

Title

Multiple genes for functional $\Delta 6$ fatty acyl desaturases (Fad) in Atlantic salmon (*Salmo salar* L.): Gene and cDNA characterization, functional expression, tissue distribution and nutritional regulation

Authors

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; LC-PUFA, long-chain polyunsaturated fatty acids; ORF, open reading frame; qPCR, quantitative (real-time) polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region; VO, vegetable oil.

Abstract

Fish are the primary source in the human food basket of the n-3 long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoate (EPA; 20:5n-3) and docosahexaenoate (DHA; 22:6n-3), that are crucial to the health of higher vertebrates. Atlantic salmon are able to synthesis EPA and DHA from 18:3n-3 through reactions catalyzed by fatty acyl desaturases (Fad) and elongases of very long fatty acids (Elovl). Previously, two cDNAs encoding functionally distinct $\Delta 5$ and $\Delta 6$ Fads were isolated, but screening of a genomic DNA library revealed the existence of more putative *fad* genes in the Atlantic salmon genome. In the present study, we show that there are at least four genes encoding putative Fad proteins in Atlantic salmon. Two genes, $\Delta 6fad_a$ and $\Delta 5fad$, corresponded to the previously cloned $\Delta 6$ and $\Delta 5$ Fad cDNAs. Functional characterization by heterologous expression in yeast showed that the cDNAs for both the two further putative *fad* genes, $\Delta 6fad_b$ and $\Delta 6fad_c$, had only $\Delta 6$ activity, converting 47 and 12 % of 18:3n-3 to 18:4n-3, and 25 and 7 % of 18:2n-6 to 18:3n-6, for $\Delta 6fad_b$ and $\Delta 6fad_c$, respectively. Both $\Delta 6fad_a$ and $\Delta 6fad_b$ genes were highly expressed in intestine (pyloric caeca), liver and brain, with $\Delta 6fad_b$ also highly expressed in gill, whereas $\Delta 6fad_c$ transcript was found predominantly in brain, with lower expression levels in all other tissues. The expression levels of the $\Delta 6fad_a$ gene in liver and the $\Delta 6fad_b$ gene in intestine were significantly higher in fish fed diets containing vegetable oil compared to fish fed fish oil suggesting up-regulation in response to reduced dietary EPA and DHA. In contrast, no significant differences were found between transcript levels for $\Delta 6fad_a$ in intestine, $\Delta 6fad_b$ in liver, or $\Delta 6fad_c$ in liver or intestine of fish fed vegetable oil compared to fish fed fish oil. The observed differences in tissue expression and nutritional regulation of the *fad* genes were discussed in relation to gene structures and fish physiology.

Introduction

Fish are the primary source in the human food basket of n-3 long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoate (EPA; 20:5n-3) and docosahexaenoate (DHA; 22:6n-3), which are crucial to the health of higher vertebrates [1-3]. Aquaculture now supplies an increasing proportion (up to 47 % in 2006) of the fish [4] and, until now, diets traditionally formulated with fish oil (FO) have ensured that farmed fish are rich in n-3 LC-PUFA [5]. However, global FO supplies are at their sustainable limit and further expansion of aquaculture requires suitable alternatives [6-8]. Vegetable oils (VOs), the principal sustainable alternative to FO, can be rich in C18 polyunsaturated fatty acids (PUFA), but lack the n-3 LC-PUFA abundant in FO [5]. Flesh fatty acid compositions of fish fed VO are characterized by increased levels of C18 PUFAs, and decreased levels of n-3 LC-PUFAs, compromising their nutritional value to the human consumer [5, 9-13]. This potentially negative effect of feeding VOs to farmed fish has prompted much investigation of the biochemistry and molecular mechanisms of the LC-PUFA biosynthesis pathway in fish including Atlantic salmon (*Salmo salar* L.) [14-22].

The biosynthesis of LC-PUFAs in vertebrates involves consecutive desaturation and elongation reactions that convert the essential fatty acids (EFAs) 18:3n-3 (α -linolenic acid) and 18:2n-6 (linoleic acid) to longer-chain, more unsaturated fatty acids of the same series, including EPA, DHA and arachidonic acid (ARA; 20:4n-6) [23]. The two main types of enzymes involved in these conversions are the elongases of very long fatty acids (Elovl) and fatty acyl desaturases (Fad). Elovl enzymes account for the condensation reaction of activated fatty acids with malonyl-CoA in the elongation multi-enzyme complex, and three members of the family Elovl2, Elovl5 and Elovl4 are involved in LC-PUFA biosynthesis in mammals [24]. On the other hand, Fad enzymes introduce double bonds in the fatty acyl chain at carbon 6 (Δ 6 Fad) or carbon 5 (Δ 5 Fad) from the carboxyl group [23]. Nutritional feeding trials and cell studies with both primary cultures (hepatocytes and enterocytes) and established cell lines have shown that salmonids, including Atlantic salmon, are

capable of producing EPA and DHA from 18:3n-3 [14,25]. Subsequent molecular studies have now revealed that Atlantic salmon express several Elovl enzymes, and cDNAs for Elovl2 and two distinct Elovl5 have been isolated and functionally characterised [16,22]. In addition, two distinct cDNAs encoding functional $\Delta 5$ and $\Delta 6$ Fads were isolated [16,18]. However, in a recent study characterising *fad* gene promoters in fish [20], screening performed on a genomic DNA library revealed the existence of more putative *fad* genes in the Atlantic salmon genome.

In the present study, we present evidence that there are at least four genes encoding putative Fad proteins in the Atlantic salmon genome, and that these do not represent alleles as each has distinct sizes and sequences of introns. Two genes corresponded to the previously cloned $\Delta 6$ and $\Delta 5$ Fad cDNAs. Here, we thus describe the cloning of the cDNAs for the two further putative *fad* genes and their phylogenetic analyses. Functional characterization by heterologous expression in yeast showed both these cDNAs have $\Delta 6$ activities. Tissue expression and nutritional regulation of the newly cloned salmon Fad cDNAs were determined by quantitative real-time PCR (qPCR).

Materials and methods

Genomic screening for fatty acyl desaturases

An Atlantic salmon genomic DNA library was constructed with the lambda FIX II/Xho I partial fill-in vector kit (Stratagene, La Jolla, USA) and probed with a full-length cDNA for salmon $\Delta 5$ Fad (gb|AF478472|). Inserts of positive recombinant phages were isolated and subcloned into the pBluescript KS II vector (Stratagene, La Jolla, USA) for sequencing (CEQ-8800 Beckman Coulter Inc., Fullerton, USA). The positive phage sequences were then assembled (BioEdit version 5.0.6, Tom Hall, Department of Microbiology, North Carolina State University, USA) to obtain the sequences and structures of all putative *fad* genes found in the Atlantic salmon genome.

Fatty acyl desaturase cDNA cloning

Preliminary results from the genomic library screening revealed the existence of four putative *fad* genes in the Atlantic salmon genome, including the genes for the previously cloned $\Delta 5$ Fad [16] and $\Delta 6$ Fad [18] cDNAs, termed $\Delta 5fad$ and $\Delta 6fad_a$, and two further uncharacterised *fad* genes. When intronic sequences were removed, alignment of the predicted cDNAs for the four Fads showed the entire open reading frame (ORF) was obtained for one of the new desaturases (subsequently termed $\Delta 6fad_b/\Delta 6$ Fad_b), whereas only partial ORF sequence from the start codon (~1000 bp) was available for the other (subsequently named $\Delta 6fad_c/\Delta 6$ Fad_c). To obtain the complete ORF sequence of Fad_c and the 3' untranslated regions (UTR) of both uncharacterized Fads, specific primers were designed (Table 1), and 3' rapid amplification of cDNA ends (RACE) PCR (FirstChoice® RLM-RACE kit, Ambion, Applied Biosystems, Warrington, U.K.) performed on cDNA synthesised from 5 µg of total brain RNA. For $\Delta 6fad_b$, a positive 3'RACE PCR fragment was obtained by two-round PCR. The first PCR was performed using the gene specific sense primer SSD6bF1 and the adapter specific 3'RACE OUTER primer, with an initial denaturing step at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, extension at 72 °C for 2 min, followed by a final extension at 72 °C for 5 min (GoTaq® Colorless Master Mix, Promega, Southampton, UK). First PCR products were used as template for nested PCR with primers SSD6bF2 and 3'RACE INNER in a 30-cycle reaction under the same thermal conditions as above. A similar approach was followed for $\Delta 6fad_c$, with first round PCR performed with primers SSD6cF1 and 3'RACE OUTER, with an initial denaturing step at 95 °C for 1 min, followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 65 °C for 30 s, extension at 68 °C for 3 min, followed by a final extension at 68 °C for 3 min (Advantage 2 Polymerase Mix®, Clontech, CA, USA). First round PCR product was then used as template for nested PCR with primers SSD6cF1 and 3'RACE INNER, with thermal conditions as above except initial denaturing was for 1 min, and annealing was at 60 °C (Advantage 2 Polymerase Mix). 3'RACE PCR products were cloned in pBluescript, sequenced, assembled with their corresponding ORF

sequences, and deposited in the GenBank database under accession numbers GU207400 ($\Delta 6fad_b$) and GU207401 ($\Delta 6fad_c$).

Sequence and phylogenetic analysis

Deduced amino acid sequences of the two newly cloned Fad cDNAs were aligned with those of human $\Delta 6$ Fad (gb|AF126799|) and fish Fads including zebrafish (*Danio rerio*) dual $\Delta 6\Delta 5$ Fad (gb|AF309556|), salmon $\Delta 5$ Fad (gb|AF478472|) and $\Delta 6$ Fad_a (gb|AY458652|) using ClustalW (Bioedit). To compare sequences two by two, the EMBOSS Pairwise Alignment Algorithms tool (<http://www.ebi.a.uk/Tools/Wise2/index.html>) was used. Phylogenetic analysis of the newly cloned salmon Fads and amino acid sequences from other organisms obtained from GenBank database using BLASTX was performed by constructing a tree using the Neighbour Joining method [26], with confidence in the resulting tree branch topology measured by bootstrapping through 1000 iterations.

Heterologous expression of desaturase ORFs in Saccharomyces cerevisiae

Comparison of cDNA nucleotide sequences of $\Delta 6fad_b$ and $\Delta 6fad_c$ enabled design of primers for PCR amplification of the respective ORFs. For $\Delta 6fad_b$, sense primer SSD6bU5F and antisense primer SSD6bU3R were designed for first round PCR, whereas sense primer SSD6cU5F and antisense primer SSD6cU3R were used for $\Delta 6fad_c$. The isolation of both $\Delta 6fad_b$ and $\Delta 6fad_c$ ORFs was achieved in a second round (nested) PCR using first PCR products primed with SSD6VF and SSD6VR containing *Hind*III and *Xho*I restriction sites, respectively (Table 1). PCR was performed using the high fidelity PfuTurbo[®] DNA polymerase (Stratagene, Agilent Technologies, Cheshire, UK), with an initial denaturing step at 95 °C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 5 min. The DNA fragments were digested with the

corresponding restriction endonucleases (New England BioLabs, Herts, UK) and ligated into a similarly restricted pYES2 yeast expression vector (Invitrogen, Paisley, UK). The purified plasmids (GenElute™ Plasmid Miniprep Kit, Sigma) containing $\Delta 6fad_b$ and $\Delta 6fad_c$ ORFs were used to transform *Saccharomyces cerevisiae* competent cells (S.c. EasyComp Transformation Kit, Invitrogen). Transformation and selection of yeast with recombinant pYES2 plasmids, yeast culture and fatty acid (FA) analyses were performed as described in detail previously [18,27,28]. Briefly, cultures of recombinant yeast were grown in *S. cerevisiae* minimal medium^{-uracil} supplemented with one of the following FA: 18:3n-3, 18:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 and 22:4n-6. The FA were added to the yeast cultures at final concentrations of 0.5 (C18), 0.75 (C20) and 1.0 (C22) mM. After 2-days, yeast were harvested and washed, and lipid extracted by homogenization in chloroform/methanol (2:1, v/v) containing 0.01% BHT as antioxidant. Methyl esters of FA were prepared, extracted, purified, and analysed by GC in order to calculate the proportion of substrate FA converted to desaturated FA product as $[\text{product area}/(\text{product area} + \text{substrate area})] \times 100$. Identities of FA peaks were based on GC retention times and confirmed by GC-MS as described previously [27,28].

Tissue distribution and nutritional regulation

Expression of the new *fad* genes was measured in tissues by quantitative real-time PCR (qPCR). Intestine, liver, white muscle, red muscle, kidney, spleen, heart, brain, gill and adipose tissue were dissected from three salmon and frozen at -70 °C. Total RNA was extracted as described above, and 1 µg of total RNA reverse transcribed into cDNA (Verso™ cDNA kit, ABgene, Surrey, UK). The fish were ~150g post-smolts held in 7 m³ seawater tanks at ambient temperature, salinity and photoperiod in the Marine Environment Research Laboratory, Machrihanish, Scotland, UK, and fed a commercial salmon feed based on fish meal and FO. The effects of diet on *fad* expression were investigated in samples from salmon post-smolts fed four diets with the same basal composition but

formulated with different oils including FO, soybean oil (SO), linseed oil (LO) or rapeseed oil (RO), as described previously [29]. At the end of the trial, 0.5 g of liver and intestine of five fish per dietary treatment were dissected and rapidly disrupted in 5ml of TRI Reagent (Ambion, Applied Biosystems) using an Ultra-Turrax homogeniser (Fisher Scientific, Loughborough, UK), and immediately frozen in liquid nitrogen and stored at -70°C prior to RNA extraction.

Statistical analyses

For tissue expression profiles, results were expressed as mean normalized values (\pm SD) corresponding to the ratio between the copy numbers of the putative *fad* transcripts and the copy numbers of the reference gene, 18s rRNA. The effects of diet on *fad* expression, expressed as the relative expression ratio of each gene in fish fed one of the VOs in relation to those fed FO (control) and normalized by two reference genes (*elf-1 α* and an EST which was previously shown to be unresponsive under the nutritional conditions tested; [29]), were analysed for statistical significance using the relative expression software tool (REST-2008[©], <http://www.gene-quantification.info/>), which employs a pair-wise fixed reallocation randomisation test (10,000 randomisations) with efficiency correction [30].

Materials

Eicosatetraenoic (20:4n-3), docosapentaenoic (22:5n-3) and docosatetraenoic (22:4n-6) acids (all > 98-99% pure) were purchased from Cayman Chemical Co., Ann Arbor, USA. Linoleic (18:2n-6), α -linolenic (18:3n-3), eicosatrienoic (20:3n-6) acids (all >99% pure), BHT, fatty acid-free BSA, galactose, 3-(hydroxymethyl) pyridine, HBSS, nitrogen base, raffinose, tergitol NP-40 and uracil dropout medium were obtained from Sigma Chemical Co. Ltd., Dorset, UK. TLC (20 x 20 cm x 0.25 mm) plates pre-coated with silica gel 60 (without fluorescent indicator) were purchased from

Merck, Darmstadt, Germany. All solvents were HPLC grade and were from Fisher Scientific, Loughborough, UK.

Results

Salmon fatty acyl desaturase gene structure

Six lambda phage salmon genomic inserts with homologous regions to the previously described salmon desaturase cDNAs were obtained after screening the library. These six phage inserts resolved into four distinct *fad* genes. Two of these corresponded to the previously cloned and functionally characterised $\Delta 5$ and $\Delta 6$ Fad cDNAs [16,18], and so were termed $\Delta 5fad$ (gb|GU294485|) and $\Delta 6fad_a$ (gb|AY736067|). The complete gene sequence for $\Delta 6fad_a$ was obtained, but only the sequence corresponding to the last five introns and exons was obtained for $\Delta 5fad$. The other two putative *fad* genes were genes for which the cDNA had not yet been cloned or characterised. After cloning and functional characterization of the cDNAs (see below), these genes were termed $\Delta 6fad_b$ (compiled from gb|GU294486-8|) and $\Delta 6fad_c$ (gb|GU294489|). All four *fad* genes had identical coding exon structures, being comprised of 12 coding exons, with a high degree of sequence identity, and sequence identity extended to intron sequence (Fig.1). Intron regions that could not be aligned across all four genes had high identity to multiple sequences in diverse salmon database entries and included degenerate salmon Class I transposons [33], or could be aligned with sequences of RepBase (<http://www.girinst.org/replib/index.html>) entries for various transposons [32]. The first coding exons of $\Delta 6fad_b$ and $\Delta 6fad_c$ contain a nine base pair insertion compared to $\Delta 6fad_a$ and $\Delta 5fad$, resulting in a three amino acid insertion in the predicted polypeptide sequences.

Comparison of the cDNA and gene sequences for $\Delta 6fad_a$ enabled the identification of an upstream 5' non-coding exon, whilst comparison of the cDNAs for $\Delta 6fad_b$ showed the presence of a further two 5' non-coding exons for $\Delta 6fad_b$. The first of these non-coding exons mapped to the

first 5' non-coding exon of $\Delta 6fad_a$. Two cDNAs were obtained for $\Delta 6fad_c$, (see section below) and comparison of these with the gene sequence indicated that these cDNAs represented alternatively spliced products, both derived from the $\Delta 6fad_c$ gene. These exons did not show similarity to the exons for $\Delta 6fad_a$ and $\Delta 6fad_b$, although they represented sequences which could be mapped elsewhere in the 5' regions of $\Delta 6fad_a$ and $\Delta 6fad_b$. One of these alternative exons was similar to the previously characterised promoter region of $\Delta 6fad_a$ [20], whilst the other appeared to consist of an inversion of a sequence immediately 5' of the first coding exon. Significantly, these exons were not located in sequenced portions of $\Delta 6fad_c$. Although the 5' regions of $\Delta 6fad_a$ and $\Delta 6fad_b$ were very similar, including the sequence and position of the promoter region, the equivalent region in $\Delta 6fad_c$ indicated the presence of an inserted repeat sequence (Fig 1).

Salmon fatty acyl desaturase cDNAs

The putative Fad cDNAs isolated from Atlantic salmon were both shown to have ORFs of 1373 bp encoding proteins of 457 amino acids, with 3'UTRs of 642 bp for $\Delta 6fad_b$ and 1408 bp for $\Delta 6fad_c$. The protein sequences included all the characteristic features of microsomal fatty acid desaturases, including three histidine boxes and an N-terminal cytochrome b₅ domain containing the haem-binding motif, H-P-G-G and two transmembrane regions (Fig. 2). These features are similar to those of other Fad cDNAs including salmon $\Delta 5$ Fad (gb|AF478472|) and $\Delta 6$ Fad_a (gb|AY458652|), the zebrafish $\Delta 6/\Delta 5$ Fad (gb|AF309556|), and the human $\Delta 5$ (gb|AF199596|) and $\Delta 6$ (gb|AF126799|) Fads. A pair-wise comparison was made between the newly cloned Atlantic salmon Fads with each other, and with other fish and human Fad sequences. The amino acid sequences predicted by the salmon putative $\Delta 6$ Fad_b and $\Delta 6$ Fad_c ORFs showed 96.7% identity to each other. When compared to other fish Fads, $\Delta 6$ Fad_b and $\Delta 6$ Fad_c amino acid sequences are 91.9 - 92.5 % identical to salmon $\Delta 6$ Fad_a and 90.4 - 90.6 % to salmon $\Delta 5$ Fad, 76.6 - 78.1 %

identical to marine fish $\Delta 6$ Fads including those from cobia (*Rachycentron canadum*) (gb|FJ440238|) and Atlantic cod (*Gadus morhua*) (gb|DQ054840|), and 65.0 - 65.6 % to the dual zebrafish $\Delta 6/\Delta 5$ Fad. Additionally, $\Delta 6$ Fad_b and $\Delta 6$ Fad_c proteins showed 63.7 - 63.9 % identity to the human $\Delta 6$ and 56.6 - 57.3 % to $\Delta 5$ Fad. Phylogenetic analysis clustered the new Atlantic salmon putative Fad sequences with other salmonid Fads, including the formerly studied Atlantic salmon $\Delta 6$ and $\Delta 5$ Fads, $\Delta 6$ Fad from rainbow trout (*Oncorhynchus mykiss*), and uncharacterised putative Fads of masou (cherry) salmon (*Oncorhynchus masou*) (Fig. 3). The salmonid Fads also clustered with other fish Fads including those of Atlantic cod, gilthead sea bream (*Sparus aurata*), turbot (*Psetta maxima*), cobia, tilapia (*Oreochromis niloticus*), European sea bass (*Dicentrarchus labrax*), Asian sea bass or barramundi (*Lates calcarifer*), zebrafish and common carp (*Cyprinus carpio*). The salmon Fads clustered more distantly from Fads of other organisms including mammalian (mouse and human), fungus (*Mortierella alpina*) and worm (*Caenorhabditis elegans*).

Functional characterization

The salmon Fad cDNAs were functionally characterized by determining the fatty acid profiles of transformed *S. cerevisiae* containing either the pYES2 vector alone or the vector with the salmon Fad cDNA inserts, grown in the presence of a variety of potential fatty acid substrates, including $\Delta 6$ substrates (18:2n-6 and 18:3n-3), $\Delta 5$ substrates (20:3n-6 and 20:4n-3) and $\Delta 4$ substrates (22:4n-6 and 22:5n-3). The fatty acid composition of the yeast transformed with the vector alone showed the main fatty acids normally found in *S. cerevisiae*, namely 16:0, 16:1 isomers, 18:0 and 18:1n-9, together with the exogenously derived fatty acids (data not shown). This is consistent with *S. cerevisiae* not possessing $\Delta 4$, $\Delta 5$ or $\Delta 6$ desaturase activities [27]. Based on GC retention time and confirmed by GC-MS, salmon $\Delta 6$ Fad_b and $\Delta 6$ Fad_c showed activity towards $\Delta 6$ substrates 18:3n-3 (Fig. 4A and B) and 18:2n-6 (Fig. 4C and D), which were converted to 18:4n-3 and 18:3n-6, respectively. The cDNA products did not possess either $\Delta 5$ or $\Delta 4$ desaturase activity as evidenced

by the lack of additional peaks representing desaturated products of 20:4n-3, 20:3n-6, 22:5n-3 or 22:4n-6 (Fig. 4E-H; Table 2). Conversion rates indicated that $\Delta 6\text{Fad}_b$ protein was more active than $\Delta 6\text{Fad}_c$ in the recombinant yeast system, with $\Delta 6\text{Fad}_b$ desaturating 47 % of 18:3n-3 to 18:4n-3, and 25 % of 18:2n-6 to 18:3n-6, whereas $\Delta 6\text{Fad}_c$ showed conversions of 12 and 7 %, respectively (Table 2). As reported for other teleost Fads, salmon $\Delta 6\text{Fad}_b$ and $\Delta 6\text{Fad}_c$ displayed more activity towards the n-3 fatty acids than n-6 (Table 2).

Fatty acyl desaturase gene expression: tissue distribution and nutritional regulation

Tissue distribution of the three $\Delta 6\text{fad}$ genes including $\Delta 6\text{fad}_a$ (gb|AY458652|) and the two newly cloned $\Delta 6\text{fad}_b$ (gb|GU207400|) and $\Delta 6\text{fad}_c$ (gb|GU207400|), was determined by qPCR performed on cDNA obtained from different tissues. The results showed that $\Delta 6\text{fad}_a$ and $\Delta 6\text{fad}_b$ genes were highly expressed in intestine (pyloric caeca), liver and brain, with gill also having high transcript copy number for $\Delta 6\text{fad}_b$ (Fig. 5). Interestingly, $\Delta 6\text{fad}_c$ transcript showed a different tissue distribution pattern, with transcripts being predominantly found in brain, with an expression level exceeding that in all other tissues by over 30-fold (Fig. 5). The regulation of $\Delta 6\text{fad}_a$, $\Delta 6\text{fad}_b$ and $\Delta 6\text{fad}_c$ in response to dietary FA composition was determined in intestine (pyloric caeca) and liver of salmon fed diets containing either FO rich in n-3 LC-PUFA (EPA and DHA) or VOs rich in C18 FA, SO (18:2n-6), LO (18:3n-3) or RO (18:1n-9). In intestine, there was significant higher expression of $\Delta 6\text{fad}_b$ transcripts in SO- and RO-fed salmon compared to the fish fed FO, with normalized expression ratios of 3.8 and 2.9, respectively (Fig. 6A). No significant differences were found between transcript levels for $\Delta 6\text{fad}_a$ and $\Delta 6\text{fad}_c$ in intestine of VO-fed fish compared to FO-fed fish, although both genes showed relative expression ratios above 1 suggesting some up-regulation in VO treatments. In liver, expression of the $\Delta 6\text{fad}_a$ gene was up-regulated by VO treatments, with significant 2.7-, 2.2- and 3.0-fold higher transcript levels in fish fed SO, LO and RO, respectively (Fig. 6B). The levels of $\Delta 6\text{fad}_b$ mRNA were also higher in liver

of fish fed SO and RO treatments and, although not statistically significant (REST-2008), this trend was consistent with the results observed in intestine.

Discussion

Prior to this study cDNAs for $\Delta 6$ and $\Delta 5$ fads had been characterised in Atlantic salmon [16,18], and despite having distinct enzymic activities, these two proteins showed a remarkable level of similarity, indicating that they had arisen within the evolutionary line leading to salmonids, and after divergence from other fish groups. This led to questions regarding the nature of the genes encoding these enzymes, and raised the possibility that they may have been allelic variants rather than distinct genes. In this study, after screening a genomic library, it is clear that salmon possess at least four fads genes. Although all of the products of these genes show the same remarkable levels of similarity (up to 97 % for $\Delta 6$ fad_b and $\Delta 6$ fad_c, for example) the evidence that these genes are distinct loci is most clear from the different exon sizes, a result of differential distribution of intronic transposable and other repeat elements. The salmon fad genes all have identical genomic coding exon structures consisting of 12 coding exons, but differ in the identity and positions of 5' non-coding exons, and in the size and sequence of 3' non-coding regions. This genomic coding organisation is identical to that previously reported for the human FADS1 ($\Delta 5$ desaturase), FADS2 ($\Delta 6$ desaturase) and FADS3 genes that also consist of 12 coding exons [34].

Although the salmon *fad* gene structures show several interesting features, particularly in the intron structures, which may eventually reveal important information on the evolutionary history of *fad* genes in salmon and fish generally, it is the heterogeneity in the 5' end of the genes that is of greatest interest for the present study. The differential splicing of the non-coding exons in this region of the $\Delta 6$ fad_a, $\Delta 6$ fad_b and $\Delta 5$ fad genes and the contrasting quite different structure of the region in the $\Delta 6$ fad_c gene may have possible functional consequences. Comparison of the

upstream sequences of *Δ6fad_a* and *Δ6fad_b* indicated a high degree of similarity, and although *Δ6fad_b* utilised an extra non-coding exon, both of these genes share a similar first exon, which in both genes is preceded by a conserved sequence, previously shown to act as a functional and PUFA-responsive promoter in *Δ6fad_a* [20]. In contrast the *Δ6fad_c* gene has a repeat element insertion in the equivalent region. This appears to have resulted in a very different 5' non-coding exon structure, including the utilisation of alternative splice sites. Although we were unable to map these exons to the available *Δ6fad_c* sequence, one of these alternative exons is an inverted repeat of a region immediately after the 5' end of the repeat element and before the first coding exon. It is possible the insertion of the repeat element has caused the repetition and inversion of the host DNA at the insertion site, and the inverted sequence has been utilised as an alternative exon. The other alternative exon comprises a sequence which matches a major portion of the promoter region of *Δ6fad_a*. Taken together this might predict that *Δ6fad_b* would be regulated similarly to *Δ6fad_a*, and that *Δ6fad_c* would be regulated differently. This prediction is supported by the gene expression data which showed substantial differences between *Δ6fad_c* and the other *Δ6fad* genes, with *Δ6fad_c* expression being very largely confined to brain whereas *Δ6fad_a* was expressed highly in intestine > liver > brain and *Δ6fad_b* was expressed in brain > intestine > gill > liver. Furthermore, *Δ6fad_c* expression showed no nutritional regulation whereas expression of *Δ6fad_a* and *Δ6fad_b* were significantly increased in liver and intestine, respectively, in fish fed diets with reduced levels of LC-PUFA.

In humans, the desaturase gene cluster (FADS1, FADS2 and FADS3) has most likely arisen by tandem gene duplication. This is on the basis that the genes are in a head to head, or head to tail arrangement, and the exon organization is nearly identical in the three family members, with each gene consisting of 12 exons and splice and acceptor sites interrupted at identical nucleotide positions within highly conserved codons [34]. The first coding exons in the salmon *Δ5fad* and *Δ6fad_a* genes are 30 bp longer than the corresponding exons in the human FADS1 ($\Delta 5$ FAD) and

FADS 2 ($\Delta 6$ FAD) genes, corresponding to the additional 10 amino acids found in several salmonid desaturases [16,18,19,35]. The other salmon *fad* genes isolated here have a further 9 additional bases in their first coding exon corresponding to an additional 3 amino acids. Therefore, after subtracting the UTR, the first coding exons contain 246 coding bp in *$\Delta 6fad_b$* and *$\Delta 6fad_c$* , and 237 bp in *$\Delta 6fad_a$* and *$\Delta 5fad$* (Table 3). In contrast, the remaining exons are exactly the same size in all the salmon *fad* genes as the human $\Delta 6$ desaturase gene (FADS2) (Table 3), with splice and acceptor sites interrupted at similar nucleotide positions, despite the lengths of the introns being very different [18,34]. Interestingly, the human FADS genes vary by 3 bp in coding exon 5 (FADS1/ $\Delta 5$ having 129 bp, FADS2/ $\Delta 6$ having 126 and FADS3 having 123 bp [34], but all the salmon genes, including *$\Delta 5fad$* , have 126 bp in coding exon 5.

The different enzymic activities of $\Delta 6$ *fad_a* and $\Delta 5$ *fad* clearly discriminate their functional roles, however the three different $\Delta 6$ *fads* may also have distinct biological/physiological roles in salmon. The activity of $\Delta 6$ *fad_a* in the heterologous yeast system was previously reported as 60 and 14 % conversion for 18:3n-3 and 18:2n-6, respectively [18] and so the activity of the three $\Delta 6$ enzymes was in the rank order $a > b > c$. Similarly, although all three were more active towards the n-3 fatty acid substrate, as observed previously for all other fish *Fad* enzymes [16,18,27,41-43], the rank order for n-3/n-6 activity ratio was $a (4.2) > b (1.9) > c (1.7)$. As mentioned above, tissue and nutritional regulation also showed functional differences, with *$\Delta 6fad_a$* being the main *fad* gene expressed in liver, and also being under nutritional regulation in that tissue. In contrast, *$\Delta 6fad_b$* was expressed highly and nutritionally regulated in intestine. Although *$\Delta 6fad_a$* was also expressed highly in intestine it showed no nutritional regulation in that tissue, and *$\Delta 6fad_b$* was not nutritionally regulated in liver. In contrast, *$\Delta 6fad_c$* was expressed predominantly in brain, as has been observed with the $\Delta 6$ *Fads* in marine fish [42,43]. Unfortunately, brain was not sampled in the earlier nutritional experiments and so we were unable to determine if *$\Delta 6fad_c$* was nutritionally regulated in that tissue. However, the data are consistent with the different *Fads* having different

functional roles in different tissues. In this respect, the high levels of expression of two of the $\Delta 6fad$ genes in both intestine and brain perhaps reflect the importance of fatty acid metabolism in these tissues, the intestine being the tissue first exposed to dietary fatty acids, and the brain having strict LC-PUFA requirements including high DHA and ARA [14]. Thus expression of both $\Delta 6fad_a$ and b in intestine, with one being regulated by dietary LC-PUFA composition is consistent with it being the first tissue to encounter and respond to dietary lipid, and high expression of both $\Delta 6fad_b$ and c in brain is consistent with the critical importance of high LC-PUFA levels in neural tissue [42-43]. The fact that $\Delta 6fad_a$, the highest activity enzyme, is the highest expressed of the genes in liver is consistent with liver being the major tissue for regulating overall body lipid metabolism. Determining how the same genes are differentially regulated in different tissues will be an interesting challenge, and presumably related to the expression, activities and regulation of transcription factors that could include sterol regulatory element binding protein-1c, liver X receptor and possibly peroxisome proliferator activated receptors and retinoid X receptors [20,44,45].

The evolutionary context of the presence of four Fads genes in Atlantic salmon is interesting. Salmonids, unlike the Actinopterygian fish species studied to date, have relatively higher activities of LC-HUFA biosynthesis and contain genes for both $\Delta 5$ and $\Delta 6$ desaturases. Actinopterygians, such as sea bass and sea bream appear not to express $\Delta 5$ activity [15] and sequenced genomes have numbers of fads genes varying from zero, in the case of pufferfish (*Takifugu rubripes*, *Tetraodon nigroviridis*), to one in the case of medaka (*Oryzias latipes*), and three in the case of stickleback (*Gasterosteus aculeatus*) [44]. It is also clear that all four genes have been duplicated relatively recently, and within the salmonid evolutionary line. Preliminary evidence from limited pedigree analyses in our laboratory (not shown) suggests that all four salmon desaturase gene are located in the same linkage group, indicating that they may have arisen as a result of segmental duplications and not as a result of the salmonid tetraploidization event

hypothesised to have occurred between 25 and 100 million years ago [36]. Regardless of the mechanism of duplication, the separate evolution by sub- or neo-functionalization of $\Delta 5$ and $\Delta 6$ desaturase activities, together with the tissue specific regulation these genes, is likely to be responsible for the high biosynthetic LC-HUFA capacity in salmon compared to other fish. It has been suggested that the relatively high LC-HUFA biosynthesis in salmonids is an evolutionary adaptation to a nutrient poor, freshwater habitat, enabling Atlantic salmon and their relatives to exist on a diet with a high input of terrestrially-derived foodstuffs that are relatively deficient in EPA and DHA. The presence of four very similar, sub-functionalized desaturase genes which group together in phylogenetic plots supports this contention. The three mammalian PUFA desaturase genes [34] are also the result of segmental duplications, followed by a sub-functionalization, giving rise to separate genes for $\Delta 5$, $\Delta 6$, and a third uncharacterised desaturase (FADS3). However, considering the phylogenetic relationships amongst vertebrate desaturases, these mammalian genes are clearly the result of a separate, earlier duplication which must have occurred in the evolutionary line leading to mammals after its split from fish. Thus, both the salmon and mammalian desaturase duplications and subsequent sub-functionalizations might represent separate evolutionary adaptations to diets containing terrestrial foodstuffs, ultimately derived from plants which are devoid of LC-PUFA.

In summary, we have demonstrated that there are at least four genes encoding putative Fad proteins in Atlantic salmon. Two genes, *$\Delta 6fad_a$* and *$\Delta 5fad$* , corresponded to the previously cloned $\Delta 6$ and $\Delta 5$ Fad cDNAs, and cDNAs for the two further genes, *$\Delta 6fad_b$* and *$\Delta 6fad_c$* , also had only $\Delta 6$ activity. Both *$\Delta 6fad_a$* and *$\Delta 6fad_b$* genes were highly expressed in intestine, liver and brain, whereas *$\Delta 6fad_c$* transcript was found predominantly in brain. The expression levels of the *$\Delta 6fad_a$*

gene in liver and the $\Delta 6fad_b$ gene in intestine were significantly higher in fish fed VO diets suggesting up-regulation in response to reduced dietary EPA and DHA. In contrast, no significant differences were found between transcript levels for $\Delta 6fad_a$ in intestine, $\Delta 6fad_b$ in liver, or $\Delta 6fad_c$ in liver or intestine of fish fed VO compared to fish fed FO. Further work is in progress to determine the mechanisms of differential expression of the fad genes in different tissues and the roles of transcription factors in regulating LC-PUFA synthesis.

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Figure legends

Fig 1. Structures of Atlantic salmon $\Delta 5$ and $\Delta 6$ fatty acyl desaturase ($\Delta 5/\Delta 6fad$) genes.

Gene structures were determined from genomic sequences from a library constructed from a single individual fish. Sequenced regions (not to scale) showing high levels of identity across all genes (using DIALIGN, <http://bibiserv.techfak.uni-bielefeld.de/dialign/>) [31] are indicated by a black line, and sequenced coding exons predicted from alignment with cDNAs are indicated by black boxes. Non-coding exon regions are indicated by unfilled boxes. Hatched lines indicate regions of the genes that have not been sequenced and grey boxes indicate exons predicted from cDNAs, but not sequenced from genomic DNA. Asterisks indicate an alternatively utilised exon predicted from cDNA sequences. Vertical arrows indicate the position of a nine base pair insertion in $\Delta 6fad_b$ and $\Delta 6fad_c$ compared to $\Delta 6fad_a$ and $\Delta 5fad$. Horizontal arrows indicate the position and orientation of an inverted repeat sequence in $\Delta 6fad_c$. The black triangle indicates the position of the promoter region for $\Delta 6fad_a$ [20]. The positions, sizes (base pairs) and similarity hit to RepBase (<http://www.girinst.org/rebase/index.html>) [32] of intron regions which could not be aligned across all four genes are indicated. These sequences all match multiple sequences in diverse salmon database entries. DTSSa1, Tss, pTSSa1 and SSTN are degenerate salmon Class I transposons [33]. Rex1, Tol2, PiggyBac, and FuguRep2 denote sequences with extensive similarity to RepBase entries for various transposons. RPT is an uncharacterised sequence with >100 high similarity hits to diverse salmonid sequences in Genbank.

Fig 2. Comparison of the deduced amino acid sequence of the newly cloned $\Delta 6$ fatty acyl desaturases from Atlantic salmon with formerly cloned *Salmo salar* $\Delta 6$ (Ss $\Delta 6Fad_a$) and $\Delta 5$ (Ss $\Delta 5Fad$) desaturases, the $\Delta 6/\Delta 5$ bifunctional desaturase from *Danio rerio* (Dr $\Delta 6/\Delta 5Fad$) and human $\Delta 6$ desaturase (Hs $\Delta 6FAD$). Deduced amino acid sequences were aligned using ClustalW. Identical residues are shaded black and similar residues are shaded grey. Identity/similarity shading was

based on the BLOSUM62 matrix, and the cut-off for shading was 70%. The cytochrome b₅-like domain is dot-underlined, the two transmembrane regions are dash-underlined, and the three histidine-rich domains are solid-underlined. The asterisks on the top mark the heme-binding motif, HPGG.

Fig.3. Phylogenetic tree comparing putative amino acid sequences of Atlantic salmon, *S. salar*, fatty acyl desaturases $\Delta 6$ Fad_b and $\Delta 6$ Fad_c, together with the formerly cloned salmon $\Delta 5$ Fad, $\Delta 6$ Fad_a, and other desaturase proteins from fish and other organisms. Desaturases that have not been functionally characterized are marked with an asterisk. The tree was constructed using the Neighbour Joining method [26] using MEGA4. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 1000 iterations.

Fig.4. Functional characterisation of the newly cloned salmon fatty acyl desaturases $\Delta 6$ fad_b (panels A, C, E and G) and $\Delta 6$ fad_c (panels B, D, F and H) in transgenic yeast (*Saccharomyces cerevisiae*) grown in the presence of $\Delta 6$ substrates 18:3n-3 (A and B) and 18:2n-6 (C and D), and $\Delta 5$ substrates 20:4n-3 (E and F) and 20:3n-6 (G and H). Fatty acids were extracted from yeast transformed with pYES2 vector containing the ORF of the putative fatty acyl desaturase cDNA as an insert. The first five peaks in all panels are the main endogenous fatty acids of *S. cerevisiae*, namely 16:0 (1), 16:1n-9 (2), 16:1n-7 (3), 18:0 (4), and 18:1n-9 (5). Substrates (“*”) and their corresponding desaturated products are indicated accordingly in panels A-H. Vertical axis, FID response; horizontal axis, retention time.

Fig. 5. Tissue distribution profile of $\Delta 6$ fad_a, $\Delta 6$ fad_b and $\Delta 6$ fad_c transcripts in Atlantic salmon, determined by RT-qPCR. Absolute copy numbers were quantified for each transcript and

normalised by absolute levels of ribosomal 18s (values shown on top of each column and represented diagrammatically in logarithmic scale). A, adipose tissue; B, brain; G, gill; H, heart; K, kidney; L, liver; PC, pyloric caeca; RM, red muscle; S, spleen; WM, white muscle.

Fig 6. Nutritional regulation of desaturases $\Delta6fad_a$, $\Delta6fad_b$ and $\Delta6fad_c$ in Atlantic salmon fed diets containing fish oil (FO), soybean oil (SO), linseed oil (LO) or rapseed oil (RO), in the intestine (A) and liver (B), determined by RT-qPCR. The results show normalised expression ratios (reference genes: *elf-1 α* and an unresponsive EST) of the target transcripts in one of the VO treatments (SO, LO or RO), in relation to the FO control treatment. Values are means with S.E. (n = 5) and asterisks represent significant differences ($P \leq 0.05$) between the column's dietary treatment and the FO treatment, for the respective transcript (REST-2008[©]).

Table 1. Sequence and annealing temperature (Tm) of the primer pairs used, size of the fragment produced and accession number of the sequence used as reference for primer design, for $\Delta 6fad_b$ and $\Delta 6fad_c$ ORF and 3'UTR cloning and for real-time quantitative PCR (qPCR) determinations of the transcript levels of Atlantic salmon $\Delta 6$ -like desaturase genes. Primers SSD6VF and SSD6VR (ORF cloning) contained restriction sites (underlined) for *Hind*III and *Xho*I, respectively.

Aim	Transcript	Primer	Primer sequence	Fragment	Tm	Accession	
3'RACE	$\Delta 6fad_b$	SSD6bF1	5'-TCCGCAATCCATCCTGATC-3'	1841 bp	62°C	AF010300	
		3'RACE OUTER	5'-GCGAGCACAGAATTAATACGACT-3'				
		SSD6bF2	5'-GATGTCAACTCACTGCATGTC-3'	1319 bp	57°C		
	$\Delta 6fad_c$	3'RACE INNER	5'-CGCGGATCCGAATTAATACGACTACTATAGG-3'				
		SSD6cF1	5'-ACTTCCAGGCCTTGCGCG-3'	2442 bp	68°C		
		3'RACE OUTER	5'-GCGAGCACAGAATTAATACGACT-3'				
ORF cloning	$\Delta 6fad_b$	SSD6cF2	5'-CAAGACTTTGAAGAAAGGCATCAT-3'	1529 bp	61°C		
		3'RACE INNER	5'-CGCGGATCCGAATTAATACGACTACTATAGG-3'				
		SSD6bU5F	5'-TTGGACAGACTGGACAGAGC-3'	1481 bp	60°C		
	$\Delta 6fad_c$	SSD6bU3R	5'-GCGATGGATTTAAAATCCCG-3'				
		SSD6VF	5'-CCC <u>AAGCTT</u> AGGATGGGGGGCGGAGGCC-3'	1395 bp	60°C		
		SSD6VR	5'-CCG <u>CTCGAGT</u> TATTATGGAGATATGCAT-3'				
qPCR	$\Delta 6fad_a$	SSD6cU5F	5'-GTGAGTGGAGCAGAGAGAACC-3'	1514 bp	59°C		
		SSD6cU3R	5'-GCAGCGATCATTATCAACACA-3'				
	$\Delta 6fad_b$	SSD6VF	5'-CCC <u>AAGCTT</u> AGGATGGGGGGCGGAGGCC-3'	1395 bp	60°C		
		SSD6VR	5'-CCG <u>CTCGAGT</u> TATTATGGAGATATGCAT-3'				
	$\Delta 6fad_c$	SSD6aF	5'-CCCCAGACGTTTGTGTCAG-3'	180 bp	60°C		
		SSD6aR	5'-CCTGGATTGTTGCTTTGGAT-3'				
	<i>elf-1α</i>	SSD6bF	5'-ATAGAGGGTTTATATAGTAGGGCC-3'	204 bp	58°C		
		SSD6bR	5'-GGTGGGACGCTAGAAGTTAA-3'				
	β -actin	SSD6cF	5'-CCCACCCCATCTTAAACT-3'	171 bp	60°C		
		SSD6cR	5'-CTGGGGTCCAAACAAGGTTA-3'				
	unresp. EST	ELF-1 α jbt2	5'-CTGCCCTCCAGGACGTTTACAA-3'	175 bp	60°C		
		ELF-1 α jbt2	5'-CACCGGCATAGCCGATTCC-3'				
18S rRNA	BACT-F	5'-ACATCAAGGAGAAGCTGTGC-3'	141 bp	56°C			
	BACT-R	5'-GACAACGGAACCTCTCGTTA-3'					
18S rRNA	B2F	5'-AGCCTATGACCAACCCACTG-3'	224 bp	60°C			
	B2R	5'-TGTTACAGCTCGTTTACCG-3'					
18S rRNA	ss18S-2F	5'-GGCGCCCCCTCGATGCTCTTA-3'	189 bp	65°C			
	ss18S-2R	5'-CCCCCGCCGTCCTCTTAAT-3'					

¹ GenBank (<http://www.ncbi.nlm.nih.gov/>)

² Atlantic salmon Gene Index (<http://compbio.dfci.harvard.edu/tgi/>)

Table 2. Functional characterization of salmon $\Delta 6$ Fad_b and $\Delta 6$ Fad_c proteins in *Saccharomyces cerevisiae*. Results are expressed as a percentage of total fatty acid substrate converted to desaturated product.

FA substrate	Product	Conversion (%)		Activity
		$\Delta 6$ Fad_b	$\Delta 6$ Fad_c	
18:3n-3	18:4n-3	47	12	$\Delta 6$
18:2n-6	18:3n-6	25	7	$\Delta 6$
20:4n-3	20:5n-3	0	0	$\Delta 5$
20:3n-6	20:4n-6	0	0	$\Delta 5$
22:5n-3	22:6n-3	0	0	$\Delta 4$
22:4n-6	22:5n-6	0	0	$\Delta 4$

Table 3.

Table 3. Exon sizes (bp) of Atlantic salmon fatty acyl desaturase (<i>fad</i>) genes				
	<i>Δ6fad-a</i>	<i>Δ6fad-b</i>	<i>Δ6fad-c</i>	<i>Δ5fad</i>
1	22 ^a	18 ^d		
2	496 ^b	320 ^e	321 ^g	
3	111	111	111	
4	198	198	198	
5	102	102	102	
6	126	126	126	
7	61	61	61	
8	77	77	77	
9	98	98	98	98
10	97	97	97	97
11	80	80		80
12	126	126		126
13	509 ^c	400 ^f		1124 ^h
^{a,d} Exon are 5' untranslated regions (UTR)				
^b Includes a 5'-UTR of 259 bp				
^c Includes a 3'-UTR of 457 bp				
^e Includes a 5'-UTR of 74 bp				
^f Includes a 3'-UTR of 348 bp (not end of gene)				
^g Includes a 5'-UTR of 75 bp				
^h Includes a 3'-UTR of 1072 bp				

Fig.1.

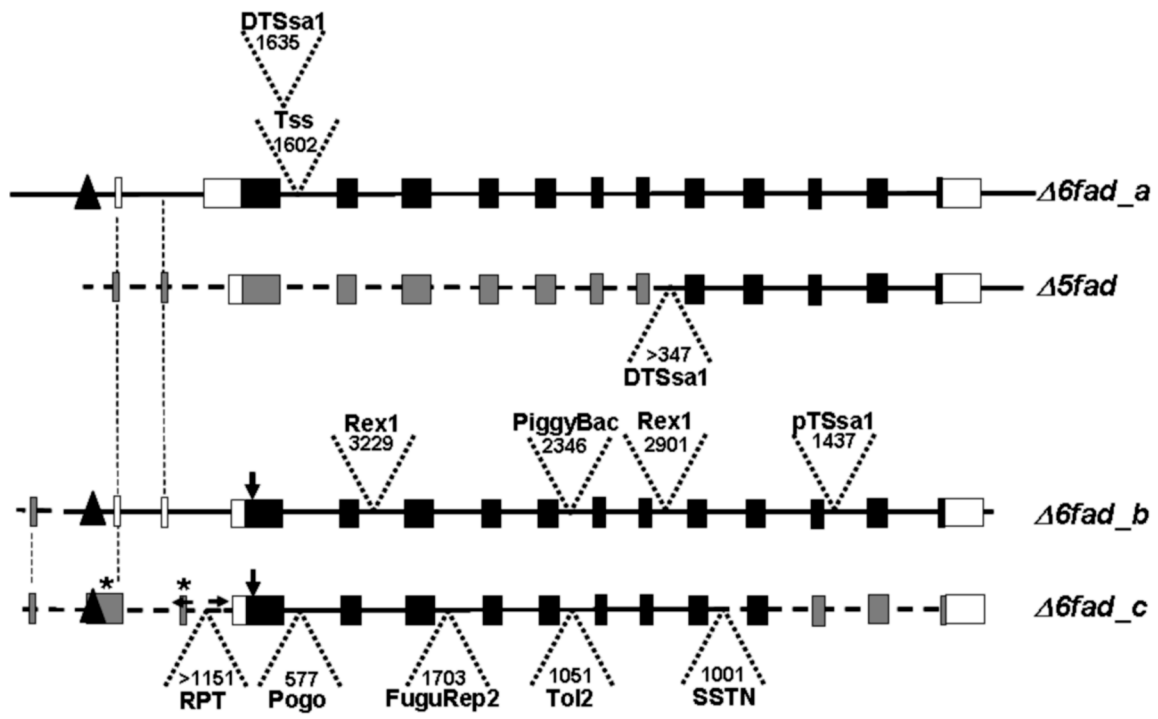


Fig.2.

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Ss A5Fad      MGGGGQQTESSEPAKGDGLEPDGGQ---GGSAVYTWEVQRHSHRSDQWLVIDRKVYINITQWAKRHPGGI 67
Ss A6Fad_a   MGGGGQQNDSEGEPAKGRGGPGGGL---GGSAVYTWEVQRHSHRSDQWLVIDRKVYINITQWAKRHPGGI 67
Ss A6Fad_b   MGGGGQQTESSEPAKGSVVVPGGGRRGGRGSSAVYTWEVQRHSHRSDQWLVIDRKVYINITQWAKRHPGGI 70
Ss A6Fad_c   MGGGGQQTESSEPAKGSVVVPGGGRRGGRGSSAVYTWEVQRHSHRSDQWLVIDRKVYINITQWAKRHPGGI 70
Dr A6A5Fad   MGGGGQQTDRITDTNG-----RFSSYTWEVQRHSHRSDQWLVIDRKVYINITQWAKRHPGGI 57
Hs A6FAD     MGRGGNQGEGAAEREVS-----VPTFSWEEDQENLRSDQWLVIDRKVYINITQWAKRHPGGI 57
          ..*.

Ss A5Fad      RVISHFAGEDATDAFSAFHLDAFVVRKFLKPLLIGELAPEPSQDHGKNAALVDFCALRDHVEREGLLR 127
Ss A6Fad_a   RVISHFAGEDATDAFSAFHPNPNFVRKFLKPLLIGELAPEPSQDHGKNAALVDFCALRNRVEREGLLR 127
Ss A6Fad_b   RVISHFAGEDATDAFSAFHPDPNFVRKFLKPLLIGELAPEPSQDHGKNAALVDFCALRDHVESEGLLR 137
Ss A6Fad_c   RVISHFAGEDATDAFSAFHPDPNFVRKFLKPLLIGELAPEPSQDHGKNAALVDFCALRDHVESEGLLR 137
Dr A6A5Fad   RILGHYAGEDATDAFSAFHPNQLVVRKFLKPLLIGELAPEPSQDRGKNAALVDFCALRERLEAEGCFK 140
Hs A6FAD     RVISHFAGEDATDAFSAFHPDLEFVGRKFLKPLLIGELAPEPSQDHGKNSKITDFRALRKTAEQDNLFK 140
          ..*.

Ss A5Fad      ARPLFFSLALGHILLEALALGLLVWVGTWSLTLCSLMLATSQSQAGWLQHDYGHLSVFKKSSWNHKL 197
Ss A6Fad_a   ARPLFFSLALGHILLEALALGLLVWVGTWSLTLCSLMLATSQSQAGWLQHDYGHLSVFKKSSWNHVL 197
Ss A6Fad_b   ARPLFFSLALGHILLEALALGLLVWVGTWSLTLCSLMLATSQSQAGWLQHDYGHLSVFKKSSWNHVL 207
Ss A6Fad_c   ARPLFFSLALGHILLEALALGLLVWVGTWSLTLCSLMLATSQSQAGWLQHDYGHLSVFKKSSWNHVL 207
Dr A6A5Fad   TQLFFALALGHILLEALAFMMVWYFEGTGWINTLIVAVILATQSQAGWLQHDYGHLSVFKSSGNHVL 210
Hs A6FAD     TNEVFLLLAHIIALESIAWFTVVEYFNGWIPTELTAFVLATSQSQAGWLQHDYGHLSVYRKPKNHVL 210

Ss A5Fad      HKFVIGHLKGASANWVNRHFQHHAKPNVFRKDPDINSLEVFVLGDTQPVEYGRKKLKYFPYHHQRYFFF 267
Ss A6Fad_a   HKFVIGHLKGASANWVNRHFQHHAKPNVLSKDPDVMLEVFVLGDKQPVEYGRKKLKYFPYHHQRYFFF 267
Ss A6Fad_b   QKFVIGHLKGASANWVNRHFQHHAKPNVFSKDPDINSLEVFVLGDKQPVEYGRKKLKYFPYHHQRYFFF 277
Ss A6Fad_c   QKFVIGHLKGASANWVNRHFQHHAKPNVFRKDPDINSLEVFVLGDTQPVEYGRKKLKYFPYHHQRYFFF 277
Dr A6A5Fad   HKFVIGHLKGASAVWVNRHFQHHAKPNVFSKDPDVMLEVFVGNVQPVEYGRKKLKYFPYHHQRYFFF 280
Hs A6FAD     HKFVIGHLKGASANWVNRHFQHHAKPNVFRKDPDVMLEVFVLGDNQPVEYGRKKLKYFPYHHQRYFFF 280

Ss A5Fad      LIGPPLIPVVFPHIQILRTMFSQRDWDVLAWMSSTYLRFFCCYYPFEGFFGSVALISFVRFLESHWFVWV 337
Ss A6Fad_a   LIGPPLIPVVFPHIQIFQTMFSQRNWDVLAWMSSTYLRFFCSYYPFEGFFGSVALISFVRFLESHWFVWV 337
Ss A6Fad_b   LIGPPLIPVVFPHIQILRTMFLRQDWDVLAWMSSTYLRFFCCYYPFEGFFGSVALISFVRFLESHWFVWV 347
Ss A6Fad_c   LIGPPLIPVVFPHIQILRTMFLRQDWDVLAWMSSTYLRFFCCYYPFEGFFGSVALISFVRFLESHWFVWV 347
Dr A6A5Fad   FIGPPLIPVVFPHIQIFHNMI SHGMVVDLWCHSYVRFELCYTQFVGVWAIILFNVEVRFLESHWFVWV 350
Hs A6FAD     LIGPPLIPVVFPHIQILMTMI VHKNWDVLAWMSSTYLRFFIYIIPFYGILGALLFLNRFLESHWFVWV 350

Ss A5Fad      TQMNHLPMENDHERHQDWLTMQLSATCNIEQSTFNDWFSGHLNFQIEHHLFPTMPRHNYELVAPLVRALC 407
Ss A6Fad_a   TQMNHLPMENDHERHQDWLTMQLSATCNIEQSTFNDWFSGHLNFQIEHHLFPTMPRHNYELVAPLVRALC 407
Ss A6Fad_b   TQMNHLPMENDHERHQDWLTMQLSATCNIEQSTFNDWFSGHLNFQIEHHLFPTMPRHNYELVAPLVRALC 417
Ss A6Fad_c   TQMNHLPMENDHERHQDWLTMQLSATCNIEQSTFNDWFSGHLNFQIEHHLFPTMPRHNYELVAPLVRALC 417
Dr A6A5Fad   TQMSHHPMNDYDEKNQDWLTMQLSATCNIEQSAFNDWFSGHLNFQIEHHLFPTMPRHNYELVAPLVRALC 420
Hs A6FAD     TQMNHLPMENDDEAYFDWFSQLSATCNIEQSTFNDWFSGHLNFQIEHHLFPTMPRHNYELVAPLVRALC 420

Ss A5Fad      EKHGVPYQVKTLLQKGMTDVVRSLLKSGDLWLDAYLHK 444
Ss A6Fad_a   EKHGVPYQVKTLLQKAIIDVVRSLLKSGDLWLDAYLHK 444
Ss A6Fad_b   EKHGVPYQVKTLLQOGLTDVVRSLLKSGDLWLDAYLHK 454
Ss A6Fad_c   EKHGVPYQVKTLLKGIIDVVRSLLKSGDLWLDAYLHK 454
Dr A6A5Fad   EKHGVPYQVKTLLYGAFADIRSLKSGDLWLDAYLHK 457
Hs A6FAD     AKHGVEYQVKTLLRALLDIRSLKSGDLWLDAYLHK 457

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Fig.3.

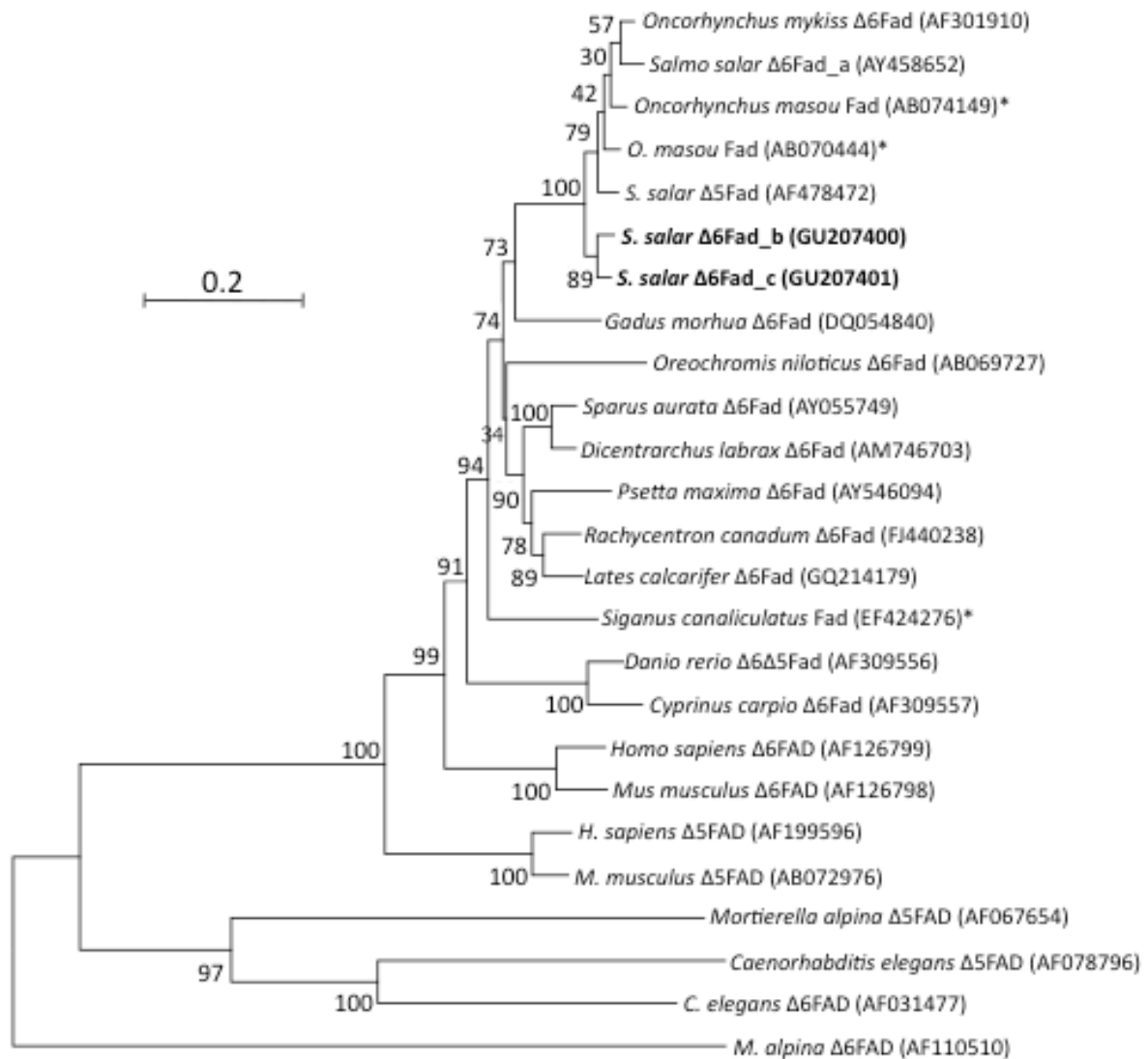


Fig 4.

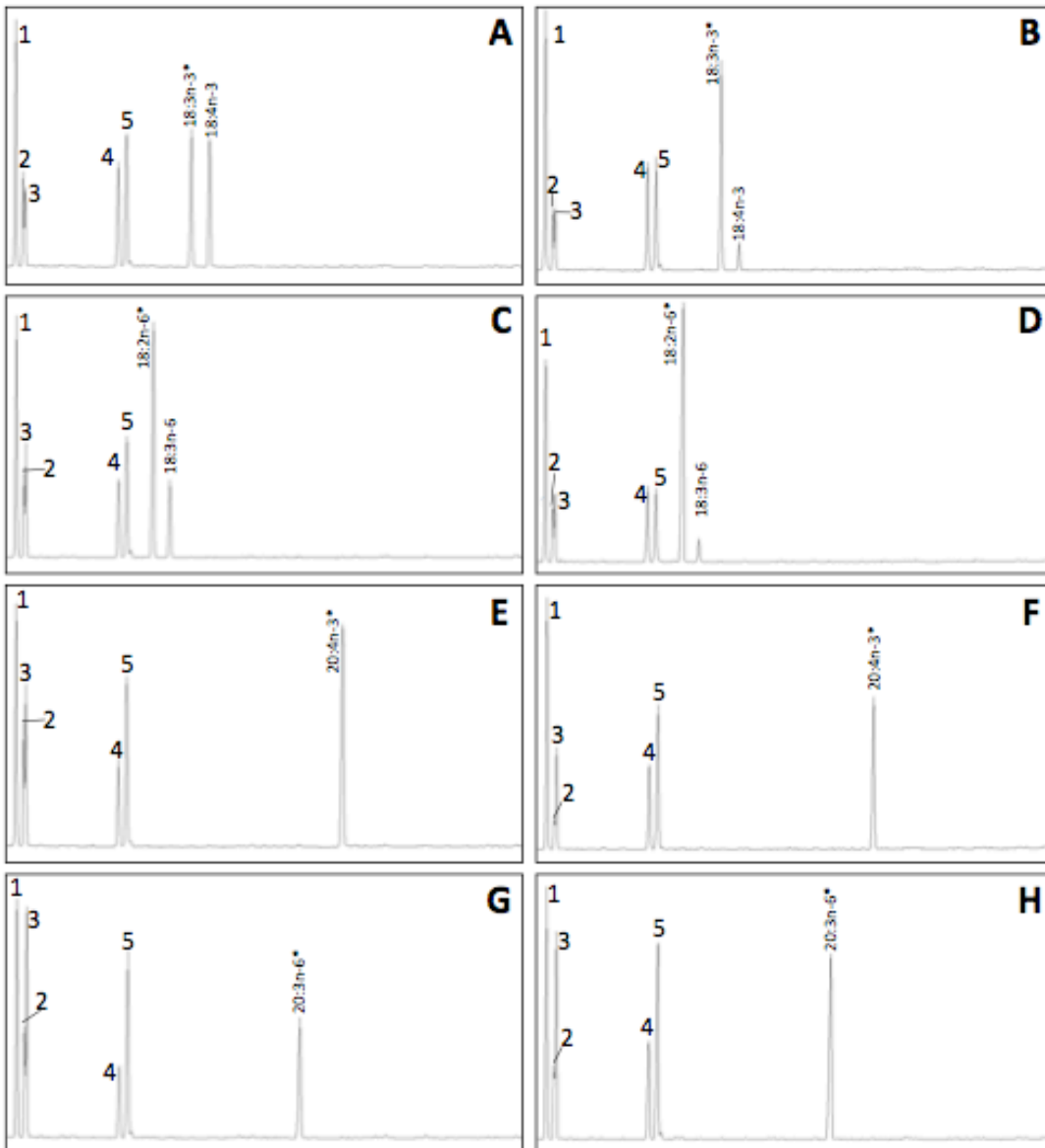


Fig. 5.

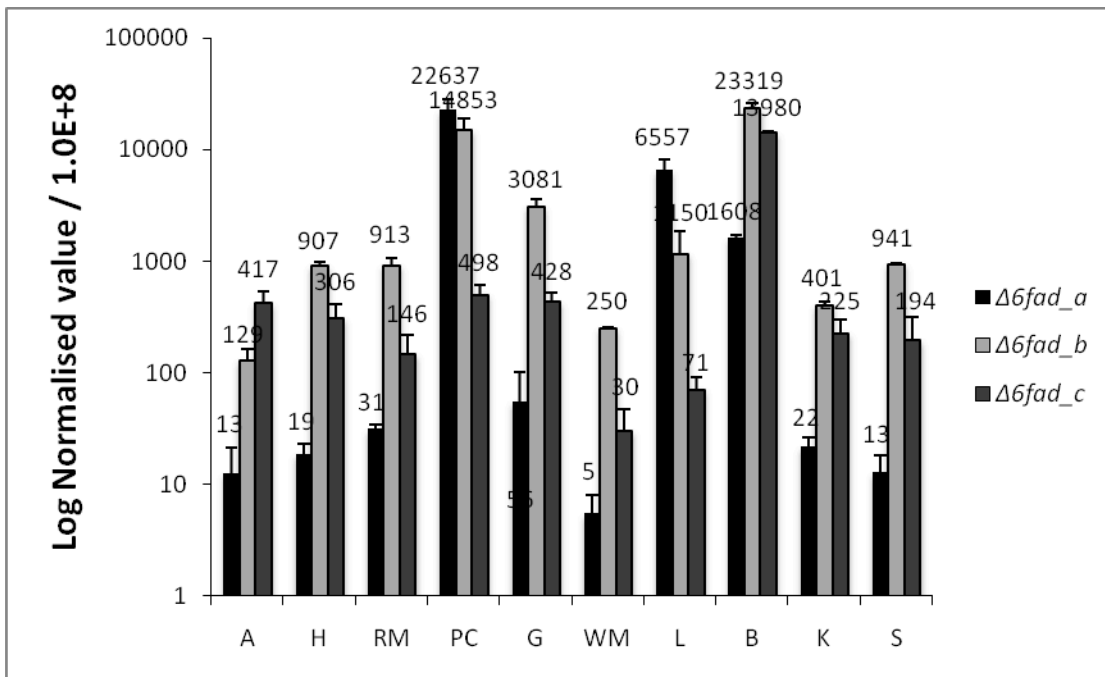


Fig.6.

