Highly Unsaturated Fatty Acid Synthesis in Vertebrates: New Insights with the Cloning and Characterisation of a Δ6 Desaturase of Atlantic Salmon

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Abbreviations: FO, fish oil; HUFA, highly unsaturated fatty acids (carbon chain length ≥ C_{20} with ≥ 3 double bonds); ORF, open reading frame; Q-PCR, quantitative (real-time) polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region; VO, vegetable oil.

ABSTRACT: Fish are an important source of the n-3 highly unsaturated fatty acids (HUFA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids that are crucial to the health of higher vertebrates. The synthesis of HUFA involves enzyme-mediated desaturation, and a Δ5 fatty acyl desaturase cDNA has been cloned from Atlantic salmon (*Salmo salar*) and functionally characterized previously. Here we report cloning and functional characterisation of a Δ6 fatty acyl desaturase of Atlantic salmon, and describe its genomic structure, tissue expression and nutritional regulation. A salmon genomic library was screened with a salmon Δ5 desaturase cDNA and positive recombinant phage isolated and subcloned. The full-length cDNA for the putative fatty acyl desaturase was shown to comprise 2106bp containing an ORF of 1365 bp specifying a protein of 454 amino acids (GenBank accession no. AY458652). The protein sequence included three histidine boxes, two transmembrane regions, and an N-terminal cytochrome b_5 domain containing the haem-binding motif HPGG, all of which are characteristic of microsomal fatty acid desaturases. Functional expression showed that this gene possessed predominantly Δ6 desaturase activity. Screening and sequence analysis of the genomic DNA of a single fish revealed that the Δ6 desaturase gene comprised 13 exons in 7965 bp of genomic DNA. Quantitative real time PCR assay of gene expression in Atlantic salmon showed that both Δ6 and Δ5 fatty acyl desaturase genes, and a fatty acyl elongase gene, were highly expressed in intestine, liver and brain, and less so in kidney, heart, gill, adipose tissue, muscle and spleen. Furthermore, expression of both Δ6 and Δ5 fatty acyl desaturase genes in intestine, liver, red muscle and adipose tissue was higher in salmon fed a diet containing vegetable oil than in fish fed a diet containing fish oil.

Highly unsaturated fatty acids (HUFA), arachidonate (AA; 20:4n-6), eicosapentaenoate (EPA; 20:5n-3) and docosahexaenoate (DHA; 22:6n-3), are crucial to the health and normal development of higher vertebrates (1-3). Fish are the most important source of n-3 HUFA for humans, but, with fisheries in decline, an increasing proportion of fish is being provided by rapidly expanding aquaculture (4). Paradoxically, aquaculture is itself dependent upon fisheries for the provision of fishmeals and oils traditionally used in the feed formulations (5). Their use ensured the high nutritional quality of farmed fish through the high levels of n-3 HUFA that fish oil and meal provided. However, feed-grade fisheries have reached sustainable limits. Along with concern over organic contaminants in fish oil, this has dictated that alternatives to fish oil must be found if aquaculture is to continue to expand and supply more of the global demand for fish (6).

The only practical, sustainable alternative to fish oils is vegetable oils, which are rich in C_{18} PUFA but devoid of the n-3 HUFA abundant in fish oils (7). Consequently, tissue fatty acid compositions in fish fed vegetable oils are characterised by increased levels of C_{18} PUFA and decreased levels of n-3 HUFA, which may reduce their nutritional value to the human consumer (8). The extent to which fish can convert C_{18} PUFA to HUFA varies, associated with their complement of fatty acid desaturase enzymes. Although Atlantic salmon (*Salmo salar* L.) are capable of producing DHA from 18:3n-3, and so express the necessary desaturase activities, the production is insufficient to maintain n-3 HUFA in fish fed vegetable oils at levels found in fish fed fish oils (9-11). Our primary hypothesis is that understanding the molecular basis of HUFA biosynthesis and its regulation in fish will enable us to optimise the activity of the pathway to ensure efficient and effective use of vegetable oils in aquaculture whilst maintaining the nutritional quality of farmed fish for the consumer.

Δ5 and Δ6 fatty acyl desaturases and elongases are critical enzymes in the pathways for the biosynthesis of HUFA. In recent years, significant progress has been made in characterizing fatty acid desaturases involved in HUFA synthesis (12). Full-length cDNAs for Δ6 desaturases have been isolated from the filamentous fungus *Mortierella alpina* (13), the nematode *Caenorhabditis elegans* (14), rat (15), mouse and human (16). Fatty acid Δ5 desaturase genes have been isolated from *M. alpina* (17) *C. elegans* (18,19) and human (20,21). Moreover, we have reported isolation of a cDNA of zebrafish (*Danio rerio*, GenBank accession no. AF309556), with high similarity to mammalian Δ6 desaturase genes. Functional analysis by heterologous expression in the yeast *Saccharomyces cerevisiae* indicated that the zebrafish gene was unique in that the cDNA encoded an enzyme having both Δ6 and Δ5 desaturase activities (22). Putative fatty acid desaturase cDNAs have now also been isolated and cloned from rainbow trout (*Oncorhynchus mykiss*, GenBank accession no. AF301910) (23) and gilthead seabream (*Sparus aurata*, GenBank accession no. AY055749) (24). Functional analysis showed that these two desaturase genes, along with cDNAs recently cloned

from common carp (*Cyprinus carpio*, GenBank accession no. AF309557) and turbot (*Psetta maximus*, GenBank accession no. AF301910) encoded basically unifunctional Δ6 fatty acid desaturase enzymes responsible for the first and possibly rate-limiting step in the biosynthesis of HUFA from 18:3n-3 and 18:2n-6 (25). Recently, a full-length cDNA for a desaturase containing 1365bp encoding 454 amino acid residues has been cloned from Atlantic salmon (GenBank accession no. AF478472). Functional analysis showed that this gene was primarily a Δ5 desaturase with virtually no Δ6 activity (26). Therefore, it was presumed that other fatty acid desaturase genes should be present in Atlantic salmon.

The objectives of the study described here were first to clone and functionally characterize a Δ6 desaturase gene of Atlantic salmon, second to describe its genomic structure and third to place it in evolutionary and physiological contexts. Therefore we detail the exon/intron organization of a salmon Δ6 desaturase gene, describe the expression profile of both Δ6 and Δ5 fatty acyl desaturase and fatty acyl elongase genes in various tissues, and demonstrate nutritional regulation of the fatty acyl desaturase genes.

MATERIALS AND METHODS

Putative desaturase cloning and its genomic organization. An Atlantic salmon genomic DNA library constructed previously with the lambda FIX II/Xho I partial fill-in vector kit (Stratagene, La Jolla, CA, USA) was probed with a full-length salmon Δ5 fatty acyl desaturase cDNA (GenBank accession no. AF478472). Inserts of positive recombinant phages were isolated and subcloned into the pBluescript KS II vector for sequencing (Stratagene, La Jolla, CA, USA). The full putative desaturase genomic nucleotide sequence was assembled using BioEdit version 5.0.6 (Tom Hall, Department of Microbiology, North Carolina State University, USA).

Total RNA was extracted from liver tissue of Atlantic salmon fed a standard extruded diet based on fish meal and fish oil using TRIzol® reagent (GibcoBRL, NY, U.S.A.). 3' RACE cDNA was synthesized using MMLV reverse transcriptase (Promega, Madison, WI, U.S.A) primed by the oligonucleotide, T7PolyT, 5'-TACGACTCACTATAGGGCGTGCAGTTTT TTTTTTTT-3'. The specific sense primer, D6P31, 5'-CAGGGGTGGGCCCGGTGGAGGGCTA-3' was designed for 3'RACE PCR based on the genomic sequence described above. This was used in conjunction with T7PolyT primer for the RACE PCR isolation of the salmon desaturase cDNA fragment predicted to contain the 3' UTR. PCR amplification was performed using the Hotstar Taq master kit (Qiagen, Crowley, West Sussex, UK) and involved an initial denaturation step at 95 ^{o}C for 15 min, followed

by 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 3 min. Final extension at 72 °C was for 10 min. 5'-RACE-cDNA was synthesized using the SMART™ RACE cDNA amplification kit (Clontech, NJ, U.S.A). The primer, SD6PPR3, 5'-GTCGCATTCCATCCCAATCC-3' was designed according to the 3'RACE PCR fragment sequence. This was used in conjunction with universal primer mix (UPM): long 5'–CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3' and short 5'-CTAATACGACTCACTATAGGGC-3' to perform 5' RACE PCR using high fidelity DNA polymerase (Roche Diagnostics Ltd., Lewes, East Sussex, UK). Amplification involved an initial step at 95 °C for 1 min and 70°C for 3 min, and 4 cycles of denaturation at 95 °C for 15 s, annealing at 62 °C for 1 min and extension at 72 °C for 1min and 30 s, followed by 27 cycles of denaturation at 95 °C for 15s, annealing at 56 °C for 30s and extension at 72 °C for 1min and 30 s. The final extension at 72 °C was for 10 min.

All RACE PCR products were cloned into the pBluescript KS II$^+$ vector for sequencing. The 3' and 5' RACE PCR fragment sequences were aligned to assemble the full nucleotide sequence of the putative desaturase cDNA using BioEdit version 5.0.6. The assembled putative fatty acyl desaturase cDNA sequence and its genomic DNA sequence were aligned to assign consensus donor and acceptor splice recognition sequences.

Heterologous expression of desaturase ORFs in Saccharomyces cerevisiae. PCR amplification was carried out to clone the salmon putative desaturase cDNA ORF. Sense primer, D6RF2, 5'-ATGGGGGGCGGAGGCCAGCAGAATGATTCAG -3', and antisense primer, D6RR1, 5'-ATGCGATGGATTAAATCCCG -3' (located in the 3'UTR) were designed for first round PCR after comparing nucleotide sequences of this putative cDNA and the Δ5 desaturase cDNA. Expression primers were designed for a second round of PCR. The sense primer, SalpYESFOR, 5'-CCCAAGCTTACTATGGGGGGCGGAGGCC–3' contains a *HindIII* site (underlined) and antisense primer, SalPYESREV2, 5'- CCGCTCGAGTCATTTATGGAGATATGCAT-3' contains an *XhoI* site (underlined). PCR was performed using high fidelity DNA polymerase (Roche Diagnostics Ltd., Lewes, East Sussex, UK) following the manufacturer's instructions. Amplification involved an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min and 30 s followed by a final extension at 72 °C for 10 min.

Following PCR, the DNA fragments were restricted with the appropriate enzymes, *HindIII* and *XhoI,* and ligated into the similarly digested yeast expression vector pYES2 (Invitrogen Ltd, Paisley, UK). Ligation products were then used to transform Top10F' *E. coli* competent cells (Invitrogen Ltd, Paisley, UK) which were screened for the presence of recombinants. Transformation of the yeast *S. cerevisiae* (strain InvSc1) with the recombinant plasmids was carried out using the S.c.EasyComp Transformation Kit (Invitrogen Ltd, Paisley, UK). Selection of yeast containing the desaturase/pYES2 constructs was on *S. cerevisiae* minimal medium (SCMM) minus uracil. Culture of the recombinant yeast was carried out in $SCMM^{-uracil}$ broth as described previously (22), using galactose induction of gene expression. Each culture was supplemented with one of the following PUFA substrates; α-linolenic acid (18:3n-3), linoleic acid (18:2n-6), eicosatetraenoic acid (20:4n-3), dihomo-γ-linoleic acid (20:3n-6), docosapentaenoic acid (22:5n-3) and docosatetraenoic acid (22:4n-6). PUFA were to added to the yeasy cultures at concentrations of 0.5 mM (C_{18}), 0.75 mM (C_{20}) and 1 mM (C_{22}) as uptake efficiency decreases with increasing chain length. Yeast cells were harvested, washed, dried, and lipid extracted by homogenisation in chloroform/methanol (2:1, by vol.) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant as described previously (22). Fatty acid methyl esters (FAME) were prepared, extracted, purified by thin layer chromatography (TLC), and analysed by gas chromatography (GC), all as described previously (22). The proportion of substrate fatty acid converted to the longer chain fatty acid product was calculated from the gas chromatograms as 100 × [product area/(product area + substrate area)]. Unequivocal confirmation of fatty acid products was obtained by GC-mass spectrometry of the picolinyl derivatives as described in detail previously (22).

Salmon tissue RNA extraction and quantitative real time PCR (Q-PCR). Tissue expression profiles and effects of diet were investigated in Atlantic salmon that had been fed one of two diets from first feeding. The diets consisted of a control in which fish oil (FO) was the only added oil and an experimental diet in which 75% of the FO was replaced by a vegetable oil blend (VO) containing rapeseed, palm and linseed oils in a 3.7 : 2 : 1 ratio. Both diets were fishmeal based and contained 48% protein, 26% lipid, 7% moisture and 8% ash as determined by proximate analyses. The fatty acid compositions of the diets (6 mm pellet) are given in Table 1. The diets were prepared by the Nutreco Aquaculture Research Centre, Stavanger, Norway and formulated to satisfy the nutritional requirements of salmonid fish (27).

Fish were sampled in November 2003, six months after seawater transfer, following 18 months on the diets, at which point the weights of the fish fed the FO and VO diets were 1250.0 ± 84.9g and 1280.0 ± 79.4g, respectively. Eight fish per dietary treatment were sampled and liver, brain, heart, kidney, gill, intestine (pyloric caeca), spleen, white and red muscle and adipose tissue were

collected, frozen immediately in liquid nitrogen and subsequently stored at –80°C before extraction. Total RNA extraction was performed as described above. Five μg of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase first strand cDNA synthesis kit (Promega UK, Southampton, UK). Gene expression of the fatty acyl Δ6 and Δ5 desaturase, and fatty acyl elongase genes in tissue from individual salmon fed the different diets was studied by quantitative RT-PCR (Q-PCR). β-Actin was used for normalization of mRNA levels. The PCR primers were designed according to Δ6 desaturase (accession no. AY458652), and the published Δ5 desaturase (accession no. AF478472), elongase (accession no. AY170327) and β-actin (accession no. AF012125) cDNA sequences. For the Δ6 desaturase, the forward primer was 5'-CCCCAGACGTTTGTGTCAG-3', and the reverse primer was 5'-CCTGGATTGTTGCTTTGGAT-3'. For the Δ5 desaturase, the forward primer was 5'-GTGAATGGGGATCCATAGCA-3', and the reverse primer was 5'-AAACGAACGGACAACCAGA-3'. For the elongase, the forward and reverse primers were 5'-TGATTTGTGTTCCAAATGGC-3' and 5'-CTCATGACGGGAACCT CAAT-3', respectively. For β-actin, 5'-ACATCAAGGAGAAGCTGTGC-3' and 5'-GACAACGGAACCTCTCGTTA-3' were the forward and reverse primers, respectively. PCR products sizes were 181,192, 219 and 141bp, respectively. The linearised plasmid DNA containing the target sequence for each gene was quantified to generate a standard curve of known copy number. Amplification of cDNA samples and DNA standards was carried out using SYBR Green PCR Kit (Qiagen, Crowley, West Sussex, UK) and the following conditions: 15 min denaturation at 95°C, 45 cycles of 15 s at 94 °C, 15 s at 55 °C and 30 s at 72 °C. This was followed by product melt to confirm single PCR products. Thermal cycling and fluorescence detection were conducted in a Rotor-Gene 3000 system (Corbett Research, Cambridge, UK). The copy numbers of the specific genes in the sample, normalised to total RNA, was used to compare expression levels between different tissues, and the ratios of copy numbers between the target genes and β-actin were calculated and used to compare the gene expression levels in fish fed the two diets.

Sequence analysis. Nucleotide sequences were determined by standard dye terminator chemistry using a Perkin Elmer ABI-377 DNA sequencer following the manufacturer's protocols (Perkin Elmer, Applied Biosystems). Deduced amino acid sequences of desaturases from various species were aligned using ClustalX and sequence phylogenies were predicted using the Neighbour Joining method (28). Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations.

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, U.S.A. Linoleic (18:2n-6), α-linolenic (18:3n-3), eicosatrienoic (20:3n-6) acids (all >99% pure), BHT, 1,1'-carbonyldiimidazole, 2,2-dimethoxypropane, 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, U.K.

RESULTS

Sequence analyses. The full length of the putative salmon desaturase cDNA (mRNA), as determined by 5' and 3' RACE PCR, was shown to be 2106bp which included a 5'-UTR of 284bp and a 3'-UTR of 457bp. Sequencing revealed that the cDNA included an ORF of 1365 bp, which specified a protein of 454 amino acids (GenBank accession no. AY458652). The protein sequence included all the characteristic features of microsomal fatty acid desaturases, including three histidine boxes and an N-terminal cytochrome b_5 domain containing the haem-binding motif, H-P-G-G (Fig.1). The protein sequence also contained two transmembrane regions. These features are similar to those of other fatty acid desaturase genes including salmon Δ5 desaturase, the zebrafish Δ6/Δ5 desaturase, and the human Δ5 (GenBank accession no. AF126799) and Δ6 (GenBank accession no. AF199596) desaturases. However, the new salmon desaturase, like the salmon Δ5 desaturase and the rainbow trout Δ6 desaturase sequences, had an insertion of 10 amino acid residues at the N-terminal end.

A pair-wise comparison was made between fish and human desaturase sequences. The amino acid sequence predicted by the salmon putative (Δ6) desaturase ORF shows 91% identity to the salmon Δ5 desaturase, and 94% identity to the trout Δ6 desaturase. The salmon cDNA shows 65% identity to that of the zebrafish Δ6/Δ5 desaturase, and 65 and 58% identity to the human Δ6 and Δ5 cDNAs, respectively.

A phylogenetic tree was constructed on the basis of the amino acid sequence alignments between the salmon fatty acyl desaturases, and 15 other desaturases of fish and mammals (Fig 2). The phylogenetic analysis clustered the new Atlantic salmon putative desaturase sequence with the Atlantic salmon Δ5 desaturase, rainbow trout Δ6 desaturase and other, as yet uncharacterised, masou (cherry) salmon (*Oncorhynchus masou*) desaturase genes, but closest to the trout Δ6 desaturase. The salmonid desaturases clustered more closely with turbot, sea bream and tilapia

(*Oreochromis nilotica*) desaturases, than with carp Δ6 desaturase and zebrafish Δ5/Δ6 desaturase. All of the fish desaturase genes clustered together, and closer to the mammalian (mouse and human) Δ6 desaturases than to the mammalian Δ5 desaturases.

Functional characterisation. The salmon desaturase cDNA was functionally characterized by determining the fatty acid profiles of transformed *S. cerevisiae* containing either the pYES vector alone or the vector with the salmon desaturase cDNA insert, grown in the presence of a variety of potential fatty acid substrates, including Δ6 substrates (18:2n-6 and 18:3n-3), Δ5 substrates (20:3n-6 and 20:4n-3) and Δ4 substrates (22:4n-6 and 22:5n-3). The fatty acid composition of the yeast transformed with the vector alone showed the four main fatty acids normally found in *S. cerevisiae*, namely 16:0, 16:1n-7, 18:0 and 18:1n-9, together with the exogenously derived fatty acids. This is consistent with *S. cerevisiae* not possessing Δ5 or Δ6 fatty acid desaturase activities (Figs. 3 and 4). The most prominent additional peaks were observed in the profiles of transformed yeast grown in the presence of the Δ6 desaturase substrates, 18:3n-3 and 18:2n-6 (Fig.3). Based on GC retention time and confirmed by GC-MS, the additional peaks associated with the presence of the salmon desaturase cDNA were identified as 18:4n-3 (Fig.3B) and 18:3n-6 (Fig.3D), corresponding to the Δ6 desaturation products of 18:3n-3 and 18:2n-6, respectively. Approximately, 60.1% of 18:3n-3 was converted to 18:4n-3 and 14.4% of 18:2n-6 was converted to 18:3n-6 in yeast transformed with the salmon desaturase (Table 2). However, a very small additional peak representing desaturated fatty acid product, as confirmed by GC-MS, was observed in the lipids of *S. cerevisiae* transformed with the desaturase cDNA when the transformed yeast was incubated with 20:4n-3 (Figs.4A and B). About 2.3% of 20:4n-3 (n-3 Δ5 activity) was desaturated by the salmon clone, but no product of desaturation of the 20:3n-6 substrate was detected, indicating no significant n-6 Δ5 desaturase activity. The desaturase cDNA did not express any Δ4 desaturase activity as evidenced by the lack of any observable additional peaks representing desaturated products of 22:5n-3 or 22:4n-6 (data not shown). Overall, therefore, the results showed that the salmon desaturase cDNA encoded enzyme was essentially a Δ6 fatty acyl desaturase, with only a very low level of Δ5 desaturase activity, and no Δ4 desaturase activity.

Genomic structure. The alignment of the Δ6 fatty acyl desaturase cDNA and the genomic sequences revealed 13 exons spanning 7965 bp of genomic DNA as illustrated in Table 3.

***F**atty acid desaturase and elongase gene expression in salmon tissues.* To identify which tissues were likely to contribute to HUFA synthesis in the Atlantic salmon, reverse transcription Q-PCR was used to examine the tissue distribution of Δ6 and Δ5 fatty acyl desaturase and fatty acyl

elongase mRNAs. The results showed that the three genes were expressed in all tissues examined, with highest expression in terms of the absolute copy numbers (mean± SD, n =8) in intestine, followed by liver and brain (Fig.5). In comparison to the Δ5 desaturase, the transcript copy abundance for the Δ6 desaturase was higher in these tissues with higher expression, but lower in tissues with lower expression, other than kidney. The transcript copy abundance for fatty acyl elongase was much lower than that for the Δ6 and Δ5 desaturases in all tissues.

The ratios of copy numbers between the target genes and β-actin were determined (means ± SD, n = 4), and the fold difference between the mean value of target gene expression in the tissue of fish fed VO calculated relative to the expression in tissues of fish fed FO (Fig 6). The results revealed that Δ6 and Δ5 fatty acyl desaturase gene expression in liver and red muscle of fish fed VO was significantly increased compared to fish fed the FO diet, whereas the expression of both desaturases in heart and spleen, and Δ5 in gill and kidney was decreased in fish fed VO (Fig.6). Expression of both desaturases in intestine and adipose tissue was also higher in fish fed VO, although with the high variation these effects were below the level of statistical significance. However, feeding VO decreased the expression of the fatty acyl elongase gene in most tissues, significantly so in heart, gill, brain, adipose, spleen and kidney (Fig.6).

DISCUSSION

Several fish desaturases have been cloned and functionally characterised in recent years. These are the bifunctional zebrafish enzyme showing both Δ6 and Δ5 desaturase activity (22), an Atlantic salmon desaturase that was shown to be predominantly an n-3 Δ5 desaturase (26), and common carp, rainbow trout, gilthead seabream and turbot desaturases that were all shown to be predominantly Δ6 desaturases (25). The bifunctional nature of the Δ6/Δ5 desaturase of zebrafish suggested that it may be a prototypic or ancestral progenitor desaturase (22,29). But the subsequent characterisation of several essentially unifunctional Δ6 fish desaturases and the salmon Δ5 desaturase indicates that the zebrafish enzyme might be atypical.

The study described here has further increased our knowledge of PUFA desaturases in fish. The cloning and functional characterisation of a predominantly Δ6 desaturase gene makes the Atlantic salmon the first fish species to be shown to have separate and distinct genes for Δ6 and Δ5 desaturases, as reported previously for *C. elegans* (14,18,19) and human (16,20,21). The salmon Δ6 desaturase clone also showed measurable, but very low, levels of Δ5 activity, and thus was similar to other fish Δ6 desaturases of carp, trout, seabream and turbot (25). But, unlike the zebrafish desaturase, which showed very significant Δ5 desaturase activity at around 70% of the Δ6 activity

291 (22), the n-3 Δ5 activity in the salmon cDNA product was only 3.8% of the Δ6 activity. It is likely
292 that the level of Δ5 desaturase activity measured is of limited physiological significance.
293 The study described here also clearly showed that the salmon Δ6 desaturase has a marked
294 preference for the n-3 substrate 18:3n-3 over the n-6 substrate 18:2n-6. A similar preference for n-3
295 fatty acid substrates rather than n-6 substrates upon heterologous expression in yeast was observed
296 previously with the zebrafish Δ6/Δ5 desaturase, salmon Δ5 desaturase (22,26), and trout, seabream,
297 carp and turbot Δ6 desaturases (25). These data are consistent with earlier enzymological studies
298 investigating the desaturation of ^{14}C-labelled fatty acid substrates in primary hepatocytes (9),
299 primary brain astrocytes (30) and established cell lines (31). Therefore, it appears that greater
300 activity towards n-3 PUFA may be a characteristic of fish fatty acyl desaturases. In contrast,
301 functional characterisation of Δ6 desaturases of other organisms including nematode, mammals,
302 fungi, mosses and higher plants failed to show a preference for either 18:3n-3 or 18:2n-6 substrates,
303 although recently Δ6 desaturases have been identified in *Primula* sp. which have a preference for n-
304 3 substrates (32). However, data of these kinds obtained from heterologous expression can only be
305 regarded as semi-quantitative as there are likely to be differences between fatty acids in, for
306 example, their uptake into organisms such as yeasts (33).
307 The present study shows unequivocally that distinct Δ6 and Δ5 desaturase genes exist in Atlantic
308 salmon, as is the case in humans, and possibly in mammals in general. However, the two salmon
309 cDNAs are very similar in that the predicted amino acid sequence encoded by the Δ6 cDNA is 91%
310 identical with that encoded by the Δ5 desaturase cDNA. In contrast, in human and *C. elegans*, the
311 two functional Δ6 and Δ5 desaturases share an amino acid identity of only 62% (20) and 45% (19),
312 respectively. Whether or not distinct Δ6 and Δ5 desaturase genes evolved from a common ancestral
313 desaturase progenitor, these data suggest that the process occurred or began more recently in the
314 evolution of Atlantic salmon than in the evolutions of human and *C. elegans*. In this regard it is
315 pertinent to note that the Atlantic salmon is partially tetraploid, with the tetraploidisation event
316 thought to have occurred 25-100 million years ago (34). However, evolution of desaturases in
317 Atlantic salmon and in fish in general remains a subject for speculation. Study of further fatty acid
318 desaturase genes of fish are indicated, and certainly other desaturases are likely to be identified in
319 fish species such as carp and trout, which have the ability to produce DHA from 18:3n-3 (35). But,
320 in marine species such as sea bream and turbot, the search for Δ5 desaturases will be particularly
321 intriguing as these species lack the ability to produce EPA and DHA from 18:3n-3. This is
322 attributed to deficiencies in Δ5 desaturation in sea bream, but to C_{18-20} elongation in turbot (36,37).
323 The salmon Δ6 desaturase showed no Δ4 desaturase activity, perhaps as expected based upon
324 the functional characterisation of all fish and mammalian Δ6 and Δ5 desaturases reported to date
325 (22,25,26,38). This is consistent with the hypothesis that the synthesis of DHA from EPA in both

mammals and fish proceeds via elongation to 24:5n-3 followed by a Δ6 desaturation rather than via Δ4 desaturation of 22:5n-3 (35,39). Heterologous expression studies of human and rat Δ6 desaturases showed that the same enzymes are active on C_{18} and C_{24} fatty acids (33,40), and the bifunctional zebrafish desaturase was also capable of desaturating C_{24} fatty acids (41). It will be interesting to determine the activities of all animal Δ6 desaturases towards C_{24} fatty acid substrates. In contrast to higher animals, production of DHA via a pathway including Δ4 desaturation appears to operate in some lower organisms such as *Thraustochytrium* sp. (42), and the algae *Euglena gracilis* (43) and *Pavlova lutheri* (44).

Genomic characterization showed that the salmon Δ6 desaturase comprised 13 exons, which is one more than that reported for the human Δ6 desaturase (45). The additional exon in the salmon gene is a small 25 bp exon at the extreme 5' end. The remaining exons are homologous to the 12 exons in the human Δ6 desaturase, except that exon 2 of the salmon gene is 30 bp longer than exon 1 in the human gene, corresponding to the additional 10 amino acids found in most salmonid desaturases. However, the remaining exons are exactly the same size as their equivalents in the human gene, and splice and acceptor sites are interrupted at similar nucleotide positions, even though the lengths of the introns are quite different. In human, there is evidence that the desaturase gene cluster has arisen by gene duplication. This is on the basis that 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 (45). Further work on the genomic organisation of fish desaturases may help to clarify the significance of the additional exon in salmon and the possible evolutionary history of desaturases, as sequence alignments alone are not conclusive (46).

The phylogenetic sequence analyses grouped the fish desaturases largely as expected based on classical phylogeny with the carp and zebrafish (Ostariophysi; cyprinids), trout and salmon (Salmoniformes; salmonidae), and tilapia, sea bream and turbot (Acanthopterygia; cichlids, perciformes and pleuronectiformes) appearing in three distinct clusters (47). However, the cloning of Atlantic salmon Δ6 desaturase has revealed that both Δ6 and Δ5 desaturases in salmonids contain additional amino acids by comparison with those of other species, having chain lengths of 454 amino acids (or 452 as in cherry salmon Des2) compared to 444 for the cyprinid (carp and zebrafish) and human desaturases (16,20,22,23,26). Furthermore, it has been reported that the desaturase cDNAs encode proteins of 445 amino acids in seabream (24) and turbot (25), one more residue than in cyprinid and human desaturases. These data support our previous observation that differences in polypeptide length are not in these cases related to function (25).

Q-PCR revealed that the expression of fatty acyl desaturase genes was highest in intestine, liver and brain, and lower in heart, gill, white and red muscle, kidney, spleen and adipose tissue.

Previously, using RT-PCR, it was shown that Δ6 desaturase of rainbow trout and sea bream was expressed in intestinal tissue (23,24). In the present study, salmon intestinal tissue had levels of Δ6 and Δ5 expression 3- and 1.5-fold greater than liver. Similarly, expression of Δ6 and Δ5 in intestine was 7.2- and 1.9-fold greater than in brain. Therefore these results suggest that intestine, the first organ to encounter dietary fatty acids, has the capacity to play an important role in the primary processing of dietary fatty acids via desaturation. Cho et al. (20) reported that human liver expressed 4-5 times more Δ5 desaturase, and 12 times more Δ6 desaturase than brain. Our results show that salmon liver contained 2.4 times more Δ6 desaturase mRNA than brain, and the Δ5 desaturase mRNA levels in liver and brain were similar. Regardless of which gene has the higher level of mRNA, the observation that all tissues investigated express detectable levels of Δ6 and Δ5 desaturase and elongase mRNAs is consistent with the important roles that desaturase and elongase enzymes play in maintaining cellular membrane HUFA. That intestine expressed such high levels of both Δ6 and Δ5 desaturase is consistent with data from *in vitro* enzyme assays in isolated enterocytes (48,49), and *in vivo* stable isotope studies (50,51), which have shown enterocytes and intestine to be sites of significant HUFA synthesis in salmonids. The level of Δ6 desaturase mRNA in highly expressing tissues was substantially greater than the amount of Δ5 desaturase mRNA, but the level of Δ6 desaturase mRNA in lower expressing tissues was lower than the amount of Δ5 desaturase mRNA. In comparison, a study of the relative abundance of Δ6 and Δ5 desaturase mRNA in various human tissues revealed that the level of Δ6 desaturase mRNA in 8 different tissues was significantly greater than the amount of Δ5 desaturase mRNA (20). This observation is particularly interesting because Δ6 is often considered the enzyme which catalyses the rate-limiting step in the synthesis of HUFA (52).

The results of this study show that the expression of Δ6 and Δ5 fatty acid desaturases is under nutritional regulation in Atlantic salmon. Thus, the expression of these genes was higher in liver and red muscle (and possibly intestine and adipose tissue) of salmon fed diets containing C_{18} PUFA-rich vegetable oil compared to fish fed diets containing HUFA-rich fish oil. Although Δ6 desaturase is regarded as the main rate-limiting step in the HUFA biosynthesis pathway, Δ6 desaturase is reported to also be under nutritional regulation in mammals (53). In a previous study, the expression and activity of fatty acyl elongase appeared to be nutritionally regulated in Atlantic salmon (54). That study showed that dietary linseed oil increased the expression of both Δ5 fatty acid desaturase and elongase genes in salmon liver (54). Similar effects of dietary linseed oil had been reported previously, with the liver transcript level of Δ6 desaturase being higher in trout fed linseed oil compared to in trout fed fish oil (23). However, the present study showed the expression and activity of the elongase decreased in most tissues of salmon fed diets containing the vegetable oil blend compared to fish fed diets containing fish oil. The precise reason for the

different responses in elongase gene expression is unclear, but may be related to differences in the fatty acid profiles of the linseed oil and VO blend diets. In the present trial, the total n-3HUFA level in the diet in which the VO blend replaced 75% of the FO was over 8%, which compares well with 9% HUFA in the diet in the previous trial in which 25% of the FO was replaced by linseed oil, a level of replacement which did not increase elongase activity (54). Elongase activity was only increased by diets in which 50-100% of FO was replaced with linseed oil, resulting in much lower levels of n-3HUFA (54).

In conclusion, the study reported here has identified and characterised a Δ6 desaturase gene in Atlantic salmon. It had measurable, but very low, levels of Δ5 desaturase activity. The salmon Δ6 desaturase gene comprises 13 exons, one more than the human Δ6 and Δ5 desaturases. Δ6 and Δ5 desaturases and elongase genes were expressed in various tissues of salmon, and highly expressed in liver, intestine and brain. Both Δ6 and Δ5 desaturase gene expression in intestine, liver, red muscle and adipose tissue were significantly increased in salmon fed vegetable oil compared to in fish fed fish oil.

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573 Legends to Figures

574 **Fig.1.** Comparison of the deduced amino acid sequence of Δ6 and Δ5 polyunsaturated fatty acyl
575 desaturases from Atlantic salmon with that of desaturases from trout, zebrafish and human.
576 Identical residues are shaded black and similar residues are shaded grey. Identity/similarity
577 shading was based on the BLOSUM62 matrix and the cut off for shading was 75%. The
578 cytochrome b_5-like domain is dot-underlined, the two transmembrane regions are dash underlined,
579 the three histidine-rich domains are solid underlined and asterisks on the top mark the haem-
580 binding motif, H-P-G-G.

581 **Fig.2.** Phylogenetic tree of Δ6 and Δ5 desaturases from salmon, and desaturases from other fish
582 species (zebrafish, cherry salmon, rainbow trout, seabream, common carp, turbot and tilapia),
583 mammals (mouse and human), fungus (*Mortierella alpina*) and nematode (*Caenorhabditis*
584 *elegans*). The tree was constructed using the N-J method using *CLUSTALX* and *NJPLOT*. The
585 horizontal branch length is proportional to amino acid substitution rate per site. The numbers
586 represent the frequencies with which the tree topology presented here was replicated after 1000
587 bootstrap iterations. Sequences marked with an asterisk are not functionally characterized.
588
589 **Fig.3.** Functional expression of the Atlantic salmon putative fatty acyl desaturase in transgenic
590 yeast (*Saccharomyces cerevisiae*) grown in the presence of Δ6 substrates, 18:3n-3 and 18:2n-6.
591 Fatty acids were extracted from yeast transformed with pYES vector without insert (A and C) or
592 containing the putative fatty acid desaturase cDNA insert (B and D). The first four peaks in panels
593 A-D are the main endogenous fatty acids of *S. cerevisiae*, namely 16:0 (1), 16:1n-7 (2), 18:0 (3) and
594 18:1n-9 (with 18:1n-7 as shoulder) (4). Peak 5 in panels A and B, and peak 7 in panels C and D are
595 the exogenously added substrate fatty acids, 18:3n-3 and 18:2n-6, respectively. Peaks 6 and 8 in
596 panels B and D were identified as the resultant desaturated products, namely 18:4n-3 and 18:3n-6,
597 respectively. Vertical axis, FID response; horizontal axis, retention time.
598
599 **Fig.4.** Functional expression of the Atlantic salmon putative fatty acyl desaturase in transgenic
600 yeast (*Saccharomyces cerevisiae*) grown in the presence of Δ5 substrates, 20:4n-3 and 20:3n-6.
601 Fatty acids were extracted from yeast transformed with pYES vector without insert (A and C) or
602 containing the putative fatty acid desaturase cDNA insert (B and D). The first four peaks in panels
603 A-D are as described in legend to Fig.3. Peak 9 in panels A and B, and peak 11 in panels C and D
604 are the exogenously added substrate fatty acids, 20:4n-3 and 20:3n-6, respectively. Peak 10 in
605 panel B was identified as the resultant desaturated product of 20:4n-3, namely 20:5n-3. Vertical
606 axis, FID response; horizontal axis, retention time.

Fig. 5. Tissue distribution of fatty acid Δ6 and Δ5 desaturase and elongase genes in Atlantic salmon. Transcript (mRNA) copy number was determined by real-time quantitative PCR (Q-PCR) as described in the Materials and Methods Section. Results are expressed as the copy numbers in 250ng of total RNA and are means ± SEM (n = 4). L, liver; H, heart; G, gill; WM, white muscle; RM, red muscle; I, intestine; B, brain; A, adipose; S, spleen; K, kidney.

Fig.6. Effect of dietary vegetable oil on the expression of fatty acid Δ6 and Δ5 desaturase and elongase genes in tissues from Atlantic salmon. Transcript (mRNA) copy number was determined by real-time quantitative RT-PCR (Q-PCR) as described in the Materials and Methods Section. The ratios of copy numbers between the target genes and β-actin were calculated as means ± SEM (n = 4). Results are expressed as the fold differences by comparison of mean values in fish fed the vegetable oil diet compared to those in fish fed the fish oil diet (FO = 1). L, liver; H, heart; G, gill; WM, white muscle; RM, red muscle; I, intestine; B, brain; A, adipose; S, spleen ; K, kidney.

Table 1

Fatty Acid Composition (Percentage of Total Fatty Acids) of Diets

	FO	VO
14:0	6.1	2.4
16:0	14.7	16.0
18:0	2.8	3.3
Total saturated[1]	24.3	21.9
16:1n-7[2]	5.0	2.0
18:1n-9	13.5	35.2
18:1n-7	2.5	2.3
20:1n-9[3]	10.4	3.6
22:1n-11[4]	14.9	4.8
24:1n-9	0.7	0.3
Total monoenes	47.0	48.2
18:2n-6	4.0	11.8
20:4n-6	0.5	0.2
Total n-6 PUFA[5]	5.1	12.2
18:3n-3	1.1	8.5
18:4n-3	2.4	0.8
20:4n-3	0.7	0.2
20:5n-3	6.7	2.8
22:5n-3	1.1	0.4
22:6n-3	10.4	4.5
Total n-3 PUFA[6]	22.4	17.3
Total PUFA[7]	28.7	29.9

Data are the means of two samples. FO, fish oil; PUFA, polyunsaturated fatty acids; VO, vegetable oil blend.[1]totals contain 15:0 present at up to 0.5%; [2]contains 16:1n-9; [3]contains 20:1n-11 and 20:1n-7; [4]contains 22:1n-9; [5]totals contain 18:3n-6, 20:2n-6, 20:3n-6 and 22:5n-6 present at up to 0.2%; [6]totals contain 20:3n-3 present at up to 0.1%; [7]totals contain C_{16} PUFA.

Table 2
Functional Characterisation of Salmon Fatty Acid Desaturase cDNA Clone in the Yeast *Saccharomyces cerevisiae*

PUFA substrates	Products	Desaturase Activity	Conversion rate (%)
α-Linolenic acid (18:3n-3)	18: 4n-3	Δ6	60.1
Linoleic acid (18:2n-6)	18: 3n-6	Δ6	14.4
Eicosatetraenoic acid (20:4n-3)	20: 5n-3	Δ5	2.3
Dihomo-γ-linoleic acid (20:3n-6)	20: 4n-6	Δ5	0
Docosapentaenoic acid (22:5n-3)	22: 6n-3	Δ4	0
Docosatetraenoic acid (22:4n-6)	22: 5n-6	Δ4	0

Conversion rates represent the proportion of substrate fatty acid converted to the longer chain fatty acid product, calculated from the gas chromatograms as 100 * [product area / (product area + substrate area)].
PUFA, polyunsaturated fatty acid.

Table 3

Exon and Intron Boundaries of Atlantic Salmon Δ6 Fatty Acyl Desaturase

Exon	Size (bp)	3' splice acceptor	5' splice donor	Intron size (bp)
1	25[a]		..AATATTGgtgagtg..	698
2	496[b]	..tttgcagCTGGCCC..	..TGCCACGgtcagta..	1127
3	111	..tttgtagGACGCAT..	..GAAAAATgtgagga..	744
4	198	..catacagGCAGTAC..	..GTCTCAGgtaccat..	228
5	102	..ctctcagTCCCAGG..	..CCTAAAGgtaggct..	345
6	126	..tttccagGGTGCCT..	..TGTAGAGgtagtta..	515
7	61	..attgcagTATGGTA..	..TTCCTCAgtaagtc..	128
8	77	..ctttcagTTGGACC..	..CTGGGTGgtgagat..	303
9	98	..tgtgaagGATCTGG..	..TCGTCAGgtaaagt..	161
10	97	..tatatagGTTTTTG..	..CATGCAGgtaacat..	1011
11	80	..gtcttagTTGAGTG..	..AACACCAgtaagtg..	383
12	126	..ctcccagTCTGTTT..	..TTGTCAGgtaagtg..	216
13	509[c]	..tctccagGTCACTG..		

[a]Exon is a 5'-UTR of 25 bp

[b]Exon includes a 5'-UTR of 259 bp.

[c]Exon includes a 3'-UTR of 457 bp.

Fig. 1

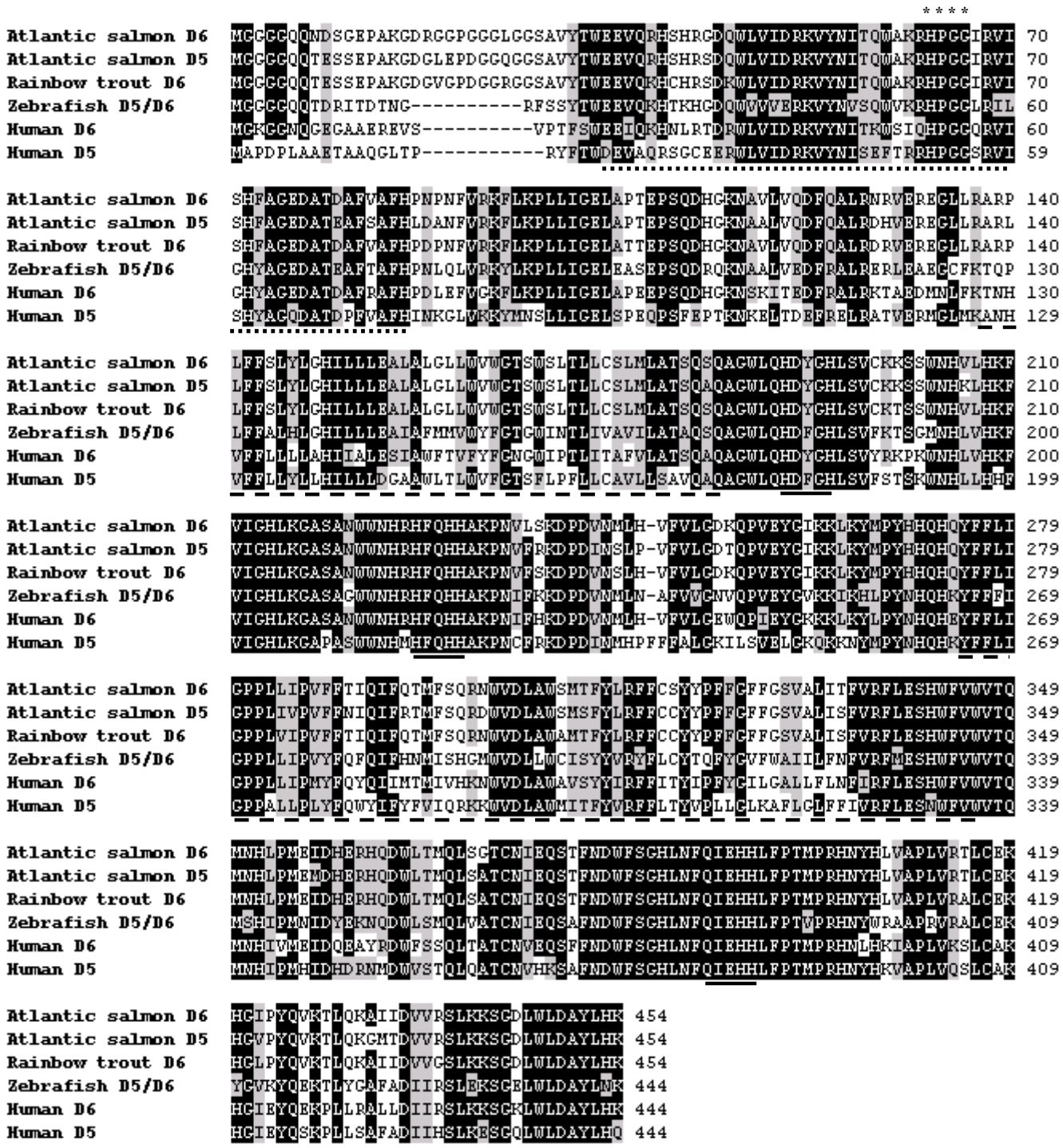
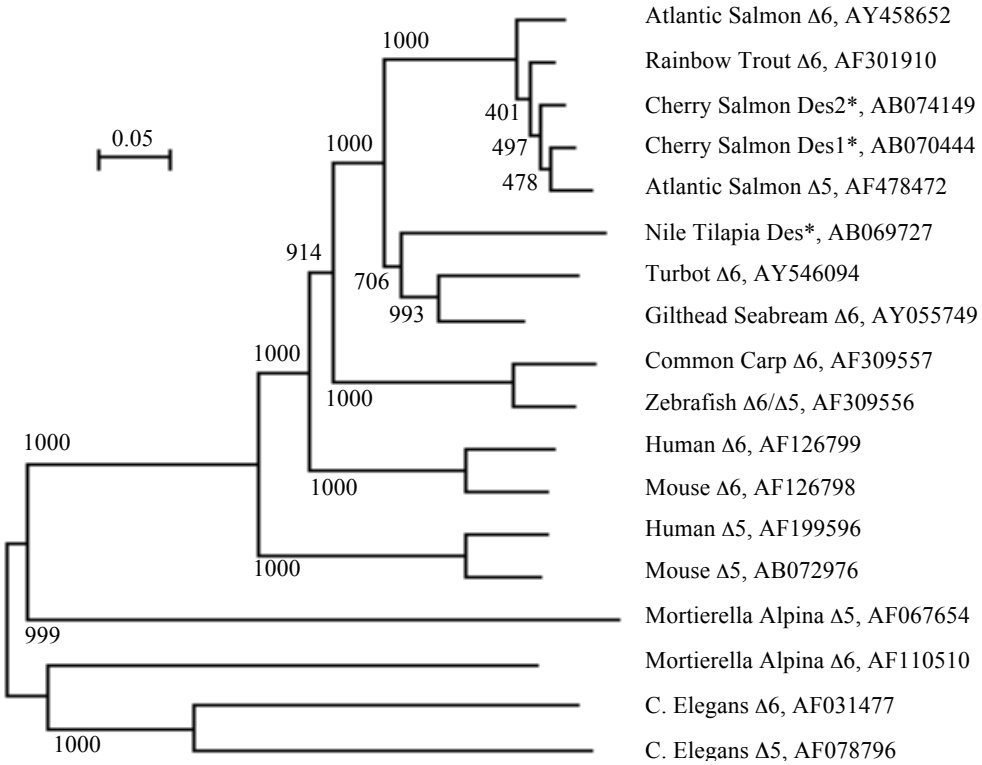

Atlantic salmon D6
Atlantic salmon D5
Rainbow trout D6
Zebrafish D5/D6
Human D6
Human D5

Fig. 2.


0.05
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401
497
478
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914
706
993
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1000
1000
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999
1000
Atlantic Salmon Δ6, AY458652
Rainbow Trout Δ6, AF301910
Cherry Salmon Des2*, AB074149
Cherry Salmon Des1*, AB070444
Atlantic Salmon Δ5, AF478472
Nile Tilapia Des*, AB069727
Turbot Δ6, AY546094
Gilthead Seabream Δ6, AY055749
Common Carp Δ6, AF309557
Zebrafish Δ6/Δ5, AF309556
Human Δ6, AF126799
Mouse Δ6, AF126798
Human Δ5, AF199596
Mouse Δ5, AB072976
Mortierella Alpina Δ5, AF067654
Mortierella Alpina Δ6, AF110510
C. Elegans Δ6, AF031477
C. Elegans Δ5, AF078796

Fig.3.

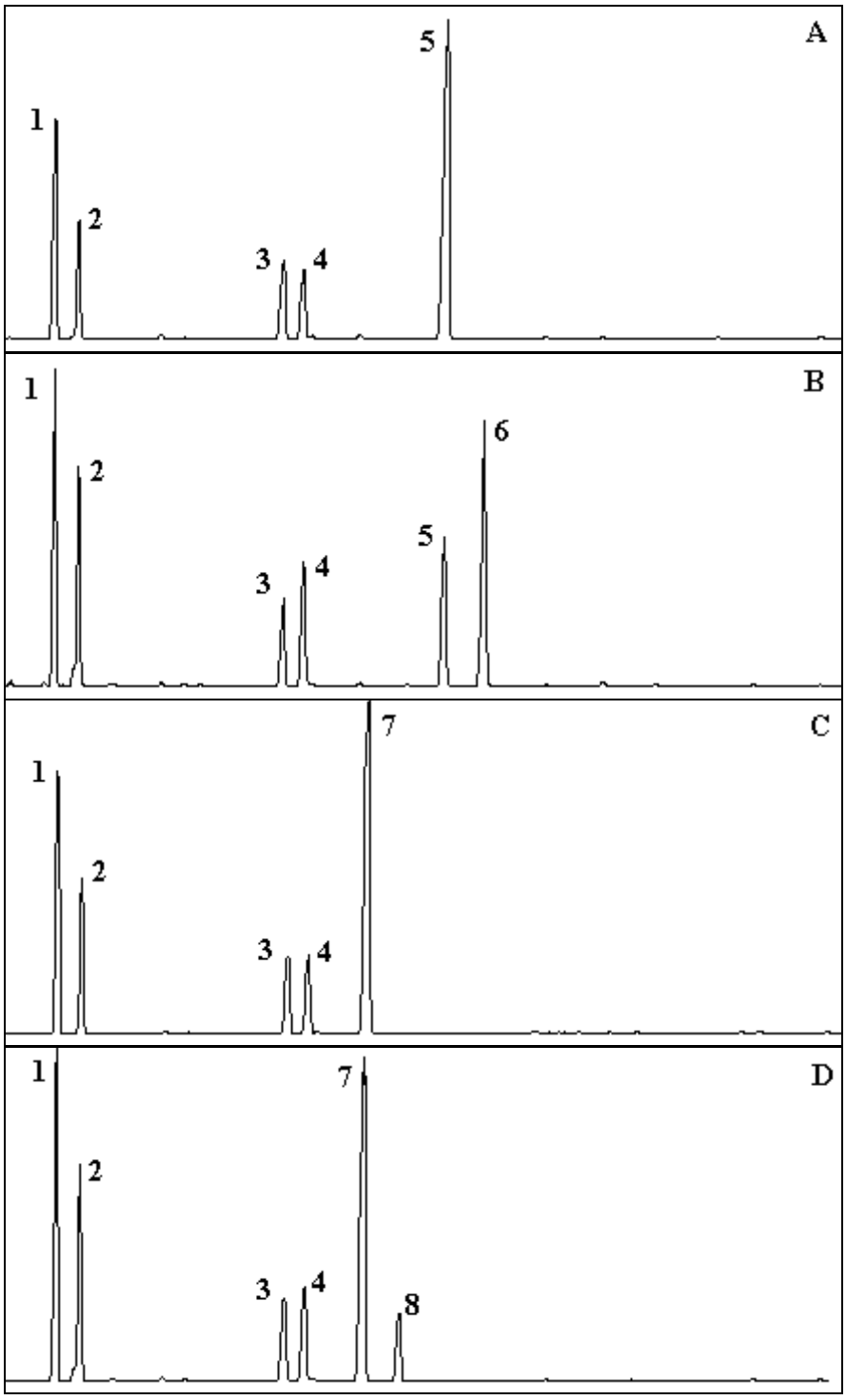


Fig.4.

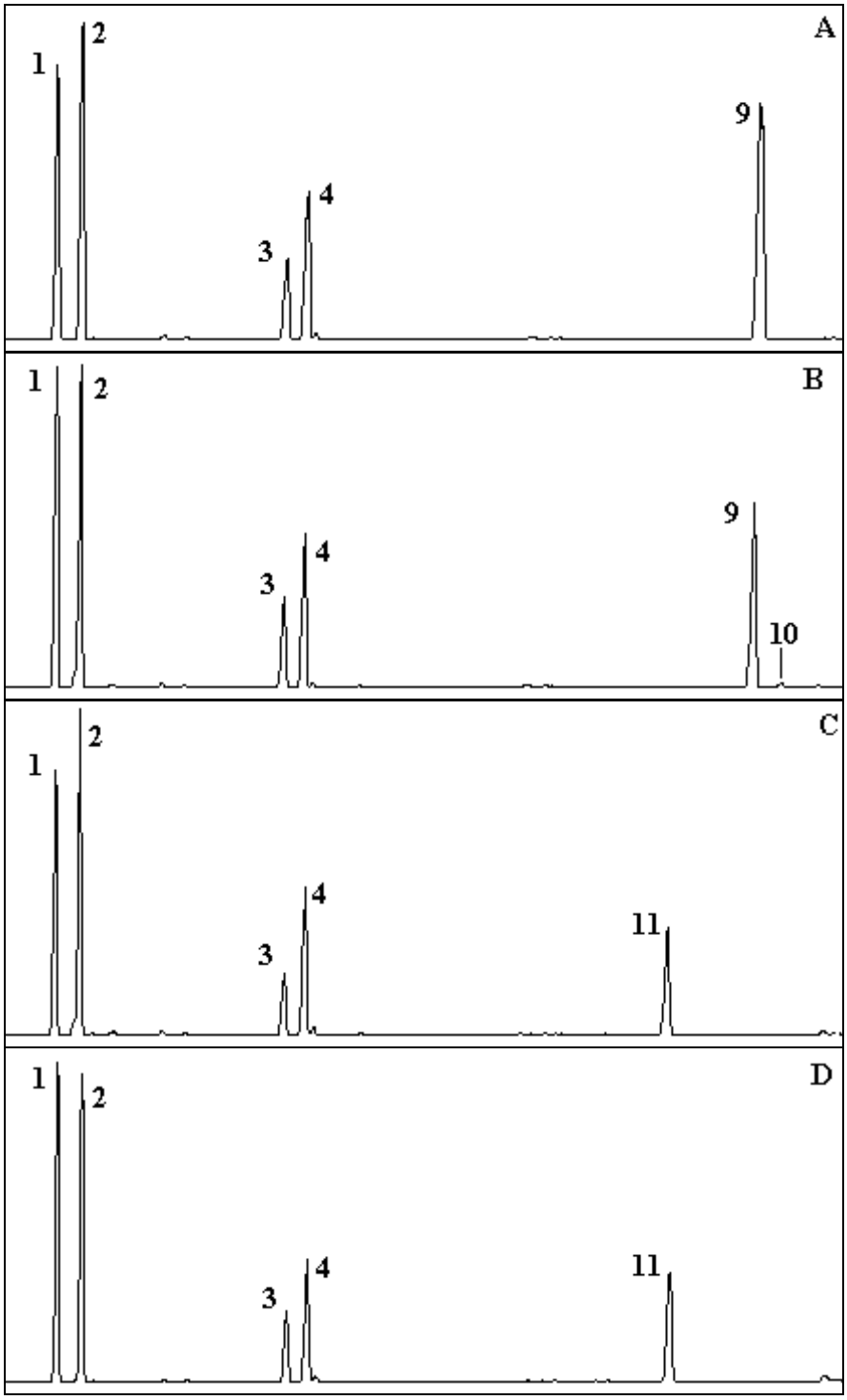
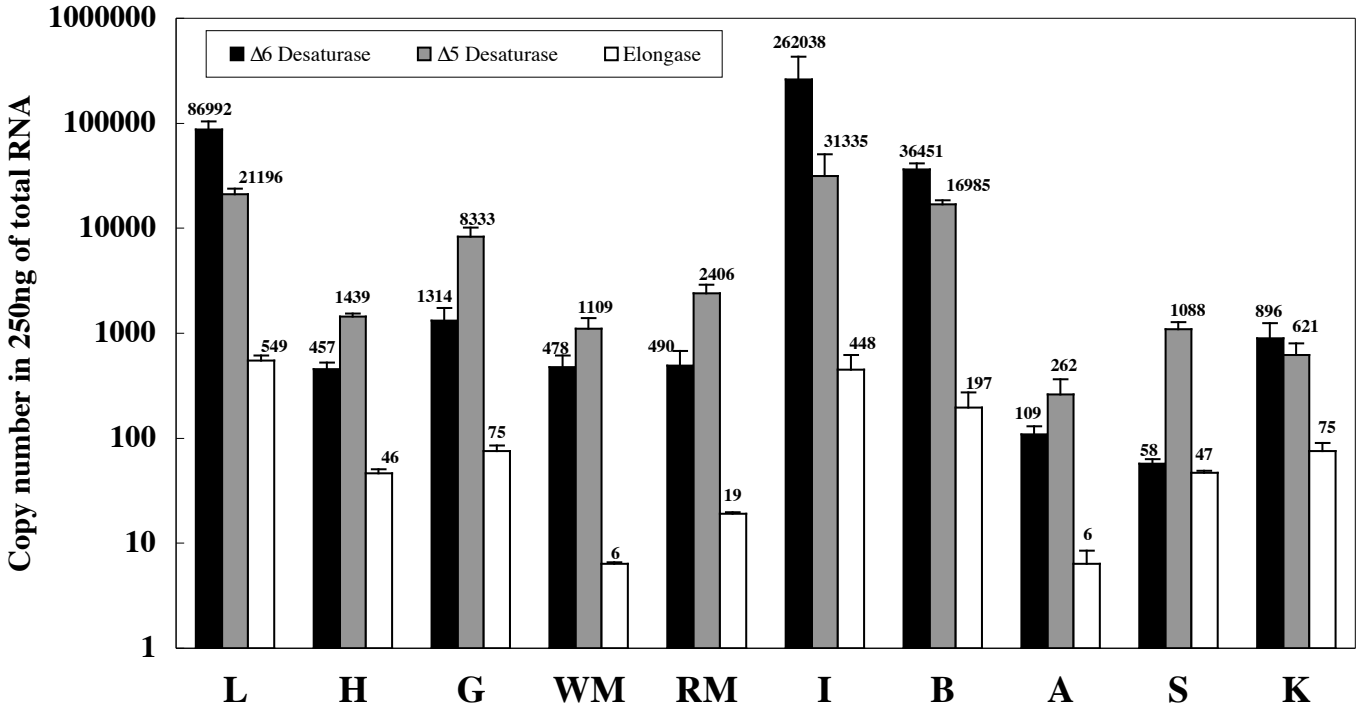


Fig. 5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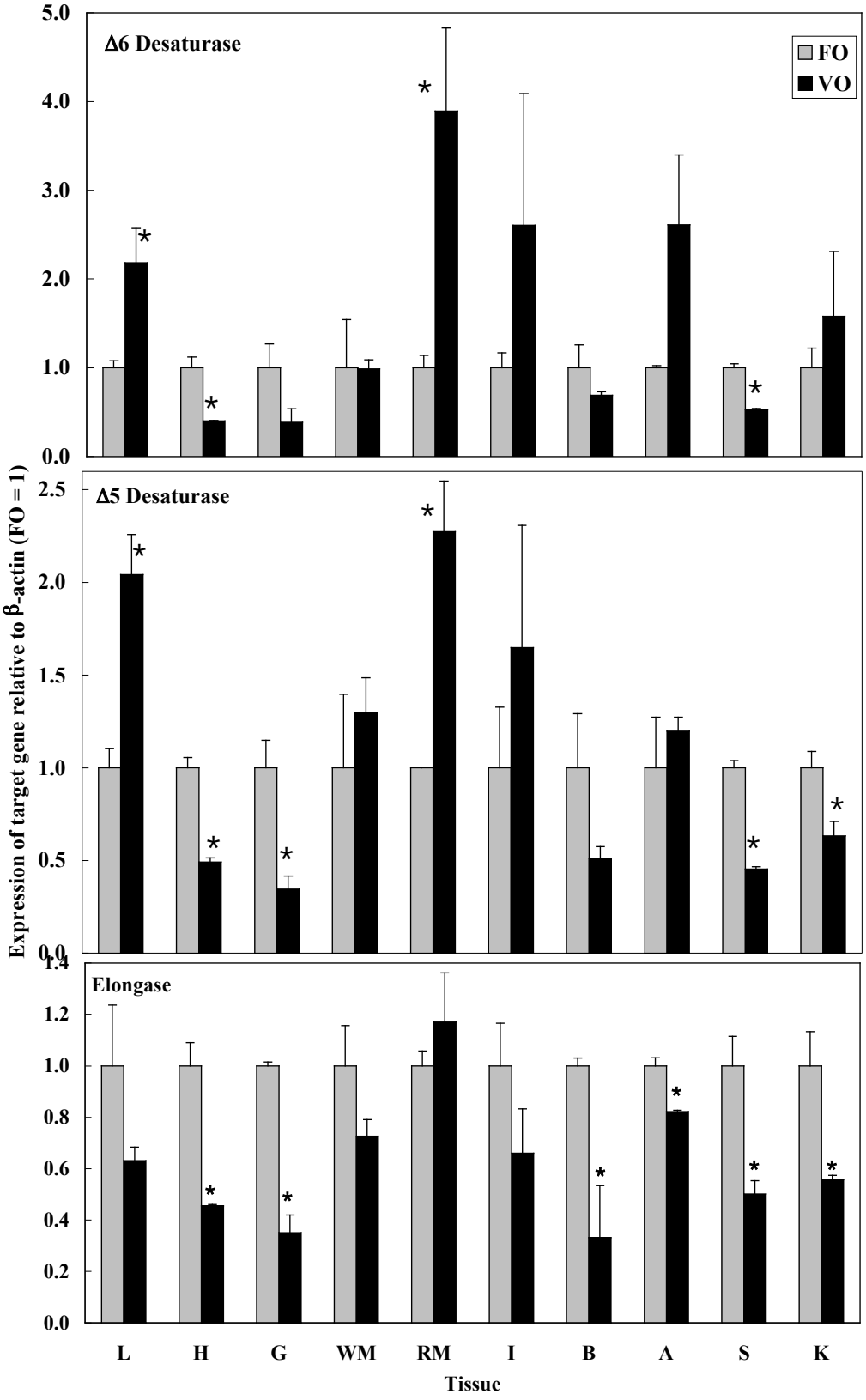
100000
10000
1000
100
10
1
Δ6 Desaturase
Δ5 Desaturase
Elongase
86992
21196
549
457
1439
46
1314
8333
75
478
1109
6
490
2406
19
262038
31335
448
36451
16985
197
109
262
6
58
1088
47
896
621
75
L
H
G
WM
RM
I
B
A
S
K

Fig.6.


Δ6 Desaturase
FO
VO
Δ5 Desaturase
Elongase
Expression of target gene relative to β-actin (FO = 1)
L
H
G
WM
RM
I
B
A
S
K
Tissue