Physiological roles of fatty acyl desaturases and elongases in marine fish: Characterisation of cDNAs of fatty acyl Δ6 desaturase and *elovl5* elongase of cobia (*Rachycentron canadum*)

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Abstract

In the present paper, we investigated the expression of fatty acyl desaturase and elongase genes in a marine teleost, cobia, a species of great interest due to its considerable aquaculture potential. A cDNA was cloned that, when expressed in yeast, was shown to result in desaturation of 18:3n-3 and 18:2n-6, indicating that it coded for a Δ6 desaturase enzyme. Very low desaturation of 20:4n-3 and 20:3n-6 indicated only trace Δ5 activity. Another cloned cDNA enabled elongation of 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6 in the yeast expression system, indicating that it had C18-20 and C20-22 elongase activity. Sequence comparison and phylogenetic analysis confirmed that it was homologous to human ELOVL5 elongase. However, the cobia Elovl5 elongase also had low activity toward C24 HUFA. The cobia Δ6 desaturase had a preference for 18:3n-3, but the elongase was generally equally active with both n-3 and n-6 substrates. Expression of both genes was 1-2 orders of magnitude greater in brain than other tissues suggesting an important role, possibly to ensure sufficient docosahexaenoic acid (DHA, 22:6n-3) synthesis in neural tissues through elongation and desaturation of eicosapentaenoic acid (EPA; 20:5n-3).

1. Introduction

Fish are the major dietary source of n-3 highly unsaturated fatty acids (HUFA), such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), for humans and, with declining fisheries, farmed fish constitute an increasing proportion of fish in the human food basket (FAO, 2006). The marine fish cobia is found worldwide in tropical, subtropical, and warm temperate seas, other than the eastern Pacific, but the commercial fishery is small due to their solitary nature (Shaffer and Nakamura, 1989; Ditty and Shaw, 1992). However, cobia is an excellent candidate for aquaculture because of its rapid growth, reaching 6-10 kg in 12-14 months, a rate around 3- to 5-fold greater than Atlantic salmon, as well as having excellent flesh quality (Liao et al., 2004; Holt et al., 2007). This species also expresses many other favourable production-related characteristics, including spawning in captivity (Caylor et al., 1994; Arnold et al., 2002; Faulk and Holt, 2006), high survival at post-weaning, ability to withstand shifts in salinity (Faulk and Holt, 2006) and responsiveness to vaccination (Lin et al., 2006). Cobia also adapts to confinement and readily accepts commercially-available extruded diets (Craig and McLean, 2005). However, while a developing cobia aquaculture industry already exists in Taiwan, the Caribbean and south-east United States, future expansion capitalising on the full potential of this species will be dependent upon increasing our knowledge of its basic nutrition and metabolism (Liao et al., 2004; Holt et al., 2007).

Furthermore, the development of cobia culture is taking place at a time of considerable expansion of aquaculture activities throughout the world that are rapidly outstripping global supplies of the principal dietary protein and oil sources, fish meal and oil, forcing changes to traditional feed formulations. Until now, high n-3 HUFA levels in flesh of farmed fish has been obtained by the use of fish oils, themselves derived from feed grade marine fisheries, in the feeds, but this is not sustainable, and will constrain continuing growth of aquaculture activities (Tacon, 2004; Pike, 2005). In consequence, alternatives to fish oil are urgently required, with vegetable oils as the prime candidates (Bell and Waagbø, 2008). However, vegetable oils are rich in short chain

polyunsaturated fatty acids (PUFA), but devoid of the n-3 HUFA abundant in fish oil (Sargent et al., 2002). As with all vertebrates, PUFA are essential in the diet of fish, but requirements vary with species, with marine fish having a dietary requirement for HUFA such as EPA and DHA, rather than shorter chain PUFA found in vegetable oil (Tocher, 2003). Feeding marine fish vegetable oil can reduce growth and lead to health problems including increased fat deposition and fatty livers, and compromised immune function (Caballero et al., 2004; Bell et al., 2005; Mourente et al., 2005). Importantly for the human consumer, it also lowers the n-3 HUFA content of the flesh compromising its nutritional value (Bell et al., 2005; Izquierdo et al., 2005; Torstensen et al., 2005).

HUFA can be biosynthesised from short-chain PUFA in reactions catalysed by fatty acyl desaturase and elongase enzymes. Synthesis of EPA is achieved by Δ6 desaturation of 18:3n-3 to produce 18:4n-3 that is elongated to 20:4n-3 followed by Δ5 desaturation (Cook, 1996), with synthesis of DHA from EPA requiring two further elongation steps, a second Δ6 desaturation and a peroxisomal chain shortening step (Sprecher, 2000). Evidence suggests that the dependence of marine fish on dietary HUFA is caused by a deficiency in the activity of one or more of the key enzymes in this pathway (Tocher, 2003). Fatty acyl desaturase cDNAs have been cloned from various fish species (Seiliez et al., 2001; Zheng et al., 2004), including a bifunctional Δ6/Δ5 desaturase from the freshwater zebrafish (Hastings et al., 2001) and separate, distinct Δ6 and Δ5 desaturases from Atlantic salmon (Hastings et al., 2005; Zheng et al., 2005). In contrast, only Δ6 desaturase cDNAs have so far been cloned from marine fish (Seiliez et al., 2003; Zheng et al., 2004; Tocher et al., 2006). Elongase cDNAs have been cloned from freshwater species, zebrafish, common carp and tilapia, salmonids, Atlantic salmon and rainbow trout, and marine species, Atlantic cod, turbot and gilthead sea bream (Agaba et al., 2004, 2005; Hastings et al., 2005).

Our overarching hypothesis is that elucidating the molecular basis of HUFA biosynthesis and regulation in marine fish will allow us to optimize the activity of the pathway to enable efficient and effective use of sustainable plant-based feedstuffs in aquaculture while maintaining the nutritional quality, in particular the n-3 HUFA content, of farmed fish for the human consumer. Our specific

objective in the present study was to characterize genes of HUFA synthesis in cobia as a first step to understanding the mechanisms underpinning the effects of vegetable oil feeding in this species. Here we describe the cDNA cloning, functional characterization and tissue distribution of a fatty acyl Δ6 desaturase and an *elovl5* elongase of cobia. Based on the results, the physiological role for these genes in a marine fish is discussed.

2. Materials and methods

2.1. Cloning of putative fatty acid desaturase and elongase from cobia

Juvenile cobia, *Rachycentron canadum*, were reared in floating sea net cages at Shen Ao Bay, near the Nan Ao Marine Biology Station of Shantou University, China. The water temperature ranged from 26 - 30°C and salinity from 25 - 33.0‰. The fish were fed a diet based on fish meal and fish oil. Total RNA was extracted from liver tissue using TRIzol® reagent (GibcoBRL, NY, USA). First strand cDNA was synthesised using MMLV reverse transcriptase (Promega, Madison, WI, USA) primed by the oligonucleotide T7polyT. Fish desaturase sequences existing in the GenBank database were aligned to enable the design of the degenerate forward primer COBDSTF and reverse primer COBDR1 for PCR isolation of a cobia desaturase cDNA fragment. Similarly, the primers COBESTF and COBESTR were designed for cobia elongase cDNA cloning. The sequences of all PCR primers used in this study are shown in Table 1. PCR amplification was performed using Thermoprime plus DNA polymerase (ABgene, Surrey, UK) under the following PCR conditions: initial denaturation at 95 ℃ for 1 min, 32 cycles of denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s and extension at 72 ℃ for 60 s. The PCR products were cloned into pBluescript KS II+ vector (Stratagene, La Jolla, CA, USA). The nucleotide sequences were determined using DTCS Kits and the CEQ™ 8800 Genetic Analysis System following the manufacturer's protocols (Beckman Coulter Inc., Fullerton, CA, USA). Following isolation of the

cDNA fragments, the specific forward primers COBD3F1 and COBE3F1, together with primer T7POLYT were used for desaturase and elongase 3' Rapid Amplification of cDNA Ends (RACE) PCR, respectively. The 3'RACE PCR were performed under the following conditions: initial denaturation at 95 ℃ for 1 min, 32 cycles of denaturation at 95 ℃ for 30 s, annealing at 56 ℃ for 30 s, and extension at 72 ℃ for 2 min. The initial PCR fragment and 3' RACE PCR fragment sequences were aligned to assemble the nucleotide sequences of the cobia putative desaturase and elongase cDNA open reading frames (ORFs) using BioEdit version 5.0.6 (Tom Hall, Department of Microbiology, North Carolina State University, NC, USA).

2.2. Functional characterization of cDNAs by heterologous expression of ORFs in Saccharomyces cerevisiae

Expression primers were designed for PCR cloning of the cobia putative desaturase and elongase cDNA ORFs. For the Δ6 desaturase, the forward primer COBDVF contained a *HindIII* site (underlined) and the reverse primer COBDVR contained a *XhoI* site (underlined). For the elongase, the forward and the reverse primers were COBEVF and COBEVR, respectively. PCR was performed using high fidelity PfuTurbo® DNA Polymerase following the manufacturer's instructions (Stratagene, La Jolla, CA, USA). Amplification involved an initial denaturation step at 95 ℃ for 2 min, followed by 30 cycles of denaturation at 95 ℃ for 30 s, annealing at 58 ℃ for 30 s, and extension at 72 ℃ for 90 s followed by a final extension at 72 ℃ for 10 min. Following PCR, the DNA fragments were restricted 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 *Saccharomyces 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 pYES2 constructs was on *S. cerevisiae* minimal

medium (SCMM) minus uracil. Culture of the recombinant yeast was carried out in SCMM-uracil broth using galactose induction of gene expression as described previously (Hastings et al., 2001). For the desaturase assay, each culture was supplemented with one of the following substrates; α-18:3n-3, 18:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 and 22:4n-6. For the elongase assay, each culture was supplemented with one of the following substrates; 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 and 22:4n-6. PUFA were added to the yeast cultures at concentrations of 0.5 mM (C18), 0.75 mM (C20) and 1 mM (C22) 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 (Hastings et al., 2001). Fatty acid methyl esters (FAME) were prepared, extracted, purified by thin layer chromatography (TLC), and analysed by gas chromatography (GC), all as described previously (Hastings et al., 2001). The proportion of substrate fatty acids converted to desaturated or elongated products 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 previously (Hastings et al., 2001).

2.3. Cobia tissue RNA extraction and quantitative real time PCR (Q-PCR)

Total RNA from liver, brain, heart, skin, gill, kidney, spleen, intestine, adipose tissue, white and red muscle of juvenile cobia were extracted 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). Expression of the fatty acyl desaturase and elongase genes in different tissues was studied by quantitative real-time PCR (Q-PCR). The PCR primers were designed according to the cloned cobia desaturase and elongase cDNA sequences. For the desaturase, the forward and reverse primers were COBDRF and COBDRR, respectively. For the elongase, the forward and reverse primers were COBERF and COBERR, respectively (Table 1).

PCR products sizes were 119 and 133 bp for desaturase and elongase, respectively. PCR amplicons were cloned into the pBluescript II KS vector (Stratagene). The linearised plasmid DNA containing the target sequence for each gene was quantified spectrophotometrically (NanoDrop ND-1000, Thermo Scientific, Wilmington, DE, USA) and serial-diluted to generate a standard curve of known copy numbers. Amplification of cDNA samples and DNA standards was carried out using SYBR Green PCR Kit (Qiagen, Crowley, West Sussex, UK) under the following conditions: 15 min denaturation at 95 ^{o}C, 45 cycles of 15 s at 94 ^{o}C, 15 s at 56 ^{o}C and 20s at 72 ^{o}C. This was followed by product melting to confirm single PCR products. Thermal cycling and fluorescence detection were conducted in a Rotor-Gene 3000 system (Corbett Research, Cambridge, UK). All reactions were carried out in triplicate and a linear standard curve was drawn, and absolute copy number of the targeted gene in each sample was calculated.

2.4. Materials

Docosapentaenoic (22:5n-3), docosatetraenoic (22:4n-6) and eicosatetraenoic (20:4n-3) acids, (> 98-99% pure) were obtained from Cayman Chemical Co., Ann Arbor, MI, USA. Linoleic (18:2n-6), α-linolenic (18:3n-3), stearidonic (18:4n-3), γ-linolenic (18:3n-6), dihomo-γ-linolenic (20:3n-6), eicosapentaenoic (20:5n-3) and eicosatetraenoic (20:4n-6) acids (all >99% pure), and BHT were from Sigma-Aldrich Co. Ltd. (Poole, U.K.). All solvents were HPLC grade and were from Fisher Scientific (Loughborough, UK).

3. Results

3.1. Sequence analyses of cobia desaturase and elongase cDNAs

The putative desaturase cDNA obtained from cobia was shown to be 1823 bp, which included a 3'-UTR of 494 bp. Sequencing revealed that the cDNA included an ORF of 1329 bp, which specified a protein of 442 amino acids (GenBank accession no. FJ440238). The protein sequence included all the characteristic features of microsomal fatty acid desaturases, including two transmembrane regions, three histidine boxes and an N-terminal cytochrome b_5 domain containing the haem-binding motif, H-P-G-G (Fig. 1). Phylogenetic analysis comparing the cobia desaturase sequence with desaturases from other fish species and human Δ5 and Δ6 desaturases, clustered the cobia most closely with turbot, sea bream, cod and tilapia and more distantly from carp, zebrafish, and salmonids (Fig. 2). A pair-wise comparison among fish desaturase sequences showed the amino acid sequence predicted by the cobia putative desaturase ORF showed greatest identity to that of sea bream Δ6 desaturase (87 %), 85 % identity to that of the turbot, 79 % to that of the Atlantic cod and 75 % identities to the Atlantic salmon Δ6 cDNAs, respectively (Table 2).

The putative elongase cDNA obtained from cobia was shown to be 1015 bp, which included a 3'-UTR of 130 bp. Sequencing revealed that the cDNA included an ORF of 885 bp, which specified a protein of 294 amino acids (GenBank accession no. FJ440239). The protein sequence included characteristic features of microsomal fatty acyl elongases, including a single histidine box redox centre motif (HXXHH) and multiple transmembrane regions (Fig. 3). The translated amino acid sequences of the elongase cDNAs cloned from cobia, along with those from other fish species, were very similar to each other and shared at least 64% sequence identity with the human ELOVL5 elongase (Table 3), with most of the sequence variation (insertions and substitutions) residing in the C-terminal region of the polypeptide (Fig. 3). Phylogenetic analysis comparing all the putative elongase sequences cloned from fish along with a range of elongases from mammals, avian, insect, fungus and nematode, clustered the cobia most closely with sea bream, turbot and tilapia, and more distantly from salmonids, Atlantic cod, catfish and zebrafish (Fig. 4). All the fish elongase sequences were clustered together and they were most similar to the mammalian (human/rodent) ELOVL5 (rat rELO1) elongase sequences.

3.2. Functional characterisation of cobia fatty acyl desaturase and elongase

The fatty acid composition of untransformed yeast *S. cerevisiae* shows four main fatty acids, namely 16:0, 16:1n-7, 18:0 and 18:1n-9 (Hastings et al., 2001). When yeast, transformed with the cobia desaturase cDNA insert, was grown in the presence of Δ6 desaturase substrates, two additional peaks were observed in the GC traces, corresponding to the exogenously added fatty acids, 18:3n-3 and 18:2n-6, and their desaturated products, 18:4n-3 and 18:3n-6, respectively (Fig. 5A and B). The GC traces of desaturase transformed yeast grown in the presence of Δ5 desaturase substrates showed large peaks for the exogenously added fatty acids, 20:4n-3 and 20:3n-6, but only traces of desaturated products, 20:5n-3 and 20:4n-6, respectively (Fig. 5C and D). In this heterologous assay system, the cobia desaturase showed a preference for the n-3 fatty acid substrates, with approximately 50.8 % of 18:3n-3 converted to 18:4n-3, and 36.6 % of 18:2n-6 converted to 18:3n-6 (Table 4). No additional peaks representing desaturated fatty acid products were observed in the lipids of transformed *S. cerevisiae* incubated with 22:5n-3 or 22:4n-6 (data not shown).

When yeast, transformed with the cobia elongase cDNA insert, was grown in the presence of fatty acid substrates for C18 elongation, four additional peaks were observed in the GC traces, corresponding to the exogenously added fatty acids, 18:4n-3 and 18:3n-6, their immediate elongated products, 20:4n-3 and 20:3n-6, and the further elongated products, 22:4n-3 and 22:3n-6 (Fig. 6A shows result for n-3 substrate). The fourth peak was 18:1n-7 indicating elongation of endogenous 16:1n-7. Small peaks were also observed (between peaks 6 and 7) that corresponded to 20:1n-9 and 20:1n-7, indicating some elongation of 18:1n-9 and 18:1n-7 (Fig. 6A). Similarly, the GC traces of elongase transformed yeast grown in the presence of substrates for C20 elongation showed additional peaks corresponding to the exogenously added fatty acids, 20:5n-3 and 20:4n-6, their immediate elongation products, 22:5n-3 and 22:4n-6, and the further elongated products, 24:5n-3

and 24:4n-6 (Fig. 6B). Transformed yeast grown in the presence of substrates for C22 elongation showed additional peaks corresponding the exogenously added fatty acids, 22:5n-3 and 22:4n-6, and minor peaks for their elongated products, 24:5n-3 and 24:4n-6, respectively (Fig. 6C). In this heterologous expression system, the cobia elongase showed similar activity towards C18 and C20 fatty acid substrates, with much lower activity towards C22 substrates (Table 5). There was no preference between the n-3 and n-6 C18 substrates, but there may be a slight preference for n-3 with the longer chain fatty acids.

3.3. Tissue expression of cobia desaturase and elongase

The absolute copy numbers of cobia fatty acyl desaturase and elongase in different tissues are shown in Fig. 7. The expression of both desaturase and elongase mRNAs was greatest by far in the brain (note logarithmic scale), followed by heart and then liver for desaturase, or liver and then heart for elongase. Lower levels of desaturase and elongase mRNA were detected in white and red muscle tissues. Desaturase mRNA was also expressed in skin and gill where elongase was not detected, and neither mRNA could be detected in intestine, spleen, kidney and adipose tissue.

4. Discussion

Research into the nutrition of cobia is required to define their precise nutritional requirements, and to develop ideal formulated diets. There have been several trials investigating aspects of lipid nutrition in cobia including dietary protein and lipid levels (Chou et al., 2001; Wang et al., 2005), digestibility (Zhou et al., 2004) and larval development (Turner and Rooker, 2005). However, it is very important to appreciate that the current activities focussed on developing a viable cobia farming industry, growing fish from egg to market size, are taking place at a time when the aquaculture nutrition industry is currently going through major changes. These changes to feed

formulations are considerable and are being forced upon the industry simply through the increasing expansion of aquaculture activities throughout the world that are rapidly outstripping global supplies of fish meal and oil (Tacon, 2004; Pike, 2005). Based on our current knowledge of marine fish nutrition, any formulated feed for cobia will have to be dependent, to some extent, upon global supplies of fish meal and fish oil (Tocher, 2003). Thus, studies have shown that 20-30% of fishmeal in cobia diets can be replaced by soybean meal without compromising growth performance but, at higher levels of substitution, growth can be significantly decreased (Chou et al., 2004; Zhou et al., 2005). Interestingly, and in contrast to salmonids, cobia do not appear to develop soybean meal-induced enteritis in the distal intestine (Romarheim et al., 2008). Similarly, replacement of fish meal with yeast protein reduced growth performance in cobia juveniles (Lunger et al., 2006, 2007). It is against this background that the present work, investigating PUFA metabolism and HUFA synthesis in cobia, is set.

Biosynthesis of n-3 HUFA in vertebrates occurs in essentially two stages with rather different kinetics. Synthesis of EPA requires Δ6 desaturation of 18:3n-3 to produce 18:4n-3 that is elongated to 20:4n-3 followed by Δ5 desaturation to produce EPA (Cook, 1996). This is essentially the pathway investigated in most early studies in relation to synthesis of HUFA in vertebrates, more specifically 20:4n-6, and the rate-limiting step was found to be the Δ6 desaturation (Brenner, 1974). Synthesis of DHA from EPA requires two further elongation steps, a second Δ6 desaturation, and a peroxisomal chain-shortening step to produce DHA (Sprecher, 2000). Thus, this second stage requires translocation of fatty acyl intermediates between microsomal and peroxisomal compartments and is consequently much slower than the synthesis of EPA from 18:3n-3. How DHA synthesis is precisely controlled and regulated is intriguing as it is an anabolic pathway (biosynthesis) although the final step involves a catabolic pathway (limited chain shortening) (Sprecher, 2000). Of further interest is that there is evidence that the Δ6 desaturation of the C24 intermediates is performed by the same Δ6 desaturase that desaturates C18 fatty acids, and is the rate-limiting step in EPA (and arachidonic acid) synthesis (De Antueno et al., 2001; D'Andrea et

al., 2002). Thus, C18 and C24 fatty acyl substrates must compete for the Δ6 desaturase enzyme, but nothing is currently known about this competition.

Isotopic studies with primary hepatocytes and cell lines have shown that the pathway for arachidonic acid and EPA synthesis is fully functional in freshwater and diadromous teleost fish species, including salmon, trout, zebrafish, carp and tilapia (Tocher and Sargent, 1990; Tocher et al., 1997, 2002; Tocher, 2003). However, production of EPA from 18:3n-3 in cell lines and primary hepatocytes from marine fish is either extremely low or completely absent (Tocher and Sargent, 1990; Tocher et al., 2006). Biochemical studies identified both Δ5 desaturation and C18-20 elongation as deficient steps in the pathway in cell lines from marine fish (Ghioni et al., 1999; Tocher and Ghioni, 1999). In the present study on cobia, we have successfully cloned two cDNAs that were shown, by heterologous expression in yeast, to be a desaturase capable of Δ6 desaturation, and an elongase capable of elongating both C18 and C20 fatty acyl substrates. These are three of the enzymatic activities required for synthesis of EPA from 18:3n-3, with Δ5 desaturase being the one missing. In the present study, PCR primers were designed based on the sequence of the only fish (salmon) Δ5 desaturase so far cloned, but no products were obtained in cobia. Previously, attempts to clone a Δ5 desaturase cDNA from another marine teleost, Atlantic cod, were also unsuccessful (Tocher et al., 2006). Only a single fatty acyl desaturase gene can be identified in the marine fish with sequenced genomes, namely Japanese medaka (*Oryzias latipes*) and pufferfish species (*Fugu rupripes* and *Tetraodon nigroviridis*). Therefore, there is increasing evidence that the inability of marine fish species to produce EPA and DHA from 18:3n-3 is due to the lack of a Δ5 desaturase gene.

The present study has demonstrated that cobia expresses functional fatty acyl Δ6 desaturase and Elovl5 elongase activities. The question must be, why? Marine fish such as the cobia inhabit a food web rich in n-3 HUFA and, therefore, receive sufficient HUFA in their natural diet to more than satisfy EFA requirements, and so there is no apparent metabolic or physiological need for HUFA synthesis from 18:3n-3 or 18:2n-6 (Sargent et al., 2002). Although there is no evolutionary pressure

to maintain activities required for the synthesis of EPA, there may an evolutionary advantage in being able to maintain, regulate or enhance DHA levels, particularly in sensitive tissues such as neural tissues, which are specifically enriched in DHA rather than EPA (Bell and Tocher, 1989). This pathway for DHA synthesis from EPA, requiring two elongations and Δ6 desaturation of 24:5n-3 has been shown to operate in rainbow trout, the only non-mammalian vertebrate where this pathway has been confirmed (Buzzi et al., 1997). The bifunctional Δ6/Δ5 desaturase in zebrafish has been shown to be capable of desaturating 24:5n-3 to 24:6n-3 (Tocher et al., 2003). Thus, there is evidence that elongation to 24:5n-3 followed by Δ6 desaturation is required for conversion of EPA to DHA in fish. Therefore, the results of the present study, clearly showing that cobia express functional Δ6 desaturase and Elovl5, that display all the enzymatic activities required, is clear evidence that DHA synthesis from EPA is possible in marine fish, even if at very low rates given the low activity of Elovl5 towards C22 fatty acyl substrates.

The results of the present study also show that the expression of both genes was 1-2 orders of magnitude greater in brain than any other tissue, suggesting an important role in neural tissues, possibly to ensure sufficient DHA despite fluctuations in dietary EPA and DHA levels, particularly at crucial times in development (Sargent et al., 2002). For instance, during larval development in marine fish there is considerable demand for DHA for neural tissue development (Mourente, 2003). Dietary deficiency of DHA resulted in larval Atlantic herring having an impaired ability to capture prey at natural light intensities (Bell et al., 1995), delayed response to visual stimuli in larval gilthead sea bream (Benitez-Santana et al., 2007), and impaired schooling behaviour in yellowtail (Masuda et al., 1998; Ishizaki et al., 2001) and Pacific threadfin (*Polydactylus sexfilis*) (Masuda et al., 2001). Previously, we have shown that 10-fold more DHA was produced from EPA than from 18:3n-3 in primary cultures of brain cells from turbot (Tocher et al., 1992). Consistent with the data in the present study on cobia, the expression of Δ6 desaturase was also an order of magnitude greater in brain than any other tissue in another marine fish, cod (Tocher et al., 2006). Therefore, the gene expression data in cobia and cod and the biochemical assay data from turbot are all

consistent with synthesis of DHA from EPA in brain being a major biological role for Δ6 desaturase and Elovl5-like elongases in marine fish, and why these genes have been retained whereas Δ5 desaturase has not. In contrast, expression of fatty acyl desaturases and elongases in salmon was highest in liver and intestine, reflecting the different role, namely the production of EPA (and subsequently DHA) from dietary 18:3n-3 (Bell et al., 2001, 2003).

The cobia Δ6 desaturase had greater activity with 18:3n-3 than 18:2n-6, which is consistent with all other fish Δ6 desaturases studied to date (Hastings et al., 2001; Zheng et al., 2004, 2005; Tocher et al., 2006). Early biochemical studies indicated that mammalian desaturases show an order of preference with n-3 > n-6 > n-9 (Brenner, 1974). In contrast, the cobia elongase showed no preference between the n-3 and n-6 C18 substrates, but did show a slight preference for the n-3 C20 substrate. This is consistent with data from cloned elongases from other marine fish, turbot and sea bream that were equally active towards 18:4n-3 and 18:3n-6, but showed a preference for 20:5n-3 over 20:4n-6, whereas the elongase from cod had greater activity with both n-6 substrates (Agaba et al., 2005). In contrast, elongases cloned from freshwater and diadromous species were more active with n-3 fatty acids irrespective of chain length (Agaba et al., 2005). The reason for the difference in substrate preference between elongases from freshwater and marine fish is not clear, but may suggest that the pathway has more importance in freshwater fish, and so there has been evolutionary pressure to produce EPA and DHA resulting in all enzymes having higher activity towards n-3 substrates. In contrast, the lesser importance of the pathway in marine fish, because of the luxus of n-3 HUFA in the diet, may have resulted in less evolutionary pressure for n-3 preferences. Taken to its extreme this argument could result in the complete loss of functionality, as it is believed has happened with the loss of Δ5 desaturase in marine fish.

Sequence comparison with other vertebrate elongases showed that the cobia elongase was similar to mammalian ELOVL5 elongases (Jakobsson et al., 2006). This was also reflected in function, with activity towards C18 fatty acyl substrates greater than that towards C20 substrates, and so we can consider the cobia elongase as an Elovl5. Nearly all the other cloned and functionally

characterised elongases from fish reported so far show sequences and functionality similar to mammalian ELOVL5 elongases (Hastings et al., 2005; Agaba et al., 2004, 2005). The fish Elovl5s have a wider substrate specificity than mammalian ELOVL5s, which elongate predominantly C18 and, to a lesser extent, C20 fatty acids but not C22, whereas most fish Elovl5-like elongases can also elongate 22:5n-3 to 24:5n-3, albeit at a very low level (Leonard et al., 2000; Inagaki et al., 2002; Agaba et al., 2005). Thus, theoretically only this one Elovl5-like elongase might be required for the production of DHA from 18:3n-3 in freshwater fish and, in marine fish, this one elongase would be all that is required to perform both the elongations of EPA necessary for the production of DHA. Nonetheless, in Atlantic salmon, a new elongase cDNA has been recently cloned, corresponding to an Elovl2 (Morais et al., 2009). Its functional characterization revealed that it elongates preferentially C20 and C22 PUFA with lower activity towards C18 PUFA, with its higher activity towards C22 LC-PUFA being the major difference in comparison to fish Elovl5 and mammalian ELOVL5 proteins. However, searches of ENSEMBL marine fish genomes, such as pufferfish, stickleback and medaka, have not revealed any *elovl2*-like genes and indicated that these genomes contain only an *elovl5* elongase.

Conclusions

We have demonstrated that cobia express a fatty acyl desaturase gene, the product of which shows substantial Δ6 desaturase activity in an heterologous yeast expression system. The Δ6 desaturase was highly expressed in brain. In addition, a fatty acyl *elovl5*-like elongase shown to elongate a range of C18, C20 and C22 PUFA in the yeast expression system, was cloned and shown to be also highly expressed in brain. Along with previous data studying HUFA synthesis in various fish species, including freshwater, diadromous and marine species, the results of this study support the hypothesis that the poor ability of marine fish to synthesise HUFA is generally not due to lack of a Δ6 desaturase or PUFA elongation activities. The data are consistent with cobia being similar to

other marine fish studied to date, indicating that only limited replacement of fish oil with vegetable oil alternatives will be possible. However, feeding trials to determine essential fatty acid requirements, and biochemical studies to measure HUFA synthesis activities, are required to support these molecular studies, and to determine the extent to which the genes cloned here have functionally meaningful activity *in vivo* and the extent to which fish oil replacement will impact upon cobia growth performance and nutritional quality.

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Legends to Figures

Fig. 1. Comparison of the deduced amino acid sequence of the fatty acyl desaturase from cobia with that of Δ6 desaturases from gilthead seabream, Atlantic cod, Atlantic salmon and human. The amino acid sequences were aligned using ClustalX, and identity/similarity shading was based on the BLOSUM62 matrix and the cut off was 75%. Identical residues are shaded black and similar residues are shaded grey. The cytochrome b_5-like domain is dot-underlined, the two transmembrane regions are dash underlined, the three histidine-rich domains are solid underlined and asterisks on the top mark the haem-binding motif, H-P-G-G.

Fig. 2. Phylogenetic tree of fatty acyl desaturases from cobia and other fish species Atlantic salmon, rainbow trout, cherry salmon, Nile tilapia, Atlantic cod, gilthead seabream, turbot, common carp and zebrafish, mammals (mouse, *Mus musculus* and human), fungus (*Mortierella alpina*) and nematode (*Caenorhabditis elegan*s). The tree was constructed using the Neighbour Joining method (Saitou and Nei, 1987) using *CLUSTALX* and *NJPLOT*. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations. Sequences marked with an asterisk are not functionally characterized.

Fig. 3. Comparison of the deduced amino acid sequence of the fatty acyl elongase from cobia with that of elongases from gilthead seabream, Atlantic cod, Atlantic salmon and human. The amino acid sequences were aligned using ClustalX, with identity/similarity shading based on the BLOSUM62 matrix and a cut off of 75%. Identical residues are shaded black and similar residues are shaded grey. Transmembrane regions (dash underlined) were predicted from a hydropathy plot taking peaks with scores > 1.6 using a scan window size of 18 (Kyte and Doolittle, 1982). The histidine box redox centre motif (HXXHH) is solid underlined.

Fig. 4. Phylogenetic tree of fatty acyl elongases from cobia and other fish species (turbot, gilthead seabream, tilapia, Atlantic salmon, rainbow trout, Atlantic cod, catfish *Clarias gariepinus*, and zebrafish), mammals (human, mouse and rat, *Rattus norvegicus*), bird (chicken, *Gallus gallus*) amphibian (*Xenopus laevis*), insect (*Drosophila melanogaster*), fungus (*M. alpina*), nematode (*C. elegans*) and plant (moss, *Physcomitrella patens*). The tree was constructed using the Neighbour Joining method (Saitou and Nei, 1987) using *CLUSTALX* and *NJPLOT*. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations.

Fig. 5. Functional characterization of the cobia putative fatty acyl desaturase in transgenic yeast (*Saccharomyces cerevisiae*) grown in the presence of Δ6 substrates 18:3n-3 (A) and 18:2n-6 (B), and Δ5 substrates 20:4n-3 (C) and 20:3n-6 (D). 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 four peaks in all panels are the main endogenous fatty acids of *S. cerevisiae*, namely 16:0 (1), 16:1n-7 (2), 18:0 (3), and 18:1n-9 (4). Peak 5 in panel A and peak 7 in panel B are the exogenously added substrate fatty acids, 18:3n-3 and 18:2n-6, respectively. Peaks 6 and 8 in panels A and B were identified as the resultant desaturated products, namely 18:4n-3 and 18:3n-6, respectively. Peaks 9 and 10 in panels C and D are the exogenously added substrate fatty acids, 20:4n-3 and 20:3n-6, respectively. Vertical axis, FID response; horizontal axis, retention time.

Fig. 6. Functional characterisation of cobia putative fatty acyl elongase in transgenic *Saccharomyces cerevisiae* grown in the presence of fatty acid substrates 18:4n-3 (panel A), 20:5n-3 (B), and 22:5n-3 (C). Fatty acids were extracted from yeast transformed with pYES2 vector containing the ORF of the putative fatty acyl elongase cDNA as an insert. Peaks 1-4 are as described in legend to Fig. 5. Peak 5 corresponds to 18:1n-7 arising from the elongation of the yeast

endogenous 16:1n-7 and the remaining main additional peaks (6-11) correspond to the exogenously added fatty acids and the products of their elongation – 18:4n-3 (6), 20:4n-3 (7), 22:4n-3 (8), 20:5n-3 (9), 22:5n-3 (10), and 24:5n-3 (11). Other minor peaks (not labelled) are 20:1n-9 and 20:1n-7, resulting from the elongation of 18:1n-9 and 18:1n-7. Vertical axis, FID response; horizontal axis, retention time.

Fig. 7. Tissue distribution of fatty acyl Δ6 desaturase and Elovl5 elongase genes in cobia. Transcript (mRNA) copy number was determined by quantitative real-time PCR (Q-PCR) as described in the Materials and Methods Section. Results are expressed as means (n = 4; SD omitted for clarity). B, brain; H, heart; L, liver; SK, skin; G, gill; WM, white muscle; RM, red muscle.

Table 1

Sequences of PCR primers utilised in this study

Primer name	Sequence (5'-3')
T7polyT	TACGACTCACTATAGGGCGTGCAGTTTTTTTTTTTT
COBDSTF	GATGGGAGGTGGAGGSCAG
COBDR1	ATCGGCAGATGATTCATCTG
COBESTF	AAATGGAGACCTTCAATCAYA
COBESTR	CAAATGTCAATCCACCCTCA
COBD3F1	GGAGCCACTCAACCTGTGGA
COBE3F1	CTCCTGTTTCAACAAACGGA
COBDVF	CCCAAGCTTAAGATGGGAGGTGGAGGCCAGCTGAC
COBDVR	CCGCTCGAGTCATTTATGGAGATATGCATCAAGCC
COBEVF	CCCAAGCTTAAAATGGAGACCTTCAATCATAAACTG
COBEVR	CCGCTCGAGTCAATCCACCCTCAATTTCTTG
COBDRF	CCGTGCACTGTGTGAGAAAT
COBDRR	GGAGATATGCATCAAGCCAGA
COBERF	ATGTGATTTCCCCAGAGGAT
COBERR	ACCATTCTGGTGCTCCTTCT

Table 2

Identity matrix showing the results of a pair-wise comparison between the identities of the amino acid sequences of the fish and human fatty acid desaturases

	Atlantic Cod Δ6	Zebrafish Δ5/ Δ6	Atlantic salmon Δ6	Atlantic salmon Δ5	Rainbow Trout Δ6	Gilthead seabream Δ6	Turbot Δ6	Human Δ6	Human Δ5
Cobia Δ6	79	71	75	76	75	87	85	65	57
Atlantic Cod Δ6		70	75	76	76	82	77	64	56
Zebrafish Δ5/ Δ6			65	64	65	68	68	65	56
Atlantic salmon Δ6				91	94	76	72	65	58
Atlantic salmon Δ5					92	77	73	63	57
Rainbow trout Δ6						76	72	65	57
Gilthead seabream Δ6							84	65	57
Turbot Δ6								62	56
Human Δ6									61

Data are percentages of amino acid residues that are identical.

Table 3

Identity matrix showing the results of a pair-wise comparison between the identities of the amino acid sequences of fish and human ELOVL5 fatty acyl elongases

	Gilthead seabream	Turbot	Nile tilapia	Rainbow trout	Atlantic Cod	Catfish	Zebrafish	Human
Cobia	91.5	87.4	81.3	83.4	75.9	76.3	74.1	70.6
Gilthead seabream		83.3	77.6	80.7	74.1	73.9	72.5	70.6
Turbot			82.0	80.7	74.7	73.8	72.9	66.9
Nile tilapia				74.6	71.9	71.1	71.5	64.2
Rainbow trout					74.2	75.9	73.2	71.9
Atlantic Cod						80.3	77.2	66.6
Catfish							76.2	68.6
Zebrafish								68.6

Data are percentages of amino acid residues that are identical.

Table 4

Activity of cobia desaturase in the yeast expression system

Fatty acid substrate	Product	Conversion (%)	Activity
18:3n-3	18:4n-3	50.8	Δ6
18:2n-6	18:3n-6	36.5	Δ6
20:4n-3	20:5n-3	2.4	Δ5
20:3n-6	20:4n-6	0.2	Δ5
22:5n-3	22:6n-3	ND	Δ4
22:4n-6	22:5n-6	ND	Δ4

Results are expressed as a percentage of total fatty acid substrate converted to desaturated product. ND, not detected.

Table 5

Activity of cobia elongase in the yeast expression system

Fatty acid substrate	Product	Conversion (%)	Activity
18:4n-3	20:4n-3	55.5	C18→20
	22:4n-3	19.2	C20→22
	24:4n-3	0.8	C22→24
	Total	75.5	
18:3n-6	20:3n-6	57.4	C18→20
	22:3n-6	17.3	C20→22
	24:3n-6	1.5	C22→24
	Total	76.2	
20:5n-3	22:5n-3	71.8	C20→22
	24:5n-3	4.4	C22→24
	Total	76.2	
20:4n-6	22:4n-6	66.2	C20→22
	24:4n-6	5.0	C22→24
	Total	71.2	
22:5n-3	24:5n-3	6.6	C22→24
22:4n-6	24:4n-6	3.8	C22→24

Results are expressed as a percentage of total fatty acid substrate converted to elongated products.

Fig.1

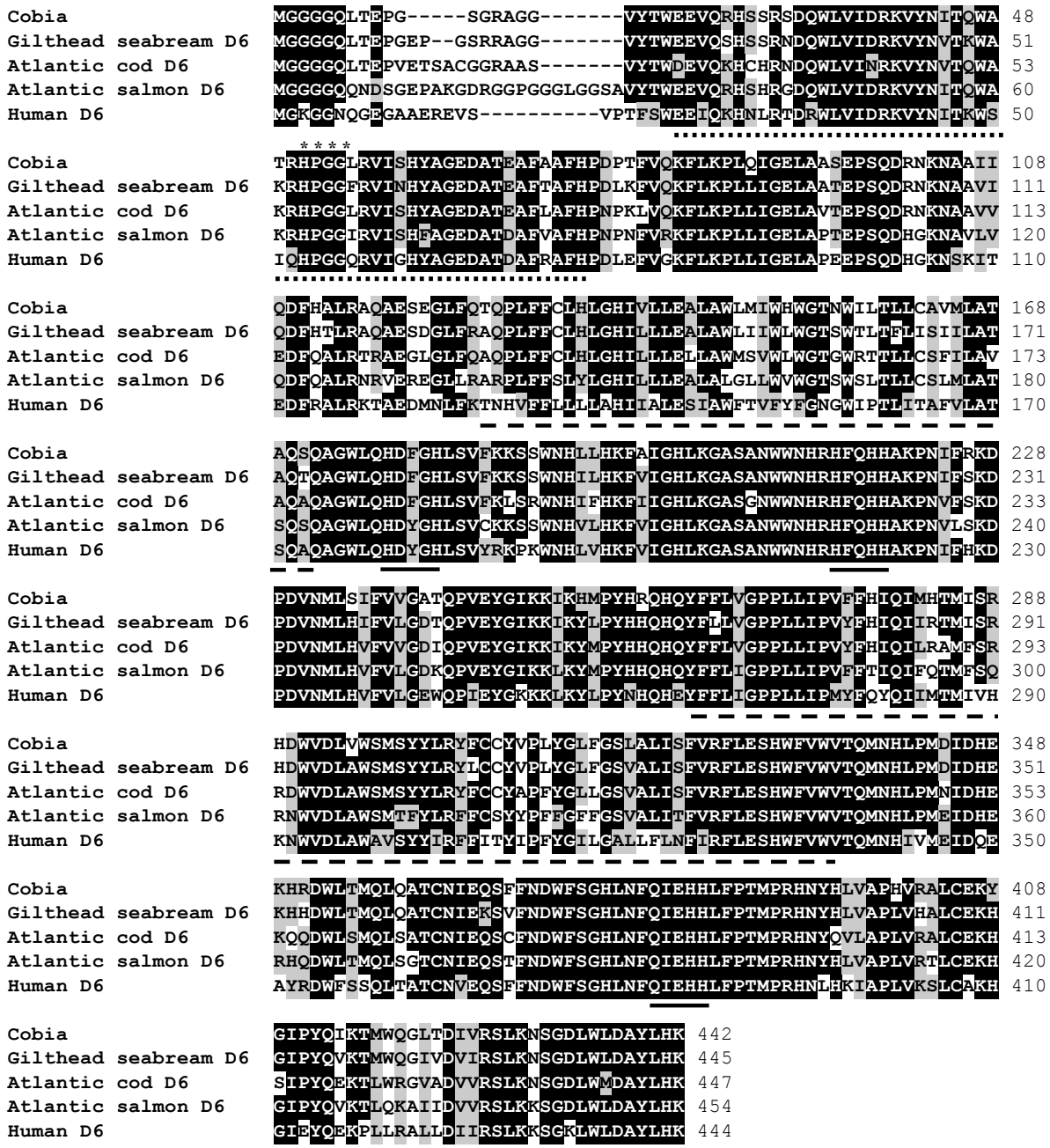
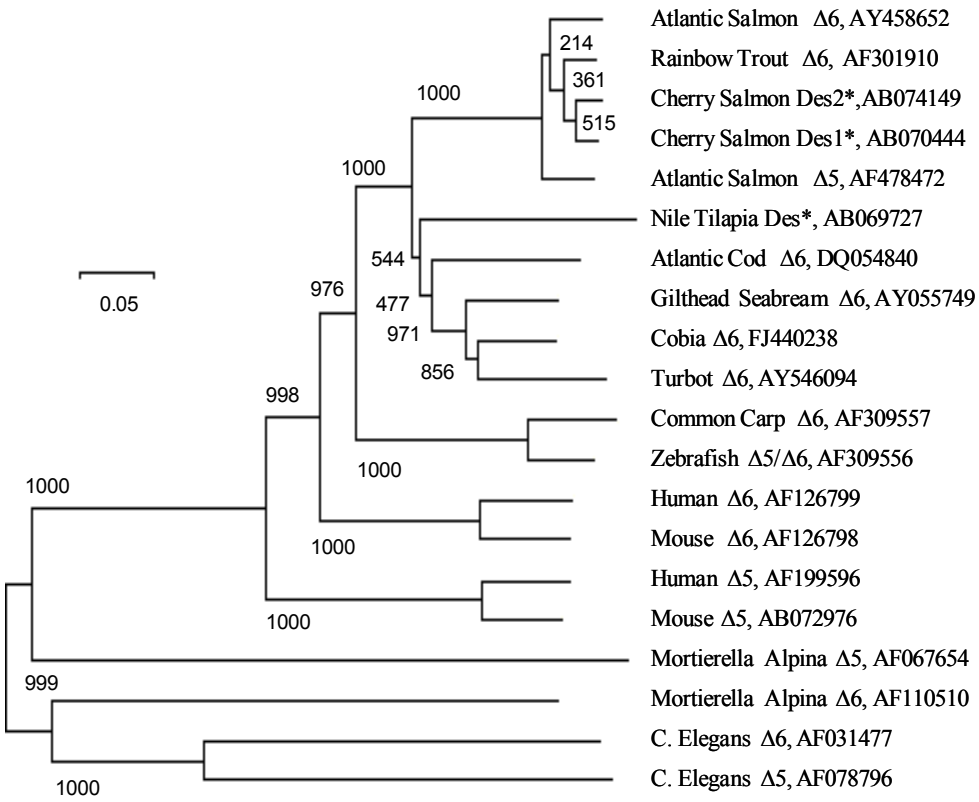

Cobia
Gilthead seabream D6
Atlantic cod D6
Atlantic salmon D6
Human D6

Fig.2.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Cherry Salmon Des1*, AB070444
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Nile Tilapia Des*, AB069727
Atlantic Cod Δ6, DQ054840
Gilthead Seabream Δ6, AY055749
Cobia Δ6, FJ440238
Turbot Δ6, AY546094
Common Carp Δ6, AF309557
Zebrafish Δ5/Δ6, 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
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1000
1000
1000
1000
999
1000
0.05

Fig.3

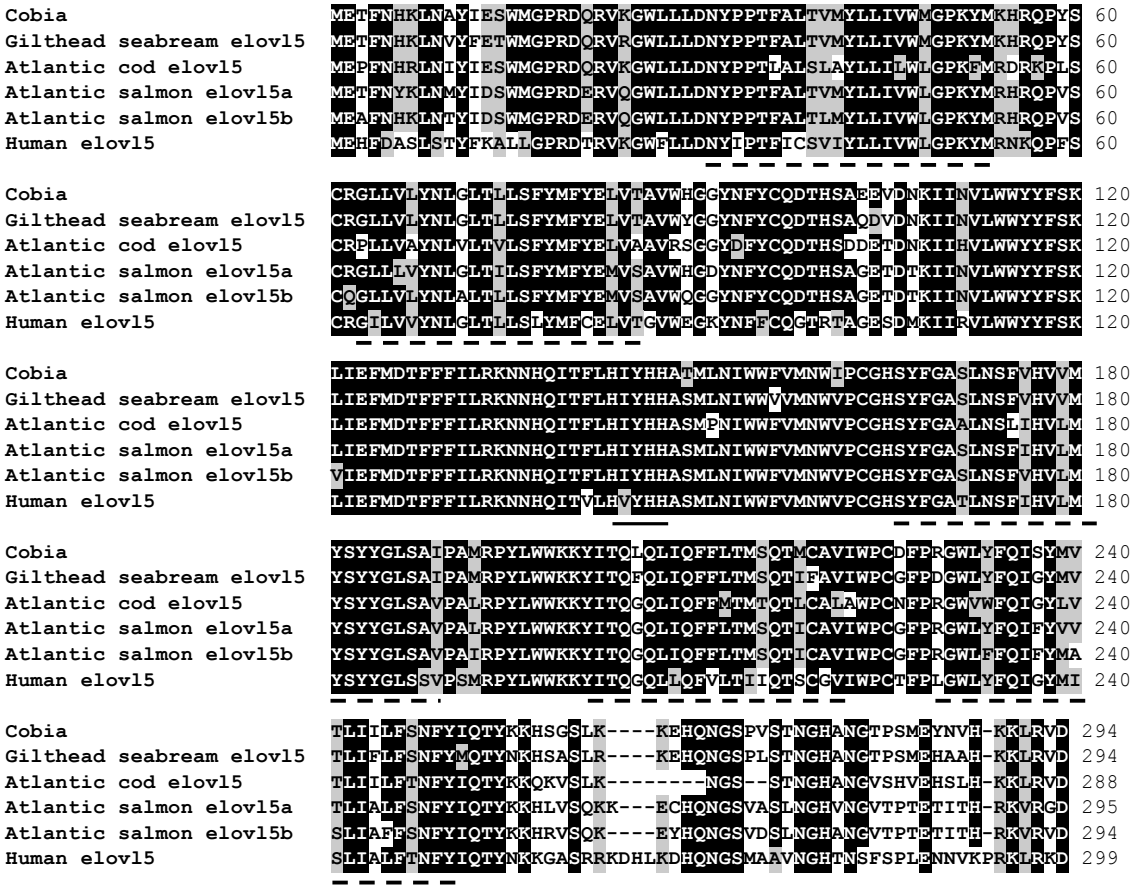
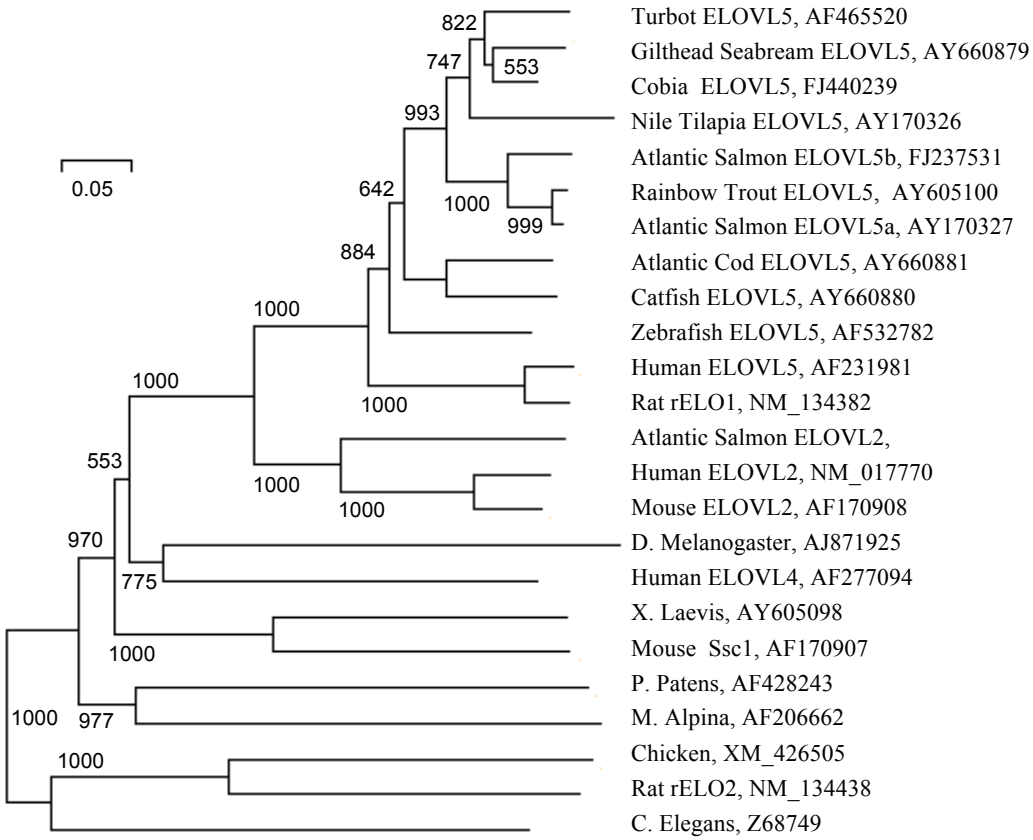

Cobia
Gilthead seabream elovl5
Atlantic cod elovl5
Atlantic salmon elovl5a
Atlantic salmon elovl5b
Human elovl5

Fig.4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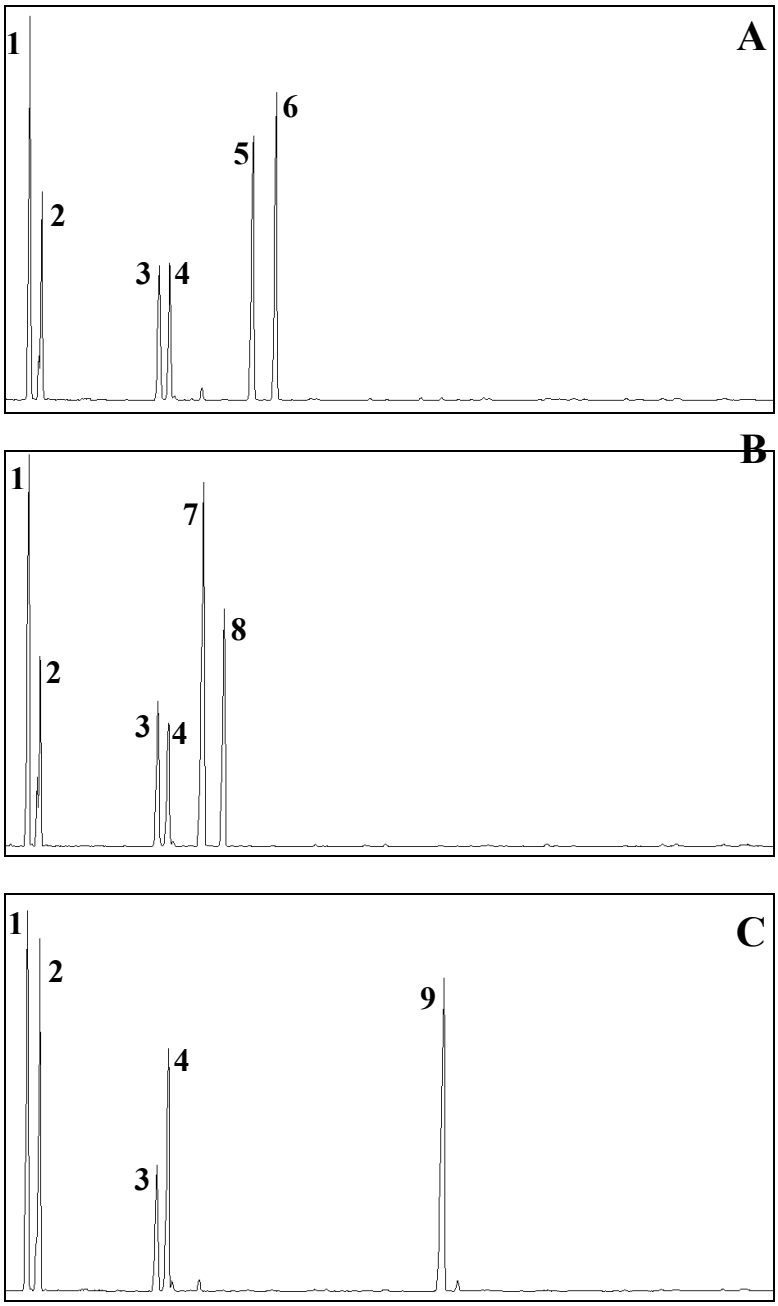
977
1000
Turbot ELOVL5, AF465520
Gilthead Seabream ELOVL5, AY660879
Cobia ELOVL5, FJ440239
Nile Tilapia ELOVL5, AY170326
Atlantic Salmon ELOVL5b, FJ237531
Rainbow Trout ELOVL5, AY605100
Atlantic Salmon ELOVL5a, AY170327
Atlantic Cod ELOVL5, AY660881
Catfish ELOVL5, AY660880
Zebrafish ELOVL5, AF532782
Human ELOVL5, AF231981
Rat rELO1, NM_134382
Atlantic Salmon ELOVL2,
Human ELOVL2, NM_017770
Mouse ELOVL2, AF170908
D. Melanogaster, AJ871925
Human ELOVL4, AF277094
X. Laevis, AY605098
Mouse Ssc1, AF170907
P. Patens, AF428243
M. Alpina, AF206662
Chicken, XM_426505
Rat rELO2, NM_134438
C. Elegans, Z68749

Fig. 5



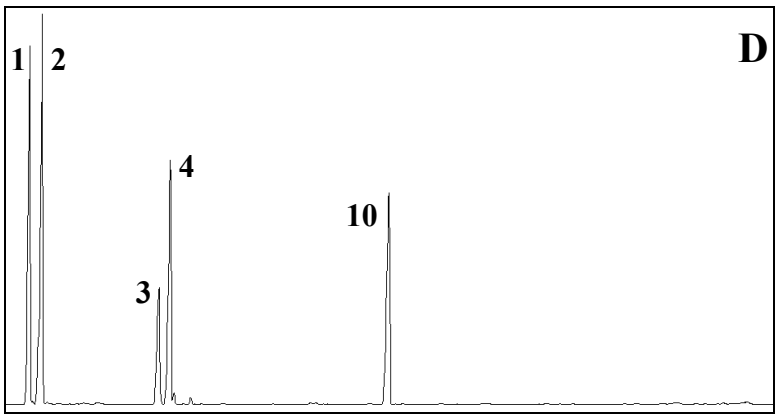

D
1
2
4
10
3

Fig.6.

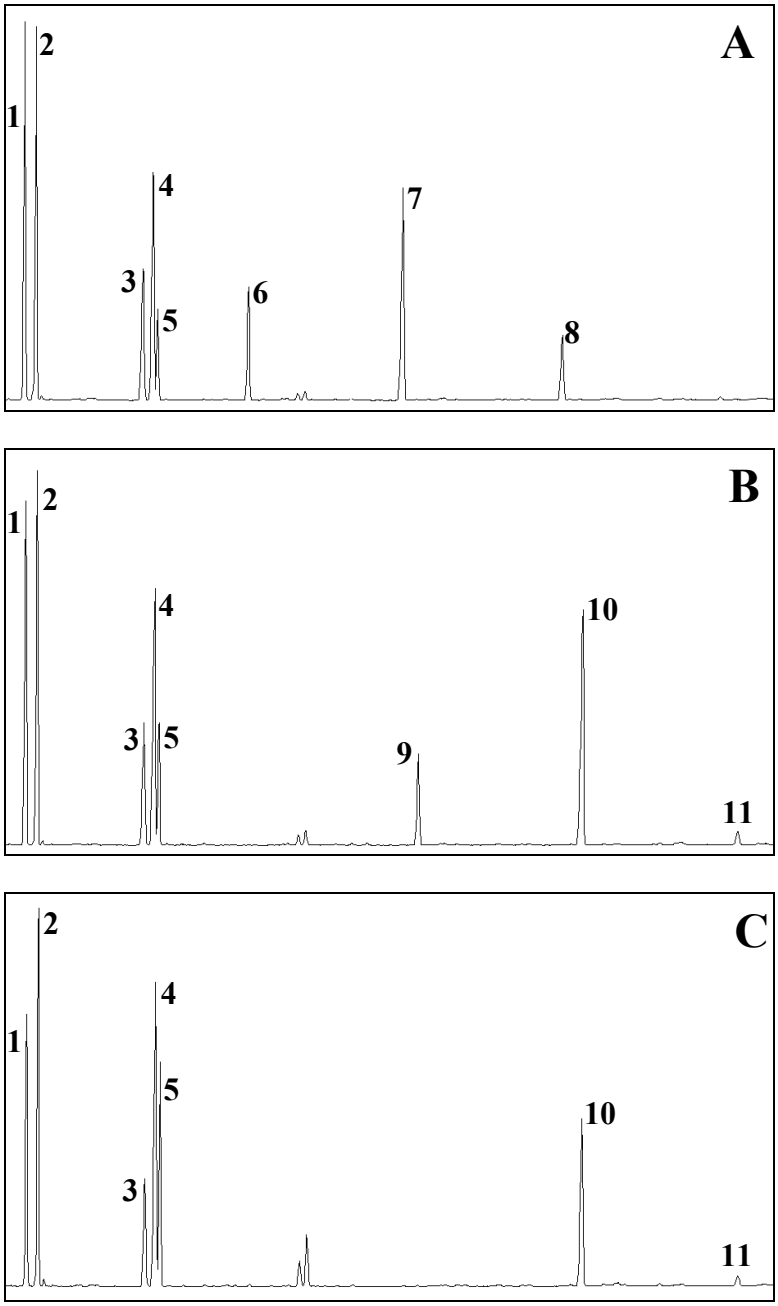
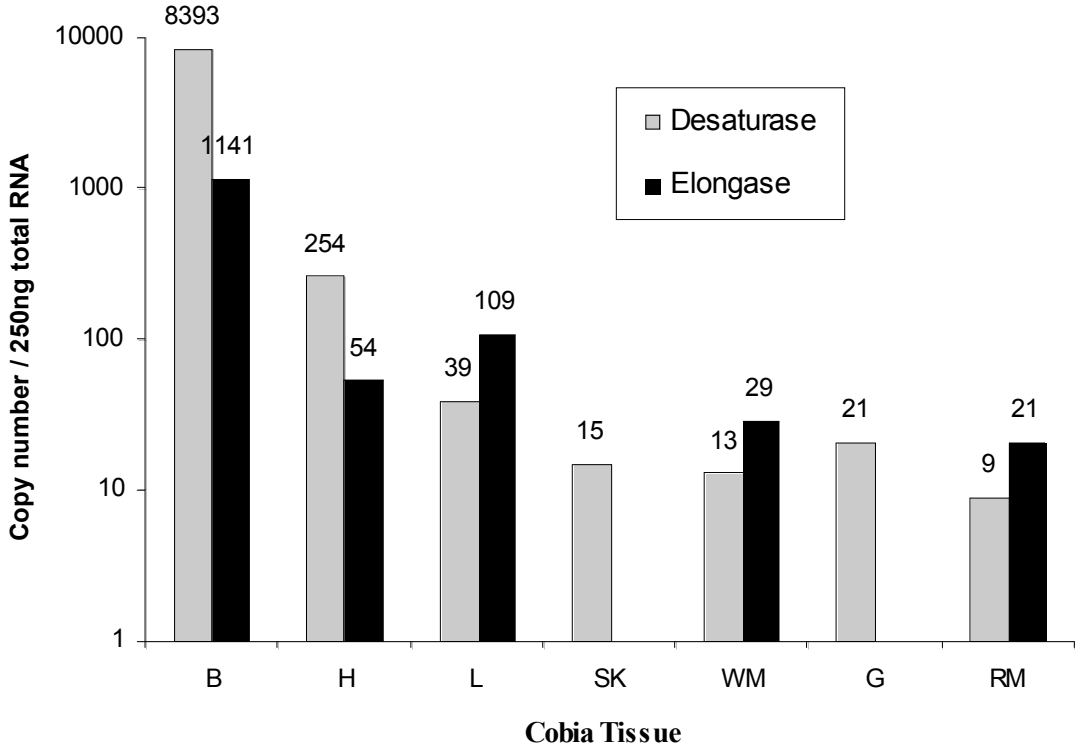


Fig. 7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Elongase
B
H
L
SK
WM
G
RM
Cobia Tissue