zone (IZ) and the outer zone (OZ) were stained (B-2). The trabecula (T) was not stained in both B-1 and B-2. Bar = 100 μm. The trabecula, IZ and OZ are identified according to Vigliano's description [35]. C. Western blot analysis of RAG1 expression in both kidney and thymus. Strong positive signals of both thymus and kidney were tested around 105 kDa.

Please cite this article in press as: Mao M-G, et al., Characterization of RAG1 and IgM (mu chain) marking development of the immune system in red-spotted grouper (*Epinephelus akaara*), Fish & Shellfish Immunology (2012), http://dx.doi.org/10.1016/j.fsi.2012.06.011

IgM (mu chain) was prone to be a secretory form (Supplementary data: Fig. 12).

3.4. Tissue distribution of RAG1 and IgM mu chain mRNA transcripts

The RAG1 mRNA transcript levels were investigated in 4-month and 1.5-year old groupers using real time PCR. Various *E. akaara* tissues including muscle, kidney, heart, thymus, brain, intestine, gonad, spleen, liver and gill were collected and tested. RAG1 mRNA was detected in the thymus and kidney, and especially in the thymus (Fig. 6A). RAG1 transcript level in both the thymus and kidney of the 1.5-year old grouper was significantly lower than that of the 4-month old grouper (Fig. 6A). The RAG1 protein in the thymus and head kidney was tested using western blot and IHC. The result showed that RAG1 protein was detected around 105 kDa in both the thymus and head kidney (Fig. 7C), and strong signals were detected in the kidney and thymus as shown in Fig. 7.

IgM mu chain mRNA was detected in most tissues except the gonad (Fig. 6B). In comparison with the 4-month old groupers, mu chain transcript levels were increased significantly in the liver, heart, gill, thymus and spleen of the 1.5-year old groupers ($P < 0.01$), but were lower in the brain and intestine (Fig. 6B).

3.5. RAG1 and IgM (mu chain) expression during development

The expression pattern of the RAG1 and IgM mu gene was investigated over the time course 0 (fertilized eggs), 1, 2, 4, 6, 9, 12, 15, 18, 23, 29, 37, 60 and 80 dph of *E. akaara* using real time PCR. β-actin was used as the endogenous control. RAG1 mRNA transcripts were first detected in the 15 dph group. Significant expression was observed in the 37, 60 and 80 dph groups (Fig. 8A). IgM mu chain mRNA transcripts were first detected in the 23 dph group, and the transcription level was increased obviously in the 29 dph group (Fig. 8B).

3.6. General morphology of the thymus

E. akaara has a pair of thymus glands, located on the superior dorsolateral corner of the gill cover close to the opercular cavity. An oval-shaped thymus was seen in 4-month old *E. akaara* (Fig. 9A). Unlike the young fish, the thymus of 1.5-year old fish (or fish over this age) was difficult to be distinguished from epithelial tissue (Supplementary data: Fig. 11). The cells observed under electron microscope are probably the thymocytes, because the thymus is essentially composed of thymocytes (lymphocytes) in the young fish [34,35]. The results showed that the thymocytes in the 4-month old *E. akaara* are more than those in the 1.5-year old but other cell types such as reticulo-epithelial cells, mucous cells and fibroblasts are less (Fig. 9B).

4. Discussion

In this study, both RAG1 and the IgM mu chain were chosen to further study development of the *E. akaara* immune system. Several aspects were addressed: the first involved the RAG1 and IgM mu chain gene in fish; and the second dealt with development of the *E. akaara* immune system. In addition, the problem of high mortality in *E. akaara* larvae was also addressed.

Alignment of *E. akaara* RAG1 sequences showed that, among vertebrates, the core region of *E. akaara* RAG1 showed high conservation, but the non-core region showed low conservation. Phylogenetic analysis confirmed that *E. akaara* RAG1 cDNA was grouped with teleost species (Supplementary data: Fig. 10). In mammals, the contribution of RAG1 to V(D)J recombination has

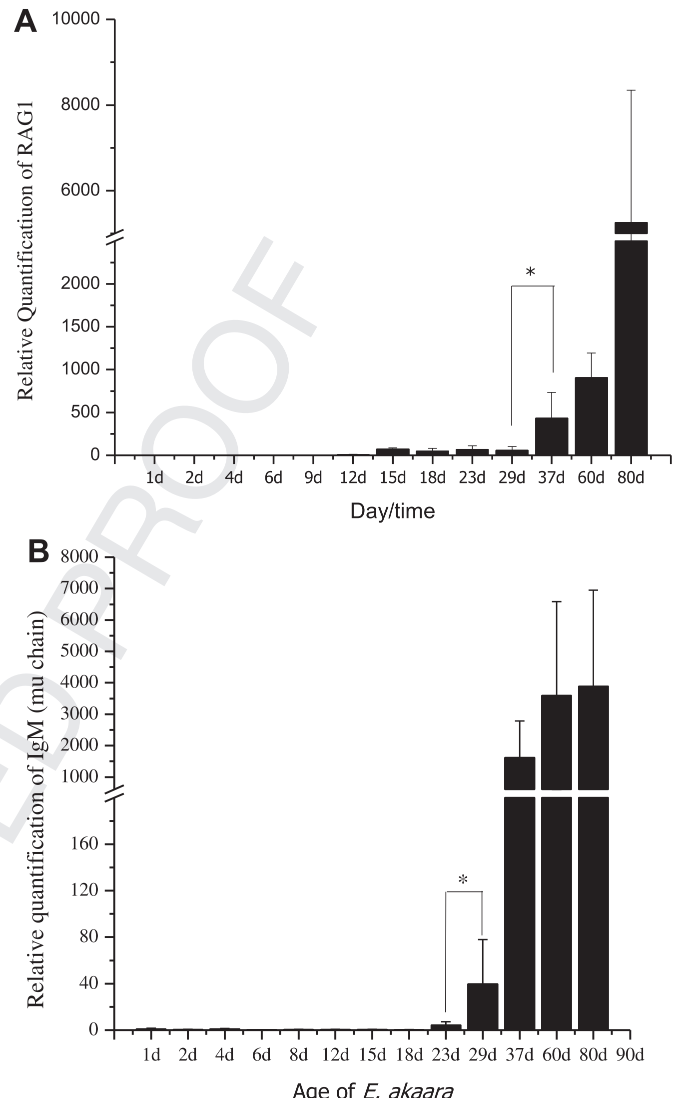

Fig. 8. Analysis of RAG1 (A) and IgM (mu chain) (B) expression during developmental stages using qPCR. Samples from eggs to 80 dph larvae were tested. β-actin gene was used as the endogenous control. Bars indicate mean ± S.E. ($n \geq 6$). A. The significant difference of RAG1 mRNA level between larvae of 29 dph and 37 dph is indicated with asterisks (*: $P < 0.05$). B. The significant difference of IgM (mu chain) mRNA level between larvae of 23 dph and 29 dph is indicated with asterisks (*: $P < 0.05$).

been well reported, however, the function and structure of RAG1 in teleost species has not yet been well studied. The majority of biochemical studies on RAG proteins have involved using fragments referred to as the core regions [36–38], which include residues 384–1008 in mouse and 271–896 in *E. akaara* (Fig. 2). Core RAG1 binds to the canonical RSS (12 or 23) with specificity for both the nonamer and heptamer conserved sequences [20,39]. The RSS nonamer is recognized by an NBR of core RAG1 residues 271–364 in *E. akaara* (Figs. 2 and 3B). The central domain residues 415–649 in *E. akaara* RAG1 could bind specifically to the RSS heptamer (Fig. 2) [40,41]. Other domains in the RAG1 core contribute to the DDE active site residues, a motif common to many recombinases and transposases, and interact with RSS elements proximal to the cleavage site and coding flank [41]. In addition, elements outside the cores are necessary for regulated protein expression and turnover. Non-core regions of the RAG1 protein include the zinc-binding C3HC4 RING finger motif and the associated C2H2 zinc finger with spanning amino acids 264–380 in mouse RAG1 and

UNCORRECTED PROOF

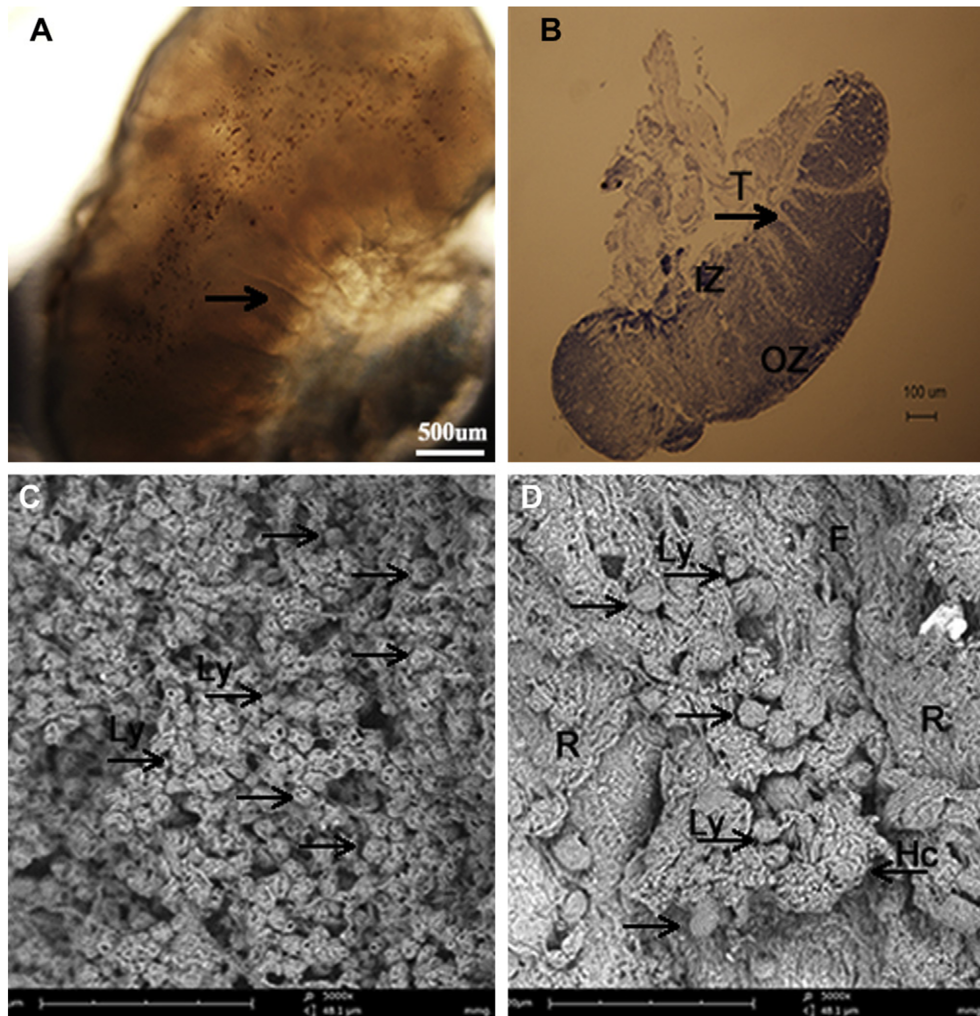

Fig. 9. Gross morphology and general organization of the thymus. A. General view of the removed thymus. Lobules are marked with arrows. Bar = 500 μm. B. General view of the thymus showing its capsule (C) and trabecule (T) limiting lobules, as well as the inner (IZ) and outer zones (OZ) according to turbot (*Psetta maxima* L.) [35]. Bar = 100 μm. C. Ultrastructure of the thymus of 4-month old *E. akaara*. Many lymphocytes (Ly) marked with arrows were seen under scanning electron microscopy. Bar = 20 μm. D. Ultrastructure of the thymus of 1.5-year old *E. akaara*. lymphocytes (Ly), Hassall's corpuscle (Hc), reticulo-epithelial cells (R) and fibroblasts (F) are shown. Bar = 20 μm. The lymphocytes and other cell type were identified according to the thymus of orange-spotted grouper [34].

142–170 in *E. akaara* (Figs. 2 and 3A) [39]. Their functions are involved in KPNA1 protein binding and a zinc-binding RING finger domain with ubiquitin ligase activity in the V(D)J recombination process [20,42]. In this study, we identified the non-core region by the C3HC4 RING finger motif and C2H2 zinc finger structure. It was easy to confirm the RAG1 core region because the core sequences were well conserved in vertebrate. Even though poor level of conservation was found in T-coffee alignment of ZDD region (Fig. 3C), the C3HC4 RING finger motif and the C2H2 zinc finger structure were well conserved. The coincident basic structure of conserved ZDD in the non-core region and the conserved core region of *E. akaara* RAG1 suggested that fishes share a similar mechanism of V(D)J function with mammals.

In teleost species, the RAG1 transcription was first examined in both the thymus and kidney of rainbow trout larvae [8], and also investigated in the same organs of zebrafish [9] and common carp [11]. In our study, when RAG1 mRNA was tested in both the head kidney and thymus, we confirmed the RAG1 protein in both using IHC and western blot, which will help us do further research on the protein levels. The results confirmed that the thymus and head kidney are the primary immune organs in teleost species, since the differentiation of early T cells and B cells occurs in both tissues. Interestingly, by comparison of the thymus between 4-month and 1.5-year old groupers, transcriptional levels of RAG1 (Fig. 6A) and the number of lymphocytes were significantly decreased according to the ultrastructure of the thymus (Fig. 9). The results indicated that like the mammal thymus, thymic atrophy also occurs in fishes.

Humoral adaptive immunity in fish is mediated by Ig, and the IgM class is the primary immunoglobulin in most teleost fish. The IgM class has been studied in many species such as salmon [21], zebrafish [22], Atlantic cod [23] and rainbow trout [24]. *E. akaara* IgM mu chain shared the basic structure of Ig with the other teleost species (Supplementary data: Fig. 13). IgM heavy chains are synthesized in two forms: the secretory form and the membrane form, the latter being integrated into the B-cell membrane. The gene elements of IgM mu chain include one leader exon (L_H), one variable exon (V_H), one diversity exon (D), one joining exon (J_H), four constant region exons (C_H 1–4) and two trans membrane exons [25]. In our study, it was found that the *E. akaara* IgM mu chain had four 'CH' but no trans membrane segments, so we concluded that the IgM (mu chain) which we cloned belonged to the secretory form. The deduced molecular mass of the mu chain was approximately 65 kDa, compared to 70 kDa in carp [26]. The distribution of IgM mu chain mRNA transcripts in most tissues of red-spotted

grouper as well as in orange-spotted grouper suggested that B cells, source of IgM, could be transported to most parts of the fish body with blood circulation. In addition, β-actin was first cloned in *E. akaara* in our experiment (GenBank accession no. HQ007251), including 1269 amino acids encoding 375 amino acids, which was employed as the endogenous control.

In this study, we focused on the expression pattern of RAG1 and IgM during the early developmental stages. The appearance of RAG1 or IgM mRNA is different from various fish species [7–12]. Because the maternal antibody exerts its function to protect fish larvae from environmental microorganisms [16], the earlier the immune related tissues are mature, the better will the larvae be protected (Supplementary data: Fig. 10). There are three 'crisis period' during grouper larval and juvenile stages [43]. The first 'crisis period' occurs during the early stage of larvae after hatching; The second occurs after disappearance of the yolk sac in the larvae; The third period usually occurs in juvenile stage by cannibalization. In *E. akaara*, the yolk sac disappears after 4 dph, and the phenomenon of cannibalization becomes serious after 20 dph during juvenile stage [44]. So the term between 4 and 20 dph is generally considered as the second "crisis period". Most organs including the immune-related tissues start to develop during this period. As we know that mass mortality in *E. akaara* juvenile occurred during this period and virus infection is one of the key reasons [2]. In this study, the immune system marked by RAG1 starts to develop around 15 dph in *E. akaara* and B cell marked by IgM mu starts to develop its function around 23 dph, which is in accordance with the point that IgM transcription will not work until RAG1 expression [20]. RAG1 started to develop its ability in immature B or T cells during 15 dph and 23 dph before endogenous Ig appearance. It was concluded that this interval was the start to develop the immune system. Generally, maternal immunity plays very important role before endogenous Ig appearance [45]. However, if maternal antibody ran out, this interval would be one "crisis period". This finding may explain the reason of mass mortality breakout during the larval stage from immunological aspect.

Disorder of V(D)J recombination leads to severe combined immunodeficiency (SCID) in mammals, characterized by a severe defect in both the T and B lymphocyte systems, and this results in the onset of one or more serious infections within the first few months of life (http://www.scid.net). RAG1 or RAG2 deficiency leads to complete blockage of B and T cell development [46], which is one of the key factors leading to SCID [47]. Interestingly, fish share both the T and B lymphocyte systems, but it is still unknown whether they suffer from SCID. Is mass mortality related to the consequence of disorders of V(D)J recombination? This conjecture needs to be further studied.

It is estimated that the mortality in *E. akaara* is usually up to 90% during larvae and juvenile stages [2], which causes farmers great losses every year. Many factors, including viral infection, a poor living environment, and bad diet may lead to the high mortality [43]. However, one point to be particularly mentioned is that the immune system of *E. akaara* has not been well understood yet. Our study provided much information concerning *E. akaara* immunology and will help us to extend our knowledge concerning the control of fish diseases. Further study on the maternal transfer of immunity is needed in order to help us understand the 'crisis period' more clearly in the future.

Acknowledgments

This work was supported by a Grant (201105027) from the Public Science and Technology Research Funds Projects of the Ocean, State Oceanic Administration of the People's Republic of China, Earmarked Fund for Modern Agro-industry Technology Research System (nycytx-50) and the Minjiang Scholar Program to K.-J. Wang (2009). Professor John Hodgkiss is thanked for his assistance with the English in this paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.fsi.2012.06.011.

References

[1] Huang W, Zhou L, Li Z, Gui JF. Expression pattern, cellular localization and promoter activity analysis of ovarian aromatase (Cyp19a1a) in protogynous hermaphrodite red-spotted grouper. Mol Cell Endocrinol 2009;307:224–36.
[2] Huang JN, Lin L, Weng SP, He JG. Molecular cloning and sequence analysis of complete coat protein gene of nervous necrosis virus from *Epinephelus akaara*. J Fish China 2005;29:429–32.
[3] Chantanachookhin C, Seikai T, Tanaka M. Comparative study of the ontogeny of the lymphoid organs in three species of marine fish. Aquaculture 1991;99: 143–55.
[4] Jósefsson S, Tatner MF. Histogenesis of the lymphoid organs in sea bream (*Spaurus aurata*, L.). Fish Shellfish Immunol 1993;3:35–49.
[5] Padrós F, Crespo S. Ontogeny of the lymphoid organs in the turbot *Scophthalmus maximus*: a light and electron microscope study. Aquaculture 1996; 144:1–16.
[6] Dos Santos NMS, Romano N, de Sousa M, Ellis AE, Rombout JHWM. Ontogeny of B and T cells in sea bass (*Dicentrarchus labrax*, L.). Fish Shellfish Immunol 2000;10:583–96.
[7] Corripio-Miyar Y, Bird S, Treasurer JW, Secombes CJ. RAG-1 and IgM genes, markers for early development of the immune system in the gadoid haddock, *Melanogrammus aeglefinus*, L. Fish Shellfish Immunol 2007;23:71–85.
[8] Hansen JD, Kaattari SL. The recombination activating gene 1 (RAG1) of rainbow trout (*Oncorhynchus mykiss*): cloning, expression, and phylogenetic analysis. Immunogenetics 1995;42:188–95.
[9] Willett CE, Cherry JJ, Steiner LA. Characterization and expression of the recombination activating genes (rag1 and rag2) of zebrafish. Immunogenetics 1997;45:394–404.
[10] Peixoto BR, Mikawa Y, Brenner S. Characterization of the recombinase activating gene-1 and 2 locus in the Japanese pufferfish, *Fugu rubripes*. Gene 2000;246:275–83.
[11] Huttenhuis HBT, Huisinga MO, Meulen T, Oosterhoud CN, Sánchez NA, Taverne-Thiele AJ, et al. Rag expression identifies B and T cell lymphopoietic tissues during the development of common carp (*Cyprinus carpio*). Dev Comp Immunol 2005;29:1033–47.
[12] Wang XL. Characterization and expression of Japanese flounder recombination activating gene (rag). Qingdao: Chinese Academy of Sciences; 2006.
[13] Zhang QY, Fan SG, Luo C. Sequence cloning and expression analysis of recombination active gene 1 and 2 in grass carp, *Ctenopharyngodon idellus*. Acta Hydrobiologica Sin 2009;33:795–803.
[14] Fan SG, Zhang QY, Luo C. Sequence cloning and expression analysis of RAG genes in goldfish. Acta Hydrobiologica Sin 2009;33:603–12.
[15] Swain P, Nayak SK. Role of maternally derived immunity in fish. Fish Shellfish Immunol 2009;27:89–99.
[16] Seppola M, Johnsen H, Mennen S, Myrnes B, Tveiten H. Maternal transfer and transcriptional onset of immune genes during ontogenesis in Atlantic cod. Fish Shellfish Immunol 2009;33:1205–11.
[17] Tonegawa S. Somatic generation of antibody diversity. Nature 1983;302: 575–81.
[18] Hesslein DGT, Schatz DG. Factors and forces controlling V(D)J recombination. Adv Immunol 2001;78:169–232.
[19] Yin FF, Bailey S, Innis CA, Ciubotaru M, Kamtekar S, Steitz TA, et al. Structure of the RAG1 nonamer binding domain with DNA reveals a dimer that mediates DNA synapsis. Nat Struct Mol Biol 2009;16:498–508.
[20] Jones JM, Simkus C. The roles of the RAG1 and RAG2 "non-core" regions in V(D)J recombination and lymphocyte development. Arch Immunol Ther Exp 2009;57:105–16.
[21] Hatten F, Fredriksen Å, Hordvik I, Endresen C. Presence of IgM in cutaneous mucus, but not in gut mucus of Atlantic salmon, *Salmo salar*. Serum IgM is rapidly degraded when added to gut mucus. Fish Shellfish Immunol 2001;11: 257–68.
[22] Danilova N, Hohman VS, Kim EH, Steiner LA. Immunoglobulin variableregion diversity in the zebrafish. Immunogenetics 2000;52:81–91.
[23] Schrøder MB, Flano E, Pilstrøm L, Jørgensen TØ. Localisation of Ig heavy chain mRNA positive cells in Atlantic cod (*Gadus morhua* L.) tissues; identified by in situ hybridisation. Fish Shellfish Immunol 1998;8:565–76.
[24] Lee MA, Bengtén E, Daggfeldt A, Rytting AS, Pilström L. Characterisation of rainbow trout cDNAs encoding a secreted and membrane-bound Ig heavy chain and the genomic intron upstream of the first constant exon. Mol Immunol 1993;30:641–8.

ARTICLE IN PRESS

[25] Hordvik I, De Vries Lindstrøm C, Voie AM, Lilybert A, Jacob J, Endresen C. Structure and organization of the immunoglobulin M heavy chain genes in Atlantic salmon, *Salmo salar*. Mol Immunol 1997;34:631–9.
[26] Vesely T, Reschova S, Pokorova D, Hulova J, Nevorankova Z. Production of monoclonal antibodies against immunoglobulin heavy chain in common carp (*Cyprinus carpio* L.). Vet Med-Czech 2006;51:296–302.
[27] Danilova N, Hohman VS, Sacher F, Ota T, Willett CE, Steiner LA. T cells and the thymus in developing zebrafish. Dev Comp Immunol 2004;28:755–67.
[28] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–25.
[29] Šali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. J Mol Biol 1993;234:779–815.
[30] Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF chimera – a visualization system for exploratory research and analysis. J Comput Chem 2004;25:1605–12.
[31] Notredame C, Higgins DG, Heringa J. T-coffee: a novel method for multiple sequence alignments. J Mol Biol 2000;302:205–17.
[32] Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 2006;22:195–201.
[33] Kiefer F, Arnold K, Künzli M, Bordoli L, Schwede T. The SWISS-MODEL repository and associated resources. Nucleic Acids Res 2009;37:D387–92.
[34] Wu JY, Lin HR. Scanning electron micrograph of the thymus of one year old orange-spotted groupers *Epinephelus coioides*. Acta Zoologica Sin 2008;54: 342–55.
[35] Vigliano FA, Losada AP, Castello M, Bermúdez R, Quiroga MI. Morphological and immunohistochemical characterisation of the thymus in juvenile turbot (*Psetta maxima* L.). Cell Tissue Res 2011;346:407–16.
[36] Sadofsky MJ, Hesse JE, McBlane JF, Gellert M. Expression and V(D)J recombination activity of mutated RAG-1 proteins. Nucl Acids Res 1993;21:5644–50.
[37] Silver DP, Spanopoulou E, Mulligan RC, Baltimore D. Dispensable sequence motifs in the RAG-1 and RAG-2 genes for plasmid V(D)J recombination. Proc Natl Acad Sci U.S.A. 1993;90:6100–4.
[38] De P, Rodgers KK. Putting the pieces together: identification and characterization of structural domains in the V(D)J recombination protein RAG1. Immunol Rev 2004;200:70–82.
[39] Bellon SF, Rodgers KK, Schatz DG, Coleman JE, Steitz TA. Crystal structure of the RAG1 dimerization domain reveals multiple zinc-binding motifs including a novel zinc binuclear cluster. Nat Struct Biol 1997;4:586–91.
[40] Landree MA, Kale SB, Roth DB. Functional organization of single and paired V(D)J cleavage complexes. Mol Cell Biol 2001;21:4256–64.
[41] Swanson PC. A RAG-1/RAG-2 tetramer supports 12/23-regulated synapsis, cleavage, and transposition of V(D)J recombination signals. Mol Cell Biol 2002; 22:7790–801.
[42] Jackson PK, Eldridge AG, Freed E, Furstenthal L, Hsu JY, Kaiser BK, et al. The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol 2000;10:429–39.
[43] He Y, Ou Y, Li J, Cai W. Advance in research on artificial breeding technique of groupers. South China Fisher Sci 2008;4:75–9.
[44] Wang H, Fang Q, Zheng L. Morphological development and growth of the larvae, juveniles and young fish of *Epinephelus akaara*. J Shanghai Fisher Univ 2001;10:307–12.
[45] Olsen YA, Press CM. Degradation kinetics of immunoglobulin in the egg, alevin and fry of Atlantic salmon, *Salmo salar* L., and the localisation of immunoglobulin in the egg. Fish Shellfish Immunol 1997;7:81–91.
[46] Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 1992;68: 869–77.
[47] Bosma MJ, Caroll AM. The SCID mouse mutant: definition, characterization, and potential uses. Annu Rev Immunol 1991;9:323–50.

UNCORRECTED PROOF

Please cite this article in press as: Mao M-G, et al., Characterization of RAG1 and IgM (mu chain) marking development of the immune system in red-spotted grouper (*Epinephelus akaara*), Fish & Shellfish Immunology (2012), http://dx.doi.org/10.1016/j.fsi.2012.06.011