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Identification of Igσ and Igλ in channel catfish, *Ictalurus punctatus*, and Igλ in Atlantic cod, *Gadus morhua*

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Abstract Immunoglobulin light (IGL) chain genes encoding σ and λ from channel catfish, *Ictalurus punctatus*, and λ from Atlantic cod, *Gadus morhua*, were identified by mining of expressed sequence tag databases, 5′-RACE and RT-PCR protocols. cDNAs for each of these IGL chains encode typical variable (V), joining (J), and constant (C) regions and Southern blot analyses and genomic sequencing show that genes encoding these isotypes, like other teleost IGL genes, are found in a cluster organization of one or two V gene segments, followed by single J and C gene segments, all in the same transcriptional orientation. However, unlike the teleost κ genes, genes encoding catfish σ and λ are few in number and the two isotypes are each encoded by only two clusters. Similarly, Atlantic cod λ genes are predicted to be encoded by two or three clusters. As expected, sequence and phylogenetic analyses comparisons demonstrate that catfish Vσ and Cσ genes are most similar to Vσ and Cσ genes of other ectothermic vertebrates. Although catfish and Atlantic cod Vλ genes cluster with other vertebrate Vλ genes, their Cλ sequences cluster in a distinct group separate from other vertebrate IGL C sequences. However, support for classifying these sequences as λ, is their V and J recombination signal sequence (RSS) organization. The catfish and Atlantic cod genes have typical λ-like RSS with the Vλ RSS consisting of heptamer-23 bp spacer-nonamer and the Jλ RSS consisting of heptamer-12 bp spacer-nonamer. This is the first report demonstrating the presence of Igλ in teleosts.

Keywords Immunoglobulin light chains · Sigma · Lambda · Isotype · Channel catfish · Atlantic cod

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Introduction

All studied jawed vertebrate species, except for birds, as represented by the galliforms (chicken, *Gallus gallus*) and anseriforms (ducks, *Anas platyrhynchos*, *Cairina moschata*), express more than one immunoglobulin light (IGL) chain isotype (Magor et al. 1994; McCormack et al. 1989; Reynaud et al. 1983, 1987; Lundqvist et al. 2006). The two mammalian IgL isotypes are classically referred to as κ and λ and were initially identified by serology approximately 40 years ago (Edelman and Gally 1962; Wu and Kabat 1970). As IGL chain isotypes were initially identified in different vertebrates there were attempts to classify them as κ- or λ-like. For example, based on sequence and recombination signal sequence (RSS) orientation, the IGL of birds was found to be related to mammalian Igλ (Gadol et al. 1976; Kubo et al. 1970) and Igκ isotypes were found in elasmobranchs and bony fish (Greenberg et al. 1993, and reviewed in Bengtén et al. 2000; Criscitiello and Flajnik 2007). However, it is now clear that many ectothermic vertebrates express more than two types of IGL chains and due to difficulties with resolving their phylogenetic relation-

ships several nomenclatures exist in parallel. Three IGL isotypes, κ (originally termed ρ), σ and λ, have been identified in the amphibian *Xenopus* (Haire et al. 1996; Schwager et al. 1991; Zezza et al. 1992), and in the elasmobranchs, four IGL isotypes have been described. Type I or sigma-cart is unique to cartilaginous fish (Criscitiello and Flajnik 2007; Fleurant et al. 2004). Type II is considered λ-like, and type III, is considered a κ-type (Hohman et al. 1992; Rast et al. 1994). More recently, a fourth IGL isotype was identified in nurse shark, *Ginglymostoma cirratum,* and found to be orthologous to the *Xenopus* Igσ isotype (Criscitiello and Flajnik 2007). In several species of teleost, three IGL isotypes have been identified, type L1, type L2, and type L3 (Coscia et al. 2008; Daggfeldt et al. 1993; Haire et al. 2000; Partula et al. 1996; Saha et al. 2004 and reviewed in Solem and Stenvik 2006). While teleost L1 and L3 (including channel catfish, *Ictalurus punctatus*, isotypes F and G; Ghaffari and Lobb 1993, 1997) clearly represent distinct isotypes with amino acid identities of ~30% to each other, phylogenetic analyses demonstrated them to be orthologs of κ light chains (Criscitiello and Flajnik 2007). In contrast, teleost L2 is orthologous to the σ isotype. Teleost Igκ and Igσ genes are organized in clusters of VL-JL-CL and for Igκ, the Vκ genes are in opposite transcriptional orientation to the Jκ and Cκ genes, while the Vσ, Jσ, and Cσ genes are all in the same transcriptional orientation. Notably, this opposite orientation for Vκ implies rearrangement occurs by inversion rather than deletion. Another difference between teleost Igκ and Igσ isotypes is that Igσ genes tend to be fewer in number than Igκ genes in the different fish examined. For example, in the rainbow trout, *Onchorynchus mykiss*, the Igκ locus is estimated to contain ~20 gene clusters (Daggfeldt et al. 1993) and the Igσ locus is estimated to consist of at least ten gene clusters (Timmusk et al. 2000). Thus, in teleost, the IGL chain genes of the κ linage have undergone extensive duplications and diversifications (Hsu and Criscitiello 2006) and are also the most abundantly expressed in peripheral blood leukocytes (PBL). It was estimated that in catfish, the F and G isotypes together represents 90% of the IGL (Lobb and Clem 1982; Lobb et al. 1984). Similarly, the ratio of type L1 to type L2 expression in rainbow trout PBL is estimated to be 85:15 (Timmusk et al. 2000). Recently, an Igσ sequence from blue catfish, *I. furcatus*, was found (Hsu and Criscitiello 2006) and annotated (Criscitiello and Flajnik 2007) and it was suggested that while primordial Igκ, Igλ, and Igσ light chain isotypes are all present in cartilaginous fish, some of these light chain lineages have been lost in different vertebrate groups. For example, Igλ appears to be present in all groups of jawed vertebrates with the exception of teleost fish (Criscitiello and Flajnik 2007), while Igκ appears to be lost among birds and microbats (microchiropterans) (Das et al. 2008) and similarly, Igσ was suggested to be present in all

cold-blooded vertebrates but absent among mammals and birds (Criscitiello and Flajnik 2007). However, a caveat is that although Igκ and Igλ have been identified in the green lizard (*Anolis carolinensis*) thus far, Igσ has not (Das et al. 2008). Here, we describe the identification, genomic organization, expression and phylogenetic relationship of Igσ and Igλ in the channel catfish and an Igλ in the Atlantic cod, *Gadus morhua*. Genes encoding each of these IGL chains are arranged in clusters consisting of one or two V, a single J and C, all in the same transcriptional orientation. However, unlike the catfish F and G isotypes, catfish Igσ, and Igλ isotypes are encoded by only one or two genomic clusters. Similarly, Atlantic cod Igλ appears to be encoded by one to three genomic clusters.

Materials and methods

Experimental animals

Channel catfish (1–2 kg) were obtained from a commercial source (ConAgra, Isola, MS) and maintained in individual tanks as previously described (van Ginkel et al. 1992). Catfish PBL were isolated from heparinized blood by centrifugation on a cushion of Ficoll-Hypaque (Lymphoprep, Accurate Chemical Corp. Westbury, NY) as described previously (Miller et al. 1994). Atlantic cod were caught by netting in the fjord Gullmaren on the Swedish west coast and spleen, pronephros (head kidney), ovary and testis were immediately snap frozen in liquid nitrogen and stored at −70°C until used.

Identification of catfish Igσ and Igλ and Atlantic cod Igλ cDNAs

A catfish Igσ sequence was obtained by RT-PCR using primers (supplementary Table 1) based on a blue catfish Igσ chain (accession number CK403931; (Hsu and Criscitiello 2006)). Using the IgT/Z C domains of rainbow trout and zebrafish (Danilova et al. 2005; Hansen et al. 2005) as queries to search catfish EST databases, a catfish Igλ-like EST (accession number CBPO4528.b1) consisting of an unrearranged V-intron-J-C was identified from an LPS-stimulated PBL cDNA library. This EST was one of four that encoded an Igλ-like sequence. The cDNA library designated CBPO (JxLPS1) was made as part of the Joint Genome Community Sequencing (JGI) program 2006: *I punctatus, I furcatus EST* project (http://www.jgi.doe.gov/sequencing/). Full-length transcripts for both catfish isotypes were subsequently obtained using 5′ and 3′-RACE protocols. An Atlantic cod Igλ fragment was obtained by using short degenerate primers corresponding to conserved regions found in vertebrate TRBV and IGL V framework (FR) 2 and FR3 regions. A larger Atlantic cod cDNA fragment was

obtained by anchored PCR from an Atlantic cod amplified spleen λgt-10 cDNA library using a gene-specific forward primer (GmVLF) and a reverse λgt-10 phage primer (see supplementary Table 1). This spleen cDNA library was prepared from poly(A)-selected mRNA using a Promega cDNA Synthesis Kit (Promega, Madison, WI) and consisted of ~10.9×10^6 independent recombinants before amplification. Full-length and larger unrearranged Atlantic cod Igλ cDNAs were then obtained by screening the spleen cDNA library using standardized methods. All cDNAs were sequenced on both strands using forward and reverse gene-specific primers (supplementary Table 1). The Atlantic cod Igσ (ES474588, EX732557, EX727050, EX727984, EY975775), rainbow trout Igλ (BX861772, CA257088), blue catfish Igλ (CK403484) and green anole Igλ (FG746006) and green anole Igκ (FG748778) sequences were identified by Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990) against the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) databases.

Sequence analysis

Nucleotide and amino acid sequences were analyzed using DNASTAR software (Madison, WI) and similarity searches were performed using BLAST analysis against the NCBI non-redundant database. Immunoglobulin domains and signal peptides were predicted using SOSUI (signal) Beta Version, SMART (http://smart.embl-heidelberg.de/) and CBS Prediction servers (http://www.cbs.dtu.dk/services). The IMGT/DomainGapAlign TOOL (http://imgt.cines.fr) or when possible IMGT/V-QUEST (Lefranc et al. 2005) were used to determine complementarity determining regions (CDR), framework (FR) and strand delimitations. Pairwise alignments were made using CLUSTALW (Thompson et al. 1997) in the MegAlign module of DNASTAR. Neighbor-joining trees with pairwise gap deletions were constructed based on the CLUSTALW alignments using MEGA 4.0 allowing bootstrap values of 1,000.

Southern blotting and genomic PCR

Genomic DNA was prepared using erythrocytes from outbred and homozygous gynogenetic catfish as described previously (Hogan et al. 1999; Miller et al. 1988). Catfish Southern blots were prepared with 5 μg genomic DNA that was digested to completion with EcoR I, Xba I or Pst I restriction enzymes, separated on 1% agarose gels and transferred by capillary action onto Hybond-N+ membranes (GE Healthcare Bio-Sciences AB, Piscataway, NJ) using standard techniques. Hybridizations were performed in Rapid-hyb buffer (GE Healthcare Bio-Sciences AB, Piscataway, NJ) at 65°C according to manufacturer's

instructions and membranes were washed at high stringency (65°C with 0.1× SSC, 0.1% SDS). The catfish V probes encompassed the FR1-FR3 region of either Igσ or Igλ and the C probes consisted of the entire C Ig domain through the stop codon of either Igσ or Igλ (see supplementary Table 1). Probes were amplified by PCR using IDPol DNA polymerase (ID Labs Biotechnology, London, Ontario) according to the manufacturer's protocol. Parameters were: 3 min 94°C, followed by 30 cycles of 94°C 30 s, 60°C 30 s, 72°C 1 min, then extension at 72°C for 10 min. The probes were random primed labeled with [^{32}P] dCTP by Megaprime labeling (GE Healthcare Bio-Sciences AB).

Atlantic cod Southern blots were prepared with 10 μg of sperm genomic DNA digested to completion with BamHI, EcoR I or Hind III. The digested DNA was electrophoresed on 0.8% agarose gels and transferred to Biodyne B nylon filters (Pall Corporation, East Hills, NY) following the manufacturer's instructions. The filters were prehybridized and hybridized in a buffer modified from Church and Gilbert (1984) at 65°C overnight and washed at high stringency conditions. Atlantic cod V and C probes were generated by digesting an Atlantic cod Igλ cDNA (#S1-41) with restriction enzymes to obtain V and C specific fragments. These fragments were then recovered on glass wool (Heery et al. 1990) and labeled with [^{32}P] dCTP using the Megaprime Labeling System (GE Healthcare Bio-Sciences AB).

IGL gene clusters were amplified from genomic DNA from a homozygous gynogenetic catfish (G25) and a total of 66 ng of DNA was used in each reaction. Amplification was performed with HotStart-IT FideliTAq DNA polymerase (USB Corporation, Cleveland OH) and parameters were: 2 min 94°C followed by 30 cycles of 94°C 30 s, 61°C 30 s 72°C 2 min followed by a final extension of 72°C 10 min (see Supplementary Table 1 for primers used). The resulting PCR products ranging from 2–2.4 kb were separated on 0.8% agarose gels, cloned into pCR4-TOPO (Invitrogen Life Technologies, Carlsbad, CA) and sequenced on both strands using vector- and gene-specific primers. Sequence analyses were performed with the DNASTAR software (Madison, WI) as described above.

Monoclonal antibodies, PBL isolation, magnetic activated cell sorting and flow cytometry

Hybridoma culture supernatant was used directly as the monoclonal antibody (mAb) source and all supernatants contained equivalent Ig concentrations as determined by ELISA (Southern Biotechnology, Birmingham, AL) and Western blot: mouse anti-catfish IgM 9E1 (IgG$_1$,κ) reacts with catfish Igμ chain, (Miller et al. 1987); mouse anti-catfish IgL 3F12 (IgG$_1$, κ) reacts with the IGL F isotype (Lobb and Clem 1982; Lobb et al. 1984); mouse anti-

catfish IgL 11A2 (IgG$_{2b}$, κ) reacts with IGL G isotype (Shen et al. 2003); and mouse anti-trout IgM 1.14 (IgG$_1$, κ) reacts with trout Igμ chains (DeLuca et al. 1983), which was used as an isotype control.

Catfish PBL were isolated from heparinized blood by centrifugation as described above and resuspended to 1×10^7 cells/ml in ice cold RPMI-1640 adjusted to catfish tonicity with 10% deonized water (cfRPMI) containing 0.03% sodium azide. To obtain specific cell populations, PBL were sorted into surface IgM enriched (sIgM$^+$) and surface IgM depleted (sIgM$^-$) fractions by magnetic activated cell sorting (MACS) with anti-catfish IgM 9E1 mAb according to the manufacturer's protocol. Briefly, 2×10^8 cells were incubated with 1 ml mAb 9E1 for 30 min on ice. After washing with cfRPMI, cells were then resuspended in 300 μl of degassed cfRPMI containing 0.5% BSA and 2 mM EDTA. Twenty microliters of goat anti-mouse IgG microbeads (Miltenyi Biotec, Gladbach, Germany) were added to 1×10^7 cells and cells were incubated for 25 min at 4°C, washed with cfRPMI, and separated into fractions using MACS LS columns (Miltenyi Biotec) according to the manufacturer's protocol. The resulting fractions were washed in cfRPMI and analyzed by flow cytometry analysis (FACS). Sub-samples of the sorted cells were also used for RNA, which was prepared as described below.

For FACS, aliquots of 1×10^6 PBL or sorted populations were incubated with 50 μl of mAb supernatant for 30 min at 4°C, washed, and then incubated with 50 μl goat anti-mouse Ig (H+L) secondary antibodies (Southern Biotechnology Associates) conjugated to phycoerythrin (PE) diluted 1:80 (v/v) for 30 min. Finally, the cells were washed, suspended in 0.3 ml of cfRPMI and analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) for single staining.

RNA preparation, RT-PCR, and 5′-RACE

Total RNA from various catfish tissues, PBL and sorted PBL populations was prepared using RNA-Bee (Tel-test Inc, Friendswood, TX) and treated with DNAse I (Invitrogen Life Technologies) according to the manufacturer's recommended protocols. One microgram was subsequently reverse transcribed into cDNA using an oligo-T primer and 200 units of Superscript III reverse transcriptase (RT; Invitrogen Life Technologies). RT-PCR was performed with specific primers for catfish membrane (m) IgM, mIgD, Igσ, Igλ, and the housekeeping gene elongation factor alpha (EF1-α; see supplementary Table 1). Typical parameters were: 3 min 94°C, followed by 30 cycles of 94°C 30 s, 60°C 30 s, 72°C 1 min, and a final extension cycle at 72°C 10 min. Annealing temperatures varied between 58–60°C depending on what primer pair was used. Amplified

products were electrophoresed on 1% agarose gels, excised, and cloned and sequenced as above. Sequence analyses were performed with the DNASTAR software (Madison, WI). For 5′-RACE analysis, PBL RNA was prepared as above and 5′-RACE was performed using the GeneRacer Kit (Invitrogen Life technologies) and gene-specific primers according to the manufacturer's recommended protocol.

Results and discussion

Catfish Igσ

Catfish Igσ transcripts were isolated from PBL using 5'-RACE and RT-PCR protocols. Of 36 RACE products, 13 contained only unspliced Cσ genes and 23 contained a Jσ gene spliced to Cσ, preceded by an intron sequence. All of the sterile JCσ transcripts contained an identical J gene consisting of a nonamer, 23-bp spacer, heptamer preceding a J segment encoding 11 amino acids. Thus, to obtain a full-length Igσ cDNA, forward primers based on the 5′-end of Vσ-like sequences identified in the EST searches were designed and used in RT-PCR in combination with reverse Cσ specific primers. The representative full-length rearranged IpIgσ1 transcript consists of 732 nucleotides encoding a 244-amino-acid polypeptide with a predicted molecular weight of 23.7 kDa. The cDNA encodes a typical leader sequence, followed by V, J and C regions (see sFig. 1a). Both the Vσ and Cσ region sequences contain the conserved cysteine residues (CYS 23, CYS 104) that form the intrachain disulfide bonds and the conserved cysteine in the C region (CYS 126) that forms the interchain disulfide bond with the IgH chain (Fig. 1a). Also, as noted for other Vσ genes (Criscitiello and Flajnik 2007), CDR1 was shorter in length than CDR2, i.e., five vs nine amino acids. The Jσ region is 11 amino acids in length and contains a typical J-glycine bulge motif of FGQG. Multiple alignments and similarity searches show that the catfish Igσ has the highest amino acid similarity to teleost L2 isotypes and Xenopus and horn shark Igσ, hence, the σ designation. As expected, the IpIgσ1 V was most similar to other teleost Vσ genes (L2 Vs), with amino acid sequence identities ranging from 58–72% when compared to rainbow trout, carp (Cyprinus carpio) and zebrafish L2 Vs. In comparison, amino acid identities with horn shark and Xenopus Vσ sequences were lower at 46% and 30%, respectively. A similar trend also occurs when comparing the Jσ and Cσ regions of the different species. Jσ amino acid identities range from 55–91%, with the teleost Jσ regions sharing the highest amino acid identities. Similarly, catfish Cσ exhibits 53–58% amino acid identity with the L2 Cσ of the three bony fish, but drops to <36% amino acid identity with the horn shark and Xenopus Cσ regions (Fig. 1b).

Southern blot analyses indicate that catfish Igσ genes, like other teleost IGL genes are found in a cluster organization. However, unlike catfish F and G genes, the Igσ genes are few in number. Previously, Southern blot analyses showed there were at least 30 V segments and 15 C segments for the G isotype, and at least 50 different

fragments hybridized with V and C probes for the F isotype (Ghaffari and Lobb 1993, 1997). Briefly, in this study, genomic DNA from five individual outbred and one gynogenetic (homozygous) catfish was hybridized with either a Vσ or Cσ probe and depending upon the restriction enzyme used, 2-6 Vσ and 2-4 Cσ hybridizing bands were

a

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   IGSV
          10        20    *        40         50          60   66 70        80         90         100    *     110
   .......|........|....|...|.........|.........|.........|ABCD.....|...|.........|.........|.........|.........|....
Ipσ:  VVTQKPPVLTLTRGQTATMDCNLGTVTDSGARWYKQVPGGVPQHVLRNYHGW-----SAPN-YGSGFSSPKFTSTHSS-KSDYKLIISNVEVGDSAVYYCKTWDSSAKEW-
CcL2: ......AI..EQK.ISV.....IKKPENYYVS....I.NAA..Y...Y..SH-----.S.DE..D....SR...KAP.-NI..Q......D.T.......C...DTA..N-
DrL2: ...S.-.I.ASK....QL.....--I.A.V....TSA...Y..Y..YSR-----..A.....S..-KS..S......D.S..........S.....GNVAV--
OmL2: A...T.S...VSTKG...FH.DITKGEGNVVI......A..Y...Y..T.SFTSS.S.DQ......DR...KAT.-DK..QF......ET.......Q......V.VH-
Gmσ:  .L..Q.S.V.....E..SI.....S.NK.R.I....I......Y..CLF.D.-----TDVK-..A.....H....YQ.-....R...N...E.......Q......R-
Xlσ:  TLSLT.SNNAVNL.ERVTLN..A.AKDGYHVHLQ..I..N...LIIAHH.SF-----TS.V--..P.ISTDRY.A.VNAAATE.QF..KKA.TAHT.H...TK.I..ISAY-
Hfσ:  TLTQP.-D..VKP.N..ILE..I..KDVYVVN....T..AA..WI.YYH.SL-----NS.T--.PS.N.DR..AVN.GHNI.Q...K....N.G.....NK.F..SSTDG
      <      FR1              ><CDR1<      FR2      >< CDR2    ><        FR3                  ><    CDR3    >
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   IGSJ
Ipσ:  VFGQGTKLIVS
CcL2: I.........N
DrL2: .........T
OmL2: ........FVT
Gmσ:  ...G...V..T
Xlσ:  ..S.SS....T
Hfσ:  S.......FLA
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   IGSC
       1   5    10   15   20   25   30   40   45  77 80   84     85        97    104     117       ↓128
   .....|...|....|....|....|....|....|....|....|....|12345|..|...|12344321|....|.....|...|.........|........|
Ipσ:  DAALPDPVLTVLPPS-SEELKSN-KATLVCLASDLAGGFADVRWLVNENSVSSGVITGSAQQQANKKFTLSSYLTIDSCDWENDKVIACEVSAGGKAALVK--IKKSECSE
CcL2: ...AA....NI.R..-R....S.S-.L.....INHMS.A.........G.....E..F....EE.PD...KM....S.E.SE.DK.TQLT...T.AS.TTS-.S-....D.RD
DrL2: ...AAP....I.R.SR...S.S-.V..L..INHMSVA.........G....E..F....E.PDH..KS..S...QRSE.DK.RELT..ATVAS.TSRAS--.R....D
OmL2: .ST.PP....I....-.D....S-.V......QM.MGY..S.TAGGTP..G.IA.SGPVP..D.T.Q...C..V.TSE.NQ....FS.K.TV.S.F.E-.D-.K.....T
Gmσ:  GSS..P....FYR..-TNP.S-S-H.A.....RQMSI...E.S....GSP.TE.TWAST.V.HPD.T.Q......SLPG..-SE.RSYT.K.TV.SSSFG-.T-HRMF...NTLE
Xlσ:  VDKF.E.A.L.F..Y-TDDNE.KDSS...T.HI.K.EVSLVN.N...IDGTT.QD..T.SNPVRESDNT.SM.....LS.K.INK.RLYS.MIKQE.SS.FISKGVKL.Q.--
Hfσ:  AQQ....SVKL.G..-AEEV.TKGAG.....VN..SM...A.S.A.DGSPT.GE.Q.SAVSRNTDNT.S.....KVPGT..SSG..YS.T.QQQ.T.SKTTATVTE.N.--
      <    A      ><    B    >< BC ><  C  ><CD ><   D  >< DE  ><   E     ><   F  >< FG  ><    G     >
```

b

IGSJ

	Carp L2	Zebrafish L2	Trout L2	Xenopus σ	Horn shark σ
Catfish σ	81.8	90.9	81.8	63.6	63.6
Carp L2		81.8	72.7	54.5	63.6
Zebrafish L2			90.9	72.7	63.6
Trout L2				63.6	72.7
Xenopus σ					63.6

IGSC

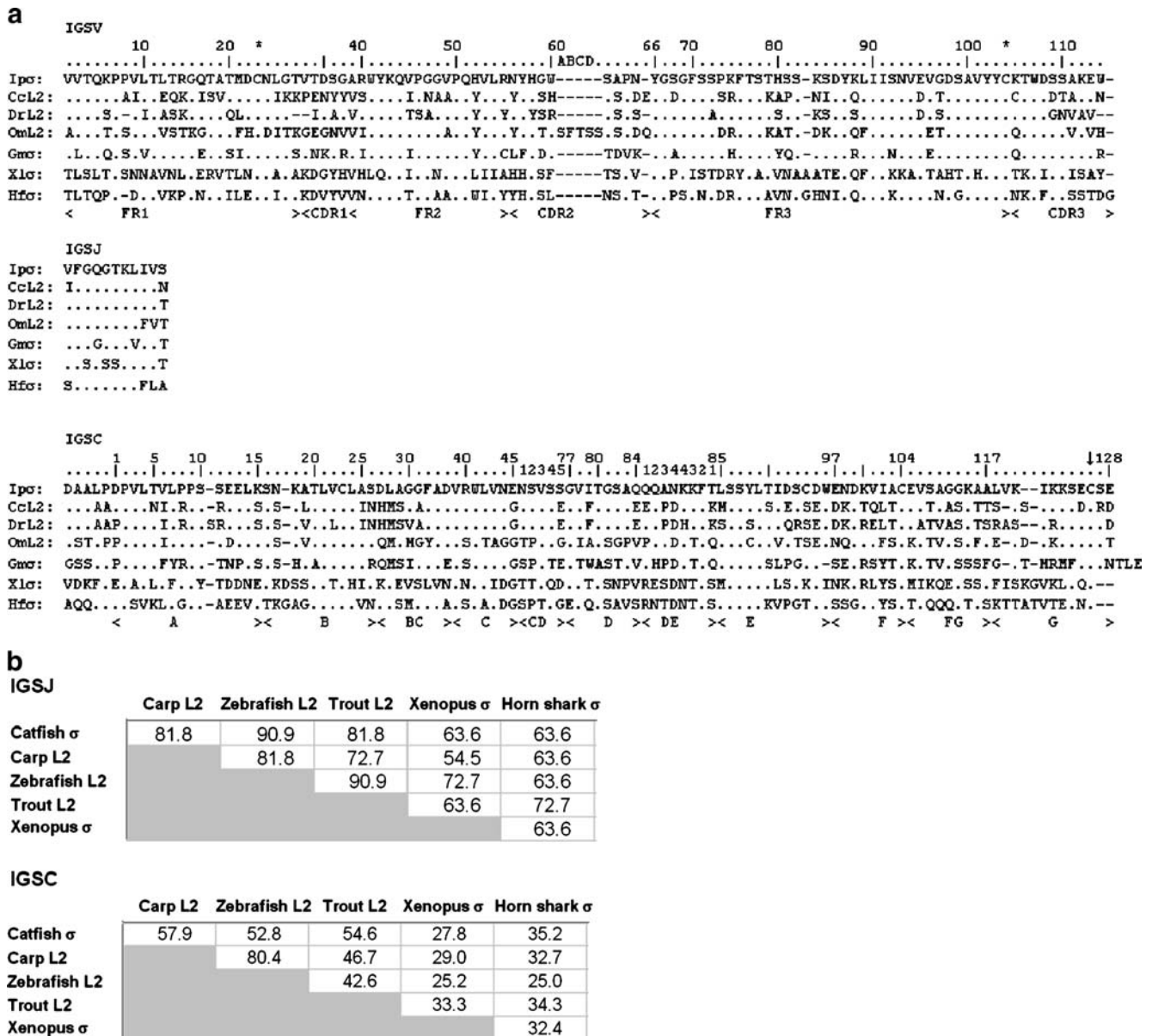|	Carp L2	Zebrafish L2	Trout L2	Xenopus σ	Horn shark σ
Catfish σ	57.9	52.8	54.6	27.8	35.2
Carp L2		80.4	46.7	29.0	32.7
Zebrafish L2			42.6	25.2	25.0
Trout L2				33.3	34.3
Xenopus σ					32.4

Fig. 1 Igσ amino acid sequence comparisons. **a** Amino acid alignments of catfish Vσ, Jσ, and Cσ regions with teleost L2, amphibian and shark Vσ and Cσ regions. The conserved cysteine residues (CYS 23 and CYS 104) that form the intrachain disulfide bonds are labeled (*) and the conserved C region cysteine (CYS 126) that forms the interchain disulfide bond with IgH is marked by (↓). Residues identical to the catfish sequence are shown as (.) and (–) represents gaps in the alignment. The amino acids numbers for the catfish sequence according to the standardized rules of the IMGT unique numbering are given above (Lefranc et al. 2005). **b** Comparison of Igσ percent amino acid identities from pairwise alignments of J and C regions. Abbreviations and GenBank accession numbers are: catfish (*Ipσ* EU872023); carp (*CcL2* V and J (AB091113) and C (BAC81202)); zebrafish (*DrL2*, AAG31698); rainbow trout (*OmL2* AAB41310); Atlantic cod (*Gmσ* V (EX732557), J and C (EC907978)); *X. laevis* (*Xlσ* AAH82413); and horn shark (*Hfσ* EF114760)
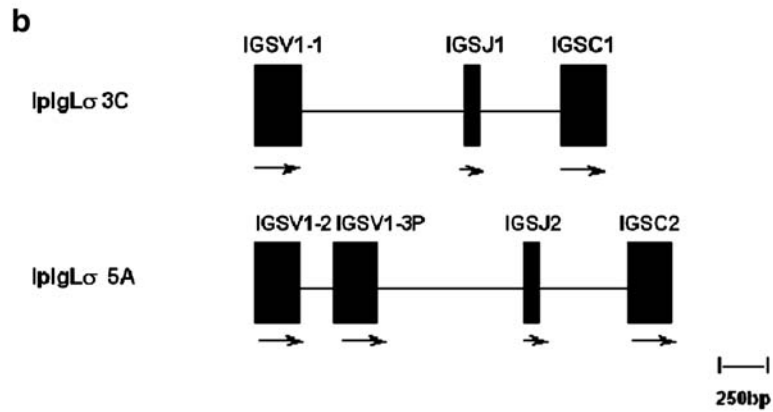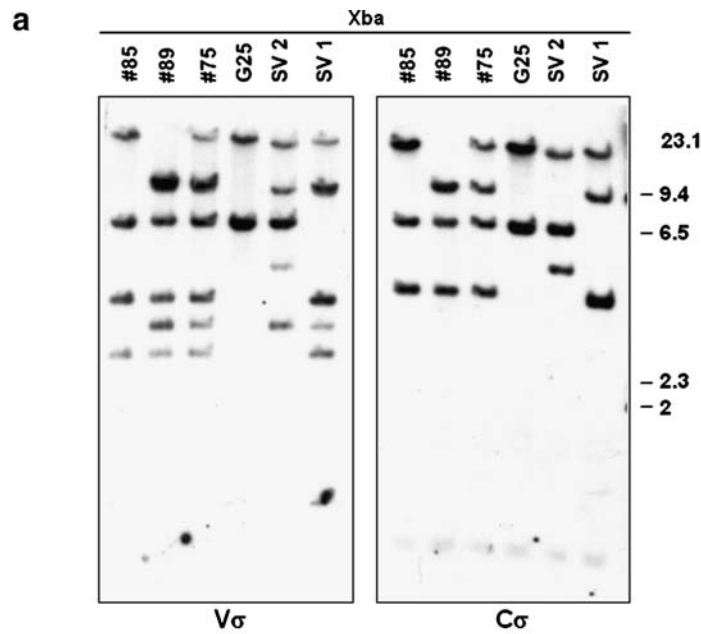
observed. The representative Southern blot of an *Xba I* digest indicates a typical Ig gene cluster organization with most of the same restriction fragments hybridizing with both V and C probes (Fig. 2a). Two Igσ clusters were amplified from gynogenetic catfish G25 genomic DNA using a Vσ forward primer and a reverse Cσ primer (Fig. 2b). The IpIgσ 3C gene cluster is 2,057 bp in length and consists of single V, J, and C genes, termed IGSV1-1, IGSJ1, and IGSC1. In contrast, the IpIgσ 5A gene cluster is 2,397 bp in length and contains two V genes, followed by single J and C genes. IGSV1-2 is 85% identical at the nucleotide level to IGSV1-1, and IGSV1-3 is a pseudogene truncated at the 5′ end. All are in the same transcriptional orientation and sequencing shows that the J and C genes in the two clusters are identical. Each of the V genes has a typical RSS of heptamer, 12-bp spacer, nonamer (Fig. 2c). Additionally three other Vσ cDNAs were amplified from outbred fish using RT-PCR and 5′-RACE (sFig. 2). Two (IGSV1-1*02 and IGSV1-1*03) are ~99% identical at the nucleotide level to IGSV1-1 and likely are alleles of this sequence. A third (IGSV1-2*02) represents an allele of IGSV1-2. Together Southern blots performed with homozygous gynogenetic DNA combined with sequencing data indicate that only two Igσ gene clusters are present in the catfish genome. This notion is further supported by sequencing of 23 RACE products from a single outbred fish, where only three different Cσ sequences, presumably alleles, with ~95% amino acid identity were identified (sFig. 3). Overall, the catfish Igσ repertoire appears very limited with two gene clusters each containing a single identical J (as well as an identical C), and either one or two V gene segments, all belonging to the same Vσ family. IGSV1-1 is located in one cluster (IpIgL σ 3C) and IGSV1-2 and IGSV1-3P are located in the second cluster (IpIgL σ 5A). In comparison, two distinct Vσ families have been identified in carp and limited sequencing suggests possibly four Jσ segments and two Cσ cDNAs, which are ~90% identical at the amino acid level (Ishikawa et al. 2004). Also, Southern blots indicate that only two to four carp Igσ clusters are present. In contrast, nine Vσ (L2) genes representing at least three Vσ families were sequenced in the rainbow trout and Southern blotting and genomic sequencing indicated that two to three Vσ genes occur in an Igσ (L2) cluster (Timmusk et al. 2000). Also, as with the catfish Igσ genes, the rainbow trout Igσ genes are in the same transcriptional orientation, and the Jσ and Cσ genes, while not identical, are quite similar with only a few amino acid differences. More recently, two different studies characterizing IGL chain gene organizations in zebrafish have shown that there are two Cσ genes in tandem with each C preceded by four or five functional Vσ genes (Hsu and Criscitiello 2006; Zimmerman et al. 2008). This discrepancy in gene segment number is likely because the

Fig. 2 Genomic analysis of IpIgσ. **a** Representative Southern blot analysis of genomic DNA from one homozygous gynogenetic (G25) and five outbred catfish. DNA was digested with *Xba I* and hybridized with either Vσ or Cσ specific probe. Size markers in kilobase pairs are on the right. **b** Representative schematics of two different IpIgσ genomic clusters that were amplified using Vσ and Cσ specific primers on gDNA isolated from gynogenetic catfish G25. Cluster IpIgLσ 3C contains a single V gene followed by a J gene segment and a C gene. Cluster IpIgLσ 5A contains two V genes located upstream of a single J gene segment and C gene. The genes are in the same transcriptional orientation. **c** Igσ V and J genes utilize typical RSS. The nucleotide sequence from the 3′ end of the V through the J gene segment of genomic cluster IpIgLσ3C is shown. Conserved heptamer and nonamer sequences are *underlined* and number of nucleotides spacing them are indicated *above* the sequence. Inferred amino acids of the partial V and J genes are shown *below* and numbers of nucleotides are on the *right*. GenBank accession number for IpIgLσ 3C (EU872024) and IpIgLσ 5A (EU872023)

annotation was performed on bacterial artificial chromosomes (BACs) from two different zebrafish libraries. However, in both studies, only two Vσ families were identified and genes from both families were associated with each of the two Cσ genes. Again, all the gene segments were in the same transcriptional orientation. Altogether, five teleost Vσ subgroups based on sequences sharing more than 75% nucleotide identity (Giudicelli and Lefranc 1999) could be identified from pairwise alignments of IGSV nucelotide sequences from catfish, carp, Atlantic cod, rainbow trout, and zebrafish (sFig. 4). The largest subgroup is represented in catfish, carp, rainbow trout, and zebrafish, while another subgroup is currently only found in carp and zebrafish and two Vσ subgroups are only present in the rainbow trout. Also, the five Atlantic cod Vσ sequences, which were identified from BLAST searches of ESTs, formed one single subgroup not represented in the other species.

Catfish and Atlantic cod IgL λ chains

RT-PCR was also used to clone and sequence catfish Igλ cDNAs. As above, forward V and reverse C region primers were designed based upon the identified catfish Igλ EST (CBPO4528.b1). Three different-sized Igλ products were amplified by RT-PCR and verified by sequencing. The longest was 916 bp and consisted of a V gene, 240-bp intron, followed by a J segment spliced to a C gene. Similarly, another product of 702 bp, was the result of mispriming by the V primer and consisted of the 3′ end of the V gene, intron, J spliced to C. As with mammalian Vλ and Jλ genes, the catfish Vλ RSS consisted of a heptamer and nonamer separated by a 23-bp intron and the Jλ heptamer and nonamer separated by a 12-bp intron. The smallest PCR product, 646 bp was a rearranged Igλ cDNA and based upon this sequence, additional 5′-RACE primers were made and used to obtain full-length catfish Igλ transcripts. The representative full-length catfish Igλ tran-

script consists of 696 nucleotides and encodes a 232-amino-acid polypeptide with a predicted molecular weight of 22.5 kDa. The cDNA encodes a typical leader sequence, followed by V, J, and C region. Both the Vλ and Cλ region sequences contain conserved cysteine residues that form the intrachain disulfide bonds and the conserved cysteine (CYS 126) in the constant region that forms the interchain disulfide bond with the IgH chain (Fig. 3a). Similarly, the

a

```
     IGLV
            15      *27     38 41              55 66           84    89    96 100  *    111
     .........|.........|....... |...|..............|...|................|.....|.....|..|..........|...
Ipλ:   VLTQEKVISVQAGQNVKILCSPSTG---SUGITUYQQK-PGKAP--TFLLYDS-TRA--SGLPSRFTYSESGS--QEYLHINGVEAEDEAVYYCVC-HNCEGS-
Omλ:   ......SL..NL.D.L..S.AV.DSS-QTUK.S..R..-A.GG.--S...A.T-....-A...N......P..GYT.....EIT......I.A.VGG.GA.V
Gmλ:   ......TL..NR.D....S.TETDSS-SNYVL...Q..-S.SP.--KY..TTTGS..-..I.....G.GTKNDKT...L...IQD....T...A.VG-.GAN-
CpII:  ....PGS..SSP.KT.T.T.TM.G.TIS.YWAS..W..-.DS...-V.VUSE.DRM.--..I.N..AG.VDS.SNKMH.T.TN.QS..ATD...AAAA--SR.-
X1III: SI..PVSE..KL.ET.R.S.TL.GASISGYHVN.....-A.NR.RYLLRF.SDSNKHQGD.V.D..SG.KDSPNNIG..T.K.ALL..D.D...ATW.--AS.-
Acλ:   TV..PAST..SL.NTIQ.S..R.GSGUSNY-VH.I...-S.EVH--RYVHGNGYN.G---P.I.N...ATA..G--TGT.T.TN.Q.....D.....W..TDNV-
Ggλ:   A...PASV.ANP.ET...T..GGS.---.Y.--.....S..S...--VTVI.SNDK.P--.DI...SG.K...--TAT.T.T..Q......F.GSYD--SYV-
Hsλ:   M...PHSV.ESP.KT.T.S.TR.S.SIVTNYVH....R-..SL.--.TVI.EDNQ.P--...V.D..SG.IDS.SNSAS.T.S.LKT....D...QSYD--SD.-
       <     FR1        >< CDR1  ><      FR2       >< ><              FR3                    >< CDR3  >
                                                CDR2

     IGLJ
Ipλ:   -YTAVFGGGTEVSF-
Omλ:   GHGTK..Q....TI-
Gmλ:   --DTG.......IL-
CpII:  P.RSI..S..KLNL-
X1III: ---CI..G..QLTVL
Acλ:   ---W......HLTVT
Ggλ:   ---GI..A..TLTVL
Hsλ:   --CY...T..K.TVL

     IGCL
           1    10   15     * 26       39    45    77    84    85 89   96        *     117       ↓
     .....|.......|....|..............|.........|.....|......|.....|12344321|...|......|.....|..........|..........
Ipλ:   AIP-SPPSLVLLAPSPSLSSG-DEVRVVCLAQGFRPDGATLSWSDNGDAVAGAEVQTSSSERQSDGTFIQSSMLKLSPERWSSGRTYTCHLNHPALSAPLSQSASAEKCS---
Omλ:   AR.P........M..AQAPL..-.KTTL.......H....S.....D.GSLT.....KGE.Q..A...YTL..L.S.PST.....Q.FT...S.S..TN...R.VNNGQ..HFG
Gmλ:   ARSP.....E.MVATQPPVP.LGGPTL....K..H.A..A....ED.AS.R.E...AGVAQ..P..SYSL..V.L.PST..R..H.FT...VS.S...S...K.V.SQQ..L--
CpII:  GS.R.-.TVSV.P..SDQITAKNMATL...VS...V.GA.EIE.TVD.-S.R.NG.E...RIQQEA.N..SV..Y.T...ASD.N.HEL.S.VVK.ETQAN..QT.I.RSS.M--
X1III: TGDVKA..VSIFP..VEEIAT-KKAT...SLSD.T.R...VK.LVD.KDQTDS-..S.GLSK...NLYME..Y.S.TADQ.LRHE...S.KVS.QG--KEII.TLKRSE.V---
Acλ:   GQ.VAH.VVS.FP..QEELKSK.KATLA..MD..H.GVVQVE.LGD.RTISSG-.E.TKPVK.G.-KY.A..Y.T..RSE.EANKS...KVT.ES--KITEKVV.QLA..---
Ggλ:   GQ.KVA.TIT.FP..KEELNEATKATL...IND.Y.SPV.VD.VID.STRS.---ETTAPQ...NSQYMA..Y.S..ASD...HE....RVT.DG--TSITKTLKRSE.----
Hsλ:   GQ.KAN.TVT.FP..SEE.QA-NKATL...ISD.Y.GAV.VA.KAD.SP.KAG-.ETTKPSK..NNKYAA..Y.S.T..Q.K.HRS.S.QVT.DG--STVEKTVAPTE..---
       <     A     >< B      >    < C >    < D  >    <   E    >< F    >        < G  >
```

b

IGLJ

	Trout λ	Cod L2	Shark II	Xenopus III	Chicken λ	Human λ
Catfish λ	46.2	66.7	38.5	45.5	36.4	63.3
Trout λ		58.3	28.6	45.5	54.5	54.5
Cod L2			41.7	41.7	50.0	54.5
Shark II				54.5	54.5	54.5
Xenopus III					75.0	58.3
Chicken λ						58.3

IGLC

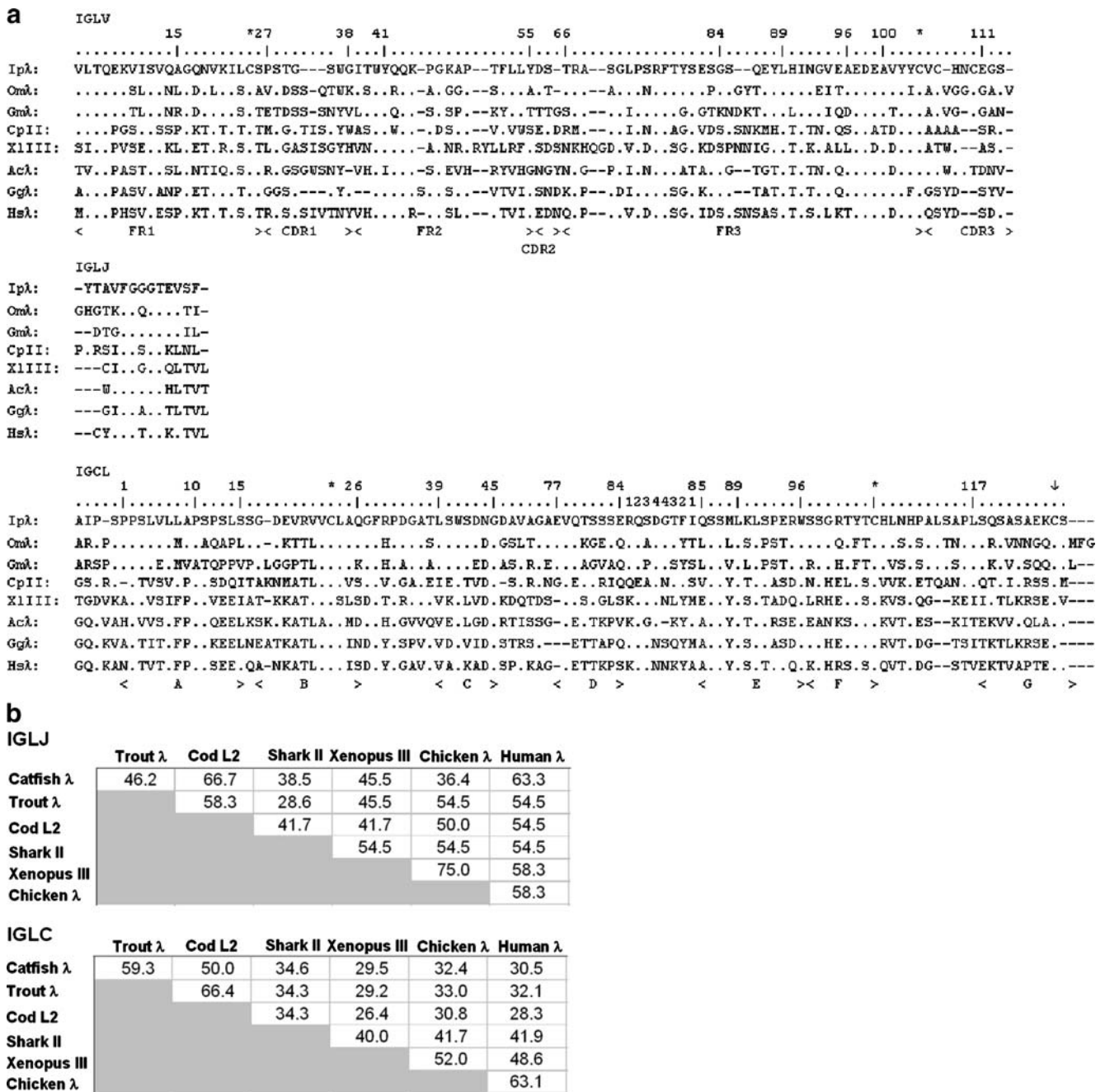|	Trout λ	Cod L2	Shark II	Xenopus III	Chicken λ	Human λ
Catfish λ	59.3	50.0	34.6	29.5	32.4	30.5
Trout λ		66.4	34.3	29.2	33.0	32.1
Cod L2			34.3	26.4	30.8	28.3
Shark II				40.0	41.7	41.9
Xenopus III					52.0	48.6
Chicken λ						63.1

Fig. 3 Igλ amino acid sequence comparisons. **a** Amino acid alignment of catfish Vλ, Jλ and Cλ regions with teleost Igλ, amphibian type III, chicken, and mammalian Igλ J and C regions. The conserved cysteine residues that form the intrachain disulfide bonds are labeled (*) and the CL region cysteine that forms the interchain disulfide bond with IgH chain is marked by (↓). Residues identical to the catfish sequence are shown as (.) and (–) represents gaps in the alignment The amino acids numbers are given above according to the standardized rules of the IMGT unique numbering (Lefranc et al. 2005). **b** Comparison of Igλ percent amino acid identities from pairwise alignments of Jλ and Cλ regions from different species. Abbreviations and Genbank accession numbers are: catfish (Ipλ, EU872025); rainbow trout (Omλ, V and J (BX861772) and C (CA257088)); Atlantic cod (Gmλ, AJ293808); sandbar shark (CpII, M81314), X. laevis type III (XlIII, L76575); Green anole (Acλ FG746006); chicken (Ggλ K00678); and human (Hsλ ABU90549)

Atlantic cod Igλ cDNA (originally termed L2) contains all the typical IGL features. It is 847 nucleotides in length and encodes a 240-amino-acid polypeptide with a predicted molecular of 23 kDa. The catfish and Atlantic cod Jλ genes are 13 and 12 amino acids in length, respectively, and like other Ig and TCR chains they encode a J-glycine bulge motif of FGxG (see sFig. 1b and c). As shown in Fig. 3a catfish Vλ also contains 2 additional cysteines at position 106 and 110 that are found conserved in rainbow trout and Atlantic cod Vλ sequences. Notably, database searches using the catfish Vλ predicted amino acid sequence identified the Atlantic cod Vλ sequence as the top related sequence (E-value, 3e−19) followed by various human, chicken, and horse Igλ V regions (E-values 9e−16 to 2e−15). Also, the CDR1 and CDR2 lengths were similar to the Vλ CDR1 and CDR2 lengths of most other species with CDR1 being longer than CDR2 (Criscitiello and Flajnik 2007). For example, the average human Vλ CDR1 length is nine amino acids and CDR2 length is four amino acids. For different teleost Vλ segments, the CDR1 length is 7-9 amino acids and CDR2 length is 2–3. We believe that CDR1 and CDR2 lengths, as suggested by Criscitiello and Flajnik 2007, combined with the RSS spacing supports the classification of this catfish IGL isotype as λ. The RSS spacer criteria has been widely used to categorize IGL isotypes and most recently it was used along with 21 other cladistic molecular markers to classify IGL chains from tetrapod species into three groups corresponding to the κ, λ, and σ isotypes (Das et al. 2008. In addition, it was recently used in combination with synteny to classify the *Xenopus tropicalis* Igλ locus (Qin et al. 2008).

In order to identify additional teleost Igλ sequences database searches using the Atlantic cod Igλ chain were performed and Igλ-like sequences from several salmonids (*Oncorhynchus nerka*, *Oncorhynchus tshawytscha*, *Salvelinus fontinalis*, *Salmo salar*), were identified and a rainbow trout Igλ sequence was included in the phylogenetic analyses. As with the catfish Igσ chains, the catfish Igλ sequences have the highest amino acid identities with the rainbow trout and Atlantic cod Vλs, Jλs and Cλs, at 55–64%, 46–67%, and 50–59%, respectively. Also, as expected, when the catfish Vλ, Jλ, and Cλ are compared to a representative V, J and C of sandbar shark Type II IgL, *Xenopus* Type III, chicken Igλ and human Igλ the amino acid identities are lower (Fig. 3b). Interestingly, Igλ transcripts were not found among the 1,609,630 zebrafish ESTs or the 32,000 carp ESTs.

Southern blots of catfish and Atlantic cod genomic DNA with Vλ and Cλ probes indicate that the Igλ locus in these two species is also of the cluster type since some of the restriction fragments hybridized with both V and C probes (Fig. 4a). Similar to the catfish Igσ locus, only two Igλ clusters have been identified in catfish. Using 5'-RACE and RT-PCR protocols three different functional Igλ VL were identified from an individual catfish. Two, IGLV1-1*02 and IGLV1-1*03, are 98% identical to each other at the nucleotide level and appear to be allelic while the third, IGLV1-2*01, with less identity (85%) is from the second Igλ cluster inferred by the Southern blot analyses (sFig. 5). The existence of two λ genomic clusters were confirmed by using two unique Vλ forward primers (O-669 and O-685) in combination with the reverse Cλ primer (O-671; sTable 1) to amplify the λ clusters from gynogenetic catfish G25 genomic DNA (Fig. 4b). One cluster IpIgλ 2A is 1,878 in length and contains single V, J, and C genes all in the same transcriptional orientation. They are designated IGLV1-1, IGLJ1, and IGLC1. The second cluster IpIgλ2E is 98% identical to IpIgλ2A at the nucleotide level and differs mainly in the V region (89% nt identity). There are also two nucleotide differences in the J sequences, resulting in amino acid changes and four nucleotide differences in the C region, one of which results in an amino acid change. Both clusters have the mammalian Igλ-type RSS and the RSS of the Atlantic cod and rainbow trout Vλ and Jλ are also of the Igλ-type. An alignment of the Igλ clusters of catfish, Atlantic cod and rainbow trout show them to be of similar size (Fig. 4c).

IgL phylogenetic analyses

It was recently proposed that vertebrate IGL chains can be classified into four distinct ancestral clans: one restricted to elasmobranchs (cartilaginous σ/type I), one in all vertebrates except teleost (λ/elasmobranch type II), one found in all ectothermic vertebrates (σ/teleost L2/elasmobranch type IV), and one in all vertebrates except birds (κ/elasmobranch type III/teleost L1 and L3; (Criscitiello and Flajnik 2007). However, as presented in this study, when teleost V regions from the previously isolated L1, L2 and L3 isotypes were compared with the newly identified catfish and Atlantic cod Vσ and Atlantic cod, rainbow trout and catfish Vλ sequences in phylogenetic analyses, three distinct clusters supported by high bootstraps values were formed (Fig. 5a). Importantly, while Vσ and Vλ sequences clustered in individual groups, the V sequences found associated with the L1 and L3 C regions were intermingled on a third branch consistent with the notion that L1/G, L3/F share a common origin and are of the κ class. In contrast, as expected, phylogenetic analyses of the different teleost IGLC sequences resulted in four individual clusters corresponding to the four teleost isotypes L1/G, σ/L2, L3/F, and λ. Moreover, the topology of the V phylogenetic tree did not change significantly when elasmobranch and tetrapod V sequences were included in the analyses; however, the bootstrap values dropped (Fig. 6a). Even so, the fact that teleost Vλs cluster with the shark type II,
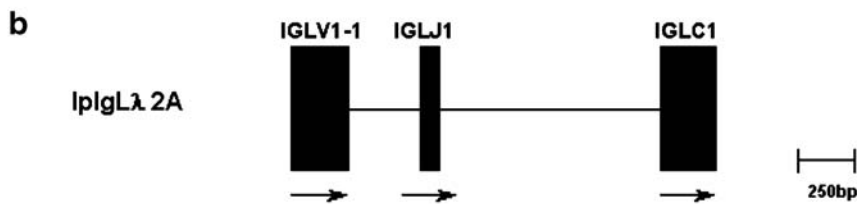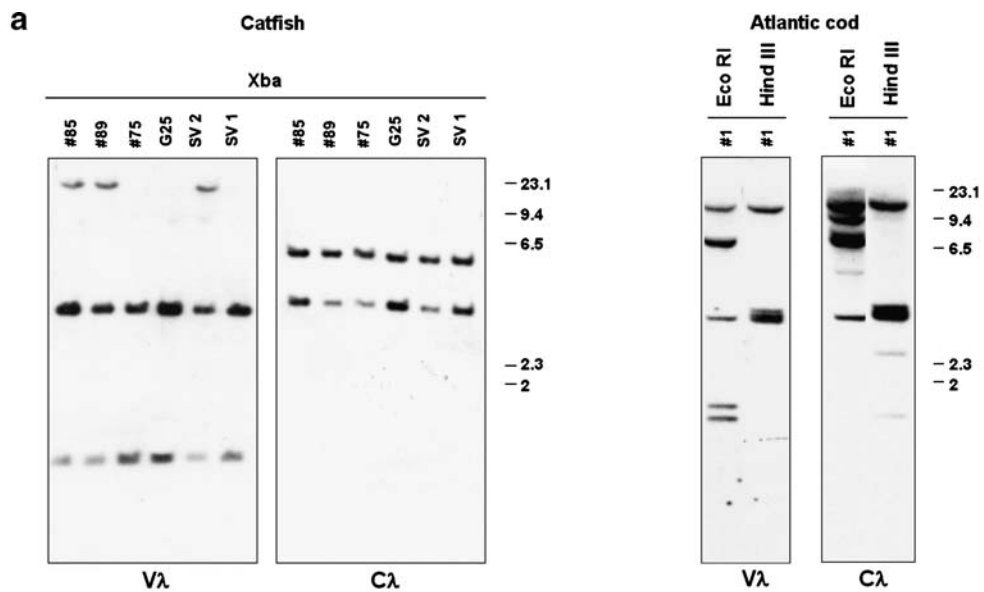
Xenopus Type III and the traditional Vλ sequences from chicken and mammals support the concept that these new teleost IGL isotypes are Igλ. Similarly, when the different vertebrate IGL C sequences were analyzed, the C of the teleost σ (type L2) chains clustered with shark and *Xenopus* Cσ sequences (Fig. 6b). Again, while not supported by high bootstrap values, the representative cartilaginous Cκ (type III) and Cλ (type II) sequences clustered with the mammalian Cκ sequences and the mammalian, *Xenopus*, and bird Cλ sequences, respectively. In contrast, the teleost Cλ as well as the κ linage C sequences (type L1/G and type L3/F), clustered in distinct groups separately from other vertebrate C sequences and appear to have diverged further from their mammalian, bird, *Xenopus*, and cartilaginous fish counterparts. Since phylogenetic analyses of IGL isotypes result in trees with low bootstrap values, Das et al. 2008 recently redefined the IGL isotypes based on 21 cladistic molecular markers. Briefly, when the molecular markers were applied to a data set of ~1,300 IGLV, 129 IGLJ and 78 IGLC sequences from 12 tetrapod species, three groups corresponding to Igκ, Igλ, and Igσ isotypes were identified (sTable 2). Here, when the same criteria were applied to the teleost IGL sequences four of the five markers identified in the IGLV region were shown to be valid (sFig. 6). For example, Igλ can be distinguished from Igκ and Igσ by the presence of a gap in amino acid position 7, Igκ has PHE at position 87 and Igσ has TYR, while Igλ does not exhibit PHE or TYR at this position. Also, from our analyses, Igσ can be distinguished by the presence of ILE at position 90 (highlighted in green in sFig. 6). However, the defined markers for IGLJ and IGLC were not so clear-cut when the teleost sequences were included in the analysis. The only valid IGLJ marker was that J sequences of the κ linage have a THR at position 117, while Jσ and Jλ sequences do not. As for the IGLC sequences, only two unique Igσ markers are observed: the absence of PHE/TYR/GLY at position 28 and PRO at position 30. Comparatively, Cλ sequences contained ARG / LYS at positions 84a combined with GLN at position 84b. Finally, the C regions of the κ isotypes, are distinguished by the presence of certain amino acid residue combinations at positions 13, 28, 81, 84a, 84g, 108, and 121. Whether these markers will be valid for a larger all-inclusive data set is not known. Nevertheless, together, our sequence and phylogenetic analyses demonstrate that teleosts possess four IgL isotypes. As previously suggested, two of these, type L1 and type L3 (which include catfish F and G) have diverged from the Igκ lineage and teleost type L2 IgL chains are of Igσ origin (Bengtén et al. 2000; Criscitiello and Flajnik 2007; Ota et al. 2000). More importantly, these findings support the prediction by Criscitiello et al. 2007 that λ light chains may be present in all vertebrate groups, but not necessarily in each species of each group.

Fig. 4 Genomic analysis of IpIgλ. **a** Representative Southern blot ▶ analysis of genomic DNA from one homozygous gynogenetic (G25) and five outbred catfish. DNA was digested with *Xba* I and hybridized with either a catfish Vλ or Cλ specific probe (*left panel*). In the right panel Atlantic cod genomic DNA was digested with either *Eco*R I, or *Hin*d III and hybridized with either an Atlantic cod Vλ or Cλ specific probe. Size markers in kilobase pairs are on the right. **b** Representative schematic of an IpIgL λ genomic cluster that was amplified using Vλ and Cλ specific primers on gDNA isolated from gynogenetic catfish G25. Cluster IpIgλ 2A contained a single VL and JL gene segment followed by a CL gene, all in the same transcriptional orientation. **c** Teleost IgL Vλ and Jλ genes utilize typical λ RSS. Nucleotide alignment from the 3′ end of a Vλ through the Jλ gene segment of a catfish, Atlantic cod, and rainbow trout Igλ genomic sequences are shown. The conserved heptamer and nonamer sequences are *underlined* and number of nucleotides spacing them are indicated *above* the sequence. Inferred amino acids of the partial Vλ and Jλ genes are shown below and numbers of nucleotides are indicated on the right. Accession numbers for IpIgLλ 2A (EU872025), rainbow trout Igλ (BX861772), and Atlantic cod Igλ (AJ293808)

Catfish Igσ and Igλ expression

At the message level functionally rearranged catfish Igσ transcripts were readily detected by RT-PCR in all catfish tissues analyzed, except for muscle by RT-PCR (Fig. 7a). Comparatively, two PCR products were consistently amplified using Igλ specific primers O-657F and O-650R (sTable 1). The larger product represented the 916-bp partially spliced unrearranged Igλ transcript and the smaller 646 bp product represented the functional rearranged catfish Igλ transcript. Both PCR products were observed in all tissues examined except brain, muscle and fin; however, the functional rearranged transcript product appeared to be more prevalent in spleen, head kidney (pronephros), trunk kidney (mesonephros), heart, and liver. As shown in Fig. 7b, message for catfish F and G light chains as well as Igσ and Igλ were detectable in freshly isolated PBL, as well as in surface (s) IgM⁺ cells, enriched by MACS-sorting. However, no G, or Igλ and negligible amounts of F message was detected in the sIgM⁻ cell population (IgM depleted), which suggest that IgM⁻ B cells preferentially express Igσ. Also, as with the catfish tissues, Igλ PCR products representing partially spliced unrearranged transcripts and rearranged transcripts were amplified from PBL and sIgM⁺ cells along with a truncated partially spliced unrearranged form due to mispriming of the V forward primer O-657F.

The initial anti-catfish F and G mAb characterization studies demonstrated that individually, these reagents react with different lymphocyte populations in catfish PBL and different immunoglobulin populations in catfish serum. Also, when they were used in combination, they immuno-precipitated >90% of purified catfish anti-DNP IgM (Lobb and Clem 1982; Lobb et al. 1984). Therefore, in order to determine if catfish PBL contained IgM⁺ cells that did not
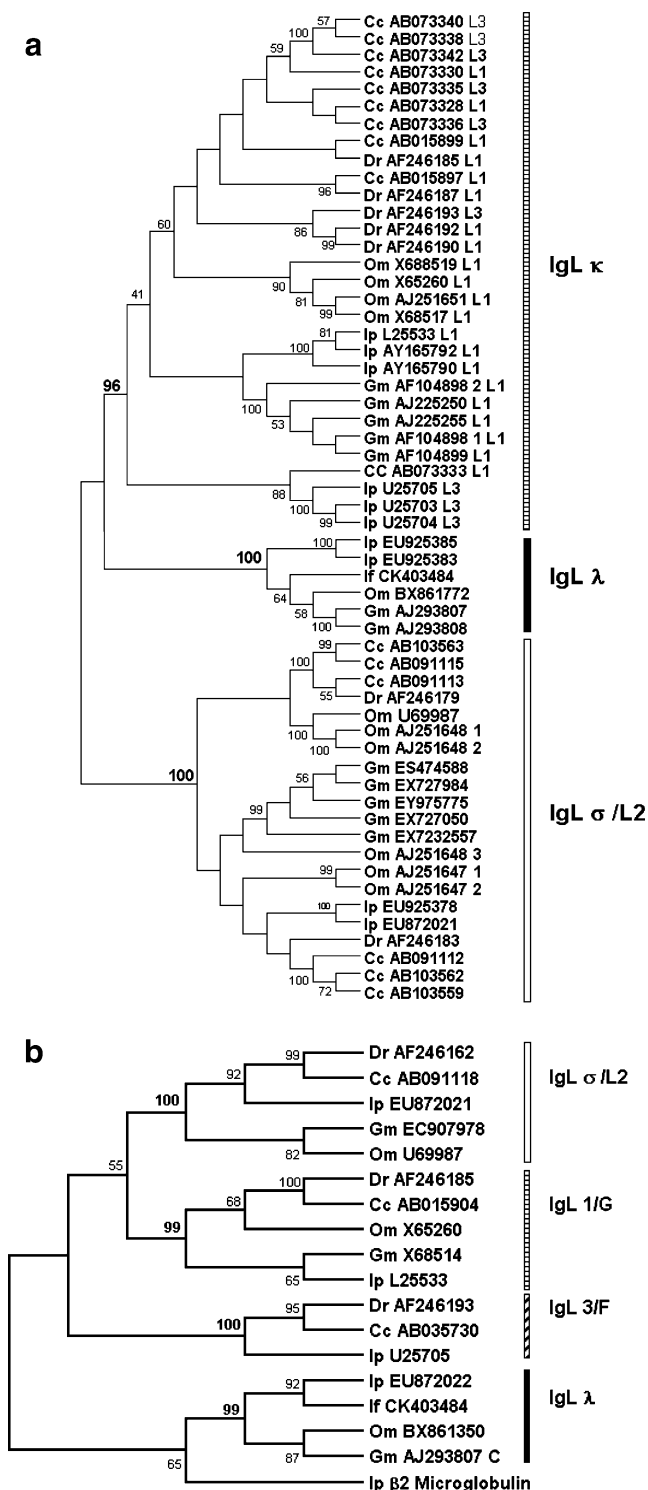
express the F or G isotypes, a negative sort with anti-F and anti-G mAbs was performed. After depleting PBL of F and G positive cells a small population of the cells was still sIgM[+] (~2%; Fig. 8a). As analyzed by flow cytometry, cells displaying the IgM[+]/F^{−}/G[−] phenotype are small, agranular,

and fall within the traditionally defined catfish lymphocyte gate (Fig. 8b). At the message level the F^{−}/G[−] population expressed message for Igλ and Igσ, as well as membrane IgM. Currently, it is unknown whether the F message present in the F^{−}/G[−] cell population is due to transcription of

a

- Cc AB073340 L3
- Cc AB073338 L3
- Cc AB073342 L3
- Cc AB073330 L1
- Cc AB073335 L3
- Cc AB073328 L1
- Cc AB073336 L3
- Cc AB015899 L1
- Dr AF246185 L1
- Cc AB015897 L1
- Dr AF246187 L1
- Dr AF246193 L3
- Dr AF246192 L1
- Dr AF246190 L1
- Om X688519 L1
- Om X65260 L1
- Om AJ251651 L1
- Om X68517 L1
- Ip L25533 L1
- Ip AY165792 L1
- Ip AY165790 L1
- Gm AF104898 2 L1
- Gm AJ225250 L1
- Gm AJ225255 L1
- Gm AF104898 1 L1
- Gm AF104899 L1
- CC AB073333 L1
- Ip U25705 L3
- Ip U25703 L3
- Ip U25704 L3

IgL κ

- Ip EU925385
- Ip EU925383
- If CK403484
- Om BX861772
- Gm AJ293807
- Gm AJ293808

IgL λ

- Cc AB103563
- Cc AB091115
- Cc AB091113
- Dr AF246179
- Om U69987
- Om AJ251648 1
- Om AJ251648 2
- Gm ES474588
- Gm EX727984
- Gm EY975775
- Gm EX727050
- Gm EX7232557
- Om AJ251648 3
- Om AJ251647 1
- Om AJ251647 2
- Ip EU925378
- Ip EU872021
- Dr AF246183
- Cc AB091112
- Cc AB103562
- Cc AB103559

IgL σ /L2

b

- Dr AF246162
- Cc AB091118
- Ip EU872021
- Gm EC907978
- Om U69987

IgL σ /L2

- Dr AF246185
- Cc AB015904
- Om X65260
- Gm X68514
- Ip L25533

IgL 1/G

- Dr AF246193
- Cc AB035730
- Ip U25705

IgL 3/F

- Ip EU872022
- If CK403484
- Om BX861350
- Gm AJ293807 C

IgL λ

- Ip β2 Microglobulin

Fig. 5 Phylogenetic analyses of teleost IGL **a** V sequences and **b** C sequences. The neighborhood-joining trees with pairwise gap deletions were drawn using MEGA4 with 1,000 bootstrap replications. Channel catfish β2-microglobulin was used as an outgroup and clusters of sequences are marked by blocks. All Genbank accession numbers are listed in the figure, except for catfish B2-microglobulin (AAC67230). Species abbreviations are: *Cc* carp, *Dr* zebrafish, *Om* rainbow trout, *Ip* channel catfish, *If* blue catfish, *Gm* Atlantic cod

logical assumption is that the apparent low expression levels of the Igσ and Igλ isotypes are because of their limited repertoire, of two to four V genes. Currently, it is not known if the Igσ$^+$ and Igλ$^+$ B cell populations can be expanded by antigen driven selection and/or if they represent populations with specialized functions. For example, it was previously shown that the ratio of catfish F and G chains changes during an immune response to dinitrophenyl (DNP): in pre-immune serum the ratio of F:G was 60:40, 1 to 2 weeks after primary immunization the F:G ratio was 80:20, and 3 weeks to 1 year later the ratio was 90:10, indicating a preferential usage of the F isotype in the anti-DNP response (Lobb 1986). However, while it is tempting to speculate that teleost Igλ or Igσ chains may have a specialized function, positive proof will only come after anti-teleost Igλ and Igσ chain antibodies become available, which will allow these respective cell populations to be isolated and functional studies to be performed.

The presence of four IgL isotypes in teleosts also raises questions concerning regulation of their expression. For example, with IgL loci organized in multi-copy clusters, how is allelic exclusion possible and do the different isotypes undergo gene rearrangements in a preferential order? More than two decades ago, two models for

Fig. 6 Phylogenetic analyses of vertebrate IGL **a** V sequences and **b** C sequences. The neighborhood-joining trees with pairwise gap deletions were drawn using MEGA4 with 1,000 bootstrap replications Channel catfish β2-microglobulin was used as an outgroup. Clusters of sequences are marked by blocks and GenBank accession numbers are: human λ (ABU90549); sheep λ (AAB94914); chicken λ (K00678); duck λ (M25726); dog λ (ABY55585); mouse λ (J00587); catfish λ (EU872022); Atlantic cod L2 (λ; CAC03754); rainbow trout λ (BX861350); *X. laevis* III (L76579); ratfish II (L25549); skate II (L25566); horn shark II (L25560); sandbar shark II (M81314); salmon L3 (AF406956); fugu L1 (AB126061); carp L1b (AB073332); human κ (AAH73764); catfish F-type (U25705); *X. laevis* ρ (V and J (L15570), C (AAA49880)); mouse κ (V and J (M34739), C (CAA47650); horse κ (X75612); zebrafish L3 (AB246193); carp L3 (AB073335); zebrafish L1 (AF246185); catfish G-type (L25533); rainbow trout L1 (X65260); Salmon L1 (F273012); horn shark III (L25561); nurse shark NS4 (GSU15144); nurse shark σ (EF114759); dogfish shark σ (CX662702); *X. laevis* σ (S78544); rainbow trout L2 (AAB41310); carp L2 (AB091113); fugu L2 (DQ471453); catfish σ (EU872021); zebrafish L2 (AF246183); horn shark σ (EF114760); Atlantic cod L1 (X68515), and catfish β2-microglobulin (AAC67230)
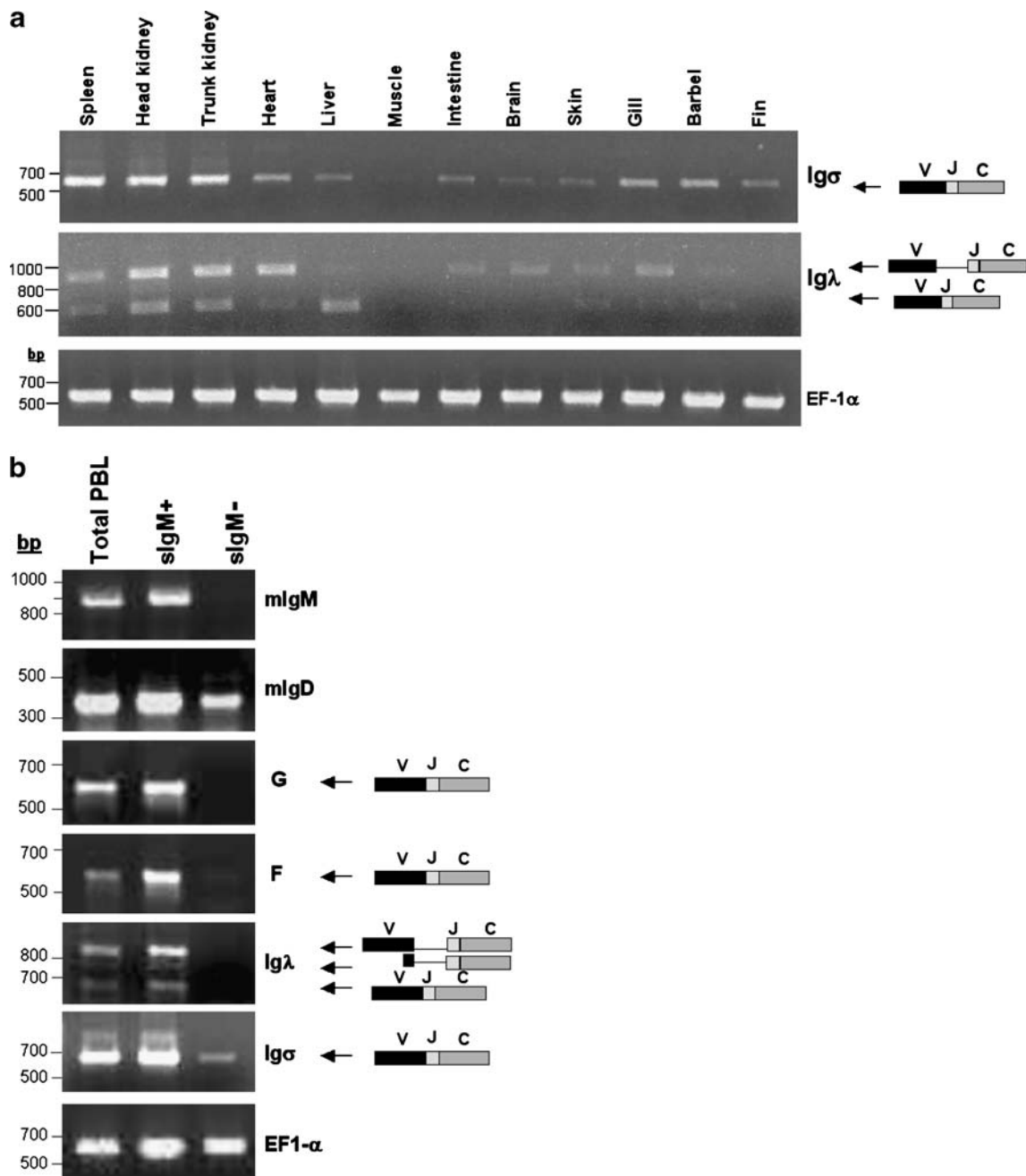
nonproductively rearranged F genes. These results combined with the RT-PCR data showing that a sIgM$^-$ cDNA pool expresses message for IgD and Igσ, but not Igλ, suggest Igσ potentially associates with both IgM and the IgD molecules, and that a small IgM$^+$ B cell population within catfish PBL expresses the Igλ isotype. Thus, a
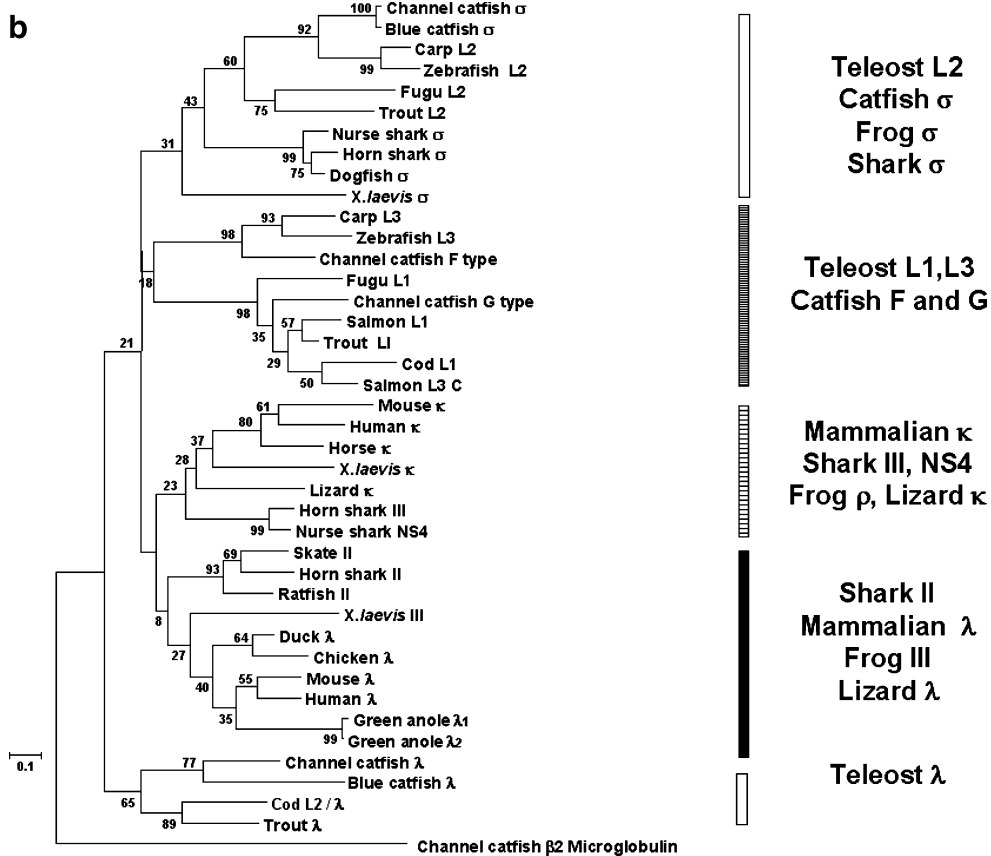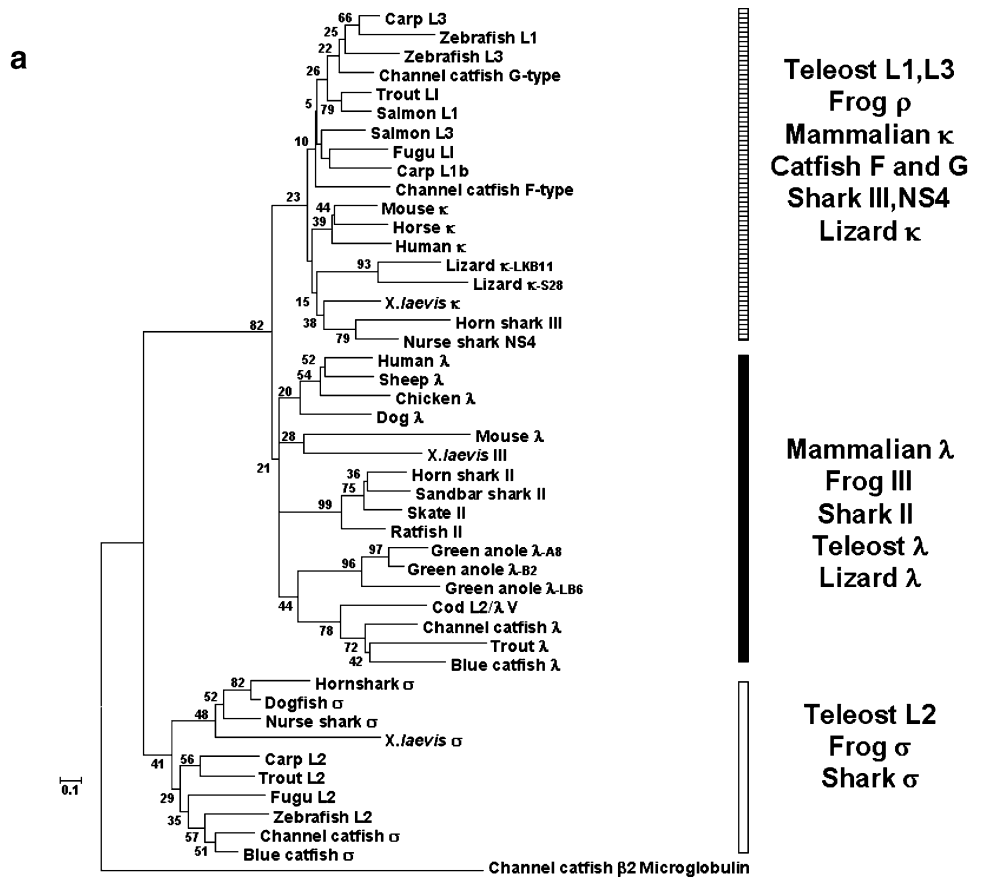
Fig. 7 RT-PCR analyses of Igλ and Igσ expression in catfish tissues and sorted PBL. **a** Total RNA was obtained from various tissues and RT-PCR was performed using VL and CL specific primers for catfish Igσ, Igλ and EF1-α. Schematics of each IgL transcript are shown next to their respective panels. A partially spliced transcript and a fully rearranged transcript was amplified using the Vλ and Cλ primers. **b** Total RNA was obtained from unfractionated PBL, MACS sorted sIgM⁺ PBL, and MACS sorted sIgM⁻ PBL from an individual catfish. RT-PCR was performed using primers specific for catfish mIgM, mIgD, G, F, Igλ, Igσ, and EF-1α. Schematics of the IGL transcripts are next to their respective panels. Base pair markers are indicated on the *left*. The resulting PCR products were verified by sequencing

regulation of mammalian Igκ and Igλ gene rearrangements were proposed, the stochastic model and the regulated/ordered model (reviewed in Mostoslavsky et al. 2004). In the stochastic model, it was assumed that both alleles of the Igκ and Igλ loci were accessible for gene rearrangements simultaneously and that due to junctional diversity, productive rearrangements on more than one allele was unlikely

(Coleclough et al. 1981). Also, it was predicted that Igκ genes were more likely to produce successful rearrangements due to the greater number of available V and J sequences and the quality of the Igκ RSS (Arakawa et al. 1996; Ramsden and Wu 1991). In contrast, the regulated/ordered model proposed that Igκ and Igλ undergo gene rearrangements through a sequential order of events, where
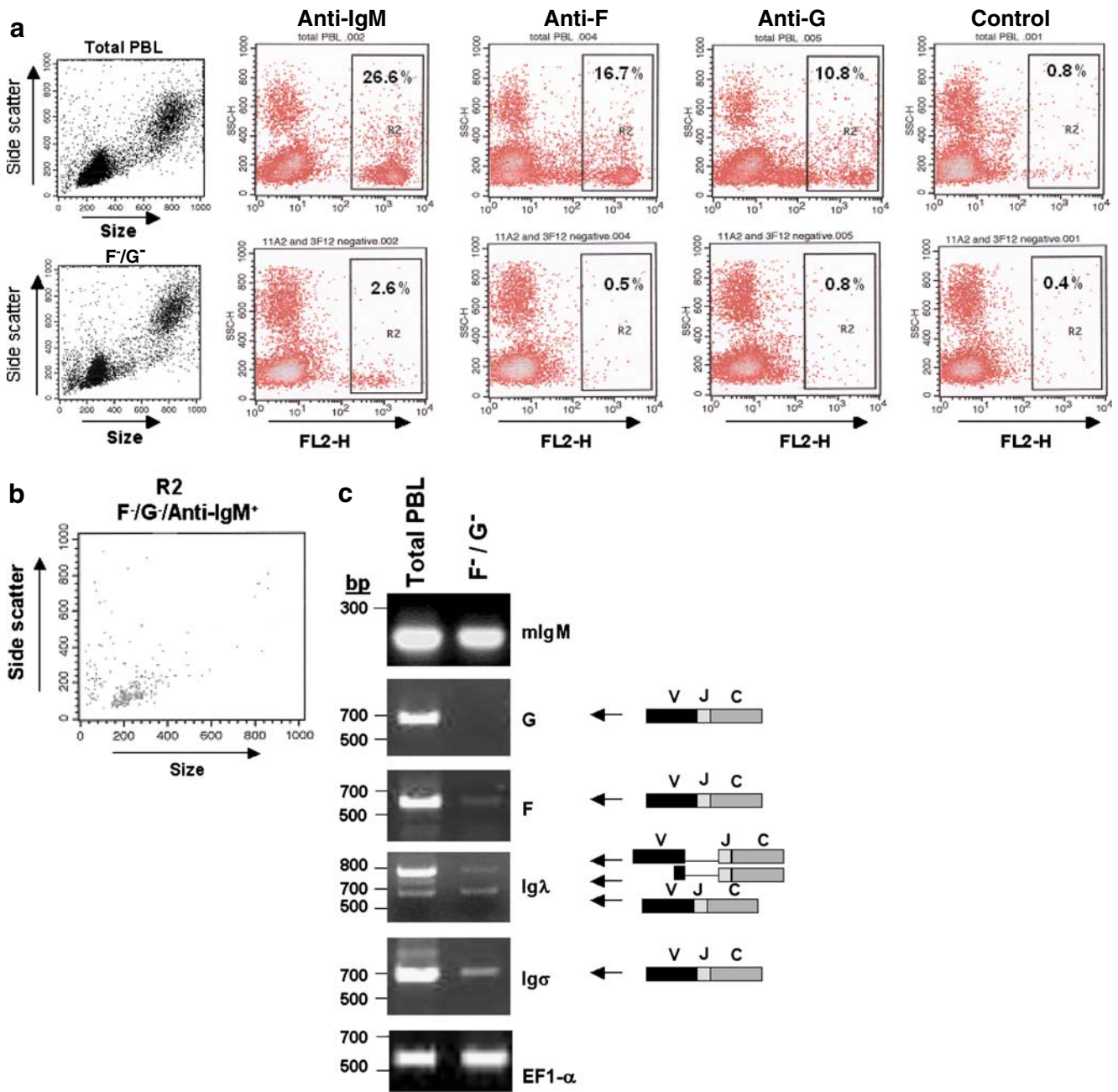
Fig. 8 IgM and IgL expression in catfish PBL. **a** Freshly isolated PBL and PBL depleted of surface F and G positive cells (F⁻/G⁻) by MACS were analyzed by flow cytometry. Aliquots from the unsorted (*top panel*) and sorted (*bottom panel*) populations were stained with anti-catfish IgM 9E1, anti-catfish F, and anti-catfish G mAbs. Anti-rainbow trout IgM 1.14 was the isotype control. The total and negatively sorted PBL populations were from the same fish. **b** IgM positive cells (~2%) from the depleted population (F⁻/G⁻) were gated and examined side and forward scatter. **c** Total RNA was obtained from PBL and the F and G depleted fraction (F⁻/G⁻) and RT-PCR was performed using primers specific for IgM, G, F, Igλ, Igσ, and EF1-α. Schematics of the IgL transcripts are next to their respective panels. Base pair size markers are indicated on the *left*. The resulting PCR products were verified by sequencing

changes in the chromatin structure first occurred on one of the Igκ alleles allowing it to attempt V–J recombination, while the other Igκ allele and the Igλ alleles remained inaccessible (Storb et al. 1981). More recently, support for the regulated/ordered model comes from the identification of several B cell developmentally controlled mechanisms,

including remodeling of the chromatin structure, demethylation of distinct alleles, and selective accessibility of recombination machinery through differential expression of transcription factors (Asenbauer et al. 1999; and reviewed in Corcoran 2005; Geier and Schlissel 2006). However, this process is not absolutely strict, while Igλ

expression is upregulated after deletion of the Igκ locus, Igλ rearrangement is not dependent on this deletion and can precede rearrangement of Igκ (Nadel et al. 1990; Oberdoerffer et al. 2003; Zou et al. 1993). That Igκ and Igλ rearrangement can occur independently is also supported by the presence of individual B cells expressing both functionally rearranged Igκ and Igλ chains (Sirac et al. 2006; van der Burg et al. 2001). The regulated/ordered model also implies that different subsets of B cells can preferentially use one or another IGL isotype, since different developmental programs could apply to different B cell subsets. Examples of preferential usage of IGL chains have been observed in *Xenopus*, where the majority IgY is associated with Igκ chains and almost never with Igσ. In contrast, Igσ chains are found associated with IgM and IgX (Hsu et al. 1991; Lee et al. 2000). Another example of preferential IGL chain usage occurs in humans, where a population of IgM^-/IgD^+ B cells present in respiratory mucosa expresses almost exclusively Igλ chains (Chen et al. 2008; Liu et al. 1996). To date, regulation of IGL chain rearrangements has not been studied in any non-mammalian vertebrate. Therefore, based on the findings presented here and as has been proposed for shark (Fleurant et al. 2004), it is tempting to speculate that the expression in catfish is stochastically driven since the IgF and IgG isotype, which are encoded by loci that combined contain 60–80 V segments combined, constitute ≥90 of the expressed IGL found in PBL and in serum (Ghaffari and Lobb 1993, 1997; Lobb and Clem 1982; Lobb et al. 1984). In comparison, the Igσ and Igλ loci possess two to four V segments, and these isotypes appear to be expressed only in 2–10% of PBL. However, based upon what is now known about IGL regulation in mice and humans, the regulation of the teleost light-chain rearrangements and expression is likely more complex.

In summary, catfish Igσ and Atlantic cod, catfish and rainbow trout Igλ cDNAs and genes have been identified. While catfish Igσ clearly shows strong homology with previously identified Igσ sequences, the teleost Igλ sequences are less conserved. Nevertheless, together with the "Igλ-type" RSS, the conserved CDR lengths, and phylogenetic analyses support the teleost Igλ classification. In contrast to the teleost Igκ type L1 and type L3 loci, the teleost Igσ and Igλ loci contain only a few VL genes suggesting that Igσ and Igλ do not contribute greatly to the overall available repertoire. Even so, studies of preferential IGL chain usage in catfish IgM^+/IgD^+, IgM^+/IgD^- and IgM^-/IgD^+ B cell populations are currently being pursued and it may be that these isotypes provide unique paratopes by combining with certain IGHV chains. Development of mAbs will allow for the functional characterization of catfish B cell $Ig\sigma^+$ and $Ig\lambda^+$ populations, as well as determinations concerning if preferential Igσ/Igλ chain usage occurs between IgM and IgD isotypes and if Igσ/Igλ expression can be expanded by antigen stimulation.

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