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Expressed IgH μ and τ transcripts share diversity segment in ranched *Thunnus orientalis*

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ABSTRACT

It is now appreciated that in addition to the immunoglobulin (Ig)M and D isotypes fish also make the mucosal IgT. In this study we sequenced the full length of Ig τ as well as μ in the commercially important *Thunnus orientalis* (Pacific bluefin tuna), the first molecular analysis of these two Ig isotypes in a member of the order Perciformes. Tuna IgM and IgT are each composed of four constant (CH) domains. We cloned and sequenced 48 different variable (VH) domain gene rearrangements of tuna immunoglobulins and grouped the VH gene sequences to four VH gene segment families based on 70% nucleotide identity. Three VH gene families were used by both IgM and IgT but one group was only found to be used by IgM. Most interestingly, both μ and τ clones appear to use the same diversity (DH) segment, unlike what has been described in other species, although they have dedicated IgT and IgM joining (JH) gene segments. We complemented this repertoire study with phylogenetic and tissue expression analysis. In addition to supporting the development of humoral vaccines in this important aquaculture species, these data suggest that the DH–JH recombination rather than the VH–DH recombination may be instructive for IgT versus IgM/D bearing lymphocyte lineages in some fish.

1. Introduction

The immunoglobulin (Ig) superfamily-based adaptive immune system evolved in cartilaginous fish (sharks and skates) and is maintained in all jawed vertebrates (Flajnik and Rumfelt, 2000). One of the major characteristics of this adaptive immune system is the production of a repertoire of antibodies through somatic V(D)J recombination of the loci that encode them. While mammals possess five functionally distinct Ig isotypes (IgM, IgD, IgG, IgA and IgE), teleost fish have only three: IgM, IgD and IgT (Danilova et al., 2005a,b; Fillatreau et al., 2013; Hansen et al., 2005a; Wilson et al., 1997).

IgT was concomitantly discovered in trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*, where it was given the appellative IgZ) and IgT or forms of Ig with IgT domains have since been described in fugu (*Fugu rubripes*) (Savan et al., 2005b), carp (*Cyprinus carpio*) (Savan et al., 2005a), and stickleback (*Gasterosteus aculeatus*) (Gambon-Deza et al., 2010). IgT perhaps exists in most teleost groups, although it has yet to be found in catfish (Bengten et al., 2006; Salinas et al., 2011) and medaka (Magadan-Mompo et al., 2011). So far IgT is an isotype restricted to bony fish, and sequence characteristics (Hansen et al., 2005a), gut localization and functional work (Zhang et al., 2010) have suggested that it is a dedicated mucosal isotype (Zhang et al., 2011), functionally analogous but not orthologous with IgX/A of tetrapods (Mashoof et al., 2013). IgT was found to be expressed in gill of Chinese perch (*Siniperca chuatsi*) (Tian et al., 2009), IgT positive cells were identified in the epithelium of trout gill lamellae (Olsen et al., 2011), and clonal IgT responses were induced to trout viral pathogens (Castro et al., 2013), all further supporting the idea of this isotype filling a mucosal role in teleost humoral adaptive immunity. The IgT encoding DH–JH–CH elements are located 5′ of the μ and δ DH–JH–CH regions in the fish genomes in which it has been studied, with most or all VH genes 5′ to the τ block (Danilova et al., 2005a,b; Gambon-Deza et al., 2010; Savan et al., 2005b). Although class switch recombination has been described in shark (Zhu et al., 2012) and fish activation-induced cytidine deaminase (AID) is competent to induce somatic hypermutation and class switch in mammalian

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cells (Wakae et al., 2006), it does not appear that teleosts employ this for Ig heavy (H) chain isotype switching, instead they use deletional VH(DH)JH rearrangement to remove τ in IgM and IgD expressing cells and differential RNA splicing to control expression of IgM and IgD (Hikima et al., 2011), the τ/μ rearrangement appearing to have influence on lineage commitment similarly to the mechanism operating at the T cell receptor αδ locus.

We recently turned our attentions to the expressed IgH transcripts of the Pacific bluefin tuna (*Thunnus orientalis*). *Thunnus* species are the most valuable global aquaculture product (Ottolenghi, 2008), yet infections from several groups of parasites plague high intensity tuna mariculture ranches (Fromentin and Powers, 2005), impeding the industry from optimal relief of fishing pressures upon wild adult stocks. In addition to their economic importance, the extreme physiological specializations of these migratory apex predators made their Ig of interest to us. Tuna are among the fastest fish and have countercurrent heat exchangers that minimize convective heat loss to maintain a form of endothermy distinct from that of birds and mammals (Block et al., 2001; Jusup et al., 2011). Specifically, we were curious whether tuna Ig harbored any special adaptations evident in their primary amino acid sequence to this rare form of fish endothermy.

Here we report the first full-length μ and τ sequences from tuna. We have analyzed representative clones of the expressed variable domain repertoire of these isotypes, performed phylogenetic analysis of the IgH genes of this modern teleost, and analyzed their relative expression in tuna primary and secondary lymphoid tissues, including the mucosal gill. Our results demonstrate that these fish employ the same Ig VH gene families as other teleosts, can use the same VH genes in both IgM and IgT heavy chains, make diverse IgH complementarity determining region (CDR)3 regions, and surprisingly employ the same DH segment in both τ and μ rearrangements in what appears to be a previously undescribed mechanism of B cell isotype determination.

2. Methods

2.1. Animals and collection of tissues

Sample tissues of spleen, gill and kidney from ranched *T. orientalis* were collected during the regular slaughter process from two different commercial tuna facilities located off the coast of Ensenada, Baja California, Mexico. At the time of harvest, fish weight and fork length were 16.2 ± 6.5 kg and 96.3 ± 14.3 cm, respectively. Samples were placed in RNAlater (Qiagen, Valencia CA), frozen in liquid nitrogen, shipped to Texas A&M on dry ice and stored at −80 °C until further use.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was purified from spleen, gill and head kidney (pronephros, or anterior kidney) (35 mg from each tissue) using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. The quantity and quality of the RNA samples were assessed by NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) respectively. Message representation of RNA was assessed by PCR of common (β-actin) and less common transcripts (TNF-α, IL1-β), using previously published primer sets (Mladineo and Block, 2009). The GeneRacer kit (Life Technologies, Grand Island, NY) with GeneRacer oligo dT and gene specific primers was used to produce 5′ rapid amplification of cDNA ends (RACE) PCR products. Pools of 3′ RACE products were synthesized by Superscript III First-Strand Synthesis SuperMix kit (Life Technologies) using the oligo dT primer.

2.3. IgH RACE PCR, cloning, and sequencing

5′ and 3′ RACE products were amplified by standard PCR using various combinations of 5′ GeneRacer (as forward primer in 5′ RACE), Oligo dT (as reverse primer in 3′ RACE), and specifically designed primers for the conserved regions encoding the C domains of *T. orientalis* IgM and IgT (as forward or reverse for 3′ RACE or 5′ RACE, respectively). Primers are listed in Supplemental Table 1. The PCR conditions were as follows: one cycle of 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 50–53 °C for 30 s, 72 °C for 2 min, followed by one cycle of 72 °C for 7 min. The amplicons were purified from a 0.8% agarose gel after electrophoresis in tris/acetic acid/EDTA (TAE) buffer, cloned into pCR II vector with the TOPO TA cloning kit (Life Technologies), and transformed into chemically competent TOP10 *Escherichia coli* cells (Invitrogen). Colonies were picked based on blue/white screening produced by X-Gal (Sigma–Aldrich, Saint Louis, MO). The plasmid DNA was purified using Zyppy Plasmid Miniprep kit (Zymo Research Corporation, Irvine, CA) and was digested with *Eco*RI (Promega, Madison, WI) to identify clones with inserts. Products for sequencing were amplified using either M13 forward or reverse primers, purified using ABI BigDye × terminator purification kit (Life Technologies), and sequenced by the DNA Technologies Core lab of the Department of Veterinary Pathobiology at Texas A&M University.

2.4. Sequence analysis of μ and τ gene rearrangements in Pacific bluefin tuna

BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and visual inspection were used to identify the Ig isotype as well as VH, JH and CH sequences of tuna amplicons based upon homology to those from representative fish and other vertebrates. The amino acid sequences were blasted to discriminate the VH segments and CH domains. SignalP 4.1 was used to determine the leader peptides (Emanuelsson et al., 2007). Three prediction methods concurred upon the cleavage site of the representative μVH (after the 18th residue) and were in less agreement for τVH (after the 20th residue) as shown in Supplemental Figs. 1 and 2. Sequences were translated with Expasy translate tool (http://web.expasy.org/translate/), and the Clustal W program in Bioedit was employed to align amino acid sequences (http://www.mbio.ncsu.edu/bioedit/bioedit.html) for figures. Sequences were managed and assembled in Bioedit and have been deposited in Genbank under accession numbers KF713322–KF713372. CDR3 length was calculated using the "CDR3 length = exclusive number of amino acids from C (of VH segment Y × C) to F (of JH segment FG × G)" IMGT formula (Lefranc et al., 2003).

2.5. Phylogenetic studies

Amino acid alignments were made with ClustalW employing gap opening penalties of 10 and gap extension penalties of 0.1 for pairwise alignments, then 0.2 for multiple alignments using a Dayhoff matrix based method (Schwarz and Dayhoff, 1979). Phylogenetic trees were constructed using MEGA 5 software (Tamura et al., 2011). Neighbor joining trees using the substitution method of Jones et al. (1992) and pairwise deletion of empty positions were constructed from alignments of VH and CH domain sequences. Trees were bootstrapped 1000 times (Koichiro Tamura et al., 2011) and were viewed and adjusted using the Treeview Software (Page, 2002).

2.6. Real time quantitative PCR

Oligo-dT transcribed cDNA samples from spleen, gill and anterior kidney were assayed for levels of μ and τ message using β-ac-

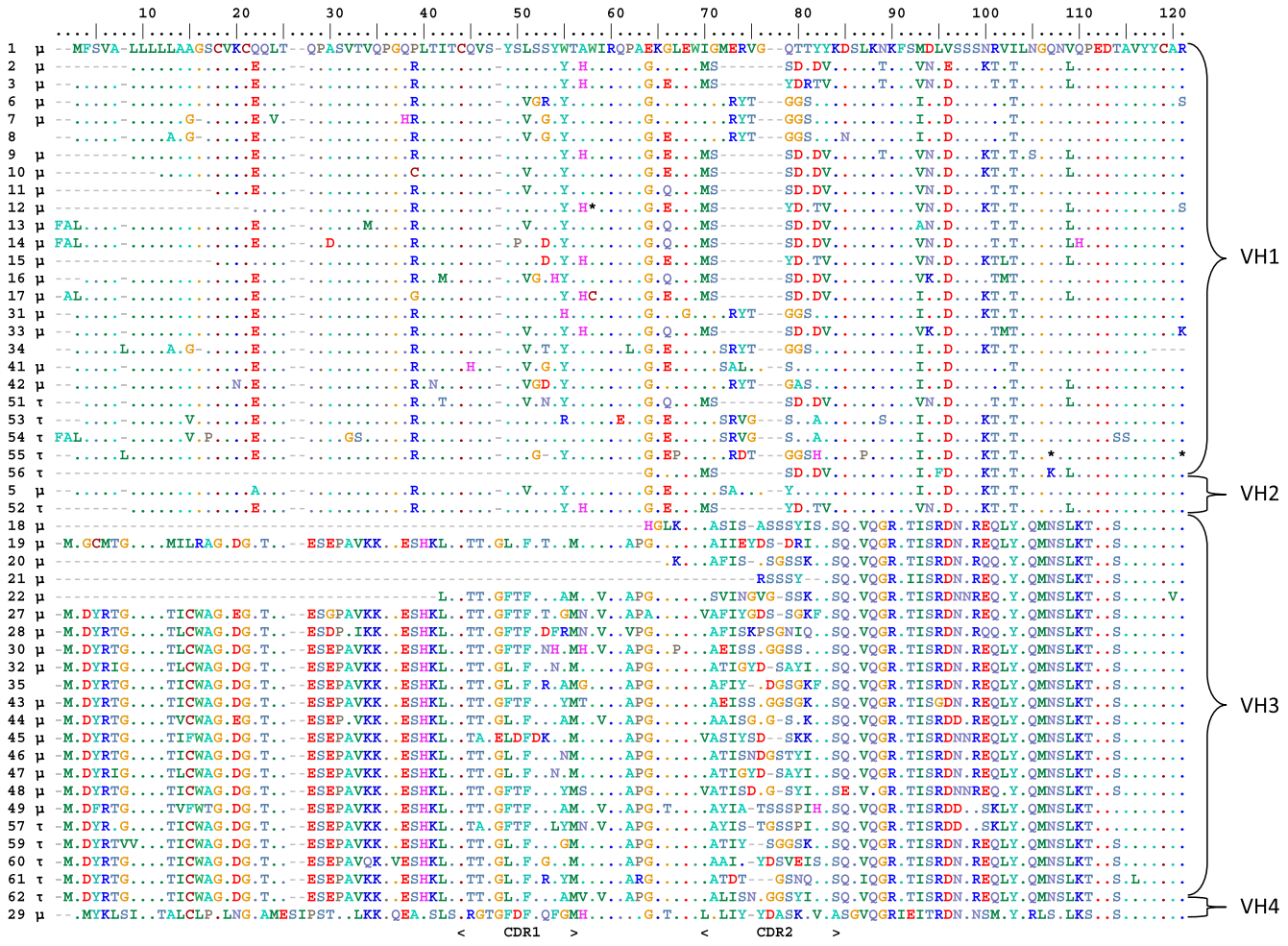

Fig. 1. Four VH families used by tuna IgH. Amino acid alignment of VH segment encoded sequences found within *T. orientalis* μ and τ cDNAs. Clone numbers are shown to the left and VH family designations are shown to the right. VH gene segment families were ascribed based upon 70% nucleotide identity in a pairwise matrix (Supplemental Fig. 2). Gaps introduced into the alignments are indicated by dashes ("–") and identity to the first sequence is indicated by a period (".") in the column. CDR1 and CDR2 are indicated below the alignment. If clone contained CH region encoding region, μ or τ is indicated at left of sequence after the clone name.

tin as a constitutively expressed control. Real-time PCR reactions were performed using 25 and 50 ng of cDNA with SYBR Advantage qPCR Premix (Clontech, Mountain View, CA) per the manufacturer's instructions. Primers were designed to span across introns. Using a Roche LightCycler 480 a three-step thermal cycling program was followed: 1 cycle at 95 °C for 5 min, then 45 cycles of 95 °C for 10 s, then 60 °C for 5 s, then 72 °C for 5 s. The Roche LightCycler software was utilized for raw data acquisition and calculation of Ct (threshold cycle) values. Changes in gene expression were estimated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with β-actin utilized as the stable reference gene for all experimental situations. The fold changes in gene expression were calculated with respect to the expression level of the genes in the anterior kidney (the primary B lymphopoietic tissue of bony fish).

3. Results

3.1. Characterization of μ cDNA of T. orientalis

The initial full length tuna μ was cloned and sequenced using a cDNA RACE library that was obtained from RNA pooled from several tuna anterior kidney, spleen and gill samples. The secretory tuna μ sequence shown in Supplemental Fig. 1 is an 1827 bp open reading frame which encodes a 609 amino acid protein containing a leader peptide of 18 residues, one Ig VH and four CH domains. The primary amino acid sequence showed two cysteine residues (and intervening tryptophan) conserved for intra-domain disulfide bond formation present in each of the Ig domains with the cysteines being spaced by approximately 70 residues in the VH domain and 60 in the CH domains. The amino-terminal cysteine in the CH1 domain forms an interdomain disulfide bond between the IgH chain to the IgL chain. The potential N-linked glycosylation site near the carboxyl terminus of the IgM chain was found at this position of the tuna IgM (Danilova et al., 2005a,b).

3.2. Characterization of tuna IgT

While sequencing 3′ RACE PCR products employing VH primers designed from μ clones we found other clones with Ig CH region amino acid sequences distinct from IgM, although they often shared a VH domain highly homologous with μ clones. The CH1 domain of these clones shares 56% amino acid identity with the CH1 of *S. chuatsi*. More primers allowed the complete cloning of the IgT encoding cDNAs, with CH3 proving to be even more definitively of the isotype (60% identical amino acids to *S. chuatsi*) (Supplemental Fig. 2).

The secretory tuna τ cDNA is composed of 1614 base pairs translating to 539 amino acids forming a leader peptide, VH domain and four CH domains. As in tuna IgM, two conserved cysteine residues and one tryptophan were identified in the VH and each CH

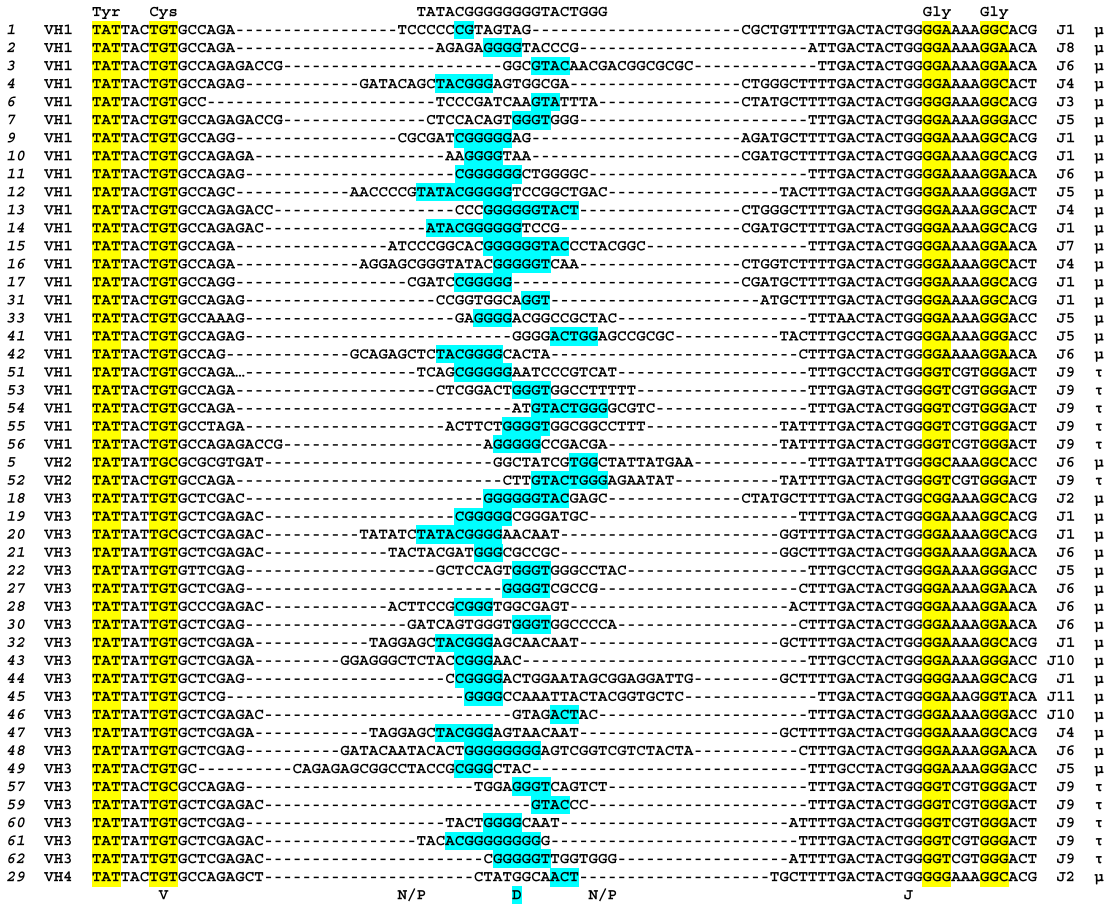

Fig. 2. Tuna VH(DH)JH junctional diversity. Nucleotide alignment arranged by VH family VH(DH)JH junctional region. Clone names and VH family are denoted on the left, JH gene and CH region is given to the right. Conserved tyrosine and cysteine codons of Y × C motif of VH segment as well as G × G glycines of JH gene are highlighted in yellow. Predicted DH segment is highlighted in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

domain which are important for folding of the β-sandwich Ig domains. There is also one conserved cysteine in the CH1 domain which forms a disulfide covalent linkage between the IgH chain to the IgL chain. The secretory tail of tuna IgT is composed of 12 amino acids.

3.3. IgHμ and τ VH, DH and JH segments

The same cDNA pools were used to examine tuna IgH μ and τ VH(DH)JH rearrangement diversity. In total 50 different sequences encoding VH domains (Fig. 1) that possessed full or partial unique VH regions were cloned, 36 spliced to μ CH regions and 11 with τ (three contained complete VH regions but were incompletely rearranged or did not splice to a CH). Based on percent identity the VH segment sequences were divided into four separate families of IgH V genes. Members of each family were more than 70% identical in their nucleotide sequences (Brodeur and Riblet, 1984; Pascual and Capra, 1991) (Supplemental Fig. 3).Analysis of the carboxy-terminal portion of the VH domains gave insight into the DH and JH gene segments used to rearrange mature VH exons. We predicted 11 different JH segments used in these clones and one DH segment (TATACGGGGGGGGTACTGGG) could be identified in the 48 unique CDR3 encoding rearrangements analyzed (Fig. 2). The one DH segment apparently was employed by both isotopes, as various stretches of the sequence (including portions at each end) are found in both μ and τ clones. The predicted DH germline nucleotide contribution to the final expressed CDR3 encoding sequence ranges from 3 to 10 with a mean of 5.5 base pairs. All three reading frame of the D segment were used (Fig. 3). The τ clones all used a dedicated JH segment (J9).

IgH CDR3 is the crucial loop in the paratope of most antibody antigen interactions. This sample of the tuna Ig heavy chains expressed at the mRNA level allowed an initial analysis of the length of IgH CDR3 of μ and τ. Table 1 shows that tuna μ clones display a broader range of CDR3 lengths (from 9 to 18aa) as well as an average of one amino acid longer CDR3 length than those found in tuna τ.

3.4. IgM and IgT CH regions of tuna

The tuna IgM CH region amino acid sequence showed the most identity to the mandarin fish (*S. chuatsi*, also known as the Chinese perch and also a member of the Order Perciformes) with 53.6% and then to the rainbow trout with 39.8% identity and presented the least with chicken (23.8% identity) amongst the sequences we included in our analysis (Fig. 4). The tuna IgT CH region has the highest identity also to that of the mandarin fish with 52.5% and the least to grass carp with 20.4% (Fig. 5). Unlike the cyprinid grass carp and zebrafish, the CH3 domain of tuna IgT conforms to the canonical immunoglobulin domain fold with cysteines and tryptophans in positions common for β-sandwich tertiary structure.

3.5. Phylogenetic analysis

To assess the phylogenetic relationship of the tuna Ig VH gene segments with those of other teleosts we created dendrograms

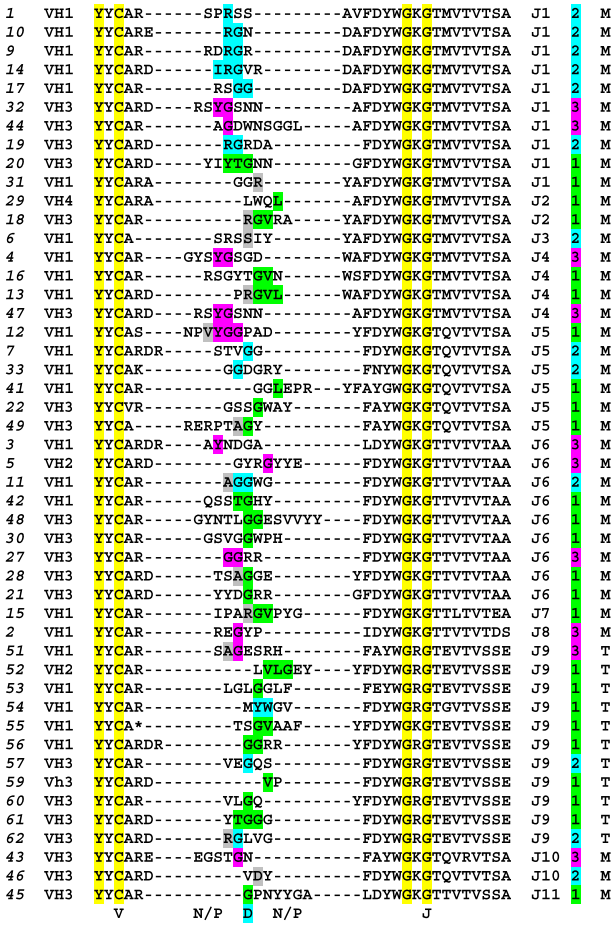

Fig. 3. Translated complementarity determining region (CDR) 3 repertoire sampling of tuna IgH. Amino acid alignment arranged by JH gene of the VH(DH)JH junctional region. Clone names and VH family are denoted on the left, JH gene, reading frame of DH used and CH region is given to the right. Conserved tyrosine and cysteine of Y × C motif of VH segment as well as G × G glycines of JH gene are highlighted in yellow. Predicted DH segment is highlighted in green, blue or magenta depending on the use of reading frame one, two or three, respectively, in panel B. Amino acids were assigned to VH, DH or JH based on at least two bases of codon matching consensus, grey highlighting indicates a residue partially encoded by D consensus that does not encode consensus amino acid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with their pairwise genetic distances (Fig. 6). The four tuna IgH V gene families interleaved amongst those VH segment sequences used by the other fish, indicating that they are using members of the same ancient VH families that have been conserved by trans-species maintenance since at least the common ancestor of these divergent fish. However tuna families VH1 and VH2 appear to have arisen from a more recent duplication in the order Perciformes.

We also explored the relationship of these new tuna IgH C regions to those of other fish and other vertebrates (Fig. 7). As expected, the tuna IgM grouped with that isotype from other fish, most closely the Perciforme trumpeter fish (*Latris lineata*). Within the IgM, teleosts group together as a sister group to all of the other vertebrates, including the shark which shares a more ancient common ancestor and would be expected to branch outside of teleosts and tetrapods. However this incongruence with the organisms' natural history is not unusual for phylogenetic analyses of teleost antigen receptors, unless balancing numbers of operational taxonomic units fill the other vertebrate classes (Criscitiello and Flajnik, 2007). IgT of tuna clusters with that isotype from other representative fish.

Table 1
CDR3 lengths in amino acids.

	IgM	IgT
Maximum	18	13
Minimum	9	8
Range	9	5
Median	12	11
Mean	12.49	11.00
Variance	3.31	2.00

3.6. IgH μ and τ relative tissue expression

Quantitative real-time PCR (Supplemental Fig. 4) was used to assess the expression of these isotypes at the mRNA level in secondary lymphoid tissues relative to the anterior kidney (the chief primary lymphoid tissue of fish (Fillatreau et al., 2013; Lam et al., 2004; Trede et al., 2004)). Relative levels of μ were higher than τ in both spleen and gill, but μ did not predominate τ to as great an extent in gill as it did in spleen. The averaged ratio of HCμ to HCτ in tuna spleen was 7.35 compared to 2.89 in the gill.

4. Discussion

4.1. Repertoire

The 20–30% sequence disparity between some VH family members in tuna suggests either ample somatic hypermutation for affinity maturation of these fish antibodies or an older divergence date of VH family members than has been seen in some other teleosts such as stickleback (Gambon-Deza et al., 2010). Families VH1 and VH2 share between 52% and 64% nucleotide identity (Supplemental Fig. 3) and appear recently diverged (Fig. 6), perhaps within a Perciformes branch including tuna.

Despite the initial report that found shorter CDR3 in trout IgM than IgT (Hansen et al., 2005a) we found a small skewing towards shorter IgT CDR3 (Table 1). We predict that this may be an effect of a different immunogenetic rearrangement mechanism involving a single shared DH gene segment that governs τ versus μ/δ in a clade including tuna and other fish (more below). IgH CDR3 often dominates antigen recognition properties of the six CDRs comprising the F_{ab} paratope (Davis, 2004; Xu and Davis, 2000). The three reading frames usually supplied by DH gene segments therefore contribute significantly to the eventual translated repertoire of antigenic specificities. Additionally, extended length of IgH CDR3 has been crucial in many clinically important antibodies against viral scourges (Kwong and Wilson, 2009; McLellan et al., 2011), and the loop has evolved into an entirely new domain in some antibodies of cattle (Wang et al., 2013). Thus, restricting the entire repertoire to rearrangements based on a single DH would be expected to place constraints on antigen recognition.

As Perciformes, tuna belong to the largest order of vertebrates that accounts for approximately 40% of all bony fishes. As *T. orientalis* is the first Perciformes to have either their IgM and T repertoire or IgH locus analyzed immunogenetically, there may be a great many fish that employ this system for Ig isotype control and B lineage commitment. As successful as the Perciformes have been in radiating to occupy most fresh and saltwater niches on Earth, the potential restriction in CDR3 length variability must not have too great a toll on the fitness of these fish.

4.2. Genomic organization

The generalized translocon configuration of the teleost IgH locus with a set of VH genes and downstream μ and δ CH regions has been confirmed in many studies (Bengten et al., 2002; Jørgen-

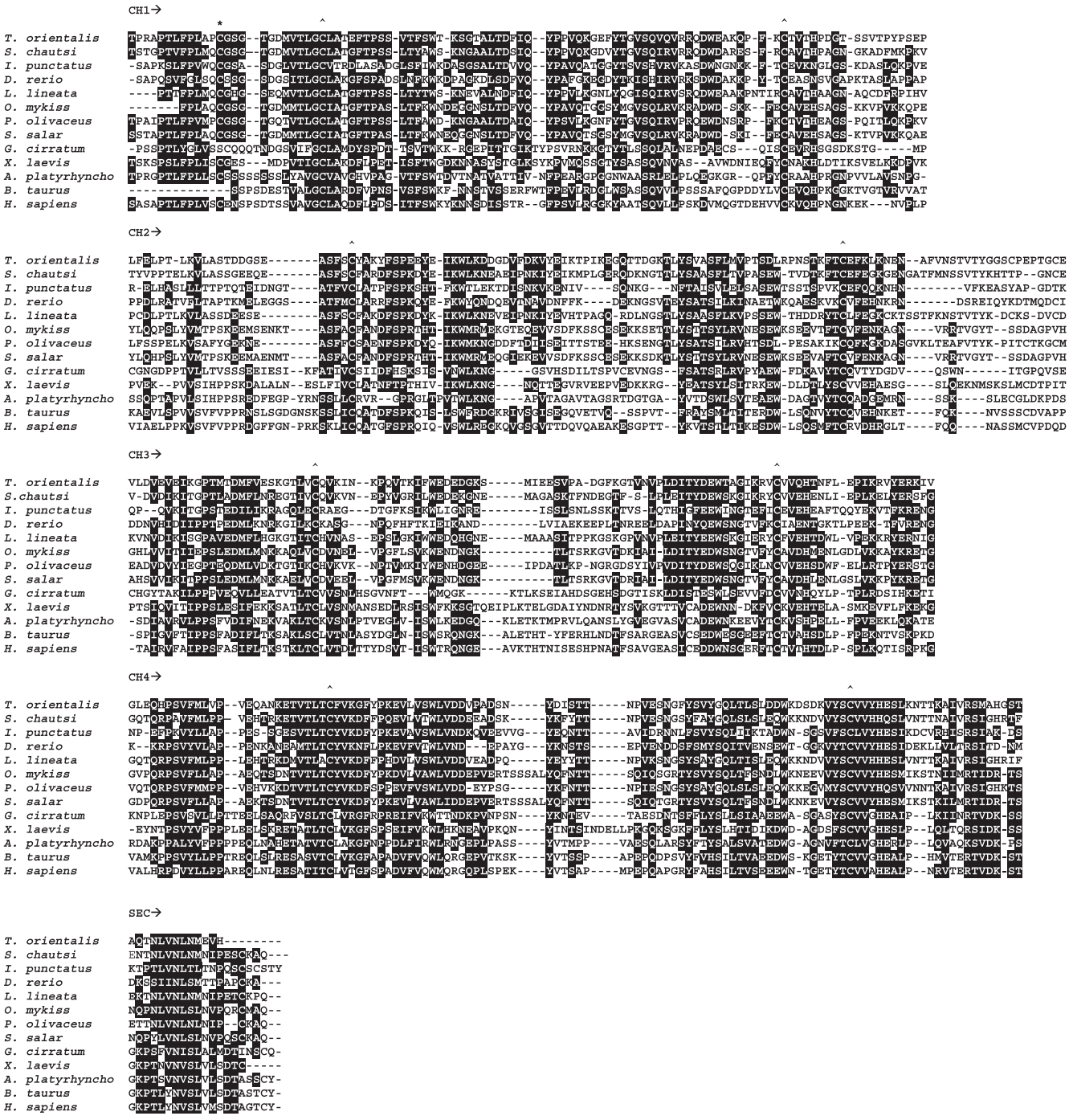

Fig. 4. Amino acid sequence alignment of the heavy chain of IgM in different vertebrate species. The conserved (identical and similar) residues are highlighted in black. Arrows indicate CH1–CH4 and the secretory tail. An asterisk (*) is above the conserved cysteine that forms a disulfide bond with the light chain, a carrot (^) is above conserved cysteines that form intra-domain disulfide bonds. Gaps are indicated by dashes. Genbank accession numbers are: AAQ14846.1 *Siniperca chuatsi* (Chinese perch), A45804 *Ictalurus punctatus* (channel catfish), AF281480_1 *Danio rerio* (zebrafish), ADC45388.1 *Latris lineata* (striped trumpeter), AAW66973.1 *Oncorhynchus mykiss* (rainbow trout), AF226284_1 *Paralichthys olivaceus* (flounder), AAB24064.1 *Salmo salar* (salmon), AAU04507.1 *Ginglymostoma cirratum* (nurse shark), AAA49774.1 *Xenopus laevis* (African clawed frog), CAC43280.1 *Anas platyrhyncho* (duck), AAN60017.1 *Bos taurus* (cattle), and AAS01769.1 *Homo sapiens* (human).

sen, 2000; Samuel Aparicio et al., 2002), but many deviations on the theme are present as catfish and medaka appear to lack τ and many fish have duplications of blocks of the locus (Fillatreau et al., 2013). Although reported in shark (Zhu et al., 2012), class switch recombination (CSR) has not been described in a teleost. However, one study showed that teleost AID could induce CSR in mouse (Barreto et al., 2005).

The IgH τ gene together with its dedicated DH–JH gene segments are located between the VH gene segment block and the (DH–JH–CH) μ cluster in zebrafish, fugu (*Takifugu rubripes*) and three-spined stickleback (*G. aculeatus*) (Danilova et al., 2005a,b, 2011; Gambon-Deza et al., 2010; Hansen et al., 2005a), or it is inserted within the VH gene segment array as in rainbow trout (Hansen et al., 2005b). Thus, in these fish the RAG mediated joining of a VH gene segment to either DH of τ or DH of μ/δ will determine whether the developing pro-B lymphocyte (using mammalian convention) becomes an IgT or IgM/D producer. Experiments in zebrafish (Schorpp et al., 2006) and trout (Zhang et al., 2010) have

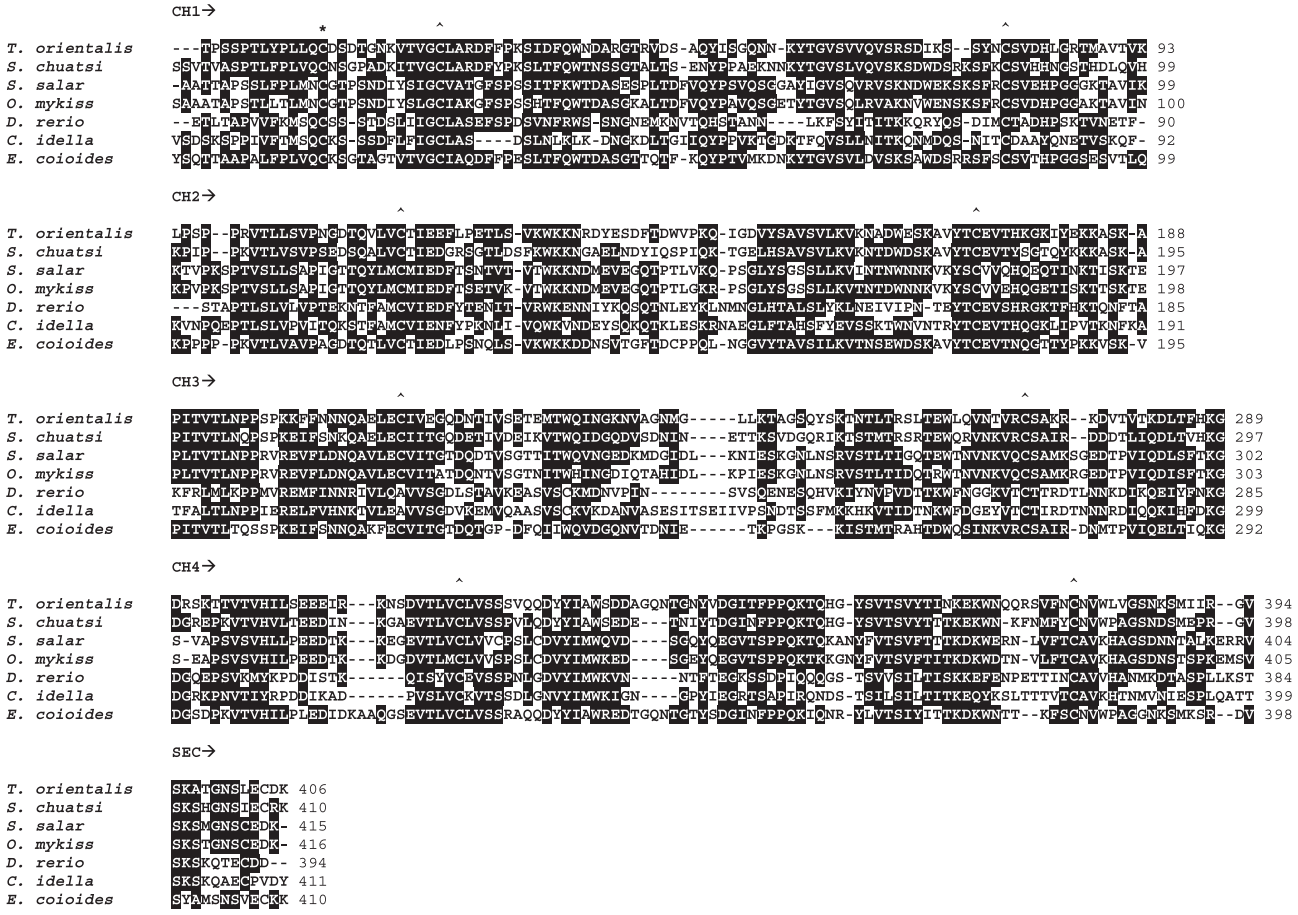

Fig. 5. Amino acid sequence alignment of the heavy chain of IgT CH domain. The conserved (identical and similar) residues are marked in black. Arrows indicate CH1–CH4 and the secretory tail. An asterisk (*) is above the conserved cysteine that forms a disulfide bond with the light chain, a carrot (^) is above conserved cysteine of intra-domain bond. Gaps are indicated by dashes. Genbank accession numbers are: ACZ54909.1 *Epinephelus coioides* (grouper), ABF19723.1 *Ctenopharyngodon idella* (grass carp), AAY42141.1 *S. chuatsi*, ACX50291. *S. salar*, AAW66981.1 *O. mykiss*, and CAI20890.1 *D. rerio*.

demonstrated heavy chain isotype exclusion at the cellular level in fish.

The repertoire data presented here suggest that something different may be occurring in tuna, however (Fig. 8). Like in other fish, VH genes appear to be shared between both τ and μ/δ. Three of the four families we found expressed in these fish clearly were used in both μ and τ, although a fourth was only found with μ. This could easily be a case of low sampling depth as VH4 appeared as a singular use in the described clones. Since this is a more parsimonious explanation than a dedicated μ VH rearranging to a shared DH segment that rearranges to dedicated JH segments, Fig. 8 depicts an array of VH gene segments that can be used in either primary transcript type.

However, unlike in other fish, both τ and μ rearrangements of tuna appear to employ the same DH gene segment. As all the tuna JH genes appear with only μ or τ (none seem to be shared), this points to an arrangement where a single shared DH can rearrange with JH segments upstream of either μ or τ to determine isotypic fate of the cell, and this DH's rearrangement to several shared VH's is not the event that stochastically determines isotype. So at least two possibilities of IgT vs. IgM/D lineage fate are now supported by data, one in which τ and μ/δ share VH genes from one block (as in zebrafish) or more than one array 5′ and 3′ to the τ elements (as in trout) but DH–JH are dedicated to isotype, and now the tuna paradigm where VH–DH are shared and JH is dedicated to isotype. In one instance (tuna) the DH–JH join would instruct lineage and in the other the VH-DH join would. Importantly, we note that genomic sequencing of the locus has not yet confirmed this organization in the tuna or the absence of additional DH that we did not sample. Interestingly, this hypothesized organization could also explain why in trout a significant difference was seen in CDR3 length and repertoire between τ and μ clones (each using dedicated DH and JH gene segments, (Castro et al., 2013)) while we do not see a great difference in tuna (sharing VH and DH and only having dedicated JH, Table 1 and Fig. 3). Future work must determine if this is truly stochastic in lymphocyte development or if there are more complex control mechanisms instructing this important juncture determining the B cell's fate.

4.3. CH regions

IgM is the most conserved isotype in jawed vertebrates and was thought to be omnipresent until the discovery of its absence in coelacanth (Amemiya et al., 2013). The tuna IgM CH region seems very consistent with its orthologs in other fish.

As also noted in other IgT sequences (Hansen et al., 2005a), there are many prolines in the region of the tuna IgT CH1/CH2 juncture which may be indicative of hinge-like flexibility. Tuna IgT CH3 seems to conform to the classical Ig superfamily β-sandwich with canonical cysteines and tryptophan positions seen in the domain of the salmonids and grouper that are important in the folding of this domain (Fig. 5) (Lesk and Chothia, 1982). The tryptophan to cysteine replacement seen in zebrafish and grass carp appears to be a cyprinid characteristic, and has been sug-

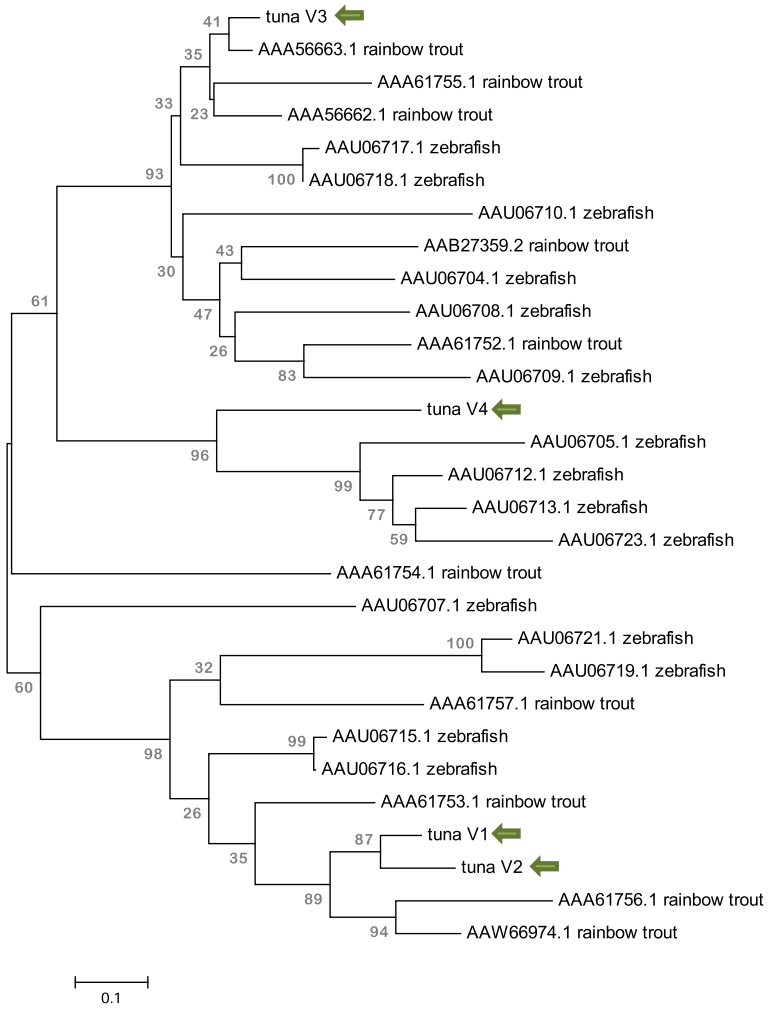

Fig. 6. Tuna IgH V genes shared with other fish. Phylogenetic analysis of representatives of the four tuna IgH V families (clone 3 for VH1, 5 for VH2, 57 for VH3 and 29 for VH4) with VH genes from two of the better studied teleost models, rainbow trout and zebrafish. Trout and zebrafish accession numbers are labeled at each branch terminus.

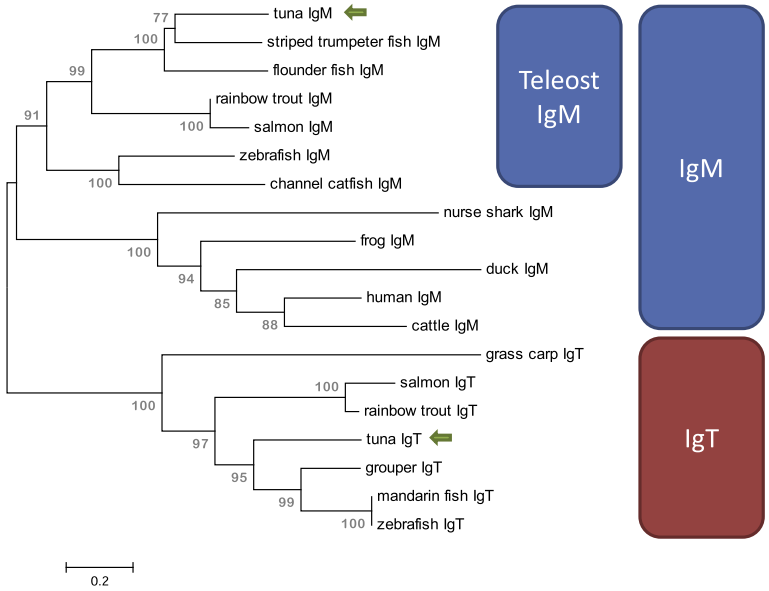

Fig. 7. Tuna IgM and IgT CH regions group with those isotypes of other teleosts. Neighbor joining phylogeny using Dayhoff matrix and 1000 bootstrap replications. Alignment and accession numbers used in tree are shown in Figs. 5 and 6.

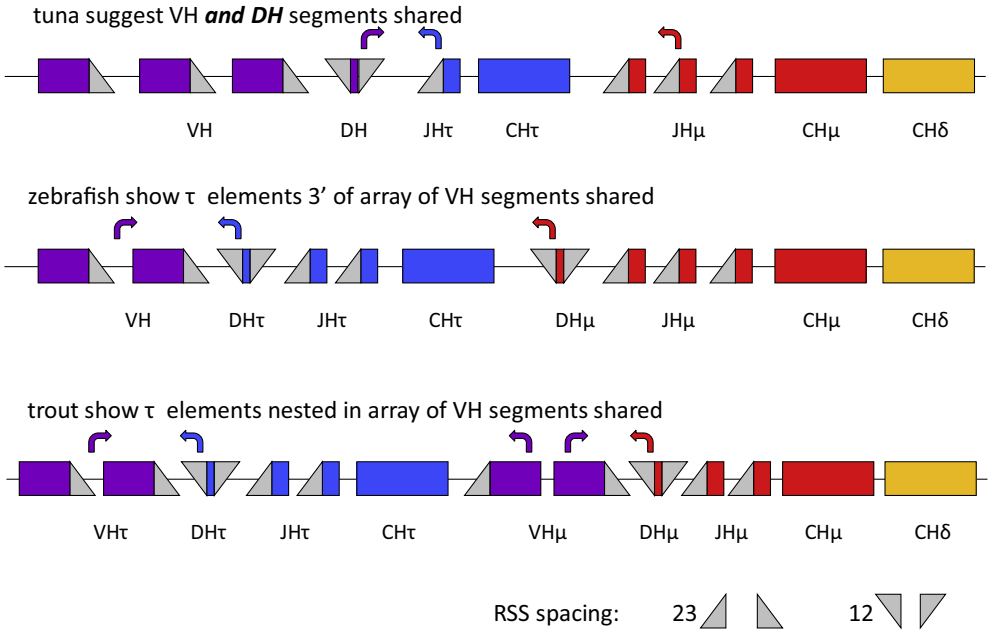

Fig. 8. Hypothetical organization of elements in the tuna IgH locus suggests a novel method of lineage determination at the fish IgH locus. Simplified cartoon showing three paradigms in the locus organization and immunogenetic control of IgH τ vs μ/δ rearrangement.

gested by modeling to still allow an immunoglobulin superfamily domain fold (Danilova and Amemiya, 2009).

In this limited sampling, we found no evidence of the IgT hybrid molecule with two CH domains identified in the common carp *Cyprinus carpio* (Savan et al., 2005a), the IgM/D hybrids (with or without VH domains) found in catfish (Edholm et al., 2010), the IgMCH1/IgTCH4 variant IgT2 in carp (Ryo et al., 2010), nor the run-on transcription secreted IgD form of trout (Ramirez-Gomez et al., 2012).

In mammalian IgM a carboxyl terminal glycosylation site in the secretory tail is important in J chain polymerization (Hohman et al., 2003; Tacchi et al., 2013), but may have distinct physiology in teleost such as catfish and zebrafish that have it (Wiersma et al., 1997). This conserved N-linked glycosylation site is part of a larger sequence motif enabling polymerization of IgM and IgA of mammals but is not present in the secretory tail of tuna IgM or IgT, although there is a conserved cysteine in IgT shared with other teleosts. Trout IgT was found as a monomer in serum but a multimer in mucus (Zhang et al., 2010), however these IgT multimers did not appear to be covalently linked as they are known to be for trout IgM (Kaattari et al., 1998). More biochemical studies are necessary to resolve the stoichiometry and functional avidity of IgT.

4.4. Expression

Isotype expression studies in tuna echo what has been determined in other fish species: IgT and IgM both are present in primary and secondary lymphoid tissues, yet more IgM than IgT, however the gap closes at mucosal sites (Hansen et al., 2005a; Ryo et al., 2010; Savan et al., 2005b; Xiao et al., 2010). IgT1 in adult zebrafish deviated from this pattern in being primarily in the head-kidney and thymus (Hu et al., 2010). The molecular data presented here could serve as a springboard for revisiting immunoglobulin studies in tuna at the protein level that were initiated in the southern bluefin (*Thunnus maccoyii*) (Watts et al., 2001). The work also opens gates to explorations of B lineage development and commitment, where molecular markers might could be adapted from fish species such as zebrafish (Zimmerman et al., 2011) and trout (Barr et al., 2011; Macmurray et al., 2013) where more work has been performed.

5. Conclusions

Endothermic birds and mammals employ immunoglobulin isotypes IgM, IgY, IgE and IgG in systemic immunity but have specialized IgA for mucosal immunity. Poikilothermic vertebrates lack IgA, although amphibians do have an orthologous mucosal isotype in their IgX. IgM had long been the primary functional immunoglobulin isotype recognized in teleost until the recent discovery of the mucosal specialization of IgT. Mucosal epithelia is the barrier breached or exploited by most internal pathogens of vertebrates, and also ectoparasites of fish (Xu et al., 2013). This penetration of mucosal defense is also true of many pathogens of concern in the tuna ranching industry, including sea lice (Hayward et al., 2009), betanodaviruses (Gomez et al., 2010) and gill platyhelminths (Colquitt et al., 2001). It is hoped that this basic molecular characterization of humoral immunity in these economically important endothermic fish will enable more studies of host–pathogen interactions and the feasibility of vaccine development for offshore ranches. Increasing the productivity of these operations by reducing infectious disease mortality will reduce pressures on wild tuna stocks and the fish species used to feed ranched tuna.

Moreover, the apparent shift of isotype determination from VH–DH recombination to DH–JH recombination at the tuna IgH locus is interesting from a fundamental standpoint of lymphocyte antigen receptor immunogenetics, and begs many questions that must be verified and queried with new algorithms (Olivieri et al., 2013) at the levels of the tuna genome, the immunoglobulin proteins, tuna B cells, development in the pronephros, and the fish's response to pathogen. If the single tuna DH gene is verified at the genome, it will be interesting to know whether this IgH locus orientation is found only within this clade of endothermic fish or a broader set of Perciformes. These studies should provide insight into the natural history and fundamental physiology of antibodies while providing much needed tools for managing the health of ranched, and thereby wild, tuna stocks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2013.10.015.

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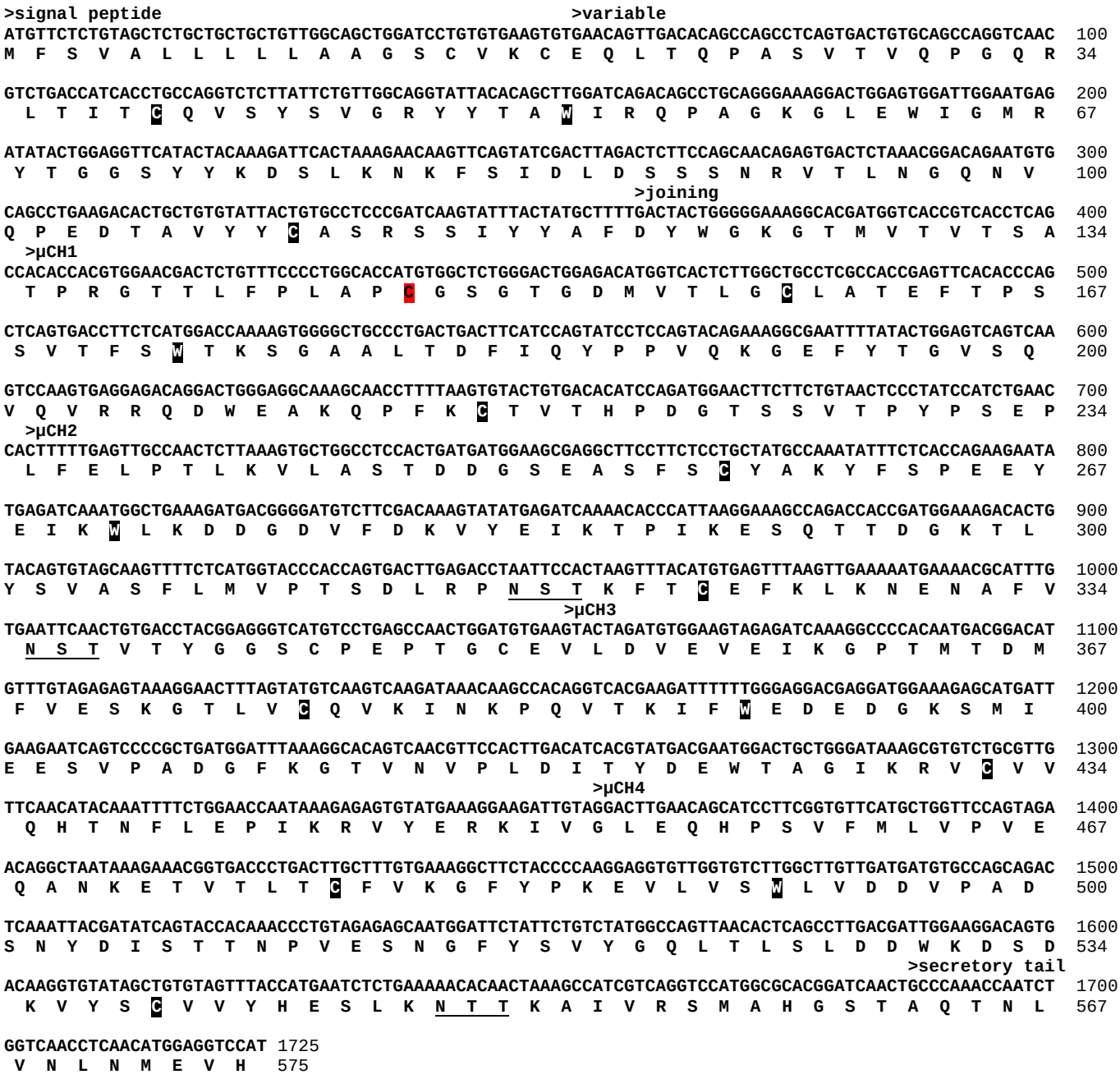
Supplemental Table 1. Primers.

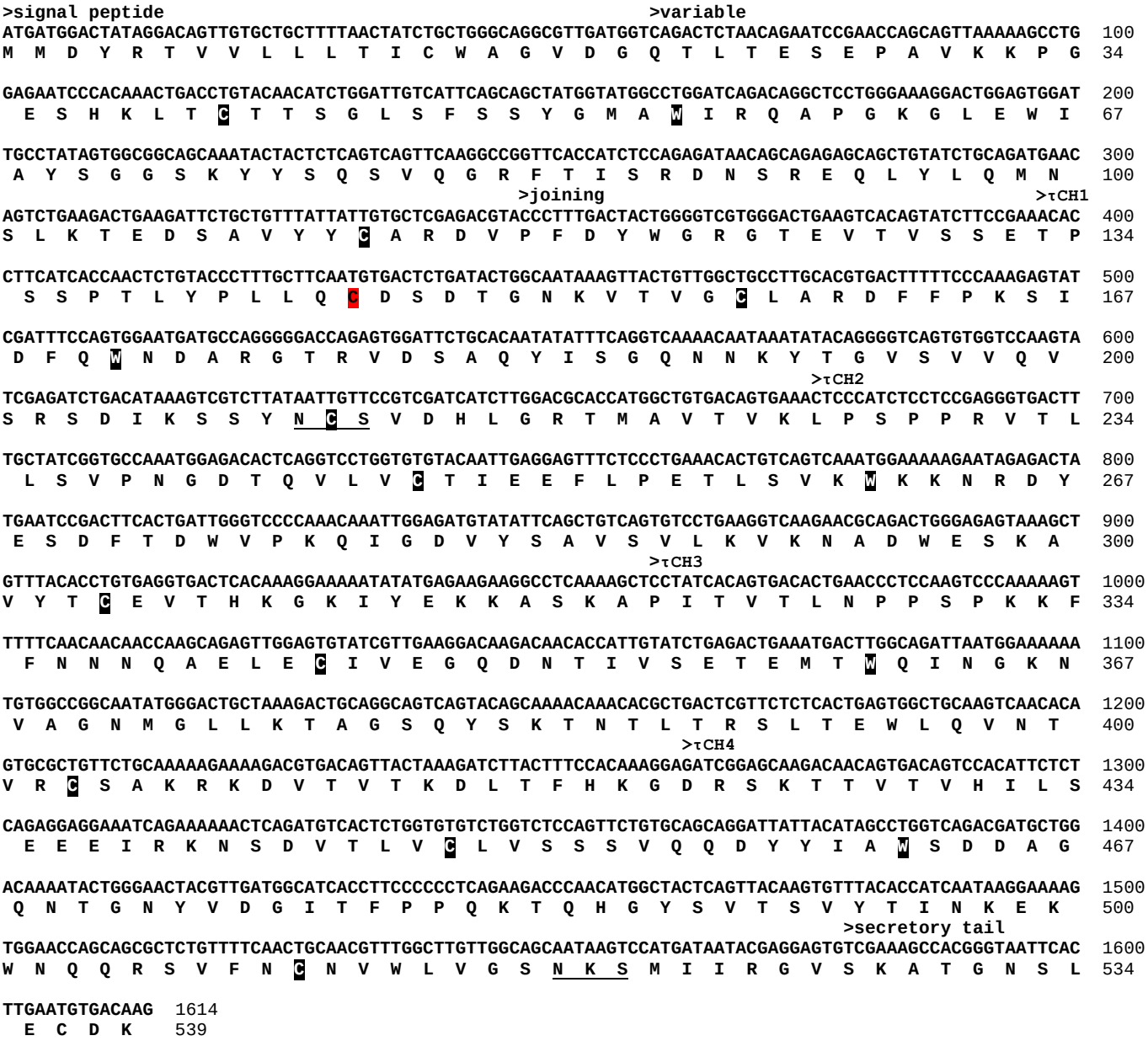
Primer ID	Primer name	Sequence (5'-3')	Designed to clone	Position	Amino acid
MFC 261	TolgMCF1	TGGATCMGACAGVMWCAGG	#6 KF713344	286-306	NGQNVQP
MFC 244	TolgMCR1	GCACARTAAAHACAGCDCTGTC	#6 KF713344	310-333	DTAVYYCA
MFC 260	TolgMCR	GCACARTAATAHACAGC	#6 KF713344	316-333	AVYYCA
MFC 340	MCF1	CCCTATTTCGCACAGACGCAACAA	#6 KF713344	1279-1302	GIKRVCVV
MFC 350	TolgMGR3F4	CTCATGGTACCCACCAGTGACTTGAGA	#6 KF713344	919- 945	LMVPTSDLR
MFC 351	TolgMGR3F5	ACTGTGACCTACGGAGGGTCATGT	#6 KF713344	1009-1032	TVTYGGSC
MFC 352	TolgMGR3F6	TTCTCTGTAGCTCTGCTGCTGCTGTTG	#6 KF713344	4- 30	FSVALLLLL
MFC 365	TolgZCR1	ACTTGGAGGGTTCAGTGTCACTGT	#59 KF713336	975- 996	TVTLNPPS
MFC 367	TolgZCR2	TGTGTTGACTTGCAGCCACTCAGT	#59 KF713336	1185-1206	TEWLQNQVNT
MFC 369	TolgZCR3	GAATGTGGACTGTCACTGTTGTCTTGCT	#59 KF713336	1278-1302	SKTTVTVHI
MFC 381	TolgMC1R4	TGTAAACTCGGTGGCGAGGCA	#6 KF713344	475- 495	CLATEFT
MFC 382	TolgMC1R5	TGGAGGATACTGGATGAAGTC	#6 KF713344	544-564	DFIQYPP
MFC 384	TolgZRC1R4	ACTGTTGGCTGCCTTGCACGTGAC	#59 KF713336	468- 489	CVGCLARD
MFC 386	TolgZRC1R6	TGGGAGTTTCACTGTCACAGCCATGGT	#59 KF713336	663- 687	TMAVTVKLP
MFC 387	TolgZRC1R7	ACCAAGATGATCGACGGA	#59 KF713336	642-657	SVDHLG

Primer name*	Sequence (5'-3')
βactin	ATCGTGGGGCGCCCCAGGCACC
βactin	GTCATCTTCTCYCTGTTGGC
TNF-α	CCAGGCRGCCATCCATTTAGAAG
TNF-α	CCGACCTCACCGCGCT
IL-1β	GGRSAGCGACATGGYRCGATTTCT
IL-1β	GGTGCTGATGTACCAGTTG

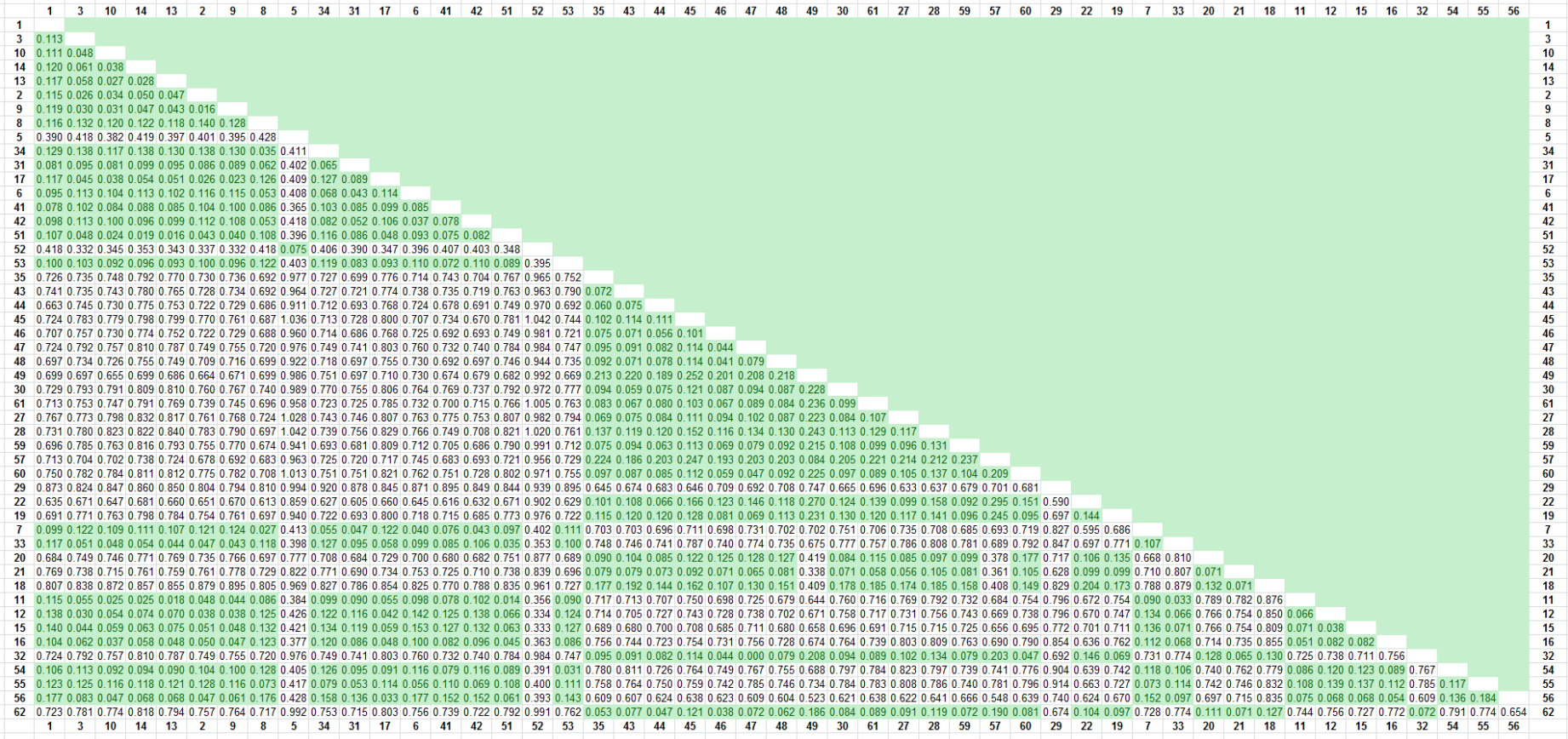
* Mladineo, I., Block, B.A., 2009. Expression of Hsp70, Na+/K+ ATP-ase, HIF-1 alpha, IL-1 beta and TNF-alpha in captive Pacific bluefin tuna (*Thunnus orientalis*) after chronic warm and cold exposure. J Exp Mar Biol Ecol 374, 51-57.



Supplemental Figure 1. Nucleic acid and deduced amino acid sequence of *T. orientalis* IgHµ full length clone 6. The start of the predicted signal peptide, VH, JH, CH domains and secretory tail are marked above the sequence. Potential N-linked glycosylation sites are underlined. Cysteines and tryptophans necessary for the Ig superfamily fold are highlighted in black, the cysteine that forms the disulfide bond to the Ig light chain is highlighted in red.

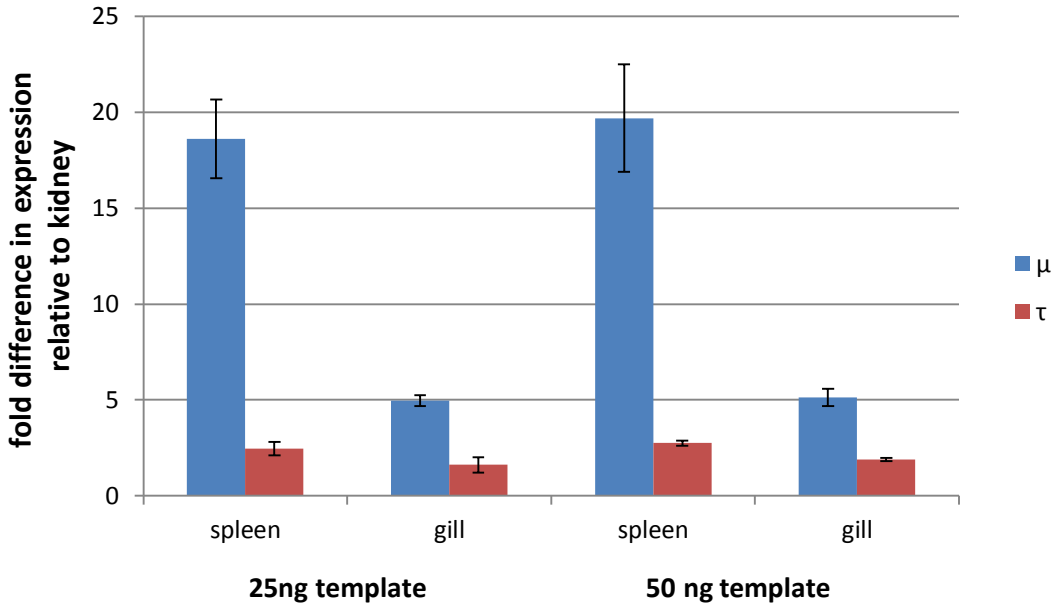


Supplemental Figure 2. Nucleic acid and deduced amino acid sequence of *T. orientalis* IgHτ full length clone 59. The start of the predicted signal peptide, VH, JH, CH domains and secretory tail are marked above the sequence. Features are annotated as in Supplemental Figure 1.



	1	3	10	14	13	2	9	8	5	34	31	17	6	41	42	51	52	53	35	43	44	45	46	47	48	49	30	61	27	28	59	57	60	29	22	19	7	33	20	21	18	11	12	15	16	32	54	55	56	
1																																																		**1**
3	0.113																																																	**3**
10	0.111	0.048																																																**10**
14	0.120	0.061	0.038																																															**14**
13	0.117	0.058	0.027	0.028																																													**13**
2	0.115	0.026	0.034	0.050	0.047																																													**2**
9	0.119	0.030	0.031	0.047	0.043	0.016																																													**9**
8	0.116	0.132	0.120	0.122	0.118	0.140	0.128																																												**8**
5	0.390	0.418	0.382	0.419	0.397	0.401	0.395	0.428																																											**5**
34	0.129	0.138	0.117	0.138	0.130	0.138	0.130	0.035	0.411																																										**34**
31	0.081	0.095	0.081	0.099	0.095	0.086	0.089	0.062	0.402	0.065																																								**31**
17	0.117	0.045	0.038	0.054	0.051	0.026	0.023	0.126	0.409	0.127	0.089																																							**17**
6	0.095	0.113	0.104	0.113	0.102	0.116	0.115	0.053	0.408	0.068	0.043	0.114																																						**6**
41	0.078	0.102	0.084	0.088	0.085	0.104	0.100	0.086	0.365	0.103	0.085	0.099	0.085																																						**41**
42	0.098	0.113	0.100	0.096	0.099	0.112	0.108	0.053	0.418	0.082	0.052	0.106	0.037	0.078																																					**42**
51	0.107	0.048	0.024	0.019	0.016	0.043	0.040	0.108	0.396	0.116	0.086	0.048	0.093	0.075	0.082																																					**51**
52	0.418	0.332	0.345	0.353	0.343	0.337	0.332	0.418	0.075	0.406	0.390	0.347	0.396	0.407	0.403	0.348																																					**52**
53	0.100	0.103	0.092	0.096	0.093	0.100	0.096	0.122	0.403	0.119	0.083	0.093	0.110	0.072	0.110	0.089	0.395																																					**53**
35	0.726	0.735	0.748	0.792	0.770	0.730	0.736	0.692	0.977	0.727	0.699	0.776	0.714	0.743	0.704	0.767	0.965	0.752																																					**35**
43	0.741	0.735	0.743	0.780	0.765	0.728	0.734	0.692	0.964	0.727	0.721	0.774	0.738	0.735	0.719	0.763	0.963	0.790	0.072																																					**43**
44	0.663	0.745	0.730	0.775	0.753	0.722	0.729	0.686	0.911	0.712	0.693	0.768	0.724	0.678	0.691	0.749	0.970	0.692	0.060	0.075																																					**44**
45	0.724	0.783	0.779	0.798	0.799	0.770	0.761	0.687	1.036	0.713	0.728	0.800	0.707	0.734	0.670	0.781	1.042	0.744	0.102	0.114	0.111																																					**45**
46	0.707	0.757	0.730	0.774	0.752	0.722	0.729	0.688	0.960	0.714	0.686	0.768	0.725	0.692	0.693	0.749	0.981	0.721	0.075	0.071	0.056	0.101																																					**46**
47	0.724	0.792	0.757	0.810	0.787	0.749	0.755	0.720	0.976	0.749	0.741	0.803	0.760	0.732	0.740	0.784	0.984	0.747	0.095	0.091	0.082	0.114	0.044																																					**47**
48	0.697	0.734	0.726	0.755	0.749	0.709	0.716	0.699	0.922	0.718	0.697	0.755	0.730	0.692	0.697	0.746	0.944	0.735	0.092	0.071	0.078	0.114	0.041	0.079																																					**48**
49	0.699	0.697	0.655	0.699	0.686	0.664	0.671	0.699	0.986	0.751	0.697	0.710	0.730	0.674	0.679	0.682	0.992	0.669	0.213	0.220	0.189	0.252	0.201	0.208	0.218																																					**49**
30	0.729	0.793	0.791	0.809	0.810	0.760	0.767	0.740	0.989	0.770	0.755	0.806	0.764	0.769	0.737	0.792	0.972	0.777	0.094	0.059	0.075	0.121	0.087	0.094	0.087	0.228																																					**30**
61	0.713	0.753	0.747	0.791	0.769	0.739	0.745	0.696	0.958	0.723	0.725	0.785	0.732	0.700	0.715	0.766	1.005	0.763	0.083	0.067	0.080	0.103	0.067	0.089	0.084	0.236	0.099																																					**61**
27	0.767	0.773	0.798	0.832	0.817	0.761	0.768	0.724	1.028	0.743	0.746	0.807	0.763	0.775	0.753	0.807	0.982	0.794	0.069	0.075	0.084	0.111	0.094	0.102	0.087	0.223	0.084	0.107																																					**27**
28	0.731	0.780	0.823	0.822	0.840	0.783	0.790	0.697	1.042	0.739	0.756	0.829	0.766	0.749	0.708	0.821	1.020	0.761	0.137	0.119	0.120	0.152	0.116	0.134	0.130	0.243	0.113	0.129	0.117																																					**28**
59	0.696	0.785	0.763	0.816	0.793	0.755	0.770	0.674	0.941	0.693	0.681	0.809	0.712	0.705	0.686	0.790	0.991	0.712	0.075	0.094	0.063	0.113	0.069	0.079	0.092	0.215	0.108	0.099	0.096	0.131																																					**59**
57	0.713	0.704	0.702	0.738	0.724	0.678	0.692	0.683	0.963	0.725	0.720	0.717	0.745	0.683	0.693	0.721	0.956	0.729	0.224	0.186	0.203	0.247	0.193	0.203	0.203	0.084	0.205	0.221	0.214	0.212	0.237																																			**57**
60	0.750	0.782	0.784	0.811	0.812	0.775	0.782	0.708	1.013	0.751	0.751	0.821	0.762	0.751	0.728	0.802	0.971	0.755	0.097	0.087	0.085	0.112	0.059	0.047	0.092	0.225	0.097	0.089	0.105	0.137	0.104	0.209																																			**60**
29	0.873	0.824	0.847	0.860	0.850	0.804	0.794	0.810	0.994	0.920	0.878	0.845	0.871	0.895	0.849	0.844	0.939	0.895	0.645	0.674	0.683	0.646	0.709	0.692	0.708	0.747	0.665	0.696	0.633	0.637	0.679	0.701	0.681																																			**29**
22	0.635	0.671	0.647	0.681	0.660	0.651	0.670	0.613	0.859	0.627	0.605	0.660	0.645	0.616	0.632	0.671	0.902	0.629	0.101	0.108	0.066	0.166	0.123	0.146	0.118	0.270	0.124	0.139	0.099	0.158	0.092	0.295	0.151	0.590																																			**22**
19	0.691	0.771	0.763	0.798	0.784	0.754	0.761	0.697	0.940	0.722	0.693	0.800	0.718	0.715	0.685	0.773	0.976	0.722	0.115	0.120	0.120	0.128	0.081	0.069	0.113	0.231	0.130	0.120	0.117	0.141	0.096	0.245	0.095	0.697	0.144																																			**19**
7	0.099	0.122	0.109	0.111	0.107	0.121	0.124	0.027	0.413	0.055	0.047	0.122	0.040	0.076	0.043	0.097	0.402	0.111	0.703	0.703	0.696	0.711	0.698	0.731	0.702	0.702	0.751	0.706	0.735	0.708	0.685	0.693	0.719	0.827	0.595	0.686																																			**7**
33	0.117	0.051	0.048	0.054	0.044	0.047	0.043	0.118	0.398	0.127	0.095	0.058	0.099	0.085	0.106	0.035	0.353	0.100	0.748	0.746	0.741	0.787	0.740	0.774	0.735	0.675	0.777	0.757	0.786	0.808	0.781	0.689	0.792	0.847	0.697	0.771	0.107																																			**33**
20	0.684	0.749	0.746	0.771	0.769	0.735	0.766	0.697	0.777	0.708	0.684	0.729	0.700	0.680	0.682	0.751	0.877	0.689	0.090	0.104	0.085	0.122	0.125	0.128	0.127	0.419	0.084	0.115	0.085	0.097	0.099	0.378	0.177	0.717	0.106	0.135	0.668	0.810																																			**20**
21	0.769	0.738	0.715	0.761	0.759	0.761	0.778	0.729	0.822	0.771	0.690	0.734	0.753	0.725	0.710	0.738	0.839	0.696	0.079	0.079	0.073	0.092	0.071	0.065	0.081	0.338	0.071	0.058	0.056	0.105	0.081	0.361	0.105	0.628	0.099	0.099	0.710	0.807	0.071																																			**21**
18	0.807	0.838	0.872	0.857	0.855	0.879	0.895	0.805	0.969	0.827	0.786	0.854	0.825	0.770	0.788	0.835	0.961	0.727	0.177	0.192	0.144	0.162	0.107	0.130	0.151	0.409	0.178	0.185	0.174	0.185	0.158	0.408	0.149	0.829	0.204	0.173	0.788	0.879	0.132	0.071																																			**18**
11	0.115	0.055	0.025	0.025	0.018	0.048	0.044	0.086	0.384	0.099	0.090	0.055	0.098	0.078	0.102	0.014	0.356	0.090	0.717	0.713	0.707	0.750	0.698	0.725	0.679	0.644	0.760	0.716	0.769	0.792	0.732	0.684	0.754	0.796	0.672	0.754	0.090	0.033	0.789	0.782	0.876																																			**11**
12	0.138	0.030	0.054	0.074	0.070	0.038	0.038	0.125	0.426	0.122	0.116	0.042	0.142	0.125	0.138	0.066	0.334	0.124	0.714	0.705	0.727	0.743	0.728	0.738	0.702	0.671	0.758	0.717	0.731	0.756	0.743	0.669	0.738	0.796	0.670	0.747	0.134	0.066	0.766	0.754	0.850	0.066																																			**12**
15	0.140	0.044	0.059	0.063	0.075	0.051	0.048	0.132	0.421	0.134	0.119	0.059	0.153	0.127	0.132	0.063	0.333	0.127	0.689	0.680	0.700	0.708	0.685	0.711	0.680	0.658	0.696	0.691	0.715	0.715	0.725	0.656	0.695	0.772	0.701	0.711	0.136	0.071	0.766	0.754	0.809	0.071	0.038																																			**15**
16	0.104	0.062	0.037	0.058	0.048	0.050	0.047	0.123	0.377	0.120	0.086	0.048	0.100	0.082	0.096	0.045	0.363	0.086	0.756	0.744	0.723	0.754	0.731	0.756	0.728	0.674	0.764	0.739	0.803	0.809	0.763	0.690	0.790	0.854	0.636	0.762	0.112	0.068	0.714	0.735	0.855	0.051	0.082	0.082																																			**16**
32	0.724	0.792	0.757	0.810	0.787	0.749	0.755	0.720	0.976	0.749	0.741	0.803	0.760	0.732	0.740	0.784	0.984	0.747	0.095	0.091	0.082	0.114	0.044	0.000	0.079	0.208	0.094	0.089	0.102	0.134	0.079	0.203	0.047	0.692	0.146	0.069	0.731	0.774	0.128	0.065	0.130	0.725	0.738	0.711	0.756																																			**32**
54	0.106	0.113	0.092	0.094	0.090	0.104	0.100	0.128	0.405	0.126	0.095	0.091	0.116	0.079	0.116	0.089	0.391	0.031	0.780	0.811	0.726	0.764	0.749	0.767	0.755	0.688	0.797	0.784	0.823	0.797	0.739	0.741	0.776	0.904	0.639	0.742	0.118	0.106	0.740	0.762	0.779	0.086	0.120	0.123	0.089	0.767																																			**54**
55	0.123	0.125	0.116	0.118	0.121	0.128	0.116	0.073	0.417	0.079	0.053	0.114	0.056	0.110	0.069	0.108	0.400	0.111	0.758	0.764	0.750	0.759	0.742	0.785	0.746	0.734	0.784	0.783	0.808	0.786	0.740	0.781	0.796	0.914	0.663	0.727	0.073	0.114	0.742	0.746	0.832	0.108	0.139	0.137	0.112	0.785	0.117																																		**55**
56	0.177	0.083	0.047	0.068	0.068	0.047	0.061	0.176	0.428	0.158	0.136	0.033	0.177	0.152	0.152	0.061	0.393	0.143	0.609	0.607	0.624	0.638	0.623	0.609	0.604	0.523	0.621	0.638	0.622	0.641	0.666	0.548	0.639	0.740	0.624	0.670	0.152	0.097	0.697	0.715	0.835	0.075	0.068	0.068	0.054	0.609	0.136	0.184																																		**56**
62	0.723	0.781	0.774	0.818	0.794	0.757	0.764	0.717	0.992	0.753	0.715	0.803	0.756	0.739	0.722	0.792	0.991	0.762	0.053	0.077	0.047	0.121	0.038	0.072	0.062	0.186	0.084	0.089	0.091	0.119	0.072	0.190	0.081	0.674	0.104	0.097	0.728	0.774	0.111	0.071	0.127	0.744	0.756	0.727	0.772	0.072	0.791	0.774	0.654	**62**
	1	**3**	**10**	**14**	**13**	**2**	**9**	**8**	**5**	**34**	**31**	**17**	**6**	**41**	**42**	**51**	**52**	**53**	**35**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**30**	**61**	**27**	**28**	**59**	**57**	**60**	**29**	**22**	**19**	**7**	**33**	**20**	**21**	**18**	**11**	**12**	**15**	**16**	**32**	**54**	**55**	**56**	

Supplemental Figure 3. Nucleotide identity matrix of VH region coding sequences to identity VH families. Clone numbers are in bold the left and right of rows and top and bottom of columns. Pairwise differences of less than 30% using nucleotide version of alignment in Figure 3 are highlighted in green and indicate inclusion in same VH family (by 70% nucleotide identity).



Supplemental Figure 4. IgH μ and τ expression in systemic and mucosal lymphoid tissues. Quantitative real-time PCR of secondary lymphoid tissue μ and τ C region mRNA expression relative to that in anterior kidney, standardized to β-actin. Two template concentrations were analyzed. Experiment performed in triplicate, error bars indicate standard deviation.