

## **Cloning and functional characterisation of polyunsaturated fatty acid elongases of marine and freshwater teleost fish**

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Abbreviations. AA, arachidonic acid, 20:4n-6); DHA, docosahexaenoic acid, 22:6n-3); EPA, eicosapentaenoic acid, 20:5n-3); HUFA, highly unsaturated fatty acids (chain length  $\geq$  C20 with  $\geq$  3 double bonds); ORF, open reading frame; PUFA, polyunsaturated fatty acids.

**Abstract:**

Enzymes that lengthen the carbon chain of polyunsaturated fatty acids are key to the biosynthesis of the highly unsaturated fatty acids, arachidonic, eicosapentaenoic and docosahexaenoic acids from linoleic and  $\alpha$ -linolenic acids. A *Mortierella alpina* cDNA polyunsaturated fatty acid elongase sequence identified mammalian, amphibian, zebrafish and insect expressed sequence tags (ESTs) in GenBank. Consensus primers were designed in conserved motifs and used to isolate full length cDNA from livers of several fish species by Rapid Amplification of cDNA Ends (RACE). The amplified cDNAs encoded putative open reading frames (ORFs) of 288-294 amino acids that were highly conserved among the fish species. Heterologous expression in yeast, *Saccharomyces cerevisiae*, demonstrated that all of the ORFs encoded elongases with the ability to lengthen polyunsaturated fatty acid substrates with chain lengths from C<sub>18</sub> to C<sub>22</sub> and also monounsaturated fatty acids, but not saturated fatty acids. There were differences in the functional competence of the elongases from different fish species. Most of the fish elongases showed a pattern of activity towards different fatty acid substrates in the rank order C<sub>18</sub> > C<sub>20</sub> > C<sub>22</sub>, although the tilapia and turbot elongases had similar activity towards 18:4n-3 and 20:5n-3. The fish elongases generally showed greater activity or similar activities with n-3 than with n-6 homologues, with the exception of the cod enzyme which was more active towards n-6 fatty acids.

*Keywords:* Fish, highly unsaturated fatty acid, elongase, cDNA, gene, chain elongation.

## 1. Introduction

Dietary long chain polyunsaturated fatty acids (PUFA), and highly unsaturated fatty acids (HUFA), are crucial to the nutritional health, physiology and reproductivity of higher vertebrates (Burr, 1981; Simopoulos, 2000). HUFA have a well-known, essential role as components of structural phospholipids of cell membranes. Docosahexaenoic (DHA; 22:6n-3) and arachidonic (AA; 20:4n-6) acids are particularly required for proper neural development and function (Lauritzen et al., 2001), and metabolites of AA and eicosapentaenoic acid (EPA; 20:5n-3), such as leukotrienes and prostaglandins, have a wide range of signalling functions (Lands, 1993). Diseases arising from HUFA deficiency are well documented in many species (Nugent, 2004; Graham et al., 2004). In fish there are degenerative lesions in the viscera, erosions of fins and gills, and poor adaptation to low temperature (March, 1993).

As with all vertebrates, PUFA are essential components of the diet in fish, but qualitative and quantitative requirements vary with species (Sargent et al., 1995, 2002). In salmonids, dietary  $\alpha$ -linolenic acid (18:3n-3) and linoleic acids (18:2n-6) alone are sufficient for optimal growth. In Nile tilapia (*Oreochromis nilotica*), both 18:2n-6 and AA are better growth promoters than are 18:3n-3 and EPA (Sargent et al., 1995, 2002). In marine fish, the dietary requirement is for HUFA rather than shorter chain PUFA. For example, turbot (*Psetta maxima*), grow optimally on EPA and DHA, but not 18:3n-3 (Sargent et al., 1995, 2002). Differences in the capacity of fish species to thrive on diets containing only the C<sub>18</sub> PUFA, 18:2n-6 and 18:3n-3, or to require dietary HUFA appear to be evolutionarily related to the nature of the fatty acid profiles present in their respective diets. Thus, fresh water fish such as tilapia whose vegetarian diet generally lacks HUFA, convert 18:2n-6 and 18:3n-3 to HUFA more efficiently than do marine species such as turbot whose predominantly carnivorous (largely piscivorous) diet is rich in HUFA (Sargent et al., 2002; Tocher 2003).

In vertebrates, biosynthesis of HUFA can proceed by sequential desaturation and elongation of 18:2n-6 and 18:3n-3. Synthesis of AA is achieved by a  $\Delta$ 6 desaturation of 18:2n-6 to produce  $\gamma$ -linolenic acid (18:3n-6) that is elongated to dihomo- $\gamma$ -linolenic acid (20:3n-6) and finally desaturated at the  $\Delta$ 5 position to produce AA (Fig.1) (Cook, 1996). The pathway for EPA synthesis from 18:3n-3 is essentially similar, but DHA synthesis requires two further elongation steps, a second  $\Delta$ 6 desaturation and a chain shortening step (Fig.1) (Sprecher, 2000). Experimental evidence suggests that the dependence of marine fish on dietary HUFA is caused by deficiency in the activity of one or more of the key enzymes,  $\Delta$ 5 and  $\Delta$ 6 fatty acid desaturases, and fatty acid elongases, required for HUFA biosynthesis (Sargent et al., 2002; Tocher, 2003). *In vivo* studies, using [1-<sup>14</sup>C]18:3n-3 supplied to the fish either in force-fed pellets or injected intraperitoneally, consistently showed the superiority of freshwater species over marine species in the biosynthesis of <sup>14</sup>C-EPA and <sup>14</sup>C-DHA (Owen et al.,

1975; Mourente and Tocher, 1993a,b, 1994). *In vitro* studies indicated major impairments in the HUFA synthetic pathway of cell lines from marine fish species, turbot (TF) and gilthead sea bream (SAF-1) in comparison to those of cell lines from salmonids (Ghioni et al., 1999; Tocher and Ghioni, 1999).

There are potentially profound implications of these differences in terms of animal nutrition, aquaculture and human health. Fish are the only major dietary source for humans of n-3HUFA and, with declining fisheries, farmed fish constitute an increasing proportion of the fish in the human diet (Tidwell and Allan, 2002). However, the current high use of fish oils, derived from feed grade marine fisheries, in aquaculture feeds is not sustainable, and will constrain continuing growth of aquaculture activities (Barlow, 2000). The only sustainable alternative to fish oils are vegetable oils which are rich in C<sub>18</sub> PUFA but devoid of the n-3HUFA abundant in fish oils (Sargent et al., 2002). Therefore, there is currently considerable interest in the HUFA biosynthetic pathway in fish to determine the effectiveness with which vegetable oils can be utilized by cultured fish species (Sargent et al., 2002).

We have previously reported the cloning and functional characteristics of desaturase and elongase enzymes in the model fresh water species zebrafish (*Danio rerio*) (Hastings et al., 2001; Agaba et al., 2004). Zebrafish elongase when expressed in yeast (*Saccharomyces cerevisiae*) lengthens all the key intermediates including 18:4n-3, EPA and 22:5n-3 required for the biosynthesis of DHA. This property is consistent with the fact that zebrafish can grow well on diets rich in vegetable oils lacking DHA, and whose hepatocytes have been shown to produce labelled DHA from [1-<sup>14</sup>C]18:3n-3 (Tocher et al., 2001). A PUFA elongase with similar activity to the zebrafish elongase has also been cloned from Atlantic salmon (*Salmo salar*) (Hastings et al., 2004). Here we extend the study of fish elongases to cover five fish species, spanning four Orders. Two species, Nile tilapia (*Oreochromis niloticus*, Perciformes) and African catfish (*Clarius gariepinus*, Siluriformes), inhabit freshwater systems and can thrive well on vegetable oil diets, and three are marine species, cod (*Gadus morhua*, Gadiformes), gilthead seabream (*Sparus aurata*, Perciformes); and turbot (Pleuronectiformes) that cannot thrive on diets lacking HUFA. Thus, we report the cloning and functional characterisation of the cDNAs of PUFA elongases from species that span the range of dietary HUFA dependency observed among fish.

## **2. Materials and methods**

### *2.1 Cloning of fish putative elongase cDNAs*

Fish elongase cDNAs were isolated by PCR amplification of cDNA derived from liver total RNA of African catfish, Nile tilapia, turbot, gilthead seabream and Atlantic cod prepared using TRIzol®

reagent (GibcoBRL, NY, U.S.A.). 5' and 3' Rapid Amplification of cDNA Ends (RACE) was achieved with the SMART<sup>TM</sup> RACE cDNA amplification kit (Clontech, NJ, U.S.A.). For initial cDNA isolation degenerate primers were designed based on conserved motifs in the putative elongase expressed sequence tag (EST) sequences of *Mortierella alpina* (AF206662), *Homo sapiens* ELOVL5 (AF231981), *Mus musculus* (AF170908), *Xenopus laevis* (BG813561), *Danio rerio* (AF532782, BF157708) and *Drosophila melanogaster* (AI113483). The forward primers UniElo1A (C CTG TGG TGG TAY TAY TT) and UniElo1AA (GAR TTY ATG GAC ACN TTC TTC TT) targeted the conserved regions VLWWYYF and EFMDTFFF, respectively and the reverse primer UniElo1B (YTG NGT RTA YTT YTT CCA CCA) targeted the conserved region WWKK. Following isolation of cDNA fragments flanked by UniElo1AA and UniElo1B, specific primers for use in 5'RACE and 3' RACE PCR were designed. All PCR products were cloned in pBlueScript SKII<sup>+</sup> vector (Stratagene, La Jolla, CA, USA) and sequenced using dye terminator chemistry in a Perkin Elmer ABI-377 DNA sequencer with associated software following the manufacturer's protocols (Perkin Elmer, Applied Biosystems). The full-length sequences were assembled and translated to the conceptual amino acid sequences. Deduced amino acid sequences were aligned using ClustalX and sequence phylogenies were predicted using the Neighbour Joining method of Saitou and Nei (1987). Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping the data through 1000 iterations.

## 2.2 Functional characterisation of fish elongase ORFs by heterologous expression in yeast, *Saccharomyces cerevisiae*

Functional characterisation of the genes was by expression of the open reading frames (ORFs) of the putative fatty acid elongase sequences in *S. cerevisiae*. Primers were designed for amplification of the ORFs of each fish elongase from the corresponding cDNA template (Table 1). These primers carried restriction sites to enable cloning into the episomal yeast expression vector pYX222 (R & D Systems Europe Ltd., Abingdon, Oxon, U.K.). Thus, the forward primers contain the Kozak translation initiation sequence (boldface) and an *EcoRI* site (underlined). The reverse primers flank or contain the termination codon and have an *XhoI* site. After PCR using high fidelity DNA polymerase mix (Roche Diagnostics Ltd., Lewes, East Sussex, U.K.), the DNA fragments were restricted with *EcoRI* and *XhoI* and ligated into similarly treated pYX222 plasmid, which was then used to transform TOP10F' *E.coli* competent cells (Invitrogen Ltd, Paisley, UK) as described previously (Hastings et al., 2001). Recombinant plasmids were prepared from at least six independent *E.coli* colonies and, after sequence confirmation, were used to transform bakers' yeast (*S. cerevisiae* strain INVSc1). Yeast transformed with non-recombinant pYX222 plasmid was used as control. Transformation of the yeast with the recombinant plasmids, and yeast culture were performed as described in detail previously (Hastings et

al., 2001). Briefly, recombinant yeast was grown in *S. cerevisiae* minimal medium<sup>-histidine</sup> (SCMM<sup>-his</sup>) supplemented with the following PUFA substrates; stearidonic acid (18:4n-3),  $\gamma$ -linolenic acid (18:3n-6), EPA (20:5n-3), AA (20:4n-6), docosapentaenoic acid (22:5n-3) and docosatetraenoic acid (22:4n-6). Yeast cells were harvested, washed and dried, and total lipid extracted with ice-cold chloroform/methanol (2:1, v/v) essentially according to Folch et al. (1957). Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification (Christie, 1982), and FAME extracted, purified by thin layer chromatography, and analysed by gas chromatography (GC) all as described previously (Hastings et al., 2001). The proportion of substrate fatty acid converted to the longer chain fatty acid product was calculated from the gas chromatograms as  $100 \times [\text{product area}/(\text{product area} + \text{substrate area})]$ . Unequivocal confirmation of fatty acid products was performed by GC-mass spectrometry of the picolinyl derivatives as described in detail previously (Hastings et al., 2001).

### 2.3 Materials

Docosapentaenoic (22:5n-3) and docosatetraenoic (22:4n-6) acids (both > 98-99% pure) were purchased from Cayman Chemical Co., Ann Arbor, U.S.A. Stearidonic (18:4n-3),  $\gamma$ -linolenic (18:3n-6), EPA (20:5n-3) and AA (20:4n-6) acids (all >99% pure), BHT, 1,1'-carbonyldiimidazole, 2,2-dimethoxypropane, fatty acid-free BSA, galactose, 3-(hydroxymethyl)pyridine, 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 precoated with silica gel 60 (without fluorescent indicator) were purchased from Merck, Darmstadt, Germany. All solvents were HPLC grade and were from Rathburn Chemicals, Peebleshire, U.K.

## 3. Results

### 3.1. Sequence analyses

A search of the public EST database identified sequences from several species including mammalian (AF231981, AF170908), avian (XM\_426505), amphibian (BG813561), fish (AF532782, BF157708) and insect (AJ871925) that were related to the *M. alpina* PUFA elongase cDNA (AF206662). By taking advantage of highly conserved motifs in the amino acid sequences, full-length elongase transcripts were isolated for each of the target fish species with ORFs of 885, 882, 885, 885 and 867 base pairs for African catfish, Nile tilapia, gilthead sea bream, turbot and cod, respectively. The predicted elongase polypeptides consist of 288 – 294 amino acid residues, and include characteristic

features of microsomal fatty acid elongases, including a single histidine box redox centre motif (HXXHH), a canonical ER retention signal (carboxyl-terminal dilysine targeting signal), and multiple transmembrane regions (Fig.2). The KXXEXXDT. QXXFLHXYHH (which contains the histidine box), NXXXHXXNYXYY and TXXQXXQ motifs are highly conserved in all PUFA elongases cloned to date (Meyer et al., 2004). The translated amino acid sequences of the elongase cDNAs cloned from the five fish species here, along with our previously cloned zebrafish and salmon sequences, are very similar to each other and share at least 61% sequence identity with the human elongase ELOVL5 (Table 2), with most of the sequence variation (insertions and substitutions) residing in the C-terminal region of the polypeptide (Fig. 2). Phylogenetic analysis by neighbour-joining comparing all the putative elongase sequences cloned from fish, along with a range (not exhaustive) of elongases from mammals, bird, insect, fungus and nematode, clustered all the fish sequences together and showed they were most similar to the mammalian (human/rodent) ELOVL5/lov15 (rat rELO2) and ELOVL2/lov12 (mouse Ssc2) elongase sequences (Fig.3).

### 3.2. Functional characterisation

The fish putative elongase cDNAs were functionally characterized by determining the fatty acid profiles of transformed *S. cerevisiae* containing either the pYX222 vector alone or the vector containing the fish elongase cDNA inserts, grown in the presence of a variety of possible fatty acid substrates including 18:4n-3 and 18:3n-6 ( $C_{18-20}$ ), 20:5n-3 and 20:4n-6 ( $C_{20-22}$ ) and 22:5n-3 and 22:4n-6 ( $C_{22-24}$ ) (see Fig.1). The fatty acid composition of the yeast transformed with the pYX222 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 whichever exogenous substrate fatty acid was added, consistent with *S. cerevisiae* not possessing PUFA elongase activity. With all the fish putative fatty acid elongase cDNAs investigated, the most prominent additional peaks were generally observed in the profile of transformed yeast grown in the presence of the  $C_{18}$  substrates, 18:4n-3 and 18:3n-6 (Figs. 4-7; Table 3). GC traces showing the fatty acid compositions of the yeast transformed with the pYX222 vector containing the putative fatty acid elongase cDNA inserts grown in the presence of 18:4n-3 are shown in Fig. 4. In addition to the major endogenous fatty acids (16:0, 16:1n-7, 18:0 and 18:1n-9, peaks 1-4), and the exogenously added substrate fatty acid (18:4n-3, peak 6) the traces show a major additional peak (peak 9) (Fig.4). Based on GC retention time and confirmed by GC-MS, the additional peak associated with the presence of the fish elongase cDNAs was identified as 20:4n-3, corresponding to the elongation product of 18:4n-3 (Fig.4). Other additional peaks were also noted including 22:4n-3 (peak 10), 18:1n-7 (peak 5) and 20:1n-9 and 20:1n-7 (peaks 7 and 8, respectively) (Fig.4). The fatty

acid compositions of transformed yeast grown in the presence of 20:5n-3 are shown in Fig. 5. In addition to the endogenous yeast fatty acids and the exogenously added substrate fatty acid (20:5n-3, peak 11) the traces show a major additional peak (peak 12) confirmed by GC retention time and GC-MS as 22:5n-3, the elongation product of 20:5n-3. When transformed yeast were grown in the presence of 22:5n-3 (peak 12), an additional peak (peak 13) was observed that was confirmed by GC-MS to be 24:5n-3, as illustrated in Fig.6. Although there were differences in the substrate specificities of the fish elongases, most showed a pattern of activity towards different fatty acid substrates in the rank order  $C_{18} > C_{20} > C_{22}$  (Table 3). However, the tilapia and turbot elongases showed similar activity towards 18:4n-3 and 20:5n-3 (Table 3). The elongase cDNA products tended to be more active towards the n-3 substrate or show similar activity with n-3 and n-6 substrates, with the exception of the cod elongase, which was more active towards the n-6 fatty acid homologues (Fig.7; Table 3).

#### 4. Discussion

Here we report the cloning and functional characterisation of several cDNAs derived from genes that are responsible for the chain elongation of PUFA in a range of freshwater and marine teleost fish species. The cDNAs encode proteins that are very similar to mammalian PUFA elongases, and have all the main structural characteristics possessed by elongases derived from other systems. These include the so-called histidine box (HXXHH), the canonical C-terminal endoplasmic reticulum (ER) retention signal, several predicted transmembrane domains and other highly conserved motifs (Meyer et al., 2004). There is remarkable conservation of the predicted amino acid sequences of the fish species, and indeed among all the vertebrates. The phylogenetic sequence analyses grouped the fish elongases into basically four clusters largely as expected based on the four main groups of modern teleosts identified in classical phylogeny with the clusters being catfish and zebrafish (Ostariophysi; siluriformes and cypriniformes), cod (Paracanthopterygii; gadiformes), trout and salmon (Salmoniformes; salmonidae), and tilapia, sea bream and turbot (Acanthopterygii; perciformes/chichlids and pleuronectiformes) (Nelson, 1994). A similar phylogenetic grouping was observed previously when the sequences of fatty acid desaturases from a similar range of teleosts were analysed (Zheng et al., 2004a). It was noteworthy that the fish PUFA elongases clustered most closely with the mammalian ELOVL5/elovl5 and ELOVL2/elovl2 elongases that have been functionally characterised and shown to be PUFA elongases. They were more distant from other mammalian elongases, such as rat rELO1 (also known as rat Elov16). The latter has been shown to be predominantly a 16:0 to 18:0 elongase (Inagaki et al., 2002) that clustered with a chicken elongase (also with elov16 homology), and human ELOVL4 and



mouse Ssc1 elongases that are not functionally characterised, but which are presumed to be involved in sphingolipid metabolism and the synthesis of very long chain fatty acids such as 26:0 (Lagali et al., 2003; Tvrdik et al., 2000).

Studies over the last few years have shown that there is a large family of fatty acid elongases, which can be perhaps broadly classified into two main groups. Elongases with activities predominantly towards saturated (and possibly monounsaturated) fatty acids and either closely linked to fatty acid synthesis ( $C_{12}$  -  $C_{16}$ ) or sphingolipid synthesis ( $C_{22}$  -  $C_{26}$ ), and elongases with activity predominantly towards PUFA with chain lengths in the  $C_{18}$  -  $C_{22}$  range. Functional characterisation by heterologous expression in *S. cerevisiae* showed that all the fish cDNAs were predominantly PUFA elongases with highest activity towards  $C_{18}$  fatty acids, but also with high activity towards  $C_{20}$  fatty acids and, in most cases, activity towards  $C_{22}$  fatty acids. The relatively loose substrate specificity of the fish elongases was highlighted by the presence of 22:4n-3 in the fatty acid profiles of *S. cerevisiae* when transformed with the catfish, tilapia, sea bream and turbot ORFs and grown in the presence of 18:4n-3, showing further elongation of 20:4n-3 produced by elongation of the exogenously added 18:4n-3. Some 24:5n-3 was also produced in yeast transformed with elongases from these species when incubated with EPA (data not shown), as previously observed with zebrafish and salmon elongases (Agaba et al., 2004; Hastings et al., 2004). Recently, an elongase was cloned from rainbow trout (*Oncorhynchus mykiss*) and shown, along with elongases from an amphibian (*Xenopus laevis*) and the sea squirt (*Ciona intestinalis*), to accept both  $C_{18}$  and  $C_{20}$  PUFA substrates (Meyer et al., 2004).

Previously, it was shown that PUFA elongases from human (ELOVL5) and rat (rELO1/elovl5) were highly active towards  $C_{18}$  PUFA, but also showed significant activity towards 20:5n-3 and 20:4n-6 (Leonard et al., 2000; Inagaki et al., 2002). In contrast, the elongases of the filamentous fungus *M. alpina* (GLELO) and the nematode *C. elegans* were highly active towards  $C_{18}$  PUFA, but showed virtually no activity towards  $C_{20}$  PUFA, consistent with these organisms not containing any significant level of  $C_{22}$  fatty acids (Parker-Barnes et al., 2000; Beaudoin et al., 2000). Two further mammalian elongases, from human (ELOVL2) and mouse (elovl2/Ssc2), have been shown to possess the ability to elongate 22:5n-3 and 22:4n-6 to 24:5n-3 and 24:4n-6, respectively (Leonard et al., 2002). The mouse elovl2 clone was also able to elongate  $C_{18}$  and  $C_{20}$  PUFA, whereas the human ELOVL2 clone was only active towards  $C_{20}$  and  $C_{22}$  PUFA (Leonard et al., 2002). Thus both mammals and fish have the ability to elongate  $C_{20}$  and  $C_{22}$  PUFA in addition to  $C_{18}$  PUFA. Humans may require two enzymes for all these elongations whereas mouse and fish appear to require only one. However, the existence of more than one PUFA elongase in human suggests that other elongases with differing substrate specificities may yet be isolated in fish.

That fish, like mammals, can chain elongate C<sub>18</sub>, C<sub>20</sub> and C<sub>22</sub> PUFA is significant in terms of HUFA synthesis. Direct synthesis of DHA from 22:5n-3, as has been described in the marine microheterotroph, *Thraustochytrium sp* (Qui et al., 2001), requires desaturation of PUFA at the  $\Delta 4$  position, but this activity has not been demonstrated in any vertebrate system (Tocher et al., 1998; Tocher, 2003). Alternatively, synthesis of DHA in rat liver was shown by Sprecher and co-workers to proceed via C<sub>24</sub> PUFA intermediates through a pathway requiring two sequential elongations of 20:5n-3 to produce 24:5n-3, which is then desaturated to 24:6n-3 before being chain shortened to 22:6n-3 (Sprecher, 2000). Thus, 24:5n-3 is an important intermediate in the biosynthesis of DHA in mammals, and so the ability of fish PUFA elongases to produce 24:5n-3 through the elongation of EPA or 22:5n-3 is consistent with this pathway for DHA synthesis also operating in fish. Biochemical studies have also supported the hypothesis that DHA synthesis proceeds via C<sub>24</sub> intermediates in fish, at least in trout (Buzzi et al., 1997).

The human elongase (ELOVL5) and rat elongase (rELO1) both also elongated monounsaturated fatty acids in addition to C<sub>18</sub> and C<sub>20</sub> PUFA (Leonard et al., 2000; Inagaki et al., 2002). The observation of 18:1n-7, and 20:1n-9 and 20:1n-7, in the fatty acid composition of *S. cerevisiae* transformed with the fish elongase ORFs in the present study indicates elongation of 16:1n-7, and also of 18:1n-9 and 18:1n-7, respectively. Therefore, these fish elongases were also able to elongate monounsaturated fatty acids in addition to PUFA, as previously demonstrated with the zebrafish and salmon elongases (Agaba et al., 2004; Hastings et al., 2004). The zebrafish elongase was also able to elongate saturated fatty acids as evidenced by the increased 18:0/16:0 ratio in transformed yeast (Agaba et al., 2004). However, this was not observed with the salmon elongase (Hastings et al., 2004), and there was no evidence that any of the fish elongases in the present study were able to elongate saturated fatty acids. Neither the mouse *elovl2* or human ELOVL2 clones were able to elongate saturated or monounsaturated fatty acids (Leonard et al., 2002). A plant (moss, *Physcomitrella patens*) PUFA elongase was cloned and characterised, and shown to be highly specific for  $\Delta 6$ -desaturated C<sub>18</sub> PUFA (18:4n-3 and 18:3n-6) and possessed no activity towards saturated and monounsaturated fatty acids (Zank et al., 2002). Very recently, two types of elongase were cloned and characterised from the algae, *Ostreococcus tauri* and *Thalassiosira pseudonana*, which were specific for either  $\Delta 6$ -C<sub>18</sub> PUFA or for  $\Delta 5$ -C<sub>20</sub> PUFA, particularly EPA (Meyer et al., 2004).

The pattern of activity shown by the elongases from catfish and sea bream were very similar to those of the previously published zebrafish and salmon elongases (Agaba et al., 2004; Hastings et al., 2004). Tilapia was slightly unusual in that the activity towards 20:5n-3 was equal to that towards 18:4n-3 and it had the highest activity towards 20:4n-6. Interestingly, the warm water species (zebrafish, catfish, tilapia and sea bream) all displayed higher activities towards the n-6 fatty acids than

the colder water species (salmon, turbot and cod). Turbot elongase was less active towards C<sub>18</sub> substrates compared to almost all the other elongases. Specifically, the ability of the turbot to elongate 18:4n-3 was substantially less than that of the salmon elongase, whereas its ability to elongate 20:5n-3 was similar to that of the salmon elongase, which is consistent with earlier biochemical studies in cell lines (Ghioni et al., 1999). Only the elongase from cod was less active than that from turbot. Thus it may be that low elongase activity may be implicated in the poor ability of these marine species to synthesise n-3HUFA. The sea bream elongase on the other hand was as active as the elongases of freshwater fish, again consistent with biochemical studies in cell lines which showed that the defect in the HUFA synthesis pathway in SAF-1 (sea bream fin) cells was due to low  $\Delta$ 5 desaturase activity and not low elongase activity (Tocher and Ghioni, 1999). There were no obvious amino acid sequence differences between the fish species to indicate the differences in fatty acid elongation activity that were observed by heterologous expression. It was noteworthy, however, that the Kozak sequence for the turbot elongase was atypical, having a C three nucleotides 5' of the ATG translation initiation codon (see Table 1) that may affect translation efficiency. However, substituting the C with an A did not affect the pattern of activity of the turbot elongase in heterologous expression although activity may be slightly enhanced (49% [A] v. 35% [C] conversion of 18:4n-3 to 20:4n-3 and 39.3% [A] v. 30.3% [C] conversion of 20:5n-3 to 22:5n-3) (results not shown).

The study of fatty acid elongases in fish is particularly relevant at the present time with the increasing use of vegetable oils, rich in C<sub>18</sub> PUFA, as replacements for n-3HUFA-rich fish oils in aquaculture (Sargent et al., 2002). Fatty acid compositions of fish fed vegetable oils are characterised by increased levels of C<sub>18</sub> PUFA and decreased levels of n-3HUFA, compromising their nutritional value to the human consumer (Bell et al., 2001, 2002; Izquierdo et al., 2003; Regost et al., 2003). Therefore, there is an urgent need to optimise the ability of fish to utilise C<sub>18</sub> PUFA and produce HUFA. Although fatty acid desaturases, such as  $\Delta$ 6 desaturase, are known to be crucial in HUFA synthesis, there is increasing awareness that elongases may also be points of regulation in the pathway. For instance, biochemical studies suggested that the biosynthesis of 20:4n-6 from 18:2n-6 in *M. alpina* may be rate-limited by the activity of the C<sub>18-20</sub> elongase (Wynne and Ratledge, 2000). Recently, the production of AA in *M. alpina* was increased by metabolic engineering through the construction of a strain overexpressing the elongase (GLELO) gene (Takeno et al., 2005). Low C<sub>18-20</sub> elongase activity had previously been observed in a turbot cell line in comparison to an Atlantic salmon cell line, suggesting it may be implicated in the inability of some marine fish to thrive on diets devoid of n-3HUFA (Ghioni et al., 1999). More recently, nutritional regulation of the expression of a PUFA elongase gene was reported in Atlantic salmon, with increased expression in fish fed diets in which fish oil was replaced by linseed oil (Zheng et al., 2004b). Gene expression of the short chain fatty acid

elongase in mice was shown to be regulated by sterol regulatory element binding protein-1 (SREBP-1) (Moon et al., 2001; Matsuzaka et al., 2002). Defects in very long chain fatty acid elongase activity have been reported in mutant (quaking and jimpy) mice (Suneja et al., 1991), and have been implicated in human disease such as Stargardt macular degeneration (Lagali et al., 2003; Karan et al., 2004). These latter examples highlight the serious consequences that deficiencies in fatty acid elongation in general can have.

In summary, full-length cDNAs for PUFA elongases were isolated from livers of several fish species. The amplified cDNA encoded ORFs of 288-294 amino acids whose sequences were highly conserved among the fish species. Heterologous expression in yeast, *S. cerevisiae*, demonstrated that the ORFs encoded elongases with the ability to lengthen PUFA with chain lengths from C<sub>18</sub> to C<sub>22</sub> and monounsaturated fatty acids, but not saturated fatty acids. Although there were no obvious amino acid sequence differences, there were differences in the functional competences of the elongases from different fish species that may contribute to the differing abilities of the species in terms of HUFA synthesis.

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## Legends to Figures.

Fig.1. Pathways of highly unsaturated fatty acid (HUFA) biosynthesis from the C<sub>18</sub> polyunsaturated fatty acids (PUFA), 18:3n-3 and 18:2n-6. Solid lines represent steps that have been shown to occur in fish. Dotted lines represent steps that have not been directly demonstrated in fish. Δ6, Δ5 and Δ4, fatty acid desaturases; Elo, fatty acid elongases; Short, peroxisomal chain shortening.

Fig.2. Comparison of the deduced amino acid sequence of fatty acyl elongases from catfish, tilapia, turbot, seabream and cod with that of elongases from zebrafish, salmon and human ELOVL5. 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 75%. Motifs highly conserved among fatty acid elongases from a wide range of organisms are boxed. Transmembrane regions (not indicated) were predicted from a hydropathy plot taking peaks with scores > 1.6 using a scan window size of 18 (Kyte and Doolittle, 1982).

Fig.3. Phylogenetic tree comparing amino acid sequences of elongases from catfish, tilapia, turbot, seabream and cod with those of elongases from other fish species (zebrafish and salmon), mammals (human, mouse and rat), 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 N-J method using *CLUSTALX* and *NJPLLOT* (Saitou and Nei, 1987). The horizontal branch length is proportional to the 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.4. Identification of fatty acid elongation products in transgenic yeast (*S. cerevisiae*) grown in the presence of 18:4n-3. Fatty acids were extracted from yeast transformed with pYX222 vector without insert (A) or containing the putative fatty acid elongase cDNA inserts from catfish (B), tilapia (C), seabream (D), turbot (E) and cod (F). The first four peaks in each panel 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), and peak 6 is the exogenously added substrate fatty acid, 18:4n-3. The main additional peaks in panels B-F represent elongated products of 18:4n-3, specifically 20:4n-3 (9) and 22:4n-3 (10). Further additional peaks observed were 18:1n-7 (5), 20:1n-9 (7) and 20:1n-7 (8). Vertical axis, FID response; horizontal axis, retention time.

Fig.5. Identification of fatty acid elongation products in transgenic yeast (*S. cerevisiae*) grown in the presence of 20:5n-3. Fatty acids were extracted from yeast transformed with pYX222 vector without insert (A) or containing the putative fatty acid elongase cDNA inserts from catfish (B), tilapia (C), seabream (D), turbot (E) and cod (F). Peaks 1-5, 7 and 8 are as described in the legend to Fig.4. Peak 11 is the exogenously added substrate fatty acid, 20:5n-3, and the main additional peak in panels B-F represents 22:5n-3 (12) the elongated product of 20:5n-3. Vertical axis, FID response; horizontal axis, retention time.

Fig.6. Identification of fatty acid elongation products in transgenic yeast (*S. cerevisiae*) grown in the presence of 22:5n-3. Fatty acids were extracted from yeast transformed with pYX222 vector without insert (A) or containing the putative fatty acid elongase cDNA inserts from catfish (B) and seabream (C). Peaks 1-5, 7, 8 and 12 are as described in the legend to Fig.5. and the additional peak in panels B and C represent 24:5n-3 (13) the elongated product of 22:5n-3. Vertical axis, FID response; horizontal axis, retention time.

Fig.7. Identification of fatty acid elongation products in transgenic yeast (*S. cerevisiae*) grown in the presence of n-6 fatty acids. Fatty acids were extracted from yeast transformed with pYX222 vector without insert (A and C) or containing the putative fatty acid elongase cDNA insert from cod (B and D). Peaks 1-5 and 7 are as described in the legend to Fig.4. Peak 14 in panels A and B represents 18:3n-6, while peak 16 in panels C and D represents 20:4n-6 (14), the exogenously added substrate fatty acids. The additional peak in panels B and D are 20:3n-6 (15) and 22:4n-6 (17), the respective elongated products of 18:3n-6 and 20:4n-6. Vertical axis, FID response; horizontal axis, retention time.

Table 1. Forward (sense) and reverse (antisense) primers used for heterologous expression

Species	Direction	Code	Primer sequence
Catfish	Sense	CatEloxA	5' <u>aagaatt</u> <b>caaaat</b> ggaaatctttaaccacag 3'
	Antisense	CatEloxB	5' cata <u>actcg</u> agaatgtacaggtcctcagtcgcc 3'
Tilapia	Sense	OniEloxA	5' <u>aagaatt</u> caagctacggtg <b>aaaaat</b> ggagacc 3'
	Antisense	OniEloxB	5' cata <u>actcg</u> agtctcaaatgtcaatccacc 3'
Turbot	Sense	TurbEloxA	5' <u>aagaatt</u> caagcttgaactttctggtg <b>caaat</b> g 3'
	Antisense	TurbEloxB	5' <u>aactcg</u> agcaaatgtcaatccaccctcagc 3'
Sea bream	Sense	SauEloxA	5' <u>aaagaatt</u> caaagggtg <b>aagat</b> ggagacc 3'
	Antisense	SauEloxB	5' ata <u>actcg</u> agacagtgagaatggggtgacg 3'
Cod	Sense	CodeloxA	5' ta <u>agaatt</u> cggtg <b>aaaaat</b> ggagccattcaatca 3'
	Antisense	CodEloxB	5' ata <u>actcg</u> agtgtacatcaatccaccctcagc 3'

The forward primers contain the Kozak translation initiation sequence (boldface) and an EcoRI site (underlined). The reverse primers flank or contain the termination codon and have a XhoI site (underlined).

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 elongases.

	Catfish	Tilapia	Salmon	Turbot	Seabream	Cod	Human
Zebrafish	76.6	69.6	75.0	72.3	74.4	73.9	67.8
Catfish	-	68.0	74.6	73.0	73.0	79.4	67.5
Tilapia	-	-	75.0	77.2	79.3	71.1	61.2
Salmon	-	-	-	80.7	81.4	75.0	67.8
Turbot	-	-	-	-	86.0	75.5	65.2
Seabream	-	-	-	-	-	77.2	68.2
Cod	-	-	-	-	-	-	65.5

Data are percentages of amino acid residues that are identical.

Table 3. Functional characterisation of fish fatty acyl elongase cDNAs

	Zebrafish		Catfish		Tilapia		Salmon		Turbot		Sea bream		Cod	
	n-3	n-6	n-3	n-6	n-3	n-6	n-3	n-6	n-3	n-6	n-3	n-6	n-3	n-6
$C_{18-20}$	85.4	70.7	87.5	78.3	82.6	71.2	71.8	42.1	34.6	36.6	85.9	84.3	7.4	15.7
$C_{20-22}$	49.4	25.6	48.1	40.8	86.6	61.8	38.9	22.6	30.3	18.1	66.8	43.9	0.8	1.5
$C_{22-24}$	4.9	0.5	2.8	0.9	1.0	tr	0.7	0.3	0.6	0.6	4.7	0.8	nd	nd

Results are presented as percentage of substrate fatty acid converted to elongated product.

Fatty acid substrates were;  $C_{18-20}$ , 18:4n-3 and 18:3n-6;  $C_{20-22}$ , 20:5n-3 and 20:4n-6;  $C_{22-24}$ , 22:5n-3 and 22:4n-6.

nd, not detected; tr, trace (< 0.05%).

**18:3n-3**



**18:4n-3** → **20:4n-3**  
Elo



**20:5n-3** → **22:5n-3** → **24:5n-3**  
Elo                      Elo



**22:6n-3** ← **24:6n-3**  
Short

**18:2n-6**



**18:3n-6** → **20:3n-6**  
Elo



**20:4n-6** → **22:4n-6** → **24:4n-6**  
Elo                      Elo



**22:5n-6** ← **24:5n-6**  
Short

Fig.2

	..... ..... ..... ..... .....	10	20	30	40	50	
Zebrafish	MEIFSHRVNS	YIDSWMGPRD	LRVTCWFELLD	DYIPTFIFTV	MYLLIVVMGP		50
Catfish	MEIFNHRIN	YIDSWMGPRD	PRVRCWILLD	NYPPTLAFIT	MYLMIIVVMGP		50
Tilapia	MEIFNHKINA	YIDSWMGPRD	QRVRCMILLD	NYPPTFALTV	MYLLIVVMGP		50
Salmon	MEIFNYKINM	YIDSWMGPRD	ERVQGWILLD	NYPPTFALTV	MYLLIVVWLP		50
Turbot	MEIFNHKINT	YIDSWMGPRD	QRVRCWILLD	NYPPTFALTV	MYLLIVVMGP		50
Sea bream	MEIFNHKLN	YFETWMGPRD	QRVRCWILLD	NYPPTFALTV	MYLLIVVMGP		50
Cod	MEIFNHRINI	YIESWMGPRD	QRVRCWILLD	NYPPTLALSL	AYLLITWLP		50
HumanELOVL5	MEIFDASIST	YFKALGPRD	TRVRCWFELLD	NYIPTFICSV	TYLLIVVWLP		50

	..... ..... ..... ..... .....	60	70	80	90	100	
Zebrafish	KYMKNRQAYS	CRALLVFNLN	CLTLISIIYMF	YELVMSVYQG	CYNFFCQNT	TH	100
Catfish	KYMRNRQEFS	CRGILVLYNL	ALTFISFYMF	YELVAGVRCG	CYNFFCQD	TH	100
Tilapia	KYMKHRQEYS	CRAVMVFYNL	GLTLISFYMF	YELVSAAWEG	CYNFYCQNT	TH	100
Salmon	KYMRHRQEVS	CRGLLVYNL	GLTLISFYMF	YELVSAVWEG	DYNFYCQD	TH	100
Turbot	KYMKNRQAYS	CRGLLVVYNL	GLTLISFYMF	YELVAVWEG	DYNFYCQD	TH	100
Sea bream	KYMKHRQEYS	CRGLLVLYNL	GLTLISFYMF	YELVAVWYQ	CYNFYCQD	TH	100
Cod	KMRDRFKELS	CRPLLVAYNL	VLTVISFYMF	YELVAAVRSG	CYDFYCQD	TH	100
HumanELOVL5	KYMRNRQEFS	CRGILVYVNL	GLTLISIIYMF	CELVTGVWEG	KYNFFCQNT	TH	100

	..... ..... ..... ..... .....	110	120	130	140	150	
			<b>K xxExxDT</b>		<b>Qxx FLHxYHH</b>		
Zebrafish	SGGDADNRMM	NVLWVYFESK	LIEFMDTFFF	ILRKNNHQIT	FLHVYHHA	SM	150
Catfish	SGGADNFI	HVLWVYFESK	LIEFMDTFFF	ILRKNNHQIT	FLHVYHHS	SM	150
Tilapia	SAGEADIKVI	NVLWVYFESK	LIEFMDTFFF	ILRKNNHQIT	FLELYHHA	SM	150
Salmon	SACETDTKI	NVLWVYFESK	LIEFMDTFFF	ILRKNNHQIT	FLHIYHHA	SM	150
Turbot	SVFEVDNKII	NALWVYFESK	LIEFMDTFFF	ILRKNFHQIT	FLELYHHA	SM	150
Sea bream	SAQDVDNKII	NVLWVYFESK	LIEFMDTFFF	ILRKNNHQIT	FLHIYHHA	SM	150
Cod	SDQETDNKII	HVLWVYFESK	LIEFMDTFFF	ILRKNNHQIT	FLHIYHHA	SM	150
HumanELOVL5	TAGESDMKII	RVLWVYFESK	LIEFMDTFFF	ILRKNNHQIT	VLEHVYHHA	SM	150

	..... ..... ..... ..... .....	160	170	180	190	200	
			<b>NxxxHxxM YxYY</b>				
Zebrafish	LN IWWFVMNW	VPCGHSYFGA	TINSFIHVL	MSYYGLS	AVP	ALRPYIWWK	200
Catfish	LN IWWFVMNW	VPCGHSYFGA	TINSFIHVL	MSYYGLS	AVP	ALRPYIWWK	200
Tilapia	LN IWWFVMNW	VPCGHSYFGA	SINSFIHVM	MSYYGLS	AVP	ALRPYIWWK	200
Salmon	LN IWWFVMNW	VPCGHSYFGA	SINSFIHVL	MSYYGLS	AVP	ALRPYIWWK	200
Turbot	LN IWWFVMNS	VPCGHSYFGA	SINSEVHV	MSYYGLS	AVP	ALRPYIWWK	200
Sea bream	LN IWWFVMNW	VPCGHSYFGA	SINSEVHV	MSYYGLS	AVP	ALRPYIWWK	200
Cod	LN IWWFVMNW	VPCGHSYFGA	AINSFIHVL	MSYYGLS	AVP	ALRPYIWWK	200
HumanELOVL5	LN IWWFVMNW	VPCGHSYFGA	TINSFIHVL	MSYYGLSS	V	ALRPYIWWK	200

	..... ..... ..... ..... .....	210	220	230	240	250	
			<b>TxxQxxQ</b>				
Zebrafish	YI'QGGCLIQI'	VLTMEQTS	CAVVWPCGF	PMGWLYFQIS	YMYVTLILLES	NFY	250
Catfish	YI'QGGCLIQI'	VLTMIQTS	CAVVWPCGF	PKGWLYFQIS	YMI TLILLES	NFY	250
Tilapia	YI'QGLCLIQI'	FLTVTQT	TLIAVWPCGF	PIGWLYFQIS	YMF TLILLEM	NFY	250
Salmon	YI'QGGCLIQI'	FLTMSQT	ICAVVWPCGF	FRGWLYFQIS	YVV TLIALES	NFY	250
Turbot	YI'QGLCLIQI'	FLTMSQT	MCVVWPCDF	FRGWLYFQIS	YVV TLILLES	NFY	250
Sea bream	YI'QGGCLIQI'	FLTMSQT	IFAVVWPCGF	PDGWLYFQIS	YMYV TLILLES	NFY	250
Cod	YI'QGGCLIQI'	FMTMTQT	ICALLWPCNF	FRGWVWFQIS	YLV TLILLET	NFY	250
HumanELOVL5	YI'QGGCLIQI'	VLTIQTS	CGVVWPCTF	PLGWLYFQIS	YMI SLIALET	NFY	250

	..... ..... ..... ..... .....	260	270	280	290		
Zebrafish	IQTYKKRSGS	RKS---DYP	N---GSVNGH	TNGVMSSEK-	IKHRKPAR	AD	291
Catfish	KQTYKKRSGA	LKS---DHR	HNAVKSNGH	SNGTTHKET-	VKQRKERG	D	294
Tilapia	IQTY--KKS	GRK---HPQ	NSSLISTNGH	ANGTPSTDF-	TAEKKIRV	D	293
Salmon	IQTYKKHLVS	QKKE---CHQ	NCSVASINGH	VNGVTPTE-	ITHRKRVR	G	295
Turbot	IQTYKKHNA	T LQK---QHP	NCSAVSRNGH	SNGTPSAEH-	MAHKKIRV	D	294
Sea bream	MQTYNKHSA	S LRK---EHQ	NCSPISTNGH	ANGTPSMEH-	AAHKKIRV	D	294
Cod	IQTYKKQKVS	LKN-----	-GS--SINGH	ANGVSHVEH-	SLHKKIRV	D	288
HumanELOVL5	IQTYNKKGAS	RRKDHKDHQ	NCSMAAVNGH	TNSFSPHENN	VKPKKIRK	D	299



Fig.3.











