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Molecular cloning and genomic characterization of novel Leptin-like genes in salmonids provide new insight into the evolution of the Leptin gene family

Anna R. Angotzi *, Sigurd O. Stefansson, Tom O. Nilsen, Raja M. Rathore, Ivar Rønnestad

Department of Biology, University of Bergen, Thormolensgate 55, Bergen 5020, Norway

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ABSTRACT

In the current study we describe the identification of novel leptin B homologous gene/s in the four salmonid species Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*). Homology modeling of *Salmo salar* (Ss) LepB1/B2 suggests that the protein satisfies parameters as long-chain four helical cytokine family and that the basic structural pattern of the protein follows that of human leptin (Zhang et al., 1997). Importantly, the docking studies suggested the SsLepB has binding affinity to the AA residues that identify the *leptin binding* and *FNIII* domains of the SsLep receptor (Rønnestad et al., 2010). Phylogenetic analyses support that LepB paralogs have most probably originated by 4R whole genome duplication (WGD) before speciation of the salmonid lineages. LepB1 and LepB2 genes are both present in the two closest relatives, the Atlantic salmon and the brown trout, while rainbow trout and charr have only preserved the long LepB1 variant in their genome. We have defined the sites of SsLepB mRNA expression at key life stages in Atlantic salmon and found that SsLepB1 and SsLepB2, although to different extent, were expressed in redundant and mostly complementary fashion in brain and gills throughout the lifecycle, suggesting that this pair of paralogs is likely undergoing early stages of subfunctionalization. Furthermore, we have quantified the expression profiles of SsLepB genes and of other two recently duplicated salmon leptins (SsLepA1, SsLepA2) during early development and show evidence that in fish, as in mammals and amphibians, leptin could play important roles in growth and development. This study provides an essential groundwork to further elucidate structural and functional evolution of this important hormone in salmonids as well as in other teleosts.

1. Introduction

In mammals, the leptin/*ob* (obese) gene codes for a protein hormone involved in a diverse range of physiological functions including appetite and body mass control (Pelleymounter et al., 1995; Banks, 2004; Neary et al., 2004), energy expenditure (Myers and Simerly, 2010), reproduction (Moschos et al., 2002; Zieba et al., 2005), neurogenesis (Bouret and Simerly, 2007), growth and development (Steppan et al., 2000; Tamashiro and Moran, 2010). Leptin actions are mostly exerted in the hypothalamus, via its ubiquitously expressed receptor (Ob-R) (Frühbeck, 2006).

The leptin gene was long thought to be specific to mammals until the recognition of the first fish leptin-like gene in the pufferfish *Takifugu rubripes*, by Kurokawa et al. (2005). This important discovery was then followed by the cloning of putative leptin orthologs in common carp (*Cyprinus carpio*), tiger salamander (*Ambyostoma tigrinum*) and African frog (*Xenopus laevis*) in the same year (Huising et al., 2006; Boswell et al., 2006; Crespi and Denver, 2006). Initial comparative studies revealed that amino acid conservation among vertebrate leptin orthologues is low, with only 13% sequence identity between pufferfish and human (Kurokawa et al., 2005). Despite the significant divergence in their primary structure, three-dimensional homology modeling predicts strong conservation of leptin protein tertiary structure from fish to mammals (Kurokawa et al., 2005; Gorissen et al., 2009; Rønnestad et al., 2010). Leptin 3D structure consists of four alpha helices that exhibit an up-up-down-down folding pattern arranged in a left-hand twisted bundle. All the helices seem to be structurally similar and run anti parallel to each other. The protein also exhibits a short strand segment and two long random-coil loops (Kline et al., 1997).

Phylogenic reconstructions based on extant fish leptin genes have proven challenging due to low amino acid conservation coupled with the acquired complexity of fish genomes following whole genome duplications (WGD) at different time points of their evolution. Unlike most vertebrates that appear to have only a single copy of leptin, duplicated leptin genes have been identified in several teleosts. Phylogenetic and syntenic studies suggest that

* Corresponding author. Fax: +47 5558 4450.
E-mail address: rita.angotzi@bio.uib.no (A.R. Angotzi).

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LepA and LepB paralogs in zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and orange-spotted grouper (*Epinephelus coioides*) are ancestral leptin duplicates (Kurokawa and Murashita, 2009; Gorissen et al., 2009; Zhang et al., 2013), as result of a WGD (3R duplication) in the last common ancestor of teleosts about 350 MYA (Meyer and Van de Peer, 2005). The few reports available on fish LepB genes show quite broad tissue mRNA distribution (brain, eye, ovary, skin, gills, muscles, liver, gut, etc.) in medaka and zebrafish (Gorissen et al., 2009; Kurokawa and Murashita, 2009) while in orange-spotted grouper mRNA transcripts were only detected in several brain regions and weakly in ovary (Zhang et al., 2013). Earlier findings have shown additional recent duplicates in Atlantic salmon (*Salmo salar*) which possess two copies of LepA (Rønnestad et al., 2010), most probably generated by lineage specific WGD, 25–100 MYA (4R) Allendorf and Thorgaard, 1984. Therefore, the 3R duplication event and the (pseudo) tetraploid state of salmonids following the 4R duplication (Danzmann et al., 2008) suggest that one or more lepB paralogs could exist in this monophyletic lineage. To gain further understanding of fish leptin genes related to genome duplications, we have identified cloned and characterized novel LepB genes from several salmonid species and used these data to infer phylogenetic relationships within the leptin family. In this work we further examine the potential of SsLepB1 and SsLepB2 loci to generate functional transcripts in several tissues of Atlantic salmon and show some interesting variations of their mRNA levels in gills and brain. Furthermore, as only one study has so far led to successful determination of leptin expression during early fish development (Liu et al., 2012), we investigate ontogeny of novel and known salmon leptin genes (LepA1, LepA2, LepB1/B2) by qPCR approach and suggest that LepA1 may play growing roles during progression of development in Atlantic salmon.

2. Materials and methods

2.1. Fish material

Eggs of Atlantic salmon of AquaGen strain were collected at Marine Harvest hatchery at Tveitevågen (Norway) at different developmental stages and from the same aquaculture brood stock. The relative age of salmon embryos was expressed in day degrees (dd, number of days multiplied by degree Celsius). Sampling and embryo dissection were performed at stages 168, 215, 262 and 332 dd.

Atlantic salmon parr (AquaGen strain; mean weight 31,8 gram, length 14,2 cm), post-smolts (mean weight 179,2 g, length 24,8 cm) and maturing Atlantic salmon (1-sea winter old, grilse) (mean weight 1435 g, length 48 cm) were obtained from the Aquatic Laboratory of the Bergen High Technology Center. Juvenile Atlantic salmon were collected from the Norwegian rivers of Alta, Imsa, Driva and Vosso. Brown trout (*Salmo trutta*) were collected from the river Driva, while juvenile Arctic charr (*Salvelinus alpinus)* and rainbow trout (*Oncorhynchus mykiss)* were obtained from the Matre research facility, Institute of Marine Research.

Samples of juvenile Atlantic salmon from a landlocked population, *Bleke*, were obtained from eggs collected from wild broodstock from lake Byglandsfjord, 200 m above present day sea level in South-central Norway.

2.2. Identification and cloning of SsLep B duplicates

We identified Atlantic salmon LeptinB-like sequence fragments in scaffolds Gnl|ti|2272277502 and gnl|ti|2264063988 from the cod (*Gadus morhua*) genome project *_Salmo salar* database_ (http://www.codgenome.no/blast/blast_new.php), using the TBLASTN algorithm and the coding sequence (cds) of Zebrafish (*Danio rerio*) LepB as query sequence (acc.No. AM901009). Genomic DNA was extracted from gills of an individual sample by DNeasy tissue Kit (Qiagen, Austin, TX, USA) and PCR amplified using a set of primers flanking the single predicted intron, with the reverse primer including the predicted stop codon at the C-terminal of the gene (http://genes.mit.edu/GENSCAN.html), (g1fw/g1rv primers, see Table 1). PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pGEMTeasy vector (Promega, Madison, WI, USA). The inserts were sequenced at the University of Bergen Sequencing Facility.

Based on intron length variability, two SsLepB-like variants were identified and subsequently extended toward their 5′UTRs by a genome walker library prepared from one individual Atlantic salmon (Genome Walker Universal Kit Clontech; Mountain View, CA, USA; g2rv/g3rv primers, Table 1).

cDNAs of SsLepB1 and SsLepB2 genes were cloned from brain and gill tissue. Total RNA was extracted with TRIzol reagent (Invitrogen, St. Louis, MI, USA) according to the manufacturer's protocol, cDNA was synthesized from 3 μg of total RNA using oligo(dT) 12–18 primer and Superscript III (Invitrogen, Carlsbad, CA, USA) by standard procedures. Primer PCR design was based on predicted coding sequences and 5′UTR regions from genomic clones. 5′UTR regions were further confirmed by cDNA RACE libraries prepared from brain and gill mRNA according to the manufacturer's protocol (Marathon™ cDNA Amplification Kit, Clontech; Mountain View, CA, USA) g6rv/g7rv primers for primary and nested 5′RACE, respectively, Table 1).

2.3. Testing SsLepB polymorphism

Potential allelic polymorphism versus duplicated loci between the two SsLepB variants was tested by running an exon-primed intron-crossing (EPIC)-PCR approach Wang et al., 2005. gDNA was extracted from gill tissue of 14 Atlantic salmon samples per river/strain analyzed (Alta, Imsa, Driva and Vosso), and of landlocked *Bleke* salmon samples (n = 10). To detect both intron variants, EPIC-PCR primer pairs were designed in their identical regions spanning the full genomic length of SsLepB1 and SsLepB2 (primers: g4fw/g4rv, Table 1). Thermal PCR conditions were as follows: 5 min at 94 °C, 30 cycles of 94 °C for 25 s, 58 °C for 30 s, 68 °C for 90 s and extension at 68 °C for 7 min.

2.4. Cloning and genomic characterization of LepB genes in other salmonid species

Identification of LepB-like duplicate numbers in arctic charr, rainbow trout and brown trout was based on a PCR homology-cloning approach using EPIC-PCR primers designed on SsLepB genomic sequences and performed with low stringency PCR conditions (5 min at 94 °C, 30 cycles of 94 °C for 25 s, 55 °C for 30 s, 68 °C for 90 s and extension at 68 °C for 7 min).

2.5. Protein prediction modeling

The 3D model for SsLepB1 was created by using Protein Homology/analogY Recognition Engine V 2.0 (PHYRE^2 server) and viewed in Rasmol. For the homology modeling human leptin was used as template (PDB-ID d1XA8), having score of 23% identity, 87% sequence coverage and 100% confidence. This satisfied the additional constraint of possible disulphide bond between the cysteine residues in the studied protein sequence. Docking with Atlantic salmon leptin receptor (SsLepR) was performed using HADDOCK (http://haddock.science.uu.nl/services/HADDOCK/haddock.php). The analysis of potential interacting residues in docked structures was done with LigPlot+ (http://www.ebi.ac.uk/thornton-srv/software/LigPlus/). SsLepR pdb file was generated from the SsLepR

Table 1
Nucleotide sequences of primers used in 5′ RACE PCR, RT-PCR, genome Walker and qPCR assays for Ss LepB1, SsLepB2, SsLepA1 and sSsLepA2, and the internal reference gene EF1α.

Primer ID	Primer sequences*	Applications
g1(Fw)	GTGGTCCACATAAAGAATTATCTGACCG	Cloning SsLepB1-B2
g1(Rv)	TCTCAGCAGATCTTAAGCTTGTCCAGGTT	Cloning SsLepB1-B2
g2(Rv)	ATTCTACTCATGTCCGTTGACCACAGA	Genome-Walker PCR
g3(Rv)	GTAGACTTTGCATGATACTTCTTTTACGTCG	
g4(Fw)	GCATGGTATTTGTATTGTTCTCCAG	EPIC-PCR/RT-PCR LepB1-B2
g4(Rv)	TCTCAGCAGATCTTAAGCTTGTCCA	
RT-E(Fw)	ATCAACATCGTGGTCATTGGCCATGTC	RT-PCR Ef1A
RT-E(Rv)	ATGTTAGGGCTAGCCTCCAGCATGTT	
g8(Fw)	TAGCAACTACAGATTGCCCCATTCAA	RT-PCR SsLepB1
g9(Fw)	TTATTAATTGTATGACTTAGTTGATTT	RT-PCR SsLepB2
g10Rw)	ATACACCTGACCACACCTGCTGTCTT	RT-PCR SsLepB1-B2
qA1(Fw)	TTGCTCAAACCATGGTGATTAGGA	qPCR LepA1
qA1(Rv)	GTCCATGCCCTCGATTAGGTTA	
qE(Fw)	ATCAACATCGTGGTCATTGGCCATGTC	qPCR Ef1A
qE(Rv)	ATCTCAGCAGCCTCCTTCTCGAACTTCT	
qA2(Fw)	TGGGAATCAAAAAGCTCCCTTCCTCTT	qPCR LepA2
qA2(Rv)	GCCTCCTATAGGCTGGTCTCCTGCA	
qB(Fw)	TCCAGATGAGGAACAACATCAAGTTGCT	qPCR LepB
qB(Rv)	GGTTCATGGGGTTGAACTCCATCTC	
g6(Rv)	AGCAACTTGATGTTGTTCCTCATCTGGA	5′ RACE SsLepB1-B2
g7(Rv)	AGAGAAGGACCACAGAGACATGCAT	

* Sequences are given in the 5′–3′ order.

sequence reported by Rønnestad et al. (2010). I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/), Modeller (http://www.salilab.org/modeller/) and SWISS-MODEL (http://swissmodel.expasy.org) were used to model the 3D structure of SsLepR. Cysteine bridge analysis was done using WHAT-IF server (http://swift.cmbi.ru.nl/servers/html/listcys.html).

2.6. Phylogenetic analysis

Novel leptin sequences isolated in this work together with those annotated in NCBI genebank were submitted to Gblock server for prediction of the most conserved amino acid blocks from multiple alignments using setting for a less stringent selection (http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=gblocks). Final alignments for the phylogenetic analysis were performed using ClustalX V1.81 software (Thompson et al., 1997) with multiple alignment parameters set at low stringency (gap opening = 1; gap extension = 0.2). Neighbor-joining (NJ) phylogenetic relationships were displayed using the NJPLOT program (Perrière and Gouy, 1996). An estimate of the robustness of the NJ tree was obtained using 100 random bootstrap replications. The human and salmon growth hormone (GH), a leptin structurally-related gene belonging to the four helical cytokine superfamily (Hill et al., 2002), were chosen as out group genes (acc.No. CAA23779; M21573).

2.7. Qualitative RT-PCR analyses of SsLepB genes

Samples of 14 different tissues from parr, postsmolt and maturing grilse stages were collected and immediately frozen on dry ice and kept at −80 °C until used. An initial RT-PCR tissue screening was performed using an assay amplifying both SsLepB variants (RT-Efw/RT-Erv and g4fw/g4rv primers for Ef1α and SsLepB genes, respectively, Table 1). PCR amplification was performed with an initial step of 94 °C for 5 min, 32 cycles of 94 °C for 25 s, 60 °C for 30 s, 68 °C for 90 s and extension at 68 °C for 7 min. Intron-flanking paralog-specific assays with forward primers designed in the 5′UTRs were subsequently employed to detect SsLepB1 and SsLepB2 expression in gill and brain (g8fw/g9fw/g10rw, Table 1). To circumvent spurious non-specific amplification, touch-down PCR was carried out as follow: 94 °C for 5 min, 15 cycles of 94 °C for 25 s, 62 °C for 90 s; 21 cycles of 94 °C for 25 s and 59 °C for 90 s and 7 min at 68 °C.

2.8. Quantitative PCR analyses of SsLep genes during development

Relative expression of Atlantic salmon leptin A1 (SsLepA1), leptin A2 (SsLepA1) and SsLepB genes during embryonic development was assessed at four different developmental stages (168, 215, 262 and 332 dd) by Real-time qPCR. For each stage analyzed, 30 embryos were dissected and total RNA extracted from triplicated samples, each with 10 pooled embryos.

SsLepA1, SsLepA2, SsLepB and Ef1α mRNAs were quantified on the CFX-96 Real-Time PCR detection system platform (Bio-Rad) using SYBR Green (QuantiTec SYBR Green PCR kit (Qiagen, Hilden, Germany). Each qPCR reaction comprised 12.5 μl 2xSYBR Green PCR Master Mix, 300 nM of forward and reverse primer, 100 ng cDNA template and nuclease-free water up to a final volume of 25 μl. To avoid potential genomic amplification in qPCR samples, only primer assays spanning exon-exon boundaries (LepA1: qA1fw/qA1rv; LepB: qBfw/qBrv; Ef1α: qEfw/qErv) or with at least one primer crossing exon-exon junction (LepA2; qA2fw/qA2rv), were designed. A serial dilution in nuclease-free water of cDNA derived from RNA pool of experimental samples was amplified to construct standard curves (2-fold dilutions) for both target and control genes. Standard curves were included in each run to determine amplification efficiency. Melting curves were recorded to evaluate specificity of amplification and lack of primer dimers. Product specificity was confirmed by evaluation of melting curves and agarose gel analysis. The qPCR conditions were as follows: 3 min at 95 °C, and amplified for 45 cycles at 95 C for 15 s and 60 C for 1 min.

2.9. qPCR data analysis

Fold change in target gene expression was determined using the "2-ddCT" method (Livak and Schmittgen, 2001), using Ef1α as a reference gene. Equal variances and normality of distributions were tested using Levene's *F*-test and normal p–p test, respectively (Zar, 1996). Data were log-transformed to better fit the assumptions of the parametric ANOVA test. Statistical significance among groups were analyzed by All-Pairwise Multiple Comparison Proce-

dures (Holm-Sidak method) and considered to be significant if $p < 0.05$. Differences between groups are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using Sigma Stat software (SPSS, Chicago, IL).

3. Results

3.1. Identification, cloning and structural organization of SsLepB genes

3.1.1. cDNA clones

The SsLepB1 and SsLepB2 cDNAs are 671 and 639 bp long respectively, both comprising a coding region of 462 bp. The open reading frames (ORFs) for polypeptides of 154 amino-acid residues start with ATG codons located at 207 and 176 bp of lepB1 and lepB2 clones, respectively. Translation initiation codon was assed by Kozak's rule (Kozak, 1991) and further confirmed by the identification of an in-frame stop codon located 9 bp upstream of the predicted start codon of both genes (Fig. 1). The cDNA sequences of SsLepB1 and SsLepB2 genes have been submitted to NCBI/GenBank with acc.No. KC464486 and KC464487). To assign gene orthology**,** the predicted peptide sequence was submitted to NCBI BLAST sequence algorithms, http://blast.ncbi.nlm.nih.gov/Blast.cgi for comparison with the non-redundant protein sequence database (BLASTP) or with the DNA sequence database translated in all six reading frames (TBLASTN). Results using both algorithms showed that the first significant hits are with the recently cloned orange-spotted grouper (JX406148), medaka (AB457589) and zebrafish (AM901009) leptin B genes (although supported by moderate *E*-values 6e-17, 1e-11 and 1e-09 respectively), followed by other vertebrate leptins. Alignments of the newly identified SsLepB sequences showed 28% of AA identity with orange-spotted grouper, 26% with both zebrafish and medaka LepB, 17% with SsLepA1/SsLepA2 paralogs and only 13–14% with human, mouse and xenopus ortholog counterparts.

3.1.2. Genomic clones

Genomic DNA fragments of 1268 bp ($n = 6$) and 1058 bp ($n = 5$) containing SsLepB genes were cloned and sequenced. Intron boundaries were determined by comparing genomic and cDNA sequences. SsLepB1 and SsLepB2 have a highly conserved organization being both encoded by two exons of the same size (exon1:162 bp; exon2: 300 bp) and interrupted by a single intron of 410 bp for SsLepB1 and 235 bp for SsLepB2. SsLepB1 and SsLepB2 5′UTRs identified by genome walker library were of 394 and 359 bp, respectively, and share 94% nt identity. Comparative sequence analysis also showed that SsLepB1 and SsLepB2 are 98% conserved in their coding sequence; thus the most striking difference is the length of the intron bordering their two exons (Fig. 1). These introns consist of a homologous and well-conserved sequence (230nt; 96% identity) and of an additional insertion only present in the SsLepB1 gene (180nt; Fig. 2). Because introns are known to be highly polymorphic non-coding regions, we wanted to elucidate if the identified SsLepB variants represent a case of intron length polymorphism at this genomic locus. We then exploited this intron length variation as genetic marker to identify potential gene polymorphism (Berrebi et al., 2005; Wang et al., 2005). In order to increase population variability, screening by EPIC-PCR was performed on a total of 66 Atlantic salmon samples collected from 4 different rivers and from Lake Byglandsfjord, in the case of landlocked salmon (*Bleke*), a population that has been

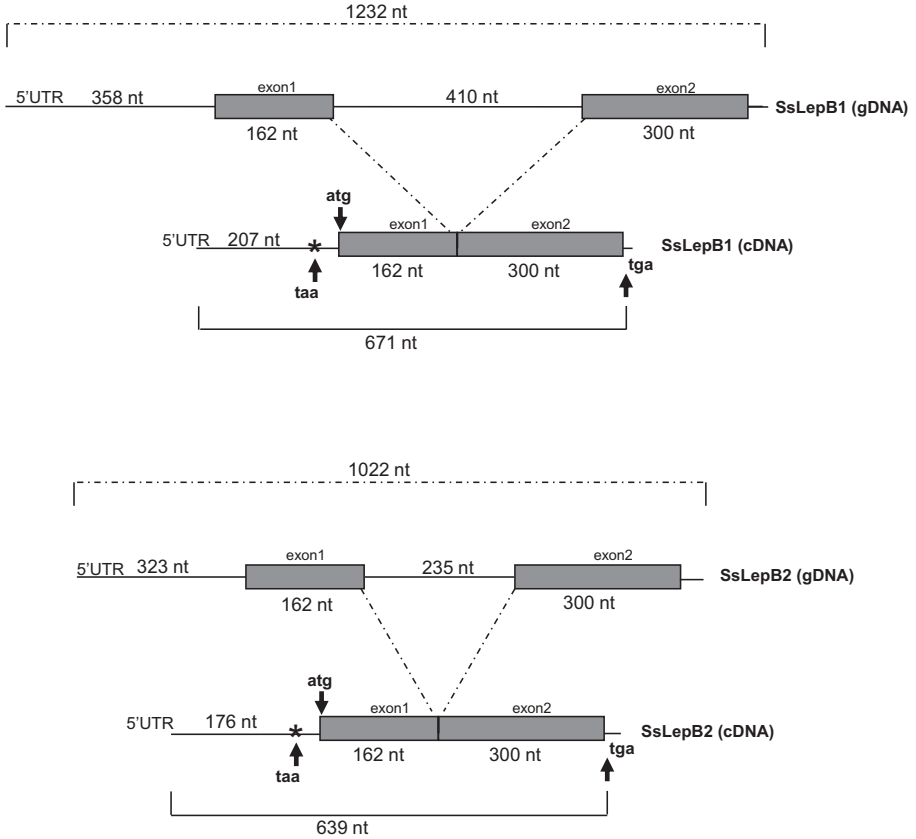

Fig. 1. Schematic representation of SsLepB1 and SsLepB2 gene structure of genomic and cDNA clones. The size of the exons, introns and 5′UTRs of both genes are represented in scale. The intron of SsLepB1 gene is bigger than that of SsLepB2, but the exons size for both genes are identical.

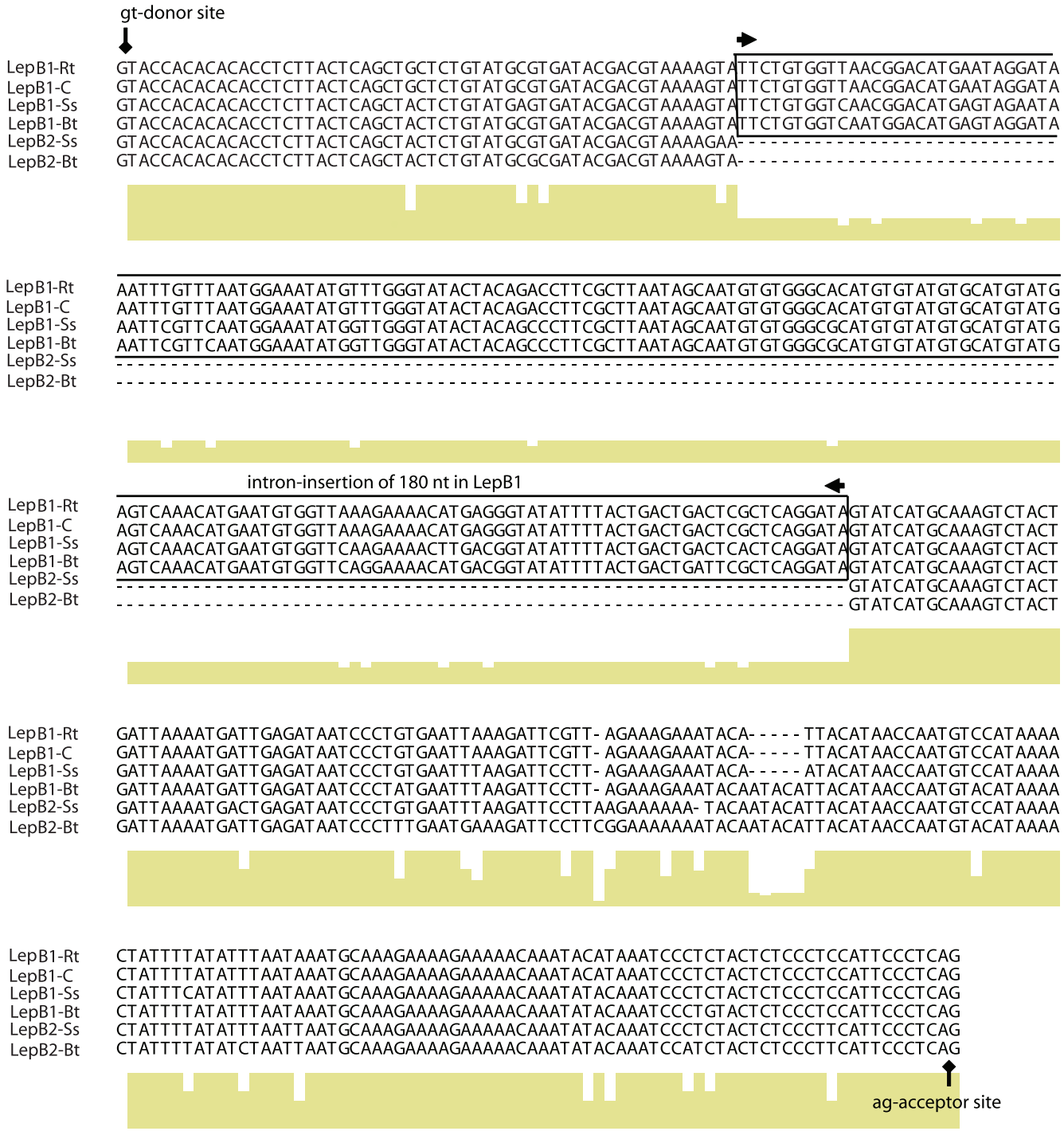

Fig. 2. Multiple alignments of the intronic sequences from the four species investigated. *Rt*, rainbow trout; *C*, charr; *Ss*, salmon; *Bt*, brown trout. Diamond bars at the 5′ and 3′ ends of the introns represent donor and acceptor splice sites, respectively. Note the intron insertion of 180 bp only present in the LepB1 gene.

isolated from anadromous populations for approximately 9000 years (Power, 1958; Behnke, 1972). We PCR-amplified salmon genomic DNA from each individual sample using primers g4fw and g4rv, which flank the LepB intron and electrophoresed the PCR products in a 1.2% agarose gel (Fig. 1aS). With no exception, in all individuals analyzed, we observed two different bands of expected size (909 and 734 bp) that after cloning and sequencing identified the long and the short SsLepB intron variants. The genomic sequences of SsLepB1 and SsLepB2 genes have been submitted to NCBI/GenBank under acc.No. JX131301 and JX131302.

3.1.3. Cloning and genomic structure of lepB genes in other salmonids

To investigate the evolutionary origin of these two putative SsLepB duplicates, we extend the search for these genes to three other salmonid species of the *Salmoninae* subfamily, rainbow trout, brown trout, and charr of the genera *Oncorhynchus*, *Salmo* and *Salvelinus*, respectively (Crespi and Fulton, 2004). PCR amplifications and subsequent cloning show that both rainbow trout and charr have preserved only the long LepB1 variant in their genome, while both copies are present in brown trout ($n = 10$ *per* species; Fig. 1bS). Comparative analyses indicate conservation in exon–

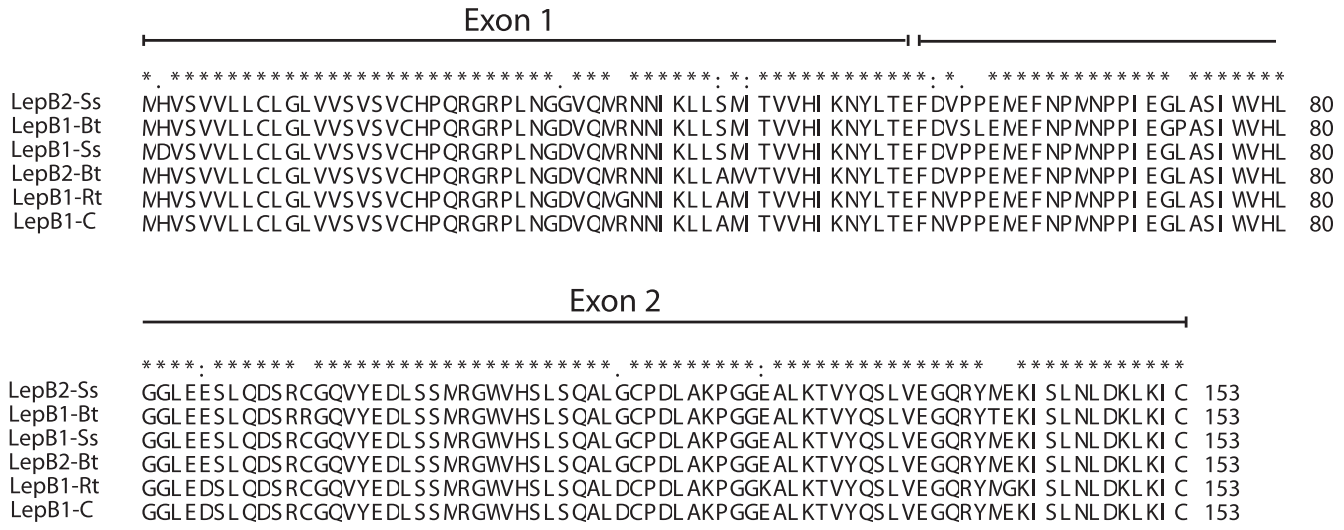

Fig. 3. Multiple alignments of LepB1 and LepB2 conceptually translated full-length amino acid sequences of *Ss*, salmon; *Bt*, brown trout; *Rt*, rainbow trout; and *C*, charr using Clustal X program. The two exons are marked with black lines above the sequences.

Table 2
Percentages of pairwise AA identities between cds of salmonid-LepB genes. The abbreviations of the species are as follows: *Ss*, salmon; *Bt*, brown trout; *Rt*, rainbow trout; *C*, charr.

	LepB2-Ss	LepB1-Bt	LepB1-Ss	LepB2-Bt	LepB1-Rt	LepB1-C
LepB2-Ss	100					
LepB1-Bt	96	100				
LepB1-Ss	98	96	100			
LepB2-Bt	98	95	98	100		
LepB1-Rt	94	92	94	95	100	
LepB1-C	96	94	96	97	98	100

intron boundaries, intron organization and sequence among LepB salmonid genes (Fig. 2, Fig. 3, Table 2). Accession numbers for these sequences are as follows: charr (LepB1 acc.No. JX131305), rainbow trout (LepB1 acc.No. JX131306), brown trout (LepB1 acc.No. JX131303; LepB2 acc.No. JX131304).

3.2. Prediction of protein features

Homology modeling of SsLepB1/2 predicts a cytokine family protein consisting of four α helices (A, B, C and D) connected by two long cross over (AB) and (CD) and one short loop (BC). The extended (AB) loop passes in front of the D helix (Fig. 4a and b). Both (AB) and (CD) loops wrap around the BD face. SsLepB protein exhibited a hydrophobic core in (AB) and (CD) loops (Fig. 4a) and is predicted to have disorder in 1–10, 30–50 (AB loop) and 90–100 (CD loop) residues. The disulphide bridge between cysteine residues, important for structural stability and biological activity, is found between 94 and 132 residues positions (Fig. 2S and Fig. 4b). Potential ligand interaction residues predicted by LigPlot+ analysis suggested that SsLepB residues of helix C (Tyr 96, Glu97, Ser100, Tyr 105), CD loop (Lys 120) and helix D (Glu 124) have binding affinity for *CHR* (cytokine homology region)/*leptin-binding domain* and fibronectin type III domain *FNIII* (residues 398 to 605) of the SsLep receptor (Fig. 3S).

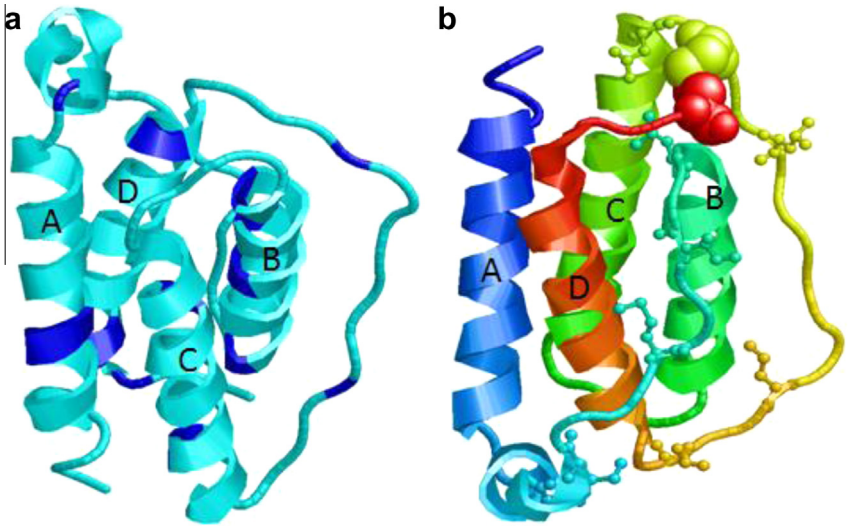

Fig. 4. (a) Predicted 3D structure of four helical SsLepB. Hydrophobic core residues are marked in blue. (b) Predicted 3D structure of SsLepB (rotated 360° up) to show hydrophobic residues in AB and CD loop (marked as ball and stick). The disulphide bridge between two cysteine residues are marked in as space-fill molecules in green and red (For interpretation of color in Fig. 4, the reader is referred to the web version of this article.).

3.3. Phylogenetic reconstruction of leptin genes family

Because leptins have highly divergent primary AA sequences among vertebrates (Denver et al., 2011), we analyzed their evolutionary relationships constructing a NJ tree by using a conserved AA sequence block identified with Gblocks server, a computer program that eliminates poorly aligned positions and divergent regions of an alignment of DNA or protein sequences (Castresana, 2000). The most conserved alignment among the input vertebrate leptin sequences ($n = 31$) comprises protein fragments of 21 AA with moderate degrees of homology, although some expected exceptions are observed (SsLepB1 vs SsLepB2, 100% of AA identity; SsLepA1 vs SsLepA2, 85%, Table 3; Fig. 4S).

The phylogenetic reconstruction revealed distinct leptin clades for mammals, fish and amphibians respectively, in agreement with classical taxonomy (Fig. 5). The topology of this tree suggests a possible evolutionary scenario with a single ancestral leptin gene before the fish-tetrapods split 408–419 MYA (Müller and Reisz, 2005) (node1), and is consistent with the duplication of an ancestral fish leptin at the base of teleost radiation, giving rise to LepA and LepB old duplicates (node2). This observation is in line with several comparative genomic studies that support the occurrence of a WGD before teleost speciation (Amores et al., 1998; Naruse et al., 2004; Meyer and Van de Peer, 2005). Moreover, LepA1/A2 and LepB1/B2 gene pairs of salmonid cluster together in two separate clades, as predicted if they arose from the recent WGD in the last common ancestor of this lineage (node 3), Allendorf and Thorgaard, 1984. As such, by definition the duplicated salmonid genes within clusters A and B would be out-paralogs to each other and co-orthologous to their corresponding unduplicated leptin in 'diploid' species (Jothi et al., 2006; Catchen et al., 2009).

3.4. RT-PCR expression of SsLepB genes in adult tissues

An initial tissue screening was performed by qualitative RT-PCR for SsLepB mRNA, using an assay detecting both paralogs mRNAs. The tissues analyzed included gill, spleen, ovary, liver, stomach, hindgut, midgut, heart, pituitary, white muscle, red muscle, belly flap, adipose tissue and brain. Expression of SsLepB genes was mainly found in gill and brain at all stages investigated, while traces of expression were also observed in red muscle and adipose tissue (Fig. 5aS). As a loading control, we determined the expression of the housekeeper gene Ef1α and found it was constitutively expressed in all tissues tested.

Primer pairs unique to the above genes were subsequently employed to amplify specific transcripts of SsLepB1 and SsLepB2 in gill and brain. We found that SsLepB1 is only expressed in brain tissue at all stages investigated (mostly in postsmolts and maturing *grilse*) while SsLepB2, albeit predominantly in gill, is redundantly expressed in both gill and brain tissues (Fig. 5bS).

3.5. Quantitative PCR expression of leptins during salmon embryonic development

In the present study we also aimed to (i) explore whether leptinB genes are potentially involved during Atlantic salmon embryogenesis and (ii) compare the temporal expression profiles of these genes during ontogeny with the two previously identified SsLepA1 and SsLepA2 paralogs (Rønnestad et al., 2010). Because amplicon-size requirements and highly conserved coding sequences adjacent to introns precluded our ability to design paralog-specific qPCR assays, we were required to use an assay amplifying both SsLepB transcripts. Analysis of the mRNA expression for these genes showed that, although in different amounts, they are all expressed during embryogenesis (Fig. 6). SsLepB and SsLepA2 transcripts do not show a significant change over development ($p > 0.05$), while the expression level of LepA1 increased gradually and significantly ($p < 0.05$, Fig. 6) with the highest levels observed at the latest stage investigated. Expression of SsLepB and LepA2 did not differ significantly from each other at stages 168, 262 and 332 dd, while LepA1 mRNA was significantly higher than LepA2 at stage 262 dd and higher than both LepA2 and SsLepB at stage 332 dd ($p < 0.05$, Fig. 6).

4. Discussion

4.1. LepB1. and LepB2: two recently duplicated genes in salmonid species

Modern salmonids are considered pseudotetraploid as they are believed to be in the process of reverting to a stable diploid state (Danzmann et al., 2008) after the postulated whole genome duplication by autotetraploidization of their last common ancestor (25–100 MYA, 4R duplication (Allendorf and Thorgaard, 1984). In this complex genetic context, allelic versus duplicated genes remains a major challenge in the characterization of their genome. Previous studies have shown that duplicated paralogs which arose from salmon WGD usually display a level of nt conservation ranging from 72% to 94% in their transcribed regions (Angotzi et al., 2008; Koop et al., 2008; Leong et al., 2010; Yasuike et al., 2010). Because of the astonishing level of amino acid identity between the two LepB variants (98%) and the fact that most of the observed sequence variations are accumulated in non coding regions (mainly in introns), the first objective was to explore for potential intron polymorphism at the LepB loci. Indeed, introns are more prone to chromosomal rearrangements like deletions and insertions that can occur independently in the two alleles. Our PCR survey of different Norwegian salmon populations revealed the presence of two SsLepB intron-variants in all individuals analyzed suggesting that they are a stable feature of the salmon genome, unrelated to

Table 3
Percentages of pairwise AA identities between SsLepB genes and some representative known vertebrate leptins as deduced by Gblock server analysis (21 AA). The abbreviations of the species are as follows: *hs*, human; *xl*, african frog; *zf*, zebrafish; *m*, medaka; *G*, orange spotted grouper; *Ss* salmon.

	Lep-hs	Lep-Xl	LepA-zf	LepB-zf	LepA-m	LepB-m	LepA-G	LepB-G	LepA1-Ss	LepA2-Ss	LepB1-Ss	LepB2-Ss
Lep-hs	100											
Lep-Xl	42	100										
LepA-zf	33	28	100									
LepB-zf	28	23	47	100								
LepA-m	14	23	52	38	100							
LepB-m	14	19	28	47	28	100						
LepA-G	33	38	47	38	52	33	100					
LepB-G	23	28	33	57	23	57	28	100				
LepA1-Ss	42	33	57	47	47	38	52	33	100			
LepA2-Ss	38	33	57	47	47	38	47	33	85	100		
LepB1-Ss	14	23	42	61	23	42	28	57	33	33	100	
LepB2-Ss	14	23	42	61	23	42	28	57	33	33	100	100

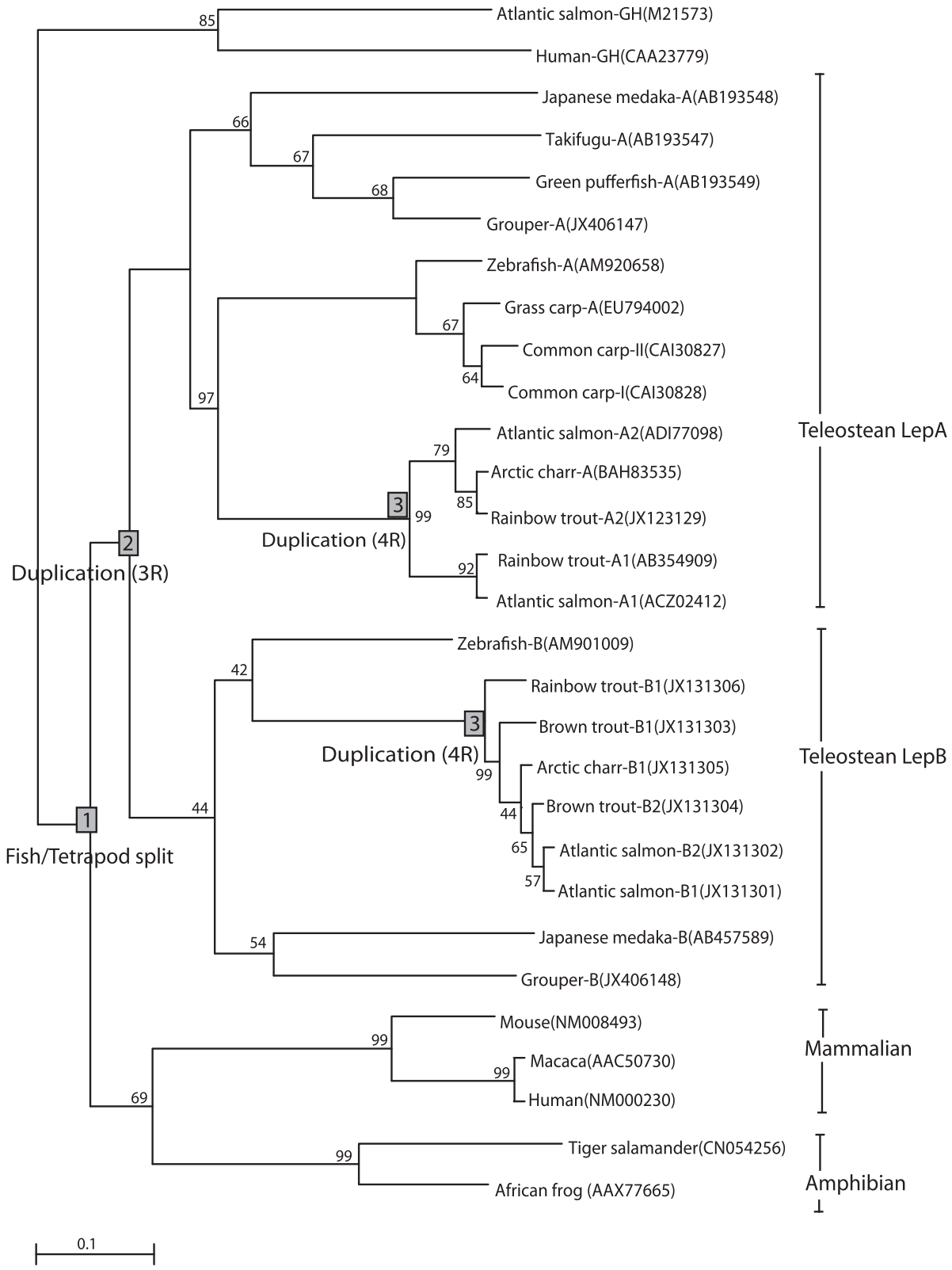

Fig. 5. Evolutionary relationships among vertebrate leptins deduced by Neighbor-joining (NJ) analysis using the conserved AA-sequence block identified with Gblocks server (Castresana, 2000). GenBank accession numbers are shown after the species name. Numbers indicate the number of times the branching was obtained from 100 bootstrap runs. Scale bar indicates the substitution rate per residue. Number 1 at the gray-marked node represents the fish-tetrapods split while nodes 2 and 3 the 3R and 4R (round) duplications in teleosts.

individual allelic polymorphism. Moreover, sequence comparisons of the 5′UTRs between the two SsLepB variants (94% nt identity) is also suggestive of two SsLepB genes with different genomic locus. Thus it is possible that these two paralogs may have mostly diverged in their non-coding sequences as result of minimal selective constraints permitting evolutionary forces to act more freely. The

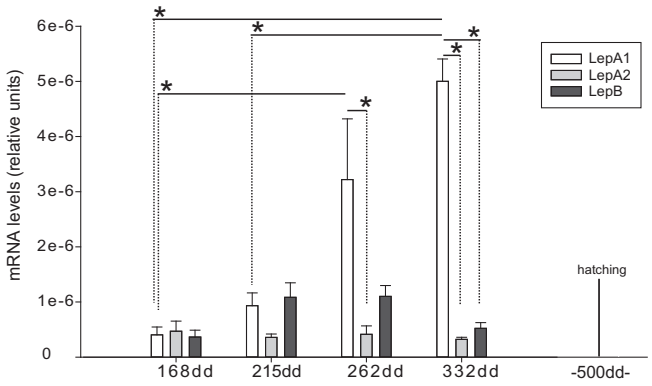

Fig. 6. SslepB, SsLepA1 and SsLepA2 mRNA expression profiles assayed by qRT-PCR normalized to Ef1α transcripts in whole salmon embryos across development. LepA1 mRNA expression increases significantly during development progression (332dd vs 168dd and vs 215 $p < 0.05$; 262dd vs 168dd: $p < 0.05$); within a given stage, LepA1 mRNA is significantly higher than LepA2 at stage 262dd ($p < 0.05$) and higher of both LepA2 and SsLepB at stage 332dd ($p < 0.05$). Error bars indicate standard error of the mean (SEM). Asterisks indicate statistically significant difference ($p < 0.05$).

duplication hypothesis of SsLepB1 and SsLepB2 is further substantiated by the finding in the present work of their orthologs in brown trout, the closest related species to salmon (Crespi and Fulton, 2004). The most parsimonious explanation calls for a LepB2 gene loss in rainbow trout and Arctic charr sometime after their speciation. Alternative scenarios would involve independent species specific LepB2 gains in salmon and brown trout, but they appear less parsimonious.

It is well known that following WGD events, duplicated chromosomes tend to retain gene orders identical to each other and to their closest ancestor over evolutionary time (syntenic conservation, (Jaillon et al., 2004; Naruse et al., 2004; Nakatani et al., 2007). Thus, to better infer orthologies and paralogies among leptin duplicates it would have been useful to take advantage of synteny analysis. Unfortunately, automated systems for the identification of conserved syntenic regions available only allow for the analysis of fully or partially assembled genomes (Catchen et al., 2009). Despite the large amounts of Atlantic salmon genomic data produced in recent years (Quinn et al., 2008; Davidson et al., 2010; Di Génova et al., 2011), the size, complexity and huge number of repeats (de Boer et al., 2007) make the assembly far from conclusive and currently not exploitable for synteny analysis.

4.2. LeptinB protein and its evolutionary conserved features

Leptin belongs to class-I helical cytokine family and possess a distinctive four α-helix bundle in the 3D structure (Coccia et al., 2010). Although identifiably distinct in primary structure, it is possible that leptin orthologs are similar in function as their tertiary structure is generally conserved (Copeland et al., 2011). Homology modeling of SsLepB in the current study suggested that the protein satisfies the required parameters for being classified as a long-chain four helical cytokine and that the basic structural pattern of the protein follows that of human leptin (Zhang et al., 1997).

Leptin structure is characterized by hydrophobic interactions that are fundamental for the conformational stability and structural integrity of the protein and like in other cytokines, exhibits a rigid hydrophobic core (Zhang et al., 1997). The hydrophobic residues at the (AB) and (CD) loops, including crucial leucine residues in the helix C, are well conserved in SsLepB1/2. Most importantly, the disulphide bridge of SsLepB cysteine residues at positions 94 and 132 is well conserved. This follows the general rule for the importance of the folding process, conformational stability and receptor binding capability (Coccia et al., 2010). The predicted SsLepB has disorder among residues 1–10, 30–50 (AB loop) and 90–100 (CD loop). Disorderness in protein residues means that there is a lack of fixed 3D structure in the putatively native state of the protein segments and this has been reported for human growth hormone (Coccia et al., 2010). It has been argued that the region will most likely assume an ordered conformation once the protein is bound to its receptor (Zhang et al., 1997).

Three receptor interacting sites on mammalian leptins (site I on the face of helix D site II on helices A and C, and site III at the N-terminus of helix D) have been mapped by mutational analysis (Peelman et al., 2004). Each of these regions shows various degree of conservation (12% to 25%) with primary amino acid sequence of SsLepB1/2. The most highly conserved stretch of amino acids among amphibians and higher vertebrates is the six residue sequence GLDFIP in AB loop of the protein (positions 38 to 43 in human leptin) for receptor binding, although this stretch of sequence was not found in SsLepB. The GLDFIP sequence is also missing in other fish leptins analyzed so far (Denver et al., 2011), and may suggest a different mechanism for binding to and activation of the leptin receptor compared with tetrapods. In *silico* studies showed a clear variation in the binding patterns of leptin/leptin receptor even within different fish and between paralogs of a given species (LepA1 versus LepA2 of salmon and leptin A versus leptin B of zebrafish and medaka, respectively) (Prokop et al., 2012). Our docking study show that residues located at helix C, D, and CD loop of SsLepB1/2 have binding affinity for residues that characterize the *CHR/leptin-binding* and *FNIII* domains of the SsLep receptor. This is in line with a recent study on murine ligand–receptor complex modeling showing that leptin binds to the C terminal of the *CHR* domain of its receptor (Mancour et al., 2012) and suggests that at least some of the ligand–receptor interactions are evolutionarily conserved.

4.3. Potential SsLepB functions in adult tissues and during embryonic development

In mammals, leptin acts mostly as an endocrine adiposity signal and its plasma levels rise in proportion to adipose tissue mass to directly affect CNS neurons and circuits to regulate food intake and energy homeostasis (Frühbeck, 2001). High levels of leptin receptors have been identified in several hypothalamic neurons expressing important orexigenic and anorexigenic factors in mammals and fish (Volkoff et al., 2005; Liu et al., 2010). This suggests an evolutionarily conserved role of fish leptins on food intake by inhibiting orexigenic pathways and stimulating anorexigenic ones (Murashita et al., 2008). Not surprisingly and in agreement with other reports on fish LepB orthologs (Kurokawa and Murashita, 2009; Zhang et al., 2013), we found SsLepB mRNA transcripts in the brain which suggests that LepB brain functions might also be exerted through paracrine and/or autocrine mechanisms.

Leptin mRNA expression in gills has also been reported in other fish species including Atlantic salmon (Kurokawa and Murashita, 2009; Gorissen et al., 2009; Rønnestad et al., 2010) although their potential role in this tissue is speculative at this point. The gill is a multifunctional organ, with a high turnover of different cell types serving important functions such as ion transport, immune responses and exchange of oxygen and metabolites (Evans et al., 2005). Leptin protein is localized in undifferentiated cells deep in the interlamellar area of gill tissue (unpublished data from our lab) and might therefore serve as paracrine factor important for cell differentiation and proliferation.

The restricted localization of SsLepB1/B2 expression to brain and gills and to brain and ovary in orange spotted grouper (Zhang et al., 2013) together with the lack of LepB expression in their

livers indicate that these genes may have more specialized function/s than their paralog counterparts (LepA genes) in these two species.

Our expression studies suggest that leptin genes may also play important roles during fish embryogenesis. The detected mRNAs of SsLepA1, SsLepA2 and SsLepB1/B2 genes are zygotically derived as maternally transmitted transcripts are already degraded at stages analyzed (Tadros and Lipshitz, 2009). The gradual increase of SsLepA1 mRNAs suggests it is required for the progressive development of normal embryos. These findings are consistent with a recent report on zebrafish embryos showing that splice blocking morpholino on leptin A and leptin receptor severely alter proper development (Liu et al., 2012). Elevated levels of leptin stimulate the breakdown of stored triglyceride as **in** vitro studies showed that in both mammals and fish leptin significantly upregulates hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) activities O'Rourke et al., 2001; Smirnova et al., 2006; Lu et al., 2012, two key genes involved in triacylglycerol mobilization. Lipids are an important energy source during ontogeny of fish, especially neutral lipids accumulated in yolk sac in the form of triacylglycerols which are primary fuel for metabolic energy requirements and formation of rapidly developing tissues (Tocher, 2003). Since triacylglycerols decrease markedly in developing salmon eggs (Cowey et al., 1985) and triacylglycerol hydrolysis is enhanced after chronic leptin treatment (Steinberg et al., 2002), it could be that a progressive increase of leptin during development may be required to boost energy production by lipolysis, utilizing triacylglycerol as a source for metabolic fuel. In addition to its role in metabolism, leptin may play contributory functions in developing embryos through promoting axon growth, synaptogenesis (Bouret, 2010) and angiogenesis (Cao et al., 2001). In line with its potential actions as growth factor, leptin is also a potent stimulator of growth hormone and prolactin (Tannenbaum et al., 1998; Tennekoon et al., 2007), both of which are important pleiotropic hormones involved in mitogenesis, growth and development (Sakai et al., 1996; Canosa et al., 2007; Zhu et al., 2007).

4.4. Redundancy and subfunctionalization of LepB genes: a mechanism of gene preservation?

The classical model originally proposed by (Ohno, 1970) predicts that once two redundant genes are generated, one retains the original function whereas its twin copy, free of selective constraints, can either accumulate deleterious mutations leading to inactivation or is affected by advantageous mutations that offer a chance to develop new functions (neo-functionalization). Since deleterious mutations occur much more frequently than advantageous ones, pseudogenization is the most likely fate for the majority of duplicated genes. Indeed, estimations based on the study of nine eukaryotes suggest that more than 90% of young duplicates degrade to pseudogenes (Lynch and Conery, 2000). Nonetheless, a recent study indicates that purifying selection is the predominant evolutionary force acting on recently duplicated salmon paralogues (Leong et al., 2010) and preservation of redundancy is a widespread phenomenon as it supports genetic robustness and versatility (Gu et al., 2003; Bozorgmehr, 2012). Since Ohno's theory, other models have been proposed to explain the evolution of duplicated genes including the subfunctionalization model proposed by Force and collegues (Force et al., 1999). According to this model, redundant duplicates are subjected to mutations which lead to a differential loss of their regulatory elements and duplicates must complement each other to retain the full set of functions present in the ancestral gene. Our RT-PCR analyses using paralog-specific assays for SsLepB genes in gills and brain may suggest both redundancy and early subfunctionalization. Indeed, tissue expression analysis in zebrafish and medaka showed that the 'unduplicated' lepB gene is expressed in both gill and brain (Kurokawa and Murashita, 2009; Gorissen et al., 2009). We found that SsLepB1 is only expressed in brain while SsLepB2 is expressed predominantly in gill, supporting the hypothesis of their preservation by complementary sites of expression to jointly fulfilling ancestral LepB role in these tissues.

Both SsLepB genes, although to very different extent, are redundantly expressed in brain and what we see may actually represent early steps of subfunctionalization with partial and still unresolved degeneration of tissue specific *cis*-acting regulatory elements in these young duplicates. Moreover, medaka and zebrafish LepB genes show a much broader tissue expression than SsLepB duplicates, being also expressed in spleen, liver, gut, heart and muscles. We could further speculate that enhancers/regulatory elements responsible of tissue-specific expression of both salmon genes may have already diverged toward degeneration or loss during evolution of the salmon. Comparative genomic and functional approaches analyzing the *cis*-regulatory elements controlling expression of duplicate and unduplicated LepB genes may provide additional information on the functional evolution of this family.

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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.ygcen.2013.03.022.

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