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Characterisation and comparison of fatty acyl Δ6 desaturase cDNAs from freshwater and marine teleost fish species

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Abstract:

Fish are the most important dietary source of the n-3 highly unsaturated fatty acids (HUFA), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), that have particularly important roles in human nutrition reflecting their roles in critical physiological processes. The objective of the study described here was to clone, functionally characterise and compare expressed fatty acid desaturase genes involved in the production of EPA and DHA in freshwater and marine teleost fish species. Putative fatty acid desaturase cDNAs were isolated and cloned from common carp (*Cyprinus carpio*) and turbot (*Psetta maximus*). The enzymic activities of the products of these cDNAs, together with those of cDNAs previously cloned from rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*), were determined by heterologous expression in the yeast *Saccharomyces cerevisiae*. The carp and turbot desaturase cDNAs included open reading frames of 1335 and 1338 base pairs, respectively, specifying proteins of 444 and 445 amino acids. The protein sequences possessed all the characteristic features of microsomal fatty acid desaturases, including three histidine boxes, two transmembrane regions, and N-terminal cytochrome b_5 domains containing the haem-binding motif, HPGG. Functional expression showed all four fish cDNAs encode basically unifunctional Δ6 fatty acid desaturase enzymes responsible for the first and rate-limiting step in the biosynthesis of HUFA from 18:3n-3 and 18:2n-6. All the fish desaturases were more active towards the n-3 substrate with 59.5%, 31.5%, 23.1% and 7.0% of 18:3n-3 being converted to 18:4n-3 in the case of turbot, trout, seabream and carp, respectively. The enzymes also showed very low, probably physiologically insignificant, levels of Δ5 desaturase activity, but none of the products showed Δ4 desaturase activity. The cloning and characterisation of desaturases from these fish is an important advance, as they are species in which there is a relative wealth of data on the nutritional regulation of fatty acid desaturation and HUFA synthesis, and between which substantive differences occur.

Keywords: Fish; Highly unsaturated fatty acid; Desaturases; cDNAs; Genes.

1. Introduction

No vertebrate species can synthesise polyunsaturated fatty acids (PUFA) *de novo*, as they lack the fatty acid desaturase enzymes required for the production of linoleate (18:2n-6) and linolenate (18:3n-3) from oleic acid (18:1n-9) (Wallis et al., 2002). However, many vertebrates can convert dietary 18:2n-6 and 18:3n-3 to long chain, highly unsaturated fatty acids (HUFA) such as arachidonic acid (20:4n-6, AA), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (22:6n-3, DHA) via a pathway involving a series of microsomal fatty acid desaturation and elongation steps (Fig.1) (Cook, 1996). The production of EPA requires $\Delta 6$ and $\Delta 5$ desaturases, and the production of DHA from EPA requires a further desaturation originally thought to be effected by a $\Delta 4$ desaturase acting on a C_{22} fatty acid intermediate, although evidence now suggests it may actually be effected by a $\Delta 6$ desaturase acting on a C_{24} intermediate (Sprecher, 2000; de Antueno et al., 2001; D'Andrea et al., 2002). Fish lipids are particularly rich in EPA and DHA (Ackman, 1980), which have critical roles in human nutrition reflecting their roles in key physiological processes (Calder and Grimble, 2002; Simopoulos, 2002). The extent to which fish species can convert C_{18} PUFA to $C_{20/22}$ HUFA varies, associated with their complement of fatty acid desaturase and elongase enzymes. Freshwater fish are capable of producing DHA from 18:3n-3 (Buzzi et al., 1996; Bell et al., 2001a) and so must express all the desaturase and elongase activities necessary for this biosynthetic pathway (Sargent et al., 2002). In contrast, marine fish are unable to produce DHA from 18:3n-3 at a physiologically significant rate (Owen et al., 1975; Sargent et al., 2002) due to apparent deficiencies in one or more steps in the pathway (Ghioni et al., 1999; Tocher and Ghioni, 1999).

Although fish are the most important food source of n-3HUFA for man, virtually every food fishery around the globe is in decline, and so an increasing proportion of fish for human consumption is being provided by rapidly expanding aquaculture (Tidwell and Allan, 2002). Paradoxically, European aquaculture, particularly of marine fish, is itself dependent upon feed grade fisheries for the provision of fish meals and oils traditionally used as the main protein and lipid sources in the feeds (Sargent and Tacon, 1999). However, stagnation in feed fisheries, along with increased demand for fish oils, has dictated that alternatives to fish oil must be found if aquaculture is to continue to expand and supply more of the global demand for fish (Barlow, 2000). The only sustainable alternative to fish oils are plant (vegetable) oils, which can be rich in C_{18} PUFA, but devoid of the n-3 HUFA abundant in fish oils (Sargent et al., 2002). Consequently, tissue fatty acid compositions in fish fed vegetable oils are characterised by increased levels of C_{18} PUFA and decreased levels of $C_{20/22}$ HUFA, compromising their nutritional value to the human consumer (Tocher et al., 2000; Bell et al., 2001b, 2002; Caballero et al., 2002; Izquierdo et al.,

2003; Regost et al., 2003). Our primary hypothesis is that understanding the molecular basis of HUFA biosynthesis and its regulation in fish will enable us to manipulate and optimise the activity of the pathway to enable efficient and effective use of vegetable oils in aquaculture.

In recent years, significant progress has been made in characterizing fatty acid desaturases involved in HUFA synthesis (Tocher et al., 1998). Full-length cDNAs for $\Delta6$ desaturases have been isolated from the filamentous fungus *Mortierella alpina* (Huang et al., 1999), the nematode *Caenorhabditis elegans* (Napier et al., 1998), rat (Aki et al., 1999), mouse and human (Cho et al., 1999a). Fatty acid $\Delta5$ desaturase genes have been isolated from *M. alpina* (Michaelson et al., 1998a), *C. elegans* (Michaelson et al., 1998b; Watts and Browse, 1999) and human (Cho et al., 1999b; Leonard et al., 2000). Using extant sequence information, we isolated a cDNA from the freshwater zebrafish (*Danio rerio*) with high similarity to mammalian $\Delta6$ desaturase genes (Hastings et al., 2001). Heterologous expression in yeast indicated that the zebrafish desaturase gene was unique in that it encoded an enzyme having both $\Delta6$ and $\Delta5$ desaturase activities, prompting us to speculate that it may represent a component of a prototypic vertebrate HUFA biosynthesis pathway. However, we recently cloned a desaturase cDNA from Atlantic salmon (*Salmo salar*) which, as shown by heterologous expression in yeast, was predominantly an omega-3 $\Delta5$ desaturase, with only very low levels of $\Delta6$ activity (Hastings et al., 2004). Thus this salmon desaturase was primarily a single function enzyme, similar to the mammalian desaturases, questioning the interpretation of the bifunctional zebrafish desaturase as a prototypic enzyme. Clearly, more information on fatty acid desaturases in fish is required.

The objective of the present study was to clone and functionally characterise desaturase genes from both freshwater and marine teleost fish species that were important in aquaculture, and in which substantial biochemical data on HUFA synthesis and metabolism was available. To this end, putative fatty acid cDNAs were isolated and cloned from common carp (*Cyprinus carpio*) and turbot (*Psetta maximus*). Along with cDNAs previously cloned from rainbow trout (*Oncorhynchus mykiss*) (Seiliez et al., 2001) and gilthead seabream (*Sparus aurata*) (Seiliez et al., 2003), the enzymic activities of all four clones were determined by heterologous expression in the yeast, *Saccharomyces cerevisiae*. The results showed that all the cDNAs encoded basically unifunctional $\Delta6$ fatty acid desaturase enzymes responsible for the first and, reportedly, rate-limiting, step in the biosynthesis of HUFA from C_{18} PUFA.

2. Materials and methods

2.1. Cloning of fish putative desaturase cDNAs

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Trout and sea bream: Putative PUFA desaturases were cloned from rainbow trout (*Oncorhynchus mykiss*, GenBank accession no. AF301910) and gilthead sea bream (*Sparus aurata*, GenBank, accession no. AY055749) based on sequence similarity to mammalian Δ6 fatty acid desaturases as described in detail previously (Seiliez et al., 2001,2003).

Carp: Liver tissue was obtained from a single fish from a stock of carp held at the Institute of Aquaculture. The RNA extraction, cDNA synthesis, primers and PCR conditions for cloning putative PUFA desaturase from carp (GenBank accession no. AF309557) were coincidentally the same as for the zebrafish desaturase as described in detail previously (Hastings et al., 2001).

Turbot: Liver tissue was obtained from a single fish from a stock of turbot held at the Institute of Aquaculture and maintained for many months on a standard pellet diet formulated with fishmeal and fish oil. RNA was extracted from liver tissue using TRIzol® reagent (GibcoBRL, NY, U.S.A.). Normal cDNA was prepared using MMLV reverse transcriptase (Promega, Madison, WI, U.S.A) primed by the oligonucleotide, 5'-GAT AGC GCC CGC GTT TTT TTT TTT TT(AGC)-3' (Not1PolyT). 5'-RACE-cDNA was synthesized using the SMART™ RACE cDNA amplification kit (Clontech, NJ, U.S.A). Primers for 5' RACE PCR were designed according to the partial nucleotide sequence of a putative desaturase cDNA from cod. This sequence was obtained by PCR using degenerate primers designed according to concensus regions around the second and third histidine boxes of published fatty acid desaturase sequences (Hastings, 2003). 5'RACE PCRs were performed using Advantage 2 cDNA polymerase (Clontech, Palo Alto, CA, U.S.A) with primers CodRev1, 5'- GCT TCT CGT GGT CTA TGT TCA T –3' for first round PCR and CodRev3, 5'- GGT GTT GGT GGT GAT AGG GCA T –3'for the nested PCR. The sequence results of the 5'RACE experiment enabled primers to be designed for 3' end RACE PCR. Sense primers Turb3RaceA, 5'- GAG CAA GGG TCT GTT TCA AGC –3' and Turb3RaceB, 5'- CCG CTG GAA TCA CTT GTT GC –3' were used to perform original and nested 3'RACE PCRs together with the antisense primer Not1polyT (as above), using the Advantage 2 cDNA polymerase.

Both 5' and 3' RACE PCR fragments were cloned into the pBluescript KS Ⅱ⁺ vector (Stratagene, La Jolla, CA, USA) using standard methods, and nucleotide sequences were determined by standard dye terminator chemistry using a Perkin Elmer ABI-377 DNA sequencer following the manufacturer's protocols (Perkin Elmer, Applied Biosystems). The full nucleotide sequence of putative desaturase was obtained by aligning the 5' and 3' RACE PCR fragment sequences.

2.2 Heterologous expression of fish desaturase ORFs in Saccharomyces cerevisae

In order to functionally characterise the genes, the open reading frames (ORFs) of the fish putative fatty acid desaturase sequences were restriction mapped to allow primers to be designed with appropriate restriction sites to aid correct orientation of insertion into the pYES2 vector (Table1). PCR was performed using High Fidelity DNA polymerase (Roche Diagnostics Ltd., Lewes, East Sussex, UK). Amplification involved an initial denaturation step at 94 $^{\circ}$C for 1 min, followed by 30 cycles of denaturation at 94 $^{\circ}$C for 30 s, annealing at 55 $^{\circ}$C (for trout, seabream and carp) and 52 $^{\circ}$C (for turbot) respectively for 30 s, and extension at 72 $^{\circ}$C for 1min 30 s. The final extension was at 72 $^{\circ}$C for 7 min. It should be noted that an additional round of PCR was required to clone the turbot desaturase. This may be due to low copy number in the turbot liver RNA preparation which was produced from fish fed a commercial fish oil and meal based diet, in comparison to the sea bream RNA which was prepared from fish fed a HUFA-deficient diet (Seiliez et al., 2003). For this additional first round PCR, primers were designed so that the first round PCR product included the open reading frame of the putative desaturase gene; sense primer, TDF1, 5'- CAG ACA CAG CAG TGA GGT GAA –3'; Antisense primer, TDR1, 5'- GAT GCG GAA GAG CAG AAA AC –3'. PCR conditions were as above mentioned but with 25 cycles.

Following PCR, the DNA fragments were restricted with the appropriate enzymes, *HindIII* and *XhoI* or *NotI,* and ligated into a similarly digested yeast expression vector pYES2 (Invitrogen Ltd, Paisley, UK). These were then used to transform Top10F' *E. coli* competent cells (Invitrogen Ltd, Paisley, UK) which were screened for the presence of recombinant plasmids. The recombinant plasmid DNAs were sequenced and the best one was used for transformation. 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 desaturase/pYES2 constructs was on *Saccharomyces cerivisiae* 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). Cultures were supplemented with one of the PUFA substrates from among the following; α-linolenic acid (18:3n-3), linoleic acid (18:2n-6), eicosatetraenoic acid (20:4n-3), dihomo-γ-linolenic acid (20:3n-6), docosapentaenoic acid (22:5n-3) and docosatetraenoic acid (22:4n-6), Yeast cells were harvested, washed and dried under oxygen-free nitrogen as described previously (Hastings et al., 2001), Lipid was extracted by homogenisation in chloroform/methanol (2:1) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, essentially according to Folch et al. (1957), and fatty acid methyl esters (FAME) prepared by addition of 1 ml toluene and 2 ml 1% methanolic sulphuric acid and incubation overnight at 50°C (Christie 1982).   The FAME were 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 acid converted to the desaturated fatty acid product was calculated from the gas chromatograms as $100 \times$ [product area/(product area + substrate area)]. Unequivocal confirmation of fatty acid products was obtained by GC-mass spectrometry of the picolinyl derivatives as described in detail previously (Hastings et al., 2001).

2.3. Materials

Eicosatetraenoic (20:4n-3), docosapentaenoic (22:5n-3) and docosatetraenoic (22:4n-6) acids (all > 98-99% pure) were purchased from Cayman Chemical Co., Ann Arbor, U.S.A. Linoleic (18:2n-6), α-linolenic (18:3n-3), eicosatrienoic (20:3n-6) acids (all >99% pure), BHT, 1,1'-carbonyldiimidazole, 2,2-dimethoxypropane, fatty acid-free BSA, galactose, 3-(hydroxymethyl)pyridine, HBSS, nitrogen base, raffinose, tergitol NP-40 and uracil dropout medium were obtained from Sigma Chemical Co. Ltd., Dorset, UK. TLC (20 x 20 cm x 0.25 mm) plates 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

Sequence information for the trout and seabream cDNAs has been published previously showing that they included open reading frames (ORFs) of 1365 and 1338 bps (including stop codons) encoding peptides of 454 and 445 amino acids, respectively (Seiliez et al., 2001,2003). Sequencing of the carp and turbot desaturase cDNAs showed that they included ORFs of 1335 and 1338 bps (including stop codons), respectively, specifying proteins of 444 and 445 amino acids (Fig.2). As with the trout and seabream proteins, the protein sequences of the carp and turbot putative desaturases possessed all the characteristic features of microsomal fatty acid desaturases, including three histidine boxes and two transmembrane regions, and N-terminal cytochrome b_5 domains containing the haem-binding motif, H-P-G-G, similar to that of other fatty acid desaturases including the previously cloned and characterised desaturases of zebrafish (GenBank accession no. AF309556), salmon (Genbank accession number AF478472), and the human Δ5 (GenBank accession no. AF126799) and Δ6 (GenBank accession no. AF199596) genes.

Phylogenetic analysis comparing all the putative desaturase sequences cloned from fish along with a variety of Δ5 and Δ6 desaturases from human, mouse, fungus and nematode, clustered all the

fish sequences together and showed they were more similar to the mammalian desaturase sequences than those of yeast and the nematode (Fig.3). The fish desaturases were also more closely related to the mammalian $\Delta 6$ desaturase sequences than mammalian $\Delta 5$ sequences. The salmonid sequences clustered together as did the two marine fish sequences from turbot and seabream. Interestingly, the Nile tilapia sequence clustered closer to the marine fish than to the other freshwater fish, carp and zebrafish, which clustered together.

3.2. Functional characterisation

The fish desaturase cDNAs were functionally characterized by determining the fatty acid profiles of transformed *S. cerevisiae* containing either the pYES vector alone or the vector containing the fish desaturase cDNA inserts, grown in the presence of a variety of possible fatty acid substrates including $\Delta 6$ substrates (18:3n-3 and 18:2n-6), $\Delta 5$ substrates (20:4n-3 and 20:3n-6) and $\Delta 4$ substrates (22:5n-3 and 22:4n-6) (see Fig.1). The fatty acid composition of the yeast transformed with the pYES 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 $\Delta 6$, $\Delta 5$ or $\Delta 4$ fatty acid desaturase activities. With all the fish fatty acid desaturase cDNAs investigated, the most prominent additional peaks were observed in the profile of transformed yeast grown in the presence of the $\Delta 6$ desaturase substrates, 18:3n-3 and 18:2n-6 (Table 2). GC traces showing the fatty acid composition of the yeast transformed with the pYES vector containing the putative fatty acid desaturase cDNA inserts grown in the presence of 18:3n-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 (18:3n-3, peak 5) the traces show an additional peak (peak 6) (Fig.4). Based on GC retention time and confirmed by GC-MS, the additional peak associated with the presence of the fish desaturase cDNAs was identified as 18:4n-3, corresponding to the $\Delta 6$ desaturation product of 18:3n-3 (Fig.4). Similarly retention time and GC-MS analysis confirmed the additional peak observed in yeast transformed with the pYES vector containing the putative fatty acid desaturase cDNA inserts grown in the presence of 18:2n-6 as the $\Delta 6$ desaturation product 18:3n-6 (data not shown). All the desaturase cDNA products were more active towards the n-3 substrate with 59.5%, 31.5%, 23.1% and 7.0% of 18:3n-3 being converted to 18:4n-3 in the case of turbot, trout, seabream and carp, respectively. In comparison, the amount of conversion of 18:2n-6 was half that of 18:3n-3 in turbot and sea bream, and even lower in trout and carp. None of the fish desaturase cDNAs expressed any $\Delta 4$ desaturase activity as evidenced by the lack of any additional peaks representing desaturated products of 22:5n-3 or 22:4n-6 being observed. However, very small additional peaks representing

desaturated fatty acid products, as confirmed by GC-MS, were observed with all the fish desaturase cDNAs when the transformed yeast was incubated with 20:4n-3 (Fig.5), and also 20:3n-6 in the cases of carp and seabream (Table 2). Overall, therefore, the results showed that all four fish desaturase cDNAs encoded enzymes that were essentially Δ6 desaturases. The enzymes also showed very low levels of Δ5 desaturase activity, but none of the enzyme products showed Δ4 desaturase activity.

4. Discussion

Prior to the present work, there were several fish desaturase cDNAs listed in the Genbank database but only two of them had been functionally characterised. These were the novel, bifunctional zebrafish cDNA showing both Δ6 and Δ5 desaturase activity (Hastings et al., 2001) and an Atlantic salmon desaturase that was shown to be predominantly an n-3 Δ5 desaturase (Hastings et al., 2004). That neither of these cDNAs were unifunctional Δ6 desaturases was surprising as they both had closer sequence homology to mammalian Δ6 desaturases than to Δ5 desaturases (Hastings et al., 2001, 2004). Therefore, there was a gap in our knowledge in that single function Δ6 desaturases had not been identified in fish, which has particular importance as Δ6 desaturase is regarded as the rate-limiting step in the HUFA synthesis pathway, at least in humans and probably mammals in general (Horrobin, 1993). The present work has significantly increased our knowledge of PUFA desaturases in fish. Functional characterisation by heterologous expression of four fish desaturase cDNA clones in the yeast *S. cerevisiae* has clearly demonstrated that they are all essentially single function Δ6 desaturase enzymes. This was consistent with all of the fish cDNAs having close homology with mammalian Δ6 desaturases. However, they also have close homology with the zebrafish and Atlantic salmon desaturases previously characterised (Hastings et al., 2001,2004).

The fish desaturase clones also showed measurable, but very low, levels of Δ5 activity. Unlike the zebrafish desaturase, which showed very significant Δ5 desaturase activity at around 70% of the Δ6 activity (Hastings et al., 2001), the n-3 Δ5 activities in trout, seabream and turbot cDNA products were only 0.6%, 0.9% and 0.2%, respectively, of the Δ6 activities. In carp, the desaturase activity towards 20:4n-3 was about 7% of the activity towards 18:3n-3 but this was from a much lower overall level. Therefore, it is possible that the levels of Δ5 desaturase activity measured are of limited physiological significance, particularly if any of these species also have separate Δ5 desaturases yet to be isolated. This is certainly the likely scenario in the case of the freshwater species carp and trout, that are known to thrive on diets containing only 18:2n-6 and 18:3n-3 as the

source of essential fatty acids (Sargent et al., 2002; Tocher, 2003). An early *in vivo* study showed that trout produced radiolabelled EPA and, especially, DHA from ^{14}C-labelled 18:3n-3 given in a food pellet (Owen et al., 1975). *In vitro* studies with trout cell cultures including both primary cultures of brain astrocytes (Tocher and Sargent, 1990a) and skin fibroblasts (Ghioni et al., 1997), and an established cell line, RTG-2 (Tocher and Sargent, 1990b), also clearly demonstrated that labelled EPA (and indeed DHA) was produced from $[1-^{14}C]18:3n-3$, indicating the presence of significant $\Delta 5$ desaturase activity. Similar cell culture studies with a carp cell line, EPC cells, showed that almost 22% of added $[1-^{14}C]18:3n-3$ was converted to $\Delta 5$-desaturated products and that culture of the cells in essential-fatty acid deficient medium doubled the recovery of radioactivity as $\Delta 5$-desaturated products (Tocher and Dick, 1999).

The situation is different with the marine fish where it is known that these species require dietary long-chain n-3HUFA such as EPA and DHA for optimal growth and survival (Sargent et al., 2002). In the same early *in vivo* study that showed about 80% recovery of radioactivity from $[1-^{14}C]18:3n-3$ as $\Delta 5$-desaturated products in trout, no radiolabelled EPA or DHA were produced from ^{14}C-labelled 18:3n-3 fed to turbot (Owen et al., 1975). A later study, using more sensitive techniques showed that only 2.6% of intraperitoneally-injected $[1-^{14}C]18:3n-3$ was recovered in $\Delta 5$-desaturated products in juvenile gilthead seabream (Mourente and Tocher, 1993). Thus, the *in vivo* biochemical studies supported the nutritional trials and implicated deficiencies in the fatty acid desaturation/elongation pathway as being central to the dietary requirement for HUFA in marine fish. Cell culture studies dissecting HUFA synthesis in fish using radiolabelled fatty acid substrates for individual desaturation and elongation steps have identified primary deficiencies in the pathway at the $\Delta 5$ desaturase in a seabream cell line (SAF-1) (Tocher and Ghioni, 1999), whereas only the C_{18-20} elongase was deficient in turbot (TF) cells which appeared to have a very active $\Delta 5$ desaturase (Ghioni et al., 1999). The results described here can be seen as consistent with these earlier findings. The seabream desaturase clone was essentially a $\Delta 6$ desaturase, but did show residual $\Delta 5$ activity that could, perhaps, account for the very low levels of $\Delta 5$ desaturation measured previously in both *in vivo* and cell culture studies (Mourente and Tocher, 1993, 1994; Tocher and Ghioni, 1999). However, although the molecular data showed the seabream $\Delta 6$ desaturase had some $\Delta 5$ activity in the yeast expression system, consistent with the earlier injection and cell studies, this activity must be too low to be of physiological significance *in vivo*. Similarly, the turbot $\Delta 6$ desaturase cloned here showed only a trace level of $\Delta 5$ activity. Taken together, the molecular data presented here and the enzymological data from earlier cell studies suggests that turbot must have a separate $\Delta 5$ desaturase enzyme, if the cell line truly reflects the *in vivo* situation in turbot.

None of the fish Δ6 desaturases showed any Δ4 desaturase activity. This was as expected based upon the functional characterisation of the previously reported fish desaturase genes, the zebrafish Δ6/Δ5 and the salmon Δ5, and all mammalian Δ6 and Δ5 desaturases reported to date (Hastings et al., 2001,2004; Pereira et al., 2003). That no Δ4 desaturase has been cloned or characterised in higher animals is, therefore, consistent with the hypothesis that the synthesis of DHA from EPA in both mammals and fish proceeds via elongation to 24:5n-3 followed by a Δ6 desaturation rather than via Δ4 desaturation of 22:5n-3 (Buzzi et al., 1996,1997; Wallis et al., 2002). Heterologous expression studies on the human and rat Δ6 desaturases have provided evidence that the same enzymes are active on C_{18} and C_{24} substrate fatty acids (deAntueno et al., 2001; D'Andrea et al., 2002). Similarly, we found that the bifunctional zebrafish desaturase was also capable of desaturating C_{24} fatty acid substrates (Tocher et al., 2003). It will be interesting to determine the activities of all animal Δ6 desaturases towards the C_{24} fatty acid substrates. In contrast to the higher animals, the Δ4 pathway for the production of DHA appears to operate in some lower organisms, based on the cloning and characterisation of Δ4 desaturases from *Thraustchytrium* sp. (Qui et al., 2001) and the algae *Euglena gracilis* (Meyer et al., 2003) and *Pavlova lutheri* (Tonon et al., 2003).

The present study clearly showed that all four desaturase genes showed a marked fatty acid preference for the n-3 substrate, 18:3n-3. A similar preference for n-3 fatty acid substrates was observed upon heterologous expression in yeast of the previously cloned zebrafish and salmon desaturases (Hastings et al., 2001, 2004). These data are entirely consistent with the results obtained in several earlier enzymological studies investigating the desaturation of ^{14}C-labelled fatty acid substrates in isolated hepatocytes (Bell et al., 1997), primary cell cultures (Tocher and Sargent, 1990a) and fish cell lines (Tocher and Sargent, 1990b). Previously however, functional characterisation of Δ6 desaturases from nematode, mammals, fungi, mosses and higher plants revealed that there was no distinct preference for either 18:3n-3 or 18:2n-6 substrates, but recently Δ6 desaturases have been identified in *Primula* sp. which have n-3 substrate preferences (Sayanova et al., 2003). It is perhaps pertinent to note that these data are to some extent only semi-quantitative as there are differences between fatty acids in their uptake into *S. cerevisiae* in terms of chain length (de Antueno et al., 2001), although there is no evidence, and no indication from our own studies, for any preference in uptake between PUFA of similar chain length, such as 18:2n-6 and 18:2n-3. In comparing desaturases between species, or different desaturases within a species, it is also important to note that, despite using the same expression vector, absolute equivalence in the levels of expressed desaturases cannot be guaranteed without enzyme-specific antibodies and Western analyses.

The phylogenetic sequence analyses grouped the fish desaturases largely as expected based on classical phylogeny with the carp and zebrafish (Ostariophysi; cyprinids), trout and salmon

(Salmoniformes; salmonidae), and tilapia, sea bream and turbot (Acanthopterygia; cichlids, perciformes and pleuronectiformes) appearing in three distinct clusters (Nelson, 1994). However, the cloning of the carp and turbot desaturases has given additional sequence information that has indicated that there are differences in fish desaturase sequences that may be considered as general trends. For instance, the data support the previously reported observation that salmonid desaturase genes appear to be different to the desaturases of other fish and mammalian desaturase genes in that they contain additional amino acids, having chain lengths of 454 aa (or 452 as in cherry salmon desaturase 2) compared to 444 for the cyprinid (carp and zebrafish) and human desaturases (Seiliez et al., 2001; Hastings et al., 2004). Furthermore, it was previously reported that the seabream desaturase clone encoded a protein of 445 amino acids which was one more residue than observed with the cyprinids and human desaturases (Seiliez et al., 2003). The turbot desaturase clone reported in the present study also encoded a protein of 445 amino acids suggesting that the additional residue may be a more general feature of fish in the phylogenetic group of percomorpha (Acanthopterygia), which includes tilapia. Although these differences are of phylogenetic interest, it is noteworthy that the differences in polypeptide size were not related to function of the desaturase enzymes. No clear pattern has emerged between chain length and function with desaturases cloned from mammals, fungus and nematodes, with $\Delta6$ in human, rat and mouse and $\Delta5$ in human all having 444 aa, $\Delta5$ in rat, mouse and *C. elegans* all having 447 aa, *C.elegans* $\Delta6$ having 443 aa, and *M. alpina* having one $\Delta6$ with 457 aa, another $\Delta6$ with 458 aa, and $\Delta5$ with 446 aa (Napier et al., 2003; Sperling et al., 2003).

Desaturases are a superfamily of enzymes that are all able to activate oxygen and use it to modify C-H bonds in diverse substrates including alkyl groups, acyl residues in amide-, thio-, or oxygen-ester linkage, carotenoids, sphingolipids, aldehydes and sterols (Sperling et al., 2003). The cloning of fatty acid desaturases from a range of organisms throughout the animal and plant kingdoms, and of varying evolutionary histories, has contributed to a greater understanding of the evolution of desaturases both in phylogenetic (Sperling et al., 2003) and mechanistic (Behrouzian and Buist, 2002,2003) terms. One important question is whether different desaturases with distinct $\Delta6$ and $\Delta5$ specificities arose by loss of activity from an ancestral progenitor desaturase possessing both activities, or by gene duplication and divergence from a unifunctional progenitor (Napier et al., 2003). In this respect, we had therefore speculated that the bifunctional zebrafish desaturase may represent a prototypic vertebrate enzyme (Hastings et al., 2001). However, the cloning and characterisation in the present study of a fatty acid desaturase from carp, phylogenetically closely related to zebrafish, was particularly interesting as it was basically unifunctional. Therefore, it is unclear whether the zebrafish desaturase represents an ancestral progenitor or a later evolutionary

adaptation. This question may remain unresolved unless other bifunctional desaturases are identified.

Pertinent to the above discussion, further fatty acid desaturase genes almost certainly remain to be identified in the fish species studied. For instance, evidence was presented recently that rat $\Delta 6$ desaturation in rat hepatocytes were catalysed by more than one $\Delta 6$ desaturase isozyme (Inagaki et al., 2003). When expression of the previously identified $\Delta 6$ desaturase gene was completely inhibited by hyperexpression of the corresponding antisense gene in the rat hepatic cell line, the conversion rates of 18:2n-6, 18:3n-3 and C_{24} substrates were all similar to control cells.

In conclusion, the present study has identified and characterised $\Delta 6$ desaturase genes in both freshwater and marine fish species. Some of these had measurable, but very low, levels of $\Delta 5$ desaturase activity of questionable physiological significance. However, although further fatty acid desaturase genes are likely to be identified in these species, the desaturases already cloned from fish species including carp, trout, turbot, sea bream and salmon is important as these are species in which there is a relative wealth of data on the nutritional regulation of fatty acid desaturation and HUFA synthesis, and in which substantive differences occur between species (Sargent et al., 2002; Tocher, 2003). Gene expression studies have shown that transcriptional regulation of desaturase occurs in fish (Seiliez et al., 2001,2003; Zheng et al., 2004), but further studies are in progress to characterise the factors such as SREBP-1c and PPARs that are likely to be important in controlling the regulation of these enzyme activities in fish (Nakamura and Nara, 2002,2003).

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Legends to Figures

Fig.1. Pathways of highly unsaturated fatty acid (HUFA) biosynthesis from the C_{18} 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. $\Delta 6$, $\Delta 5$ and $\Delta 4$, fatty acid desaturases; Elo, fatty acid elongases; Short, peroxisomal chain shortening.

Fig.2. Comparison of the deduced amino acid sequence of polyunsaturated fatty acyl desaturases from carp, rainbow trout, sea bream and turbot with that of desaturases from zebrafish, salmon and human. 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%. The dotted line denotes the cytochrome b_5 domain, with asterisks above to indicate the haem-binding motif. The dashed line denotes the two transmembrane regions, and the solid underlines the three histidine boxes.

Fig.3. Phylogenetic tree of desaturases from carp, rainbow trout, sea bream and turbot, other fish species (zebrafish, salmon and tilapia), mammals (mouse and human), fungus (*Mortierella alpina*) and nematode (*Caenorhabditis elegan*s). The tree was constructed using the N-J method using *CLUSTALX* and *NJPLOT*. The horizontal branch length is proportional 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 desaturation products in transgenic yeast (*Saccharomyces cerevisiae*) grown in the presence of 18:3n-3. Fatty acids were extracted from yeast transformed with pYES vector without insert (A) or containing the putative fatty acid desaturase cDNA inserts from carp (B), rainbow trout (C), seabream (D) and turbot (E). The first four peaks in panels A-E are the main endogenous fatty acids of *S. cerevisiae*, namely 16:0 (1), 16:1n-7 (2), 18:0 (3) and 18:1n-9 (with 18:1n-7 as shoulder) (4). The next peak (5) in each panel is the exogenously added substrate fatty acid, 18:3n-3, and the final peak (6) is the resultant desaturated product, namely 18:4n-3. Vertical axis, FID response; horizontal axis, retention time.

Fig.5. Identification of fatty acid desaturation products in transgenic yeast (*Saccharomyces cerevisiae*) grown in the presence of 20:4n-3. Fatty acids were extracted from yeast transformed with pYES vector without insert (A) or containing the putative fatty acid desaturase cDNA inserts from carp (B), rainbow trout (C), seabream (D) and turbot (E). The first four peaks in panels A-E

are the main endogenous fatty acids of *S. cerevisiae*, namely 16:0 (1), 16:1n-7 (2), 18:0 (3) and 18:1n-9 (with 18:1n-7 as shoulder) (4). The next peak (5) in each panel is the exogenously added substrate fatty acid, 20:4n-3, and the final peak (6) is the resultant desaturated product, namely 20:5n-3. Vertical axis, FID response; horizontal axis, retention time.

Table 1. List of primers used for heterologous expression

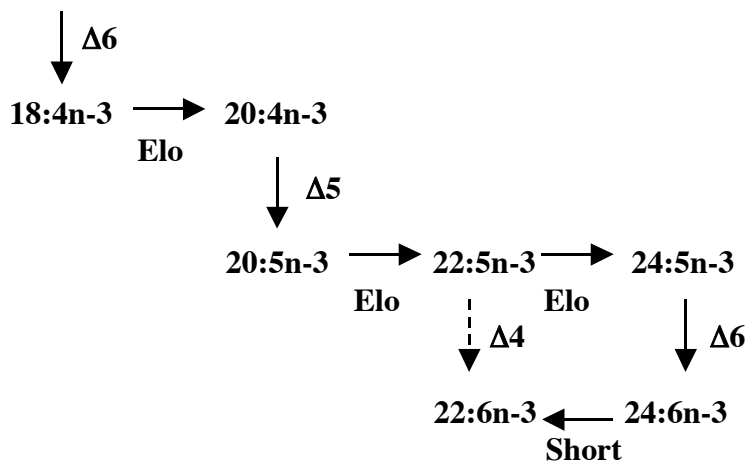| Fish species	Primer	Primer name	Restriction enzyme	Oligonucleotide sequence
Rainbow Trout	Sense	OMDES1S	HindIII	5'-CGG AAT TC<u>A AGC T T</u>A AGA TGG GGG GCG GAG GTC A-3'
	Antisense	OMDES1A	*XhoI*	5'- GCT CTA GA<u>C TCG AG</u>T TAT TTA TGG AGA TAC GCA TC-3'
Gilthead seabream	Sense	SAUDES1S	HindIII	5'-CGG AAT TC<u>A AGC T</u> T<u>A</u> AGA TGG GAG GTG GAG GCC A-3'
	Antisense	SAUDES1A	*XhoI*	5'- GCT CTA GA<u>C TCG AG</u>T CAT TTA TGG AGA TAA GCA TC-3'
Carp	Sense	ZF2AHindIII	HindIII	5'- CCC <u>AAG</u> <u>CTT</u> ACT ATG GGT GGC GGA GGA CAG -3'
	Antisense	CpRevNotI	Not I	5'- ATA GTT TA<u>G CGG CCG</u> CTT ATT CGT TGA GGT ACG C -3'
Turbot	Sense	TURBPYESFOR3	HindIII	5'- CGG AAT TC<u>A AGC T</u> T<u>A</u> AGA TGG GAG GTG GAG GCC A -3'
	Antisense	TURBPYESREV2	*XhoI*	5'- GCT CTA GA<u>C TCG AG</u>T CAT TTA TGG AGA TAT GCA TC -3'

Table 2. Functional characterisation of fish fatty acid desaturase cDNA clones in the yeast *Saccharomyces cerevisiae* .

Desaturase activity	PUFA substrates	PUFA products	Conversion rate			
			Common Carp	Rainbow Trout	Gilthead Seabream	Turbot
$\Delta6$	18:3n-3	18:4n-3	7.0	31.5	23.1	59.5
$\Delta6$	18:2n-6	18:3n-6	1.5	3.6	12.2	31.2
$\Delta5$	20:4n-3	20:5n-3	0.5	0.2	0.2	0.1
$\Delta5$	20:3n-6	20:4n-6	0.4	0.0	0.1	0.0
$\Delta4$	22:5n-3	22:6n-3	0.0	0.0	0.0	0.0
$\Delta4$	22:4n-6	22:5n-6	0.0	0.0	0.0	0.0

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

Fig.1

18:3n-3

   ↓ Δ6

18:4n-3 ⟶ 20:4n-3
    Elo

             ↓ Δ5

          20:5n-3 ⟶ 22:5n-3 ⟶ 24:5n-3
              Elo   Elo

                       ↓ Δ4   ↓ Δ6

                22:6n-3 ⟵ 24:6n-3
                       Short

18:2n-6

   ↓ Δ6

18:3n-6 ⟶ 20:3n-6
    Elo

             ↓ Δ5

          20:4n-6 ⟶ 22:4n-6 ⟶ 24:4n-6
              Elo   Elo

                       ↓ Δ4   ↓ Δ6

                22:5n-6 ⟵ 24:5n-6
                       Short

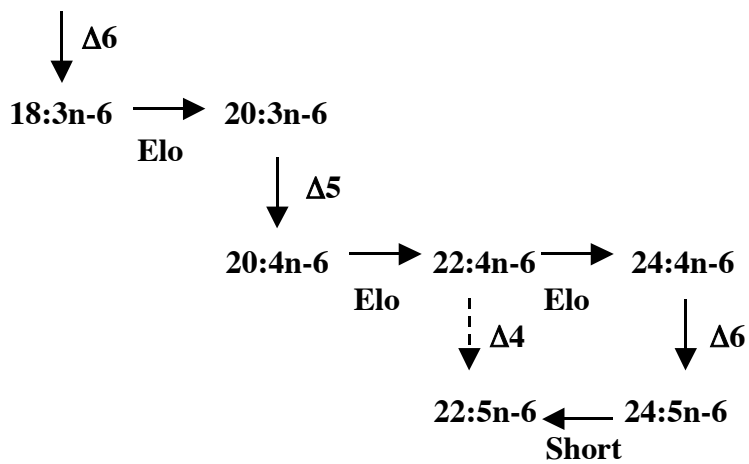**Fig.2.**

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                                                                            ****
Turbot D6            MGGGGQLTEQGET---------GSKRAGCVYTWEEVQSHSSRTDQWLVIDRKVYNTTQWAKRHPGGFHVI 61
Gilthead seabream D6 MGGGGQLTEPGEP---------GSRRAGGVYTWEEVQSHSSRNDQWLVIDRKVYNVTKWAKRHPGGFRVI 61
Rainbow trout D6     MGGGGQQTESSEPAKGDGVGPDGGRGGSAVYTWEEVQKHCHRSDKWLVIDRKVYNITQWAKRHPGGIRVI 70
Common carp D6       MGGGGQQTDRITG----------TNGRFGTYTWEEVQKHTKFGDQWIEVERKVYNVSQWVKRHPGGVRIL 60
Atlantic salmon D5   MGGGGQQTESSEPAKGDGLEPDGGQGGSAVYTWEEVQRHSHRSDQWLVIDRKVYNITQWAKRHPGGIRVI 70
Zebrafish D5/D6      MGGGGQQTDRITD----------TNGRFSSYTWEEVQKHTKHGDQWVVVERKVYNVSQWVKRHPGGLRIL 60
Human D6             MGKGGNQGEGAAER---------EVSVPTFSWEEIQKHNLRTDRWLVIDRKVYNITKWSIQHPGGQRVI 60
Human D5             MAPDPLAAETAAQG----------LTPRYFTWDEVAQRSGCEERWLVIDRKVYNISEFTRRHPGGSRVI 59

Turbot D6            SHYAGQDATEAFTAFHPDLKFVQKFLKPLLIGELAATEPSQDRNKNAALVQDFHTLFVKAESKGLFQARP 131
Gilthead seabream D6 NHYAGEDATEAFTAFHPDLKFVQKFLKPLLIGELAATEPSQDRNKNAAVIQDFHTLRAQAESDGLFRAQP 131
Rainbow trout D6     SHFAGEDATDAFVAFHPDPNFVRKFLKPLLIGELATTEPSQDHGKNAVLVQDFQALRDRVEREGLLRARP 140
Common carp D6       GHYAGEDATEAFTAFHPDLPLVRKYMKPLLIGELEASEPSQDRQKNAALVEDFRALRERLEAEGCFKTQP 130
Atlantic salmon D5   SHFAGEDATEAFSAFHLDANFVRKFLKPLLIGELAPTEPSQDHGKNAALVQDFQALRDHVEREGLLRARL 140
Zebrafish D5/D6      GHYAGEDATEAFTAFHPNLQLVRKYLKPLLIGELEASEPSQDRQKNAALVEDFRALRERLEAEGCFKTQP 130
Human D6             GHYAGEDATDAFRAFHPDLEFVGKFLKPLLIGELAPEEPSQDHGKNSKITEDFRALRKTAEDMNLFKTNH 130
Human D5             SHYAGQDATDPFVAFHINKGLVRKYMNSLLIGELSPEQPSFEPTKNKELTDFRELRATVERMGLMKANH 129

Turbot D6            LFFCLHLGHIVLLEALAWLIIWVWGTNWILTFLCALLMTIAQSQAGWLQHDFGHLSVFKQSPWNHLLQKF 201
Gilthead seabream D6 LFFSLHLGHILLLEALAWLIIWLWGTSWTLTFLISIILATAQTQAGWLQHDFGHLSVFKKSSWNHILHKF 201
Rainbow trout D6     LFFSLYLGHILLLEALALGLLWVWGTSWSLTLLCSLMLATSQSQAGWLQHDYGHLSVCKTSSWNHVLHKF 210
Common carp D6       LFLILHLSHILLLEAIALMMVWYLGTGWINTAIVAVLLATAQSQAEWLQHDFGHLSVFKTSPWNHLVHKF 200
Atlantic salmon D5   LFFSLYLGHILLLEALALGLLWVWGTSWSLTLLCSLMLATSQQAGWLQHDYGHLSVCKKSSWNHKLHKF 210
Zebrafish D5/D6      LFFALHLGHILLLEAIAFMMVWYFGTGWINTLIVAILLATAQSQAGWLQHDFGHLSVFKTSGMNHLVHKF 200
Human D6             VFFLLLLAHIIALESIAWFTVFYFGNGWIPTLITAFVLATSQAQAGWLQHDYGHLSVYRKPKWNHLVHKF 200
Human D5             VFFLLYLLHILLLDGAAWLTLWVFGTSFLPFLLCAVLLSAVQAQAGWLQHDFGHLSVFSTSKWNHLLHHF 199

Turbot D6            AIGHLKGASANWWNHRHFQHHARTNIFRKDPDVNMLN-IFVIGATQPVEYGVKKIKHMPYHRQHQYFFLV 270
Gilthead seabream D6 VIGHLKGASANWWNHRHFQHHAKPNIFSKDPDVNMLH-IFVLGDTQPVEYGIKKIKYLPYHQHQYFLLV 270
Rainbow trout D6     VIGHLKGASANWWNHRHFQHHAKPNVFSKDPDVNSLH-VFVLGDKQPVEYGIKKLKYMPYHQHQYFFLI 279
Common carp D6       VVGHLKGASAGRWNHRHFQHHAKPNVFKKDPDVNMLN-AFVAGKVQPVEYGVKKIKHLPYHQHKYFFFI 269
Atlantic salmon D5   VIGHLKGASANWWNHRHFQHHAKPNVFRKDPDINSLP-VFVLGDTQPVEYGIKKLKYMPYHQHQYFFLI 279
Zebrafish D5/D6      VIGHLKGASAGWWNHRHFQHHAKPNIFRKDPDVNMLN-AFVVGNVQPVEYGVKKIKHLPYHQHKYFFFI 269
Human D6             VIGHLKGASANWWNHRHFQHHAKPNIFHKDPDVNMLH-VFVLGEWQPIEYGKKKLKYLPYHQHEYFFLI 269
Human D5             VIGHLKGAPASWWNHQMHFQHHAKPNCFRKDPDINMHPFFFALGKILSVELGKQKKNYMPYNWQHKYFFLI 269

Turbot D6            GPPLLIPVYFQMQLMNSIISRHDWVDLGWSMSYYLRFFCCYIPMYGLFGSVALIIFVRFLESHWFVWVTQ 340
Gilthead seabream D6 GPPLLIPVYFHIQIIRTMISRHDWVDLAWSMSYYLRYLCCYVPLYGLFGSVALISFVRFLESHWFVWVTQ 340
Rainbow trout D6     GPPLVIPVFFTIQIFQTMFSQRNWVDLAWAMTFYLRFFCCYPFFGFGSVALISFVRFLESHWFVWVTQ 349
Common carp D6       GPPLLIPVYFQFQIFHNMIAHGLWVDLAWCISYYVRYFLCYTQYYGVFWAVILFNFVRFLKSHWFVWVTQ 339
Atlantic salmon D5   GPPLIVPVFFNIQIFRTMFSQRDWVDLAWSMSFYLRFFCCYPFFGFGSVALISFVRFLESHWFVWVTQ 349
Zebrafish D5/D6      GPPLLIPVYFQFQIFHNMISHGMWVDLLWCISYYVRYFLCYTQFYGVFWAIILFNFVRFMESHWFVWVTQ 339
Human D6             GPPLLIPMYFQYQIIMTMIVHKNWVDLAWAVSYYIRFFITYIPFYGILGALLLFNFIRFLESHWFVWVTQ 339
Human D5             GPPALLPLYFQWYIFYFVIQRRKWVDLAWMITFYVRFFLTYVPLLGLKAFLGLFFIVRFLESNWFVWVTQ 339

Turbot D6            MNHLPMDIDHEKHKDWLTMQLQATCNIEQSFFNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPQVRALCAK 410
Gilthead seabream D6 MNHLPMDIDHEKHKDWLTMQLQATCNIEKSVFNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVHALCEK 410
Rainbow trout D6     MNHLPMEIDHERHQDWLTMQLSATCNIEQSTFNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVRALCAK 419
Common carp D6       MSHIPMQIDYEKHQDRLSMQLVATCNIEQSTFNDWFSGHLNFQIEHHLFPTMPRHNYWRAAPHVRELCAK 409
Atlantic salmon D5   MNHLPMEMDHERHQDWLTMQLSATCNIEQSTFNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVRTLCEK 419
Zebrafish D5/D6      MSHIPMNIDYERKNQDWLSMQLVATCNIEQSAFNDWFSGHLNFQIEHHLFPTVPRHNYWRAAPRVRALCEK 409
Human D6             MNHIVMEIDQEAYRDWFSSQLTATCNVEQSFFNDWFSGHLNFQIEHHLFPTMPRHNLHKIAPLVKSLCAK 409
Human D5             MNHIPMHIDHDRNMDWVSTQLQATCNVHKSAFNDWFSGHLNFQIEHHLFPTMPRHNYHKVAPLVQSLCAK 409

Turbot D6            YGITYQVKTMWQGLADVFRSLKTSGELWLDAYLHR 445
Gilthead seabream D6 HGIPYQVKTMWQGIVDVIRSLKNSGDLWLDAYLHR 445
Rainbow trout D6     HGLPYQVKTLQKAIIDVVGSLKKSGDLWLDAYLHR 454
Common carp D6       YGIKYQBKTLQGAFADVVRSLEKSGEIWLDAYLNE 444
Atlantic salmon D5   HGVPYQVKTLQKGMTDVVRSLKKSGDLWLDAYLHR 454
Zebrafish D5/D6      YGVKYQBKTLYGAFADIIRSLEKSGELWLDAYLNK 444
Human D6             HGIEYQBKPLLRALLDIIRSLKKSGKLWLDAYLHR 444
Human D5             HGIEYQSRPLLSAFADIIHSLKESGCQLWLDAYLHQ 444
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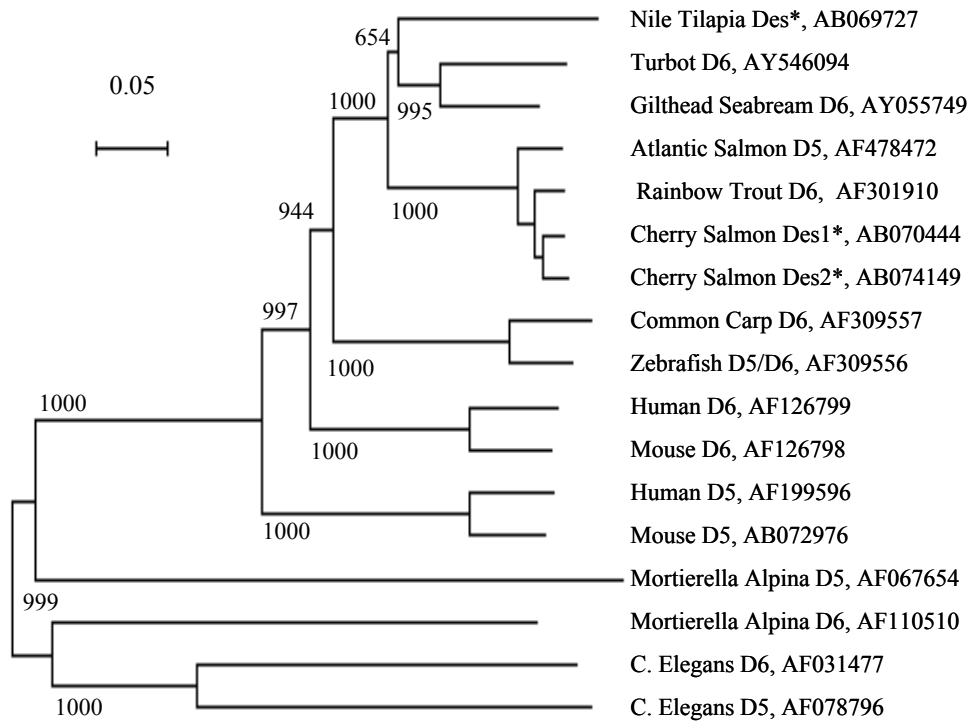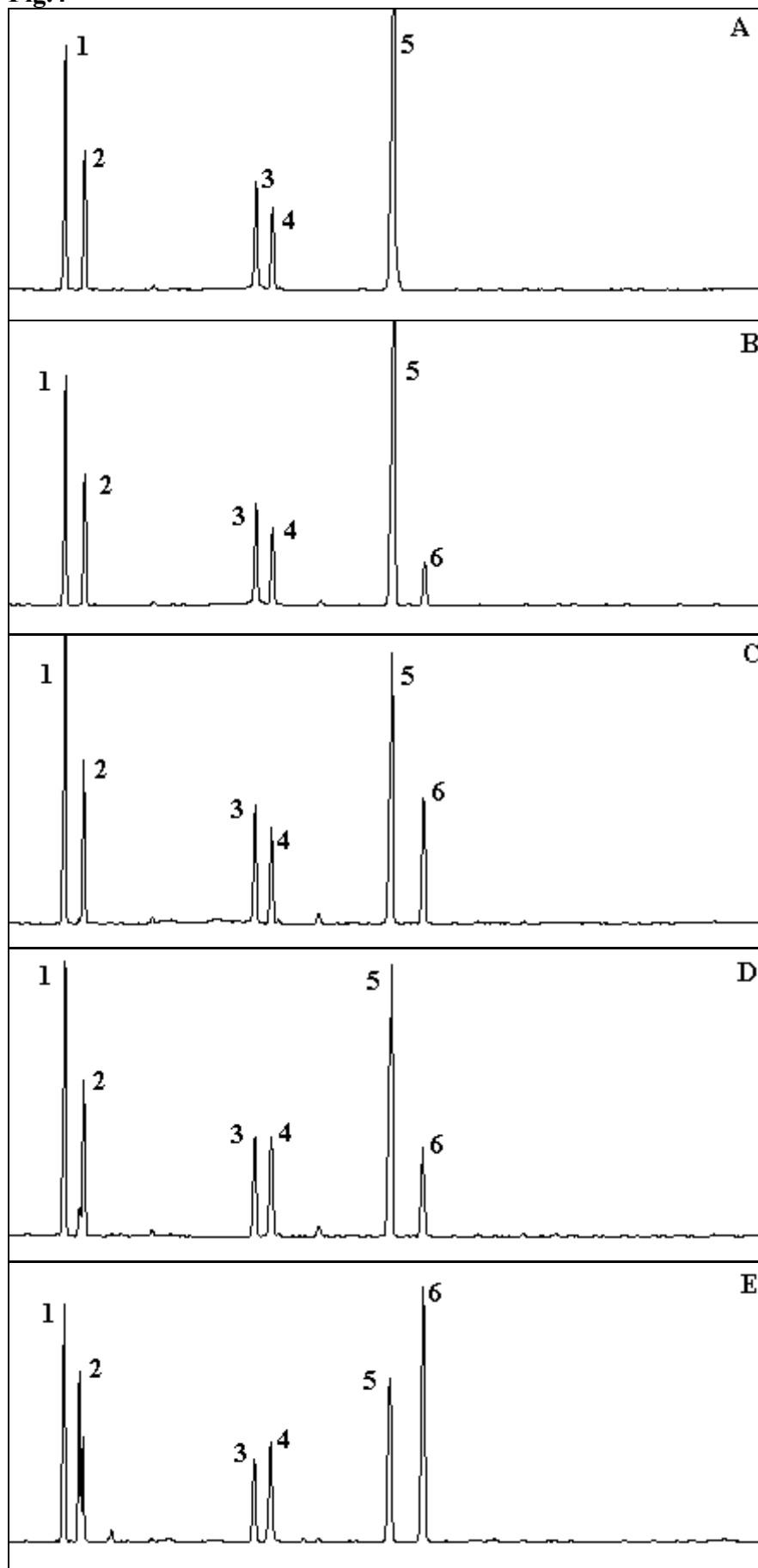
Fig. 3

0.05

Nile Tilapia Des*, AB069727
Turbot D6, AY546094
Gilthead Seabream D6, AY055749
Atlantic Salmon D5, AF478472
Rainbow Trout D6, AF301910
Cherry Salmon Des1*, AB070444
Cherry Salmon Des2*, AB074149
Common Carp D6, AF309557
Zebrafish D5/D6, AF309556
Human D6, AF126799
Mouse D6, AF126798
Human D5, AF199596
Mouse D5, AB072976
Mortierella Alpina D5, AF067654
Mortierella Alpina D6, AF110510
C. Elegans D6, AF031477
C. Elegans D5, AF078796

Fig.4

Fig. 5