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**Cloning, Tissue Expression Analysis, and Functional Characterization of  
Two  $\Delta 6$ -Desaturase Variants of Sea Bass (*Dicentrarchus labrax* L.)**Santigosa, E. <sup>1</sup>; Geay, F. <sup>1</sup>; Tonon, T. <sup>2,3</sup>; Le Delliou, H. <sup>1</sup>; Kuhl, H. <sup>4</sup>; Reinhardt, R. <sup>4</sup>; Corcos, L. <sup>5,6</sup>,  
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**Abstract:**

Fish are the main source of the n-3 highly unsaturated fatty acids, which are crucial for human health. Their synthesis from C<sub>18</sub> precursors is mediated by desaturases and elongases, but the activity of these enzymes has not been conclusively established in marine fish species. This study reports the cloning, tissue expression, and functional characterization of a sea bass (*Dicentrarchus labrax* L.)  $\Delta 6$ -desaturase and one of its splicing variants. Two cDNAs with open reading frames of 1,346 and 1,354 bp were cloned and named D6D and D6D-V, respectively. Both deduced protein sequences (445 and 387 amino acids, respectively) contained two transmembrane regions and the N-terminal cytochrome b<sub>5</sub> domain with the HPGG motif characteristic of microsomal desaturases. D6D presents three histidine-rich regions, whereas in D6D-V, an insertion of eight nucleotides in the boundaries of exons 10 and 11 modified the third histidine-rich domain and led to insertion of a premature STOP codon, resulting in a shorter predicted protein. Quantitative real-time polymerase chain reaction assay of gene expression showed that D6D was highly expressed in the brain and intestine, and to a lesser extent, in muscle and liver; meanwhile, D6D-V was expressed in all tissues tested, but at level at least 200-fold lower than D6D. Functional analysis in yeast showed that sea bass D6D encodes a fully functional  $\Delta 6$ -desaturase with no residual  $\Delta 5$ -desaturase activity. This desaturase does not exhibit a clear preference for n-3 versus n-6 C<sub>18</sub> substrates. Interestingly, D6D-V is a nonfunctional protein, suggesting that the C-terminal end is indispensable for protein activity.

**Keywords:** Sea bass (*Dicentrarchus labrax*) - Desaturase - HUFA biosynthesis - Fish - EPA

## 1. Introduction

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The decrease in worldwide fisheries recorded in the last years (Tidwell and Allan, 2001; Pauly et al., 2005 ; Brunner et al., 2009), in conjunction with aquaculture expansion (FAO, 2006), leads to the utilization of vegetable oils rich in C<sub>18</sub> PUFA in aquafeeds (Bell and Waagbø, 2008). However, the capacity of marine fish species to bioconvert the vegetable C<sub>18</sub> precursors linoleic (LN; 18:2n-6) and linolenic (LNA; 18:3n-3) acids into long-chain highly unsaturated fatty acids (HUFA; chain length  $\geq$  C<sub>20</sub> with  $\geq$  3 double bonds) eicosapentanoic (EPA; 20:5n-3), docosahexanoic (DHA; 22:6n-3) and arachidonic (AA; 20:4n-6) acids is controversial. In freshwater species such as Atlantic salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*), the desaturation/elongation pathway is under nutritional regulation. Thus, when these fish species are fed a diet lacking fish oil, they are capable of modulating the activity of the enzymes to produce the long-chain HUFAs (Tocher et al., 2006, Zheng et al., 2005). In contrast, most marine fish species, when deprived of long-chain HUFAs, are not capable of completing the desaturation/elongation steps that allow the synthesis of EPA, DHA and AA and, consequently, they have a dietary requirement for long-chain HUFA. In this regard, the low level of EPA and DHA biosynthesis from C<sub>18</sub> vegetable precursors in sea bream hepatocytes has been related to a low  $\Delta$ 5-desaturase activity (Tocher and Ghioni, 1999), whereas in the turbot TF cell line, the HUFA elongation/desaturation pathway seems to be impaired in the C<sub>18</sub> to C<sub>20</sub> elongation step (Ghioni et al., 1999). In the particular case of sea bass (*Dicentrarchus labrax* L.), the flesh fatty acid profile is impoverished in long chain n-3 and n-6 fatty acids when fish are fed vegetable-oil diets (Mourente et al., 2005b; Mourente and Bell, 2006), with a concomitant decrease of nutritional value. However, previous studies have proved that this fish species possess the desaturase/elongase activities necessary to produce EPA, AA and DHA from C<sub>18</sub> fatty acids (Mourente and Dick, 2002; Mourente et al., 2005a), even if the conversion rates are extremely low. In this context, it is important to better characterise the different enzymes (elongases and desaturases) involved in HUFA synthesis pathway in order to improve the use of vegetable oils lacking long chain HUFA for marine fish aquafeeds.

Fatty acid desaturases introduce double bounds in selected positions of the acyl chains (Cook, 1996). Particularly,  $\Delta$ 6-desaturases are the first enzymes involved in the biosynthesis of long-chain HUFA from the C<sub>18</sub> n-3 and n-6 precursors. Over the last few years, some desaturases have been isolated and characterized from different fish species (supplementary data 1). Thus, full length cDNAs encoding  $\Delta$ 6-desaturases from rainbow trout (*Oncorhynchus mykiss*), sea bream (*Sparus aurata*), Atlantic salmon (*Salmo salar*), cherry salmon (*Oncorhynchus masou*), carp (*Cyprinus carpio*), turbot (*Scophthalmus maximus*), Nile tilapia (*Oreochromis niloticus*), cod (*Gadus morhua*) and cobia (*Rachycentron canadum*) are now available. In addition,  $\Delta$ 5-desaturases from Atlantic salmon and cherry salmon, as well as a zebrafish (*Danio rerio*) desaturase with both  $\Delta$ 5/ $\Delta$ 6 activities, have been characterized (Hastings et al., 2001). These enzymes contain three histidine boxes, up to three hydrophobic domains, and a N-terminal cytochrome b<sub>5</sub>-like domain (Shanklin et al., 1994). A high percentage of identity (around 90%) between the  $\Delta$ 5- and  $\Delta$ 6-desaturase cDNAs of the same species have been reported (Zheng et al., 2005).

To get deeper insight into the desaturation capacity of sea bass (*Dicentrarchus labrax* L.), the objective of this study was to clone the  $\Delta$ 6-desaturase, to survey its expression profile in different tissues, and finally to functionally characterize this enzyme in order to elucidate its role in the HUFA synthesis pathway of this fish species.

## 1. Materials and Methods

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### 2.1. Cloning of putative fatty acyl desaturase from sea bass.

Total RNA was extracted from sea bass larvae (Day 45 post hatching) fed with a fish-meal/fish-oil diet using the TRIzol® reagent (Invitrogen, Ltd., USA). Five micrograms of RNA were reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) primed by the oligonucleotide Reverse 0 (Table 1). Available fish desaturase sequences (supplementary data 1) were aligned to design a degenerate Forward 1 primer (Table 1). Reverse 1 primer was designed after alignment of sequences contained in sea bass partial clones obtained from multi-tissue cDNA libraries described in *sigenae* databases (cdn22p0006m07, cdnp0002m05 and cdn24p0001j11; <http://public-contigbrowser.sigenae.org:9090>). These primers were used for PCR amplification in a TC-512 instrument (Techné, UK) under the following conditions: initial denaturation 10 min at 94°C, 37 cycles of 1 min denaturation at 94°C, 45 s annealing at 55°C and 1 min 30 s at 72°C for elongation, and a final extension at 72°C for 10 min. A nested PCR was then performed using Forward 2 and Reverse 2 primers (Table 1) to improve amplification specificity. The expected fragment was then purified, cloned into the pCRTM II-TOPO plasmid and used for transformation of ONE Shot™ competent cells (Invitrogen). The fragment was sequenced and found to contain 845 bp.

From this sequence, the specific primers 5'RACE1, 5'RACE2 and 5'RACE3 (Table 1) were designed and used to obtain the 5'-UTR region with the 5'/3'RACE kit (Roche, USA) following the manufacturer's instructions. After cloning and sequencing the isolated DNA as described above, the 5'-UTR specific primer Forward 3 (Table 1) was designed. This primer was combined with the 3'-UTR Reverse 3 primer, designed according to sequences contained in sea bass available clones, to obtain the full length ORF by using the Advantage® cDNA Polymerase Mix (Clontech Laboratories, Inc., USA) under the following conditions: 10 min at 94°C, 40 cycles of 1 min denaturation at 94°C, 45 s annealing at 62°C and 1 min 45 s at 72°C for elongation, and a final extension at 72°C for 10 min. Cloning and sequencing of the PCR products revealed the presence of two highly homologous cDNAs that were named sea bass-D6D and sea bass-D6D-V respectively. All PCR and RACE products were sequenced by Millegen (France).

For comparative analyses, the deduced amino acid sequences of  $\Delta 6$ -desaturases from various species were aligned using ClustalX, and sequence phylogenies were predicted using the Neighbor Joining method (Saitou and Nei, 1987).

## 2.2. Sea bass tissue RNA extraction, and quantitative real time PCR (qrt-PCR).

Nine tissues (brain, pyloric caeca, anterior intestine, posterior intestine, muscle, liver, kidney, heart and skin) of two adult fish fed with a commercial fish-meal/fish-oil diet were isolated and total RNA extraction was immediately performed using TRIzol® reagent. Twelve micrograms of total RNA were reverse-transcribed into cDNA by using the QuantiTect Reverse Transcription Kit (QIAGEN, Germany). Expression of both sea bass-D6D and sea bass-D6D-V was studied by qrt-PCR using specific primers (Table 1). PCR product sizes for sea bass-D6D and D6D-V were 138 and 150 bp respectively. Amplification from cDNA samples was performed using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA) under the following conditions: 1 min 30 s of initial denaturation at 95°C, 45 cycles of 30 s at 95°C and 1 min at 65°C. This was followed by product melt to confirm single PCR products. EF1 was used as a housekeeping gene for normalization of mRNA levels. Thermal cycling and fluorescence detection were conducted in a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad).

## 2.3. Heterologous expression of sea bass desaturases cDNA in yeast

Forward 3 and Reverse 3 primers (Table 1) were used for the amplification of the ORFs of each desaturase transcript (D6D and D6D-V) from cDNA template synthesised from larvae total RNA. PCR products were cloned into the pYES2.1 TOPO expression plasmid (Invitrogen) to obtain the recombinant plasmids pYESD6D and pYESD6D-V, which were then used to transform TOP10F' *E. coli* (Invitrogen). After sequence confirmation, S.

*cerevisiae* strain INVSc1 (Invitrogen) was transformed with one of the recombinant plasmids according to the pYES2.1TOPO@TA Expression Kit manufacturers instructions. Yeast transformed with the empty pYES2.1 plasmid was used as control. Transformants containing pYES2.1, pYESD6D or pYESD6D-V were selected on solid minimal medium plates lacking uracil.

For functional expression, cultures were grown at 25 °C in minimal medium containing 0.67% (w/v) nitrogen base, 0.19% (w/v) dropout medium, and 2% raffinose (w/v). Expression of the transgene was induced at an OD<sub>600</sub> of 0.2 by supplementing with galactose to 2% (w/v) and tergitol to 1% (w/v) final concentration. At that time, the appropriate fatty acids were added as follows (final concentration): 0.5 mM of 18:3n-3, 0.5 mM of 18:2n-6, or 1 mM of 20:4n-3 (Eicosatetraenoic acid, ETA). Incubation was carried out at 25 °C for 3 days in a shaking incubator.

## 2.4. Fatty acid analysis

Yeast cells were harvested by centrifugation (10,000 g, 15 min). Total lipid extraction was performed according to Folch et al. (1957) with dichloromethane replacing chloroform. Briefly, cells were homogenised in 2 mL of 2:1 dichloromethane:methanol (v/v) containing 0.01% butylated hydroxytoluene (BHT). Cell debris were discarded by centrifugation (14,000 g, 10 min, 4°C). Eight hundred µL of NaCl 0.75% were added to the supernatant and samples were decanted for 1h at RT. Then, the bottom phase was transferred into a microcentrifugation tube for saponification (90°C, 3 min) using 0.5 mL 2M KOH-methanol. Two mL of hexane were added, and after centrifugation (630 g, 10 min) the supernatant was recovered and the hexane evaporated. Five hundred microliters of 6N HCl and 2 mL of hexane were added, and after centrifugation (630 g, 10 min) the supernatant was recovered and evaporated. To prepare fatty acid methyl esters (FAME), 1 mL HCl 2.5% in MeOH was added (90°C, 3 min), and after addition of 1mL double distillate water and of 2 mL hexane, samples were centrifuged (630 g, 10 min) and the supernatant was recovered, evaporated and diluted in 0.5mL of hexane. FAME were separated by GLC (Clarus500 Perkin-Elmer with a flame ionisation detector, BPX 70 capillary column: 30 m x 0.22 mm i.d. x 0.25 µm film thickness). The percentage of conversion (conversion rate) of each FA substrate was calculated as follows:  $100 \times (\text{Product area} / (\text{Substrate area} + \text{Product area}))$ .

## 2. Results

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### 3.1. Cloning of putative fatty acyl desaturase from sea bass

Starting from partial cDNA sequences obtained from several clones of a multi-tissue library, the sequence of a putative desaturase transcript was completed by 5' and 3'RACE. This allowed us to identify two transcripts of 1346 and 1354 nucleotides respectively, named sea bass-D6D (GenBank accession no. EU647692) and sea bass-D6D-V (GenBank accession no. EU439924). The alignment of the two cDNA sequences (Fig. 1) revealed an insertion of 8 nucleotides at the boundaries of exon 10 and exon 11 in the D6D-V cDNA, which interrupted the amino acid translation just after exon 10, and gave a deduced amino acid sequence of 387 aa rather than 445 aa for D6D. The alignment of the  $\Delta 6$ -desaturase cDNA and the genomic sequences (obtained by 454 sequencing of the *D. labrax* BAC clone bassbac-102L2) revealed 12 exons spanning 7158 bp of genomic DNA as illustrated in figure 1. All splice sites except the donor site of intron 5 (GC..AG) exhibit the consensus splice site GT..AG. The cDNA aligned completely to the genomic DNA and resulted in only 1 nucleotide mismatch in the 3'UTR at position 59 (A in cDNA, G in gDNA; data not shown).

The comparison of the sea bass D6D deduced amino acid sequences with zebrafish, mouse and human  $\Delta 6$ -desaturase (Fig. 2) revealed an identity of 68, 64 and 65%, respectively. In addition, two transmembrane domains, the typical desaturase N-terminal cytochrome b5-like domain (HPGG), as well as the three characteristic membrane-bound histidine motifs

(HDxGH, HFQHH and QIEHH), were well conserved in sea bass D6D transcript. In the alternative splicing transcript (D6D-V), the third histidine motif was replaced by the QIEHQ sequence (Fig. 2). A pair-wise comparison between fish and human desaturase sequences ((supplementary data 2)) showed that sea bass amino acid sequences exhibit identity ranging from 82 to 94% with marine fish, while values were lower with sequences isolated from salmonids (around 76%) and zebrafish (68%). In addition, about half of the residues were identical between the sea bass and the human desaturases. Phylogenetic analysis comparing both sea bass sequences with characterized and putative  $\Delta 5$  and  $\Delta 6$ -desaturases identified in fish showed that sea bass D6D and D6D-V sequences cluster with cobia, sea bream and turbot sequences rather than with salmonids, carp and zebrafish desaturases (Fig. 3).

### 3.2. D6D and D6D-V gene expression in several sea bass tissues

D6D and D6D-V expression in sea bass tissues (brain, liver, pyloric caeca, anterior intestine, posterior intestine, muscle, kidney, heart and skin) was monitored by quantitative PCR analysis (Fig 4.). Results show that D6D was mainly expressed in brain and intestine, followed by muscle and liver, while its expression was weak in the other tissues considered for the analysis. Sea bass D6D-V expression was maximal in the anterior intestine, followed by posterior intestine, brain and pyloric caeca. Interestingly, the absolute Ct values recorded for D6D (ranging from 19 to 22) in comparison to those obtained for D6D-V (between 28 and 30) showed that the splicing variant was expressed at a level at least 200-fold lower than D6D (data not shown).

### 3.3. Heterologous expression of sea bass desaturase cDNA in yeast

To complete our analysis we functionally characterised both newly identified sea bass desaturases by heterologous expression in *S. cerevisiae* (Table 2). Their activity and specificity was assessed by feeding recombinant yeast expressing pYESD6D or pYESD6D-V with potential  $\Delta 6$  (18:2n-6 and 18:3n-3) and  $\Delta 5$  (20:4n-3) desaturase substrates. The fatty acid composition of the yeast transformed with the control vector (pYES2.1) showed the presence of the main fatty acids currently found in the yeast host (16:0, 16:1n-7, 18:0 and 18:1n-9), together with the exogenously fed fatty acids (see fatty acids profiles in supplementary data 3). In experiments with the pYESD6D-transformed yeast, additional peaks were observed when 18:3n-3 and 18:2n-6 were added to the culture medium. These new fatty acids, Stearidonic acid (STD) 18:4n-3 and Gamma-linolenic acid (GLA) 18:3n-6 respectively, were identified by gas chromatography (GC) analysis after comparison with the profiles obtained in presence of pure standards (data not shown). The percentage of conversion ranged from 7.4 to 10.4 for the LNA and from 5.7 to 8.2 for the LN according to the different replicates of the feeding experiments (Table 2). This observation suggested that the sea bass D6D exhibits no significant preference between C18 n-6 and n-3 PUFA. Concerning yeast transformed with the pYESD6D-V plasmid, the percentage of conversion was nil for LNA and LN. No EPA was found when ETA (20:4n-3) was added in the induction medium in presence of any constructs.

## 3. Discussion

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Data presented in this work represent the first information on cloning, tissue distribution, and functional characterisation of a sea bass desaturase. This is an important step in the understanding of the molecular basis of marine fish HUFA synthesis

The D6D sea bass cDNA ORF encoded for a 445 amino acid protein with high similarity to fish fatty acyl desaturases, being more similar to sea bream (Seilliez et al., 2003), turbot

(Zheng et al., 2004) and cobia (Zheng et al., 2009) than to salmonid sequences, as revealed by amino acid pair-wise comparison and phylogenetic analysis. The functional characterization of the protein in yeast confirmed the sea bass enzyme as a  $\Delta 6$ -desaturase, with no preference for either the omega-3 substrate 18:3n-3 or the omega-6 linoleate. Similar observations have been done after the functional characterisation of mammalian, fungal and moss desaturases (Aki et al., 1999; Cho et al., 1999; Kajikawa et al., 2004; Kaewsuwan et al., 2006), with the exception of the plant family *Primula* sp.  $\Delta 6$ -desaturase and the *Mantoniella squamata*  $\Delta 6$ -desaturase that preferentially desature the n-3 substrates (Hoffmann et al., 2008; Sayanova et al., 2003). In contrast, the selectivity for n-3 rather than for n-6 substrates is clear for the zebrafish bifunctional  $\Delta 5/\Delta 6$ -desaturase (Hastings et al., 2001), as well as for other fish  $\Delta 6$ -desaturases (*Oncorhynchus mykiss*, *Sparus aurata*, *Cyprinus carpio* and *Psetta maximus*; Zheng et al., 2004). Whilst differences in yeast uptake have been described when studying fatty acids of different carbon length (De Antueno et al., 2001), no preference should exist in the uptake when comparing fatty acids with the same chain length.

The percentage of conversion of 18:3n-3 to 18:4n-3 measured for the sea bass D6D ranged from 7.4 to 10.4 for the LNA and from 5.7 to 8.2 for the LN. This poor desaturation capacity could explain, at least partially, sea bass impairment in the bioconversion of the C<sub>18</sub> fatty acids provided by vegetable oil-based diets (Mourete et al., 2005a).

Some of the fish  $\Delta 6$ -desaturases characterized so far presented a low capacity to desaturate the  $\Delta 5$  substrates 20:4n-3 and 20:3n-6, with conversion rates from 0.2% in rainbow trout (Zheng et al., 2004), sea bream (Zheng et al., 2004) or turbot (Zheng et al., 2004) to 2.3% in Atlantic salmon (Zheng et al., 2005) or cobia (Zheng et al., 2009). In our report, no  $\Delta 5$  activity was detected in transgenic yeasts containing the D6D sea bass desaturase genes when cultivated in presence of 20:4n-3, indicating that, like Atlantic cod (Tocher et al., 2006), the sea bass  $\Delta 6$ -desaturase protein was not capable of desaturate 20:4n-3 at the  $\Delta 5$  position, or that this activity was extremely low and thus was not detectable under the conditions tested.

Results obtained in this work, in conjunction with the existing literature concerning the controversial HUFA synthesis efficiency in marine fish species (Sargent et al., 2002), and especially in sea bass (Mourete and Dick, 2002; Mourete et al., 2005a), allow us to hypothesise on the occurrence of  $\Delta 5$ -desaturase activity. Firstly, the low activity detected for sea bass liver (Mourete et al., 2005a) could be related to an unknown gene encoding for a  $\Delta 5$ -desaturase, and thus responsible for the trace amounts of EPA (20:5n-3) and AA (20:4n-6) detected in hepatocyte cultures in the presence of C<sub>18</sub> precursors (Mourete et al., 2005a). The existence of an enzyme with  $\Delta 5$ -desaturation capacity is supported by the fact that different desaturases with distinct  $\Delta 6$  and  $\Delta 5$  specificities have been reported for freshwater species (Hastings et al., 2005), even if the origin of the two independent desaturase genes is unclear (Zheng 2004; Napier et al. 2003). However, the low capacity of marine fish species to synthesize long chain HUFA from C<sub>18</sub> precursors could also be related to an absence of the  $\Delta 5$ -desaturase gene. In this regard, to the best of our knowledge, no marine fish  $\Delta 5$ -desaturase proteins have been reported. Moreover, preliminary analysis of the results from 'shot gun' genome sequencing suggests the existence of a unique desaturase gene in sea bass (data not shown). If this is the real situation, it is possible to hypothesize that the zebrafish bifunctional desaturase might have evolved in a unifunctional  $\Delta 6$ -desaturase by changes in catalytic residues which would have abolished the  $\Delta 5$  activity in marine fish species. The difference in evolution of desaturase activities in freshwater and marine fish could be related to the fact that marine fish natural diet is rich in EPA and DHA while freshwater fish diet is usually poor in long chain HUFA. Therefore, the freshwater species need to cover their requirements by the desaturation and elongation of C<sub>18</sub> precursors (Sargent et al., 1995, 1999).

Recent studies in sea bass HUFA pathway have shown the occurrence of numerous splicing variants for the D6D gene. Thus, in addition to the D6D-V detected in the present work (GenBank accession no. EU439924), five other variants exist in the NCBI database (GenBank accession no. AM746707, AM746706, AM746705, AM746704, AM746703). Despite the high homology between the two sequences identified in this study, the D6D-V

splicing variant indicated that the C-terminal end was indispensable for the activity of the protein. In addition, even if the physiological occurrence of these splicing variants was doubtful because of the lower expression levels of the variant when compared to the main desaturase transcript, the qrt-PCR analysis performed in our study demonstrated that the D6D-V variant was expressed in several tissues from juvenile sea bass (brain, intestine, muscle, liver, kidney, heart and skin). Concerning its role in regulation of  $\Delta 6$ -desaturase activity, it is possible that the D6D-V, like the other identified variants, could decrease substrate availability by trapping it into the enzyme active site that would be preserved in the variant proteins. This would then diminish the apparent activity of the main protein. The strong expression of the D6D gene in brain is in concordance with previous results obtained for Atlantic cod (Tocher et al., 2006) and cobia (Zheng et al., 2009). In addition, the higher level of transcripts detected in intestine when compared to liver is in agreement with the level of enzymatic activity monitored in vitro by Mourente et al. (2005a), who have measured a stronger desaturation rates in enterocytes than in hepatocytes. Further studies will be needed to more fully address the correlation between D6D expression levels with desaturation capacity in different sea bass organs.

In summary, we reported here the first characterisation of the sea bass  $\Delta 6$ -desaturase, and showed its high level of expression in the brain and intestine. This work also demonstrates the existence of a predicted 387 amino acid non-functional splicing variant expressed in a wide range of sea bass tissues. The functional  $\Delta 6$ -desaturase did not exhibit substrate selectivity towards n-3 and n-6 C<sub>18</sub> substrates, and no  $\Delta 5$ -desaturase residual activity was detected by yeast heterologous expression. The low conversion rate determined for sea bass  $\Delta 6$ -desaturase could, at least partially, explain the low HUFA biosynthesis when this species is fed vegetable-based diets. The discovery of this limited step in the HUFA synthesis process for sea bass may enable to manipulate and optimise the activity of the pathway to allow efficient and effective use of vegetable oils in aquaculture.

In fact, whilst the sea bass  $\Delta 6$ -desaturase cDNA sequence has a strong homology with those of other fish species exhibiting better conversion rates, it is not excluded that the dysfunction of the enzyme can be due to mutation at the level of the nucleotide sequence. Accordingly, the use of transgenic fish to commercial ends not being accepted by consumers at the present time, it is however possible to consider the selection of sea bass family exhibiting better rates of  $\Delta 6$ -desaturase activity as soon as  $\Delta 6$  desaturase gene polymorphism will be determined. In addition, regarding other potential limiting steps in sea bass HUFA synthesis pathway such as bioconversion of ETA into EPA, other oil resources rich in HUFA such as microalgae may be considered in aquafeeds.



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## Figures

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Figure 1

Fig. 1. Exon/Intron boundaries of sea bass D6D and D6D-V gene (exon1-12). Location of different exons is shown by alternation of white/grey areas. 5' and 3' extremities of intronic sequences are indicated in lower case letters. Hash in the boundaries of exon5/exon6 denotes a non consensus splice donor and the size of each intron is indicated between brackets. Asterisks correspond to the nucleotides conserved between the two sea bass cDNA sequences. Predicted amino acid translation noted in the boundaries of exon10/exon11 shows the stop in the translation of the D6D-V cDNA.

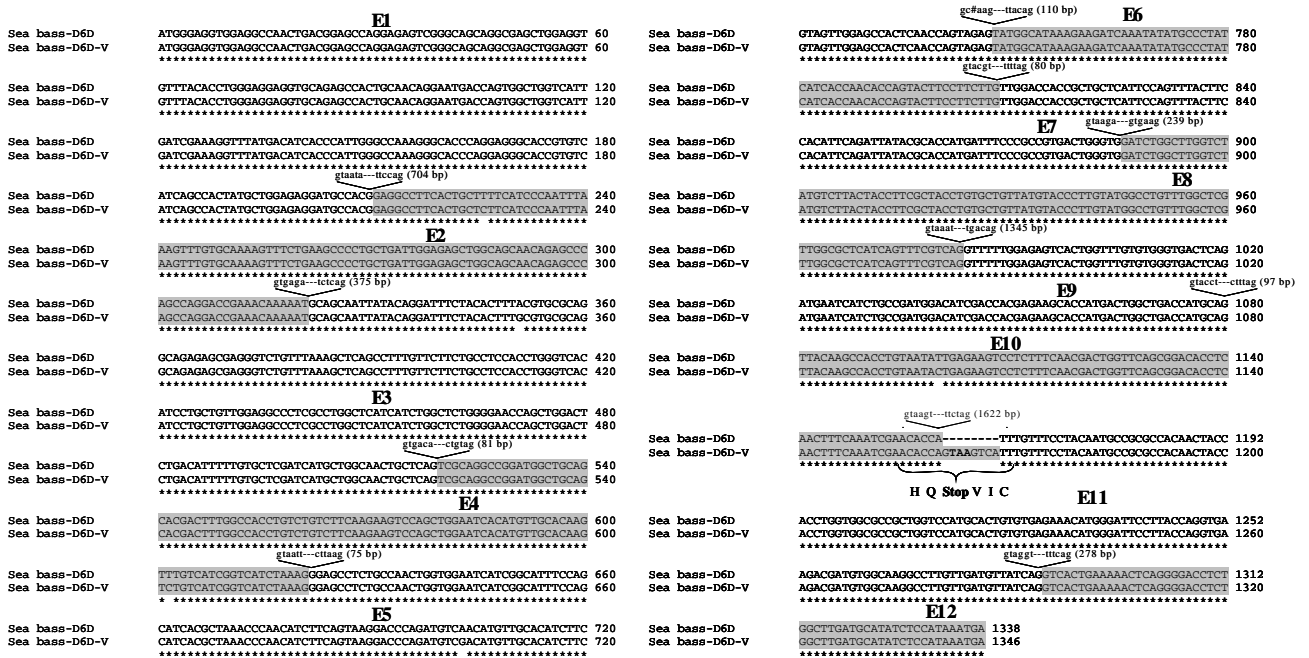


Figure 2  
 Fig. 2. Alignment of the deduced amino acid sequences from sea bass D6D and D6D-V with desaturases from zebrafish (GenBank accession No.Q9DEX7), mouse (GenBank accession No.NP062673) and human (GenBank accession No.NP004256). The cytochrome b5-domain is dot-underlined. Putative transmembrane domains are shown in shaded areas, and three histidine-rich regions are boxed. The asterisks indicate the heme-binding motif (HPGG), and two highly conserved histidine positions, are in bold.

\*\*\*

Mouse-D6D MGKGGNQEGEGSTERQAPMP-TFRWEEIQKHNLRTDRWLVIDRKVYNVTKWSQRHPGGHRV 59  
Human-D6D MGKGGNQEGEGAAEREVSVP-TFSWEEIQKHNLRTDRWLVIDRKVYNITKWSIQHPGGQRV 59  
Sea bass-D6D MGGGGQLTEPGESGSRAGGVYTWEEVQSHCNRNNDQWLVIDRKVYDITHWAKGHPGGHRV 60  
Sea bass-D6D-V MGGGGQLTEPGESGSRAGGVYTWEEVQSHCNRNNDQWLVIDRKVYDITHWAKGHPGGHRV 60  
Zebrafish-D5/6D MGGGGQQTDRITDTNGRFS-SYTWEVQKHTKHGDQVWVVERKVYNVSVQVVKRHPGGLRI 59  
\*\* \*\* : : . . : \*\* : \* \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \*

Mouse-D6D IGHYSGEDATDAFRAFHLDDLDFVKGFLKPLLI GELAPEEPSLDRGKSSQITEDFRALKKT 119  
Human-D6D IGHYAGEDATDAFRAFHPDLEFVKGFLKPLLI GELAPEEPSQDHGKNSKITEDFRALRKT 119  
Sea bass-D6D ISHYAGEDATEAFTAFHPNLKFVQKFLKPLLI XGELAAATEPSQDRNKNAAI IQDFYTLRAQ 120  
Sea bass-D6D-V ISHYAGEDATEAFTALHPNLKFVQKFLKPLLI GELAAATEPSQDRNKNAAI IQDFYTLRAQ 120  
Zebrafish-D5/6D LGHYAGEDATEAFTAFHPNLQLVVRKYKPLLI GELEAASEPSQDRQKNAALVEDFRALRER 119  
: \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \*

Mouse-D6D AEDMNLFKTNHFFLLLSHIIVMESLAWFILSYFGTGWIP TLVTAFLV LATSQAQAGWLQ 179  
Human-D6D AEDMNLFKTNHVFLLLLAHLIALESIAWFTV FYFGNGWIPTLITAFVLATSQAQAGWLQ 179  
Sea bass-D6D AESEGLFKAQPLFFCLHLGHILLLEALAWLI IWXWGT SWTLTFLCSIMLATAQSQAGWLQ 180  
Sea bass-D6D-V AESEGLFKAQPLFFCLHLGHILLLEALAWLI IWLWGT SWTLTFLCSIMLATAQSQAGWLQ 180  
Zebrafish-D5/6D LEAEGCFKTPPLFFALHLGHILLLEAIAFMMVWVYFGTGWINTLIVAVILAATAQSQAGWLQ 179  
\* . \*\* : : \*\* \* \* \*\* : : \* : \* \* : : \* : \* \* : : \* : \* \* : : \* : \* \* : \*

Mouse-D6D HDYGHLSVYKKS IWNHVHVKFVIGHLKGASANWNNHR HFQHHA KPNIFHKDPDIKSLHVF 239  
Human-D6D HDYGHLSVYRKPKNHNLVHKFVIGHLKGASANWNNHR HFQHHA KPNIFHKDPDVNMLHVF 239  
Sea bass-D6D HDFGHLSVFKKSSWNHMLHKFVIGHLKGASANWNNHR HFQHHA KPNIFSKDPDVNMLHIF 240  
Sea bass-D6D-V HDFGHLSVFKKSSWNHMLHKFVIGHLKGASANWNNHR HFQHHA KPNIFSKDPDVNMLHIF 240  
Zebrafish-D5/6D HDFGHLSVFKTSGMNHLVHKFVIGHLKGASAGWNNHR HFQHHA KPNIFKDPDVNMLNAF 239  
\*\* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \*

Mouse-D6D VLGEWQPLEYGGKKLKYPYNHQHEYFFLIGPPLLI PMYFQYQ IIMTMSRRDWDVLAWA 299  
Human-D6D VLGEWQPIEYGGKKLKYPYNHQHEYFFLIGPPLLI PMYFQYQ IIMTMI VHKNVVLAWA 299  
Sea bass-D6D VVGATQPVEYGIKKIKYMPYHHQH QYFLLVGPPLLI PVYFHIQ IIRTMISRRDWDVLAWS 300  
Sea bass-D6D-V VVGATQPVEYGIKKIKYMPYHHQH QYFLLVGPPLLI PVYFHIQ IIRTMISRRDWDVLAWS 300  
Zebrafish-D5/6D VVGNVQPVEYGVKKIKHLPYNHQHKYFFF IGPPLLI PVYFQFQ IIFHNMI SHGMVWDLWC 299  
\* : \* \*\* : \* \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \*

Mouse-D6D ISYYMRFYTYIPFYGILGALVFLNF I RFLESHWVWVVTQMNHLVMEIDL DHDYRDWFFSSQ 359  
Human-D6D VSYIRFFITYIPFYGILGALLFLNF IRFLESHWVWVVTQMNHLVMEIDQ EAYRDWFFSSQ 359  
Sea bass-D6D MSYYLRYLCCYVPLYGLFGSLALISFVRFLESHWVWVVTQMNHLPM DIDHEKHHDWLTMQ 360  
Sea bass-D6D-V MSYYLRYLCCYVPLYGLFGSLALISFVRFLESHWVWVVTQMNHLPM DIDHEKHHDWLTMQ 360  
Zebrafish-D5/6D ISYYVRYFLCYTQFYGVFWA IILFNFVRFMESHWVWVVTQMSHIPMNIDYEKNQDWLSMQ 359  
: \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \* : \*

Mouse-D6D LAATCNVEQSFFNDWFSGLNFIQIEHHI FPTMPRHNLHKIAPLVKSLCAKHGIEYQEKPL 419  
Human-D6D LTATCNVEQSFFNDWFSGLNFIQIEHHI FPTMPRHNLHKIAPLVKSLCAKHGIEYQEKPL 419  
Sea bass-D6D LQATCNIEKSSFFNDWFSGLNFIQIEHHI FPTMPRHNYHLVAPLVHALCEKHGIPYQVKT 420  
Sea bass-D6D-V LQATCNTEKSSFFNDWFSGLNFIQIEHQ----- 387  
Zebrafish-D5/6D LVATCNIEQSAFNDWFSGLNFIQIEHHI FPTVPRHNYWRAAPRVRALCEKYGVKYQEKTL 419  
\* \* \* \* \* : \* \* \* \* \* : \* \* \* \* \* : \* \* \* \* \* : \* \* \* \* \* : \* \* \* \* \*

Mouse-D6D LRALIDIVSSLKSGELWLDAYLHK 444  
Human-D6D LRALLDIIRSLKKS GKLWLDAYLHK 444  
Sea bass-D6D WQGLVDVIRSLKNSGDLWLDAYLHK 445  
Sea bass-D6D-V -----  
Zebrafish-D5/6D YGAFADIIRSLKSGELWLDAYLNK 444

Figure 3

Fig. 3. Phylogeny of desaturase deduced amino acid sequences. The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using CLUSTALX and NJPLOT. 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. Arrows highlight the location of sea bass delta-6 desaturase variants. Asterisks denote desaturase sequences that have not been functionally characterized.

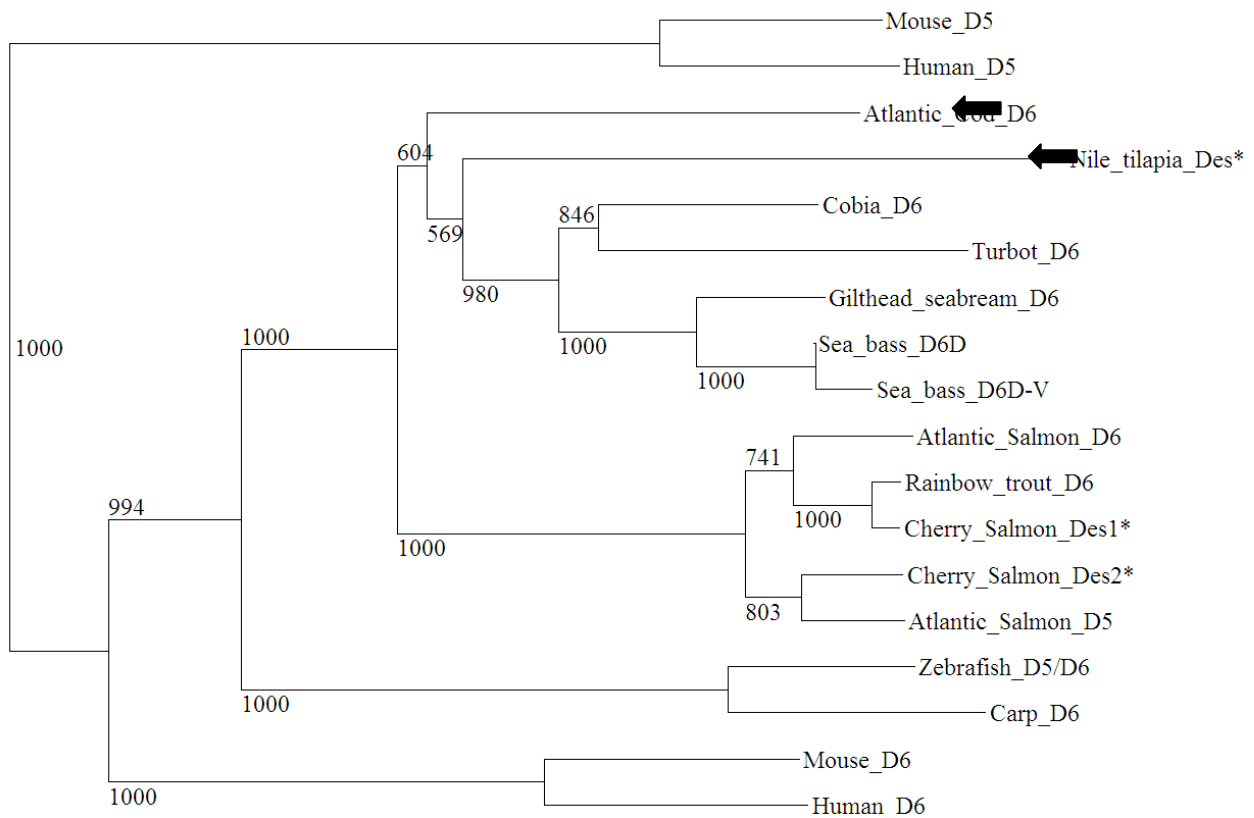
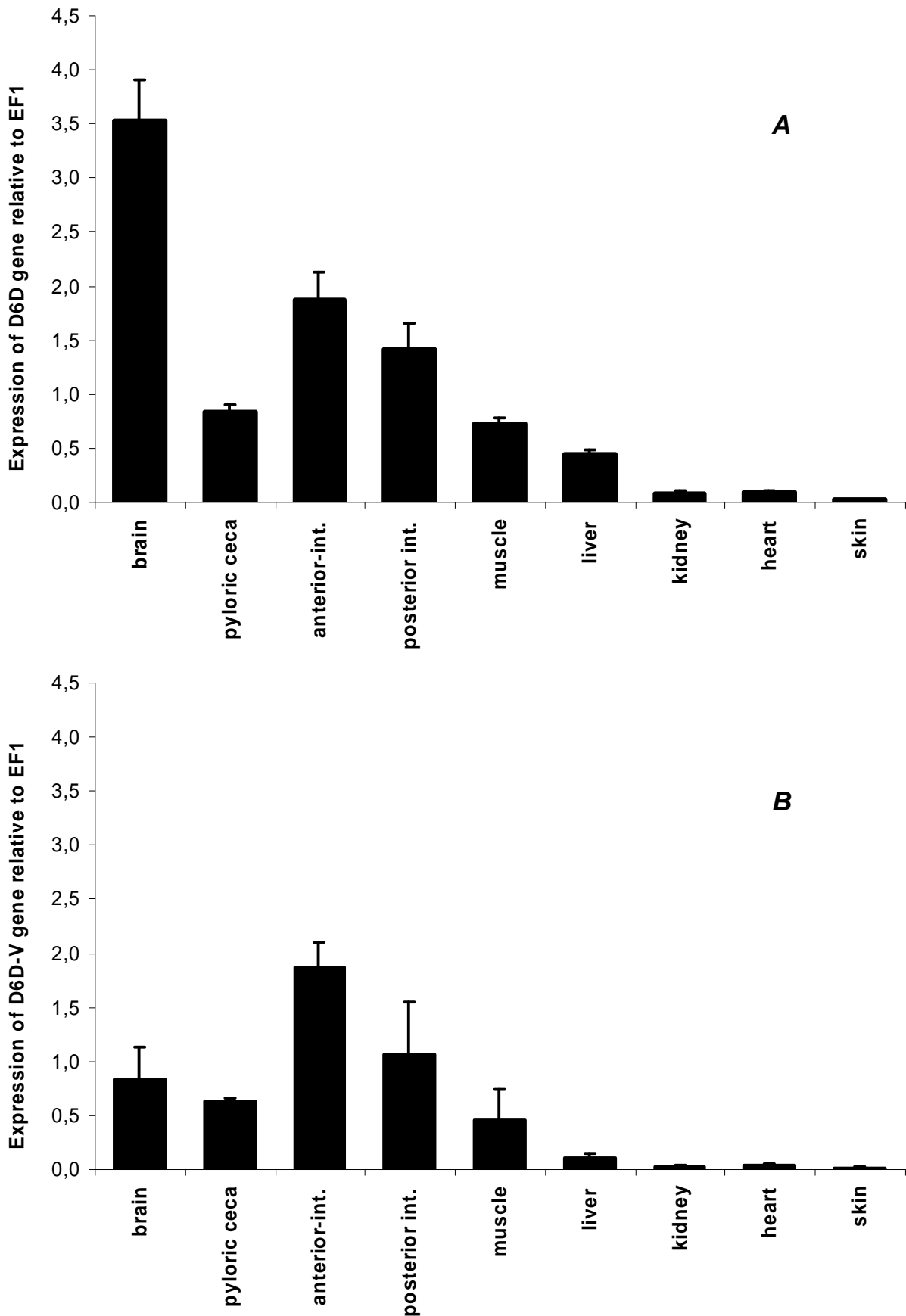


Figure 4

Fig. 4. Sea bass D6D (A) and D6D-V (B) gene expression in several tissues. Levels of relative D6D or D6D-V expression were determined by qRT-PCR and after normalisation by the expression of EF-1 housekeeping gene.



## Tables

Table 1. Primers used in this study.

Use	Primer (5'-3')
Partial cDNA cloning	Reverse 0: ACCCACACAAACCAGTGACTCT <sup>a</sup>
	Forward 1: ATGGGKGGCGGAGGHCAGCAGA <sup>b</sup>
	Reverse 2: ACCAGTGACTCTCCAAAAATYT <sup>a</sup>
	Forward 2: KGGCGGAGGHCAGCAGA <sup>b</sup>
	Reverse 2: TGTGGAAGTAAACTGGAATGAG <sup>a</sup>
5' UTR cloning	5'RACE1: CCTTTCGATCAATGACCAGCC <sup>c</sup>
	5'RACE2: CATTCTGTTGCAGTGGCTC <sup>c</sup>
	5'RACE3: CGCCTGCTGCCCGACTCTCC <sup>c</sup>
Full-length cDNA cloning	Forward 3: TTTAGTGTAGGCTTCAGGTGG <sup>c</sup>
	Reverse 3: GGTACAGGGTAGAGAATACRATTGG <sup>b</sup>
qrt-PCR	D6D-F: CATCGACCACGAGAAGCACC <sup>c</sup>
	D6D-R: GGCATTGTAGGAAACAAATGGTG <sup>c</sup>
	D6D-V-F: GATGGACATCGACCACGAGAA <sup>c</sup>
	D6D-V-R: CATTGTAGGAAACAAATGACTTAC <sup>c</sup>

<sup>a</sup> Specific sea bass primers designed from database clones.

<sup>b</sup> Degenerate primers designed from available fish desaturases.

<sup>c</sup> Specific sea bass primers designed from sequences identified in this study.



Table 2. Percentage of LN (18:3n-3), LNA (18:2n-6) and ETA (20:4n-3) conversion into STD (18:4n-3), GLA (18:3n-6) and EPA (20:5n-3) as measured after analysis by Gas Chromatography of fatty acid contents in yeast transfected by different clones. pYES2-Empty: Expression vector pYES2 without any insert. pYESD6D: Expression vector pYES2 including D6D cDNA. pYESD6D-V: Expression vector pYES2 including D6D-V cDNA. ND= Not Detectable. Each feeding experiment being performed in triplicate, this table represents results of a representative experiment.

Clone tested	Substrate FA fed, % found	Product FA, % found	Conversion, %
pYES2-Empty	18:3n-3, 46.78%	18:4n-3, ND	ND
pYES2-Empty	18:2n-6, 48.53%	18:3n-6, ND	ND
pYES2-Empty	20:4n-3, 12.34%	20:5n-3, ND	ND
pYESD6D	18:3n-3, 51.37%	18:4n-3, 6.01%	10.4%
pYESD6D	18:2n-6, 45.51%	18:3n-6, 4.05%	8.17%
pYESD6D	20:4n-3, 9.28%	20:5n-3, ND	ND
pYESD6D-V	18:3n-3, 64.9%	18:4n-3, ND	ND
pYESD6D-V	18:2n-6, 50.7%	18:3n-6, ND	ND
pYESD6D-V	20:4n-3, 12.42%	20:5n-3, ND	ND

Supplementary data 1. Available fish fatty acyl desaturase sequences and GenBank Accession Numbers. An asterisk denotes a desaturase sequence that has not been functionally characterized. The percentage of conversion (conversion rate) of each FA substrate was calculated as follows:  $100 \times (\text{Product area} / (\text{Substrate area} + \text{Product area}))$ .

Species	Type	GenBank Accession No	Authors submission	conversion rate				Functional Characterization
				18:3n-3	20:3n-6	18:2n-6	20:4n-3	
Rainbow trout ( <i>Oncorhynchus mykiss</i> )	Δ6	AF30191	Seilliez et al, 2001	31.5	ND	3.6	0.2	Seilliez et al, 2001
sea bream ( <i>Sparus aurata</i> )	Δ6	AY055749	Seilliez et al, 2003	23.1	0.1	12.2	0.2	Seilliez et al, 2003
Atlantic salmon ( <i>Salmo salar</i> )	Δ6	AY458652	Zheng et al, 2005	60.1	ND	14.4	2.3	Zheng et al, 2005
Cherry salmon ( <i>Oncorhynchus masou</i> )	Δ6*	AB070444	Yoshizaki et al. (unpublished)	-	-	-	-	Yoshizaki et al. (unpublished)
Carp ( <i>Cyprinus carpio</i> )	Δ6	AF309557	Hastings et al., 2001	7.0	0.4	1.5	0.5	Hastings et al., 2001
Turbot ( <i>Scophthalmus maximus</i> )	Δ6	AY546094	Zheng et al, 2004	59.5	ND	31.2	0.1	Zheng et al, 2004
nile tilapia ( <i>Oreochromis niloticus</i> )	Δ6*	AB069727	Sirisuay et al. (unpublished)	-	-	-	-	Sirisuay et al. (unpublished)
Cod ( <i>Gadus morhua</i> )	Δ6	DQ054840	Tocher et al, 2006	33.5	ND	17.5	ND	Tocher et al, 2006
Cobia ( <i>Rachycentron canadum</i> )	Δ6	FJ440238	Zheng et al., 2009	50.8	0.2	36.5	2.4	Zheng et al., 2009
Atlantic salmon ( <i>Salmo salar</i> )	Δ5	AF478472	Hastings et al, 2005	0.6	0.9	0.4	10.2	Hastings et al, 2005
Cherry salmon ( <i>Oncorhynchus masou</i> )	Δ5*	EU098126	Yoshizaki and Shusa (unpublished)	-	-	-	-	Yoshizaki and Shusa (unpublished)
Zebrafish ( <i>Danio rerio</i> )	Δ5/Δ6	AF309556	Hastings et al., 2001	29.4	8.3	11.7	20.4	Hastings et al., 2001
Sea bass ( <i>Dicentrarchus labrax</i> )	Δ6	EU647692	Terova et al., 2008 (unpublished)	10.4	ND	8.2	ND	Santigosa et al., 2009
Sea bass ( <i>Dicentrarchus labrax</i> )	Δ6	EU439924	Geay et al., 2008 (unpublished)	ND	ND	ND	ND	Santigosa et al., 2009



Supplementary data 3. GC analysis of FAME from yeast transformed with the pYES2 empty plasmid (A, B) or pYES-D6D plasmid (C, D, E) and incubated in the presence of 0.5 mM 18:3n-3 (A, D); 0.5 mM 18:2n-6 (B, E) and 1 mM 20:4n-3 (C). Peaks 1, 2, 3, and 4 correspond to the four endogenous FA in *Saccharomyces cerevisiae* (16:0, 16:1n-7, 18:0 and 18:1n-9, respectively). The exogenously added FA substrates are represented by peaks 5 (18:3n-3), 6 (18:2n-6) and 7 (20:4n-3); peaks 8 and 9 account for 18:4n-3 and 18:3n-6 respectively, which were identified as the desaturated products produced in presence of 18:3n-3 and 18:2n-6. Each feeding experiment was performed in triplicate, and results of a representative experiment are shown.

