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Cloning and functional characterization of Δ6 fatty acid desaturase (FADS2) in Eurasian perch (*Perca fluviatilis*)

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ABSTRACT

The Eurasian perch (*Perca fluviatilis*) is a freshwater carnivorous species of high interest to diversify inland aquaculture. However, little is known about its ability to bioconvert polyunsaturated fatty acids (PUFAs) from plant oils into long chain polyunsaturated fatty acids (LC-PUFAs). In this study, special attention has been given to the fatty acid desaturase 2 (FADS2) which is commonly described to be a rate-limiting enzyme of the LC-PUFA biosynthesis. This work reports on the cloning, tissue expression and functional characterization of the Eurasian perch *fads2*, but also on the cloning of two alternative splicing transcripts named *fads2-AS1* and *fads2-AS2*. The *fads2* cDNA cloned is composed of an open reading frame (ORF) of 1338 nucleotides (nt) and encodes a protein of 445 amino acids. This deduced amino acid sequence displays the typical structure of microsomal FADS2 including two transmembrane domains and an N-terminal cytochrome b_5 domain with the "HPGG" motif. Quantitative real-time PCR assay of *fads2*, *fads2-AS1* and *fads2-AS2* expressions revealed that the *fads2* transcript was mainly expressed in the liver and intestine and exhibited a typical gene expression pattern of freshwater species while *fads2-AS1* and *fads2-AS2* genes were highly expressed in the brain, followed by the liver and intestine. Functional characterization of Eurasian perch FADS2 in transgenic yeast showed a fully functional Δ6 desaturation activity toward C_{18} PUFA substrates, without residual Δ5 and Δ8 desaturase activities.

1. Introduction

Fish are the main dietary source of long chain polyunsaturated fatty acids (LC-PUFAs) such as eicosapentaenoic acid (EPA, 20:5n−3) and docosahexaenoic acid (DHA: 22:6n−3) from the n−3 series, and the arachidonic acid (ARA, 20:4n−6) from the n−6 series. These LC-PUFAs are well known to be essential for fish development, and also provide some benefits for human health by reducing cardiovascular and neurological diseases (Fernandes and Venkatraman, 1993). In response to the worldwide stagnation of fisheries, the aquaculture activity rapidly increased throughout the world since the 1990s to cover the consumer's demand. Unfortunately, the high n−3 LC-PUFA concentrations in farmed fish flesh are maintained by the use of fish oils, themselves largely produced from feed grade marine fisheries. This situation is not sustainable in the long term since it further reduces the marine fish stocks. The use of terrestrial plant ingredients to replace fish oil and fish meal in the fish farmed nutrition has been investigated since the 90s. However, contrary to fish oil, vegetable oils are deprived in LC-PUFA but are rich in fatty acids with 18 carbon atoms. Most of these oils contain a significant proportion of polyunsaturated fatty acids (PUFAs), some being particularly rich in linoleic acid (LA, 18:2n−6), while a few contain mostly α-linolenic acid (ALA, 18:3n−3). Thus, the possibility of using transgenic terrestrial plant with endogenous LC-PUFA biosynthesis capacities has been examined recently (Ruiz-Lopez et al., 2015).

The C_{18} PUFA can be converted into LC-PUFA through a succession of desaturation and elongation reactions, described as the "Sprecher pathway" in vertebrates (Sprecher et al., 1995). Synthesis of EPA and ARA respectively from ALA and LA precursors involves a Δ6 desaturation (FADS2) to produce 18:4n−3 and 18:3n−6, then an elongation (ELOVL5) to 20:4n−3 and 20:3n−6 respectively and then followed by a Δ5 desaturation (FADS1) (Cook, 1996). An alternative pathway involving an elongation of the C_{18} precursor to produce 20:3n−3, followed by a Δ8 desaturation catalyzed by FADS2 has been described in many teleost fish (Monroig et al., 2011). However, as demonstrated by the authors, marine species exhibited higher Δ8 desaturation activity than freshwater/diadromous fish. According to the "Sprecher pathway", the synthesis of DHA from EPA is achieved by two elongation steps, followed by a second Δ6 desaturation step, and a final β-oxidation reaction. Additionally to this conventional pathway, a shorter pathway to produce DHA from EPA through a Δ4 desaturation and an elongation has been recently described in the white-spotted spinefoot (*Siganus*

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canaliculatus) (Li et al., 2010), the Senegalese sole (*Solea senegalensis*) (Morais et al., 2012), the pike silverside (*Chirostoma estor*) (Fonseca-Madrigal et al., 2014) and the striped snakehead (*Channa striata*) (Kuah et al., 2015). Among teleosts, it is well established that marine fish species have a very low LC-PUFA biosynthesis capacities from PUFA precursors compared with anadromous and freshwater/diadromous fish species such as salmonids and zebrafish. The main hypothesis proposed to explain this difference is a loss of LC-PUFA endogenous biosynthesis capacity in response to trophic position. Indeed, most carnivorous fish species obtain their LC-PUFA directly through the ingestion of their LC-PUFA-rich preys (Zheng et al., 2004). At the molecular level, this loss of function is attributed to deficiencies of desaturase and/or elongase enzymatic activities (Tocher, 2003).

To improve the knowledge on LC-PUFA synthesis ability in fish, several fatty acid desaturase cDNAs have been cloned in fish species (Seiliez et al., 2001; Zheng et al., 2004), including a single bifunctional Δ6/Δ5 desaturase gene from the freshwater zebrafish (*Danio rerio*) (Hastings et al., 2001) and distinct *fads2* and *fads1* genes from the cartilaginous catshark (*Scyliorhinus canicula*) (Castro et al., 2012). With the exception of zebrafish, only *fads2* cDNAs have been cloned from teleost fish such as Atlantic salmon (*Salmo salar*) (Hastings et al., 2004; Zheng et al., 2005), Atlantic cod (*Gadus morhua*) (Tocher et al., 2006), European seabass (*Dicentrarchus labrax*) (Gonzáles-Rovira et al., 2009; Santigosa et al., 2011), gilthead sea bream (*Sparus aurata*) (Zheng et al., 2004) and cobia (*Rachycentron canadum*) (Zheng et al., 2009). However, as it has been described above, some of these FADS2 proteins display a diversification of their desaturation activities (Δ4, Δ5 and Δ8 desaturation activities) which might be associated to their respective environments and their specific food.

Currently, the endogenous potential of freshwater carnivorous fish to biosynthesize LC-PUFA is still unclear. It is suggested that the supply of LC-PUFA from preys could possibly impair the LC-PUFA biosynthesis capacity in these species. Investigations on the bioconversion of ALA and LA in freshwater carnivorous species including Northern pike (*Esox lucius*) and Murray cod (*Maccullochella peelii peelii*) have so far presented contradictory results (Henderson et al., 1995; Francis et al., 2007). However, the recent functional characterization of one *fads2* gene with additional Δ4 desaturase activity toward 22:5n − 3 in the striped snakehead might suggest endogenous LC-PUFA biosynthesis capacities in this carnivorous freshwater species (Kuah et al., 2015). The endogenous LC-PUFA biosynthesis potential was investigated in Eurasian perch (*Perca fluviatilis*) because of the interest of this species in the diversification of the inland aquaculture in Europe (Kestemont and Dabrowski, 1996; Henrotte et al., 2011). The relatively high DHA concentrations recorded in the flesh of Eurasian perch after a nutritional challenge with different LC-PUFA levels in diets suggested a relative high endogenous potential of this species to synthesize LC-PUFA from C_{18} precursors (Xu and Kestemont, 2002; Henrotte et al., 2011). However, to date, there is no information available in Eurasian perch regarding the functionality of genes involved in LC-PUFA biosynthesis.

The main objective of the present study was to clone and characterize the *fads2* transcript from Eurasian perch as a first step to understand the molecular mechanisms implicated in the response of this species to vegetable oil feeding.

2. Material and methods

2.1. Tissue samples

Five Eurasian perch juveniles maintained in the facilities of the University of Namur (Belgium) and fed a fish-based diet were euthanized with an overdose of MS-222 (aminobenzoic acid). The liver, brain, anterior intestine, posterior intestine, kidney, muscle, gill, heart and spleen were sampled from each fish and directly stored at − 80 °C.

2.2. Cloning of putative fatty acid desaturase from Eurasian perch

Total RNA from each liver was extracted using 1 mL of Extract-all® reagent (Eurobio, Courtaboeuf, France) following the manufacturer's instructions. Based on the nucleic acid concentration measured with a Nanodrop 2000 spectrophotometer (Thermo Scientific Wilmington, USA), 20 μg of total RNA was treated with the RTS DNAse™ kit (MO BIO Laboratories, Carlsbad, CA) to avoid gDNA contaminations. Finally, 1 μg of total RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The identification of Eurasian perch *fads2* transcript was investigated through a cDNA mix obtained with a blend of 10 μL of each liver template (total of 50 μL).

Degenerate primers, named Fads-deg-F and Fads-deg-R, were designed from multiple alignments with orthologous sequences of desaturases from the GenBank database (*D. labrax*: ACD10793; *S. aurata*: AAL17639; *D. rerio*: NP_571720; *Oncorhynchus mykiss*: AF478472; *Cyprinus carpio*: AAG25711; *S. salar*: AF478472; *Homo sapiens*: NP_037534.3) (Table 1). PCR amplification was performed using the proofreading polymerase from the Advantage® 2 Polymerase Mix (Clontech Laboratories, Mountain View, CA, USA) under the following conditions: 2 min initial denaturation at 94 °C, 37 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C and 1 min elongation at 68 °C, and 2 min of final extension at 68 °C. Migration of PCR products in a 1.2% agarose gel allowed the identification of a single amplicon at the expected length. The PCR product was purified with the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) according to the

Table 1
Primers used in the different steps of this study.

Aim	Transcript	Primer name	Primer sequence (5′–3′)	Annealing temperature (T) (°C)
Partial cDNA cloning	Fads2	Fads-deg-F1	ACVGAGCCSAGCCAGGACC	66.0
		Fads-deg-R1	KTGATTCATCTGWGTCACCCA	47.3
5′ UTR cloning	Fads2	Fads-5′-R1	GCCTCCAGCAGAAGGATGTG	64.0
		Fads-5′-R2	AGAAGAACAAAGGCCGAGCTCGA	52.0
3′ UTR cloning	Fads2	Fads-3′-F1	CACCATGATTTCCCGCCGTGA	51.2
		Fads-3′-F2	TGCGCTACCTGTGCTGTTCTATA	50.2
ORF cloning	Fads2	Fads2-dig-F	CCCAAGCTTATGGGAGGTGGAGGCCAGCT	62.1
		Fads2-dig-R	GCGGGATCCTCATTTATGCAGATATGCATC	56.5
qPCR	Fads2	Fads2-qPCR-F	CCACCTGGGTCACATCCTTC	64.0
		Fads2-qPCR-R	GCATGATGGCGCTCAGAAAG	62.0
		Fads2-AS1-qPCR-F	CAATGCCGGTTTCTCTTCAT	58.0
		Fads2-AS1-qPCR-R	TTAGCGTGATGCTGGAAATG	53.5
		Fads2-AS2-qPCR-F	TCTTCAGTAAGAACCCTGATGTC	54.0
		Fads2-AS2-qPCR-R	TATTCCTGYGAAGTTTTCA	54.0
	Ef1α	EF1α-qPCR-F	GGAAATTCGTCGTGGATACG	60.0
		EF1α-qPCR-R	GGGTGGTTCAGGATGATGAC	62.0
	β-Actin	β-Actin-qPCR-F	ACCTTCTACAACGAGCTGAGAGTT	50.6
		β-Actin-qPCR-R	AGTGGTACGACCAGAGGCATAC	51.6

manufacturer's instructions, cloned into the pGEM®-T Easy Vector (Promega, Madison, USA), and then, transformed in *Escherichia coli* competent cells. After an overnight incubation on the LB plate at 37 °C, a successful transformant was picked from the plate and then cultured overnight in liquid LB medium. Plasmids were extracted from the cultures with the NucleoSpin® Plasmid kit (Macherey-Nagel, Düren, Germany). Finally, the fragment was sequenced (Macrogen Europe, Amsterdam, The Netherlands) and found to have a 738 bp length for the desaturase transcript.

From this sequence, specific primers named Fads-5′-R1 and Fads-5′-R2 for the 5′ RACE reaction, and Fads-3′-F1 and Fads-3′-F2 for the 3′ RACE reaction, were designed to obtain the cDNA full length of the desaturase transcript (Table 1). Amplification of cDNA ends was done with the SMARTer™ Race cDNA Amplification kit (Clontech Laboratories) according to the instructions. After cloning and sequencing the isolated cDNA fragments as described above, the specific primers Fads2-FL-F and Fads2-FL-R were designed in the 5′ and 3′ UTR regions in order to amplify the *fads2* full-length ORF. The PCR reaction was done with the Advantage cDNA polymerase Mix under the following conditions: 2 min initial denaturation at 94 °C, 37 cycles of 45 s denaturation at 95 °C, 45 s annealing at 55 °C, 2 min elongation at 68 °C, and 5 min of final extension at 68 °C. The PCR fragments were cloned into the pGEM®-T Easy Vector (Promega) and sequenced (Magrogen). Finally, several clones revealed a cDNA of 1338 nt named *fads2* (GenBank: KM924433) and two alternative splicing named *fads2-AS1* and *fads2-AS2*.

```
FADS2 P.fluviatilis MGGGGQLTEPEEP---------ISGRAAGVYTWEEVQSHCNRNDQWLVIDRKVYNITQWA
FADS2 S.aurata      MGGGGQLTEPGEP---------GSRRAGGVYTWEEVQSHSSRNDQWLVIDRKVYNVTKWA
FADS2 O.mykiss      MGGGGQQTESSEPAKGDGVGPDGGRGGSAVYTWEEVQKHCHRSDKWLVIDRKVYNITQWA
Fads2/1 D.rerio     MGGGGQQTDRITD----------TNGRFSSYTWEEMQKHTKHGDQWVVVERKVYNVSQWV
Fads2 H.sapiens     MGKGGNQGEGAAE----------REVSVPTFSWEEIQKHNLRTDRWLVIDRKVYNITKWS
                    ** **:  :                    ::***:*.*  : *:*:*::*****:::*

FADS2 P.fluviatilis KRHPGGIRVISHYAGEDATEAFTAFHPDLKFVQKFLKPLQIGELAATEPSQDRNKNATII
FADS2 S.aurata      KRHPGGFRVINHYAGEDATEAFTAFHPDLKFVQKFLKPLLIGELAATEPSQDRNKNAAVI
FADS2 O.mykiss      KRHPGGIRVISHFAGEDATDAFVAFHPDPNFVRKFLKPLLIGELATTEPSQDHGKNAVLV
Fads2/1 D.rerio     KRHPGGLRILGHYAGEDATEAFTAFHPNLQLMRKYLKPLLIGELEASEPSQDRQKNAALV
Fads2 H.sapiens     IQHPGGQRVIGHYAGEDATDAFRAFHPDLEFVGKFLKPLLIGELAPEEPSQDHGKNSKIT
                     :**** *:::*:******:** ****: ::: *:**** **** . *****: **: :

FADS2 P.fluviatilis QDFHTLRAQVESEGLFRARPLFFCLHLGHILLLEALAWLIIWVWGTSWTLTFLSAVILAT
FADS2 S.aurata      QDFHTLRAQAESDGLFRAQPLFFCLHLGHILLLEALAWLIIWLWGTSWTLTFLISIILAT
FADS2 O.mykiss      QDFQALRDRVEREGLLRARPLFFSLYLGHILLLEALALGLLWVWGTSWSLTLLCSLMLAT
Fads2/1 D.rerio     EDFRALRERLEAEGCFKTQPLFFALHLGHILLLEAIAFVMVWYFGTGWINTLIVAVILAT
Fads2 H.sapiens     EDFRALRKTAEDMNLFKTNHVFFLLLLAHIIALESIAWFTVFYFGNGWIPTLITAFVLAT
                    :**::**   *  . ::::. :** * *.**: **::*   :: :*..*  *:: :.:***

FADS2 P.fluviatilis TQLQAGWLQHDFGHLSVFKKSSWNHLLHKFVIGHLKGASANWWNHRHFQHHAKPNIFSKD
FADS2 S.aurata      AQTQAGWLQHDFGHLSVFKKSSWNHILHKFVIGHLKGASANWWNHRHFQHHAKPNIFSKD
FADS2 O.mykiss      SQSQAGWLQHDYGHLSVCKTSSWNHVLHKFVIGHLKGASANWWNHRHFQHHAKPNVFSKD
Fads2/1 D.rerio     AQSQAGWLQHDFGHLSVFKTSGMNHLVHKFVIGHLKGASAGWWNHRHFQHHAKPNIFKKD
Fads2 H.sapiens     SQAQAGWLQHDYGHLSVYRKPKWNHLVHKFVIGHLKGASANWWNHRHFQHHAKPNIFHKD
                    :* ********:***** :..  **::**************.**************:* **

FADS2 P.fluviatilis PDVNMLHVFVVGETQPVEYGIKKIKYMPYHHQHKYFFLVGPPLLIPVYFHIQIIHTMISR
FADS2 S.aurata      PDVNMLHIFVLGDTQPVEYGIKKIKYLPYHHQHQYFLLVGPPLLIPVYFHIQIIRTMISR
FADS2 O.mykiss      PDVNSLHVFVLGDKQPVEYGIKKLKYMPYHHQHQYFFLIGPPLVIPVFFTIQIFQTMFSQ
Fads2/1 D.rerio     PDVNMLNAFVVGNVQPVEYGVKKIKHLPYNHQHKYFFFIGPPLLIPVYFQFQIFHNMISH
Fads2 H.sapiens     PDVNMLHVFVLGEWQPIEYGKKKLKYLPYNHQHEYFFLIGPPLLIPMYFQYQIIMTMIVH
                    **** *: **:*: **:*** **:*::**:***:**:::****:**::*  **: .*: :

FADS2 P.fluviatilis RDWVDLAWSLSYYLRYLCCSIPMFGLFGSVVFISFIRFLESHGFVWVTQMSHLPMDIDHE
FADS2 S.aurata      HDWVDLAWSMSYYLRYLCCYVPLYGLFGSVALISFVRFLESHWFVWVTQMNHLPMDIDHE
FADS2 O.mykiss      RNWVDLAWAMTFYLRFFCCYYPFFGFFGSVALISFVRFLESHWFVWVTQMNHLPMEIDHE
Fads2/1 D.rerio     GMWVDLLWCISYYVRYFLCYTQFYGVFWAIILFNFVRFMESHWFVWVTQMSRIPMNIDYE
Fads2 H.sapiens     KNWVDLAWAVSYYIRFFITYIPFYGILGALLFLNFIRFLESHWFVWVTQMNHIVMEIDQE
                      **** *.:::*:*::     ::*.: :: ::.*:**:*** *******.:: *:** *

FADS2 P.fluviatilis KNQDWLSMQLQATCNIKQSLFNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVRALCEKH
FADS2 S.aurata      KHHDWLTMQLQATCNIEKSVFNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVHALCEKH
FADS2 O.mykiss      RHQDWLTMQLSATCNIEQSTFNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVRALCEKH
Fads2/1 D.rerio     QNQDWLSMQLVATCNIEQSAFNDWFSGHLNFQIEHHLFPTMPRHNYWRAAPRVRSLCEKY
Fads2 H.sapiens     AYRDWFSSQLTATCNVEQSFFNDWFSGHLNFQIEHHLFPTMPRHNLHKIAPLVKSLCAKH
                      :**:: ** ****:::* ************************    ** *::** *:

FADS2 P.fluviatilis GVPYQVKTLWQGFADVFRSLKHSGDLWLDAYLHK
FADS2 S.aurata      GIPYQVKTMWQGIVDVIRSLKNSGDLWLDAYLHK
FADS2 O.mykiss      GLPYQVKTLQKAIIDVVGSLKKSGDLWLDAYLHK
Fads2/1 D.rerio     GVKYQEKTLYGAFADIIRSLEKSGELWLDAYLNK
Fads2 H.sapiens     GIEYQEKPLLRALLDIIRSLKKSGKLWLDAYLHK
                    *: ** *.:  .: *:. **::**.*******:*
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Fig. 1. Alignment of the deduced amino acid sequence of the fatty acid desaturase 2 from Eurasian perch. The heme-binding motif "HPGG" of the cytochrome b_5-domain is underlined while the two transmembrane domains, including the four TM-helices, are shaded. The three histidine-rich regions are framed. The two highly conserved histidine positions are in bold.

2.3. Phylogenetic analysis

The deduced amino acid sequence of the newly cloned Eurasian perch *fads2* transcript was aligned with FADS1 and FADS2 orthologues. The FADS2 amino acid sequence from *Mortierella alpina* was used as outgroup sequence to root the tree. The tree was constructed by the neighbor-joining method (Saitou & Nei, 1987) using PhyML and MEGA5 softwares. Confidence in the resulting phylogenetic tree branch topology was measured with 1000 bootstrap iterations.

2.4. Hydropathy profiles

Hydropathy profiles were built with several programs, such as DAS (BRC, Hungarian Academy of Sciences, Hungary), HMMTOP (IE, Hungarian Academy of Sciences, Hungary), Phobius (SBC, Stockholm University, Sweden), PredictProtein (Technische Universität München, Germany & Columbia University at New York, USA), SOSUI (Applied Physics, Nagoya University, Japan), SPOCTOPUS (SBC, Stockholm University, Sweden), TM-Finder (SickKids, Toronto University, Canada), TMHMM (CBS, Technical University of Denmark, Denmark), TMpred (EMBnet, University Nijmegen Medical Centre, The Netherlands) and TopPred (BITC, Institut Pasteur & RPBS, Université Paris VII-Diderot, France). The accuracy of model helicity and hydrophobicity was assessed by consensus building.

2.5. Functional characterization of Eurasian perch fads2 cDNA by heterologous expression in yeast

The specific sequence corresponding to the ORF of the Eurasian perch *fads2* transcript was amplified by PCR with the primer pairs named Fads2-dig-F/Fads2-dig-R containing HindIII and BamHI restriction sites (Table 1). PCR was performed with the Advantage cDNA polymerase Mix from a mixture of liver cDNA as template. PCR conditions were an initial denaturation step at 94 °C for 5 min, followed by 38 cycles of 30 s denaturation, 30 s annealing at 56 °C, 2 min elongation at 68 °C, followed by a final step of elongation for 5 min at 68 °C. The PCR product with the expected length was cloned into the pGEM®-T Easy Vector (Promega). Several recombinant plasmids were subsequently extracted, purified and sequenced to allow the identification of the *fads2* transcript. 2 μg recombinant plasmid containing *fads2* cDNA was digested with the corresponding restriction enzymes (Promega), ligated into the pYES2 expression vector (Invitrogen) with the T4 DNA ligase (Promega) and transfected into *E. coli* competent cells. After verification of the sequence of the recombinant pYES2 plasmid, *Saccharomyces cerevisiae* strain INVSc1 (Invitrogen, Gent, Belgium) was transformed with the pYES2-*fads2* recombinant plasmid according to the manufacturer's instructions. Yeasts transformed with the recombinant plasmid were selected on solid minimal medium plates without uracil.

The cultures transformed with recombinant plasmids were grown at 30 °C in minimal medium containing 0.67% (w/v) nitrogen base (Sigma-Aldrich, St. Louis, MO, USA), 0.19% (w/v) drop out medium, and 2% (w/v) raffinose. Expression of the transgene was induced by adding 2% (w/v) galactose and 1% (w/v) tergitol in a culture diluted at an initial OD_{600} of 0.3 in 5 mL of final volume. For each assay, all the fatty acids tested as substrate were previously dissolved in absolute ethanol and supplemented at the appropriate concentration in the medium culture described above. The Eurasian perch desaturase functionality was tested with the following fatty acids: 18:3n − 3 (0.5 mM), 18:2n − 6 (0.5 mM), 20:3n − 6 (0.5 mM), 20:3n − 3 (0.5 mM), 20:4n − 3 (0.5 mM) and 24:5n − 3 (1 mM). All the incubations were carried out at 30 °C for 2 days in a shaking agitator. After incubations, the cultures were centrifuged for 1 min at 5000 ×*g* and washed with 5 mL of sterile water. After another centrifugation for 1 min at 5000 ×*g*, the cultures were suspended in 1 mL of sterile water and stored at − 20 °C.

2.6. Fatty acid analysis from transgenic yeasts

Total cellular lipids were extracted according to a method adapted from the one described in details by Schneider et al. (2012). Briefly, 800 μL of yeast culture suspended in sterile water as described

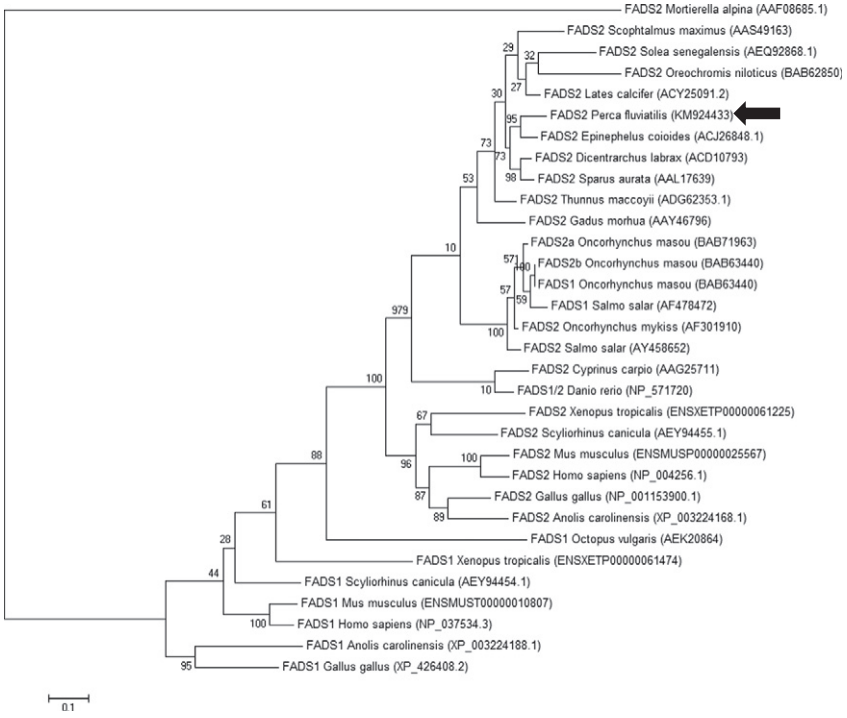
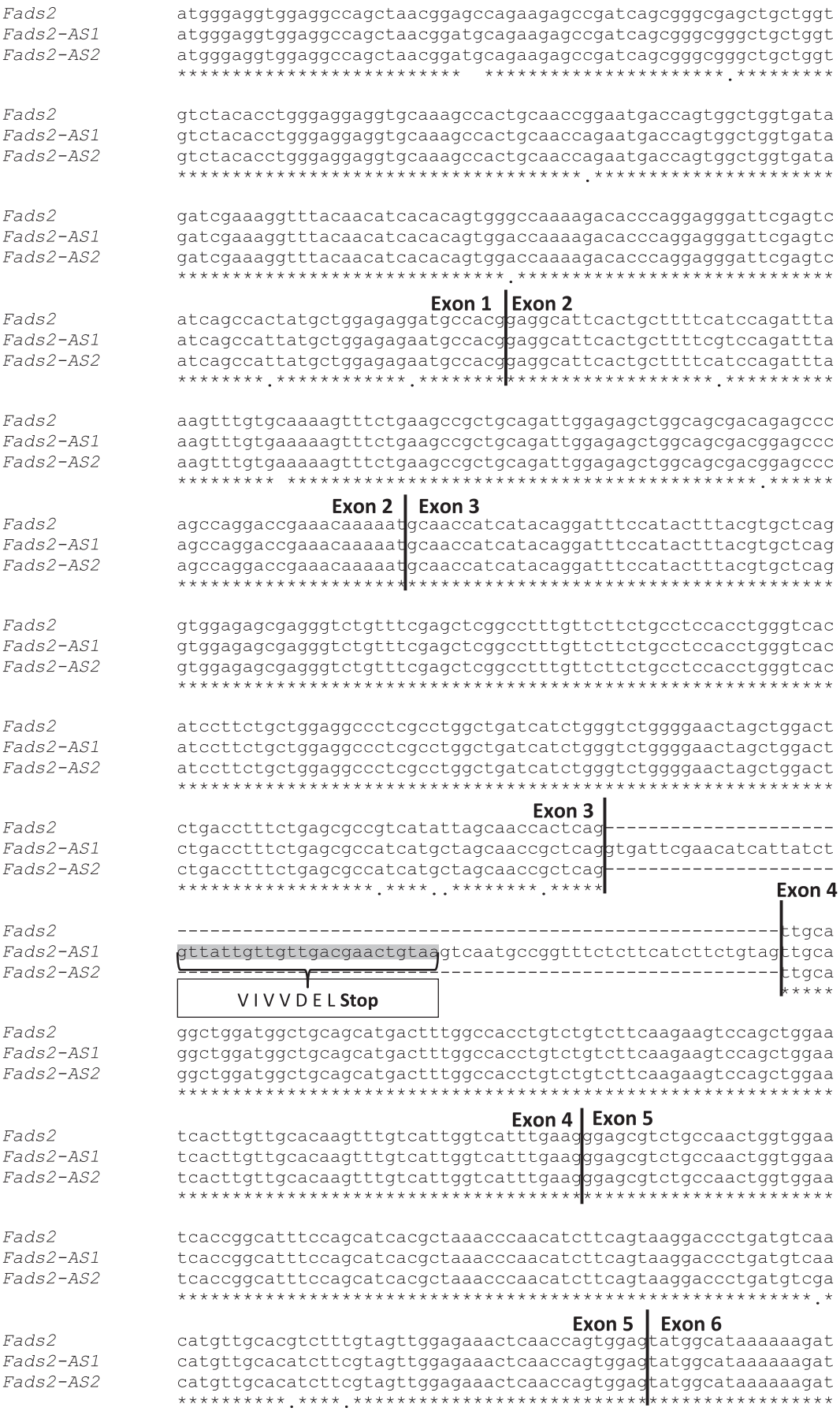


Fig. 2. Phylogeny of FADS1 and FADS2 deduced amino acid sequences. The functional FADS2 protein of *Perca fluviatilis* is indicated with an arrow. The tree was constructed by the neighbor-joining method (Saitou & Nei, 1987) using PhyML and MEGA5 softwares. The horizontal branch length is proportional to amino acid substitution rate per site. The values represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations. The FADS2 amino acid sequence from *Mortierella alpina* was used as outgroup sequence to root tree.


Fads2
Fads2-AS1
Fads2-AS2
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Exon 6
V I V V D E L Stop

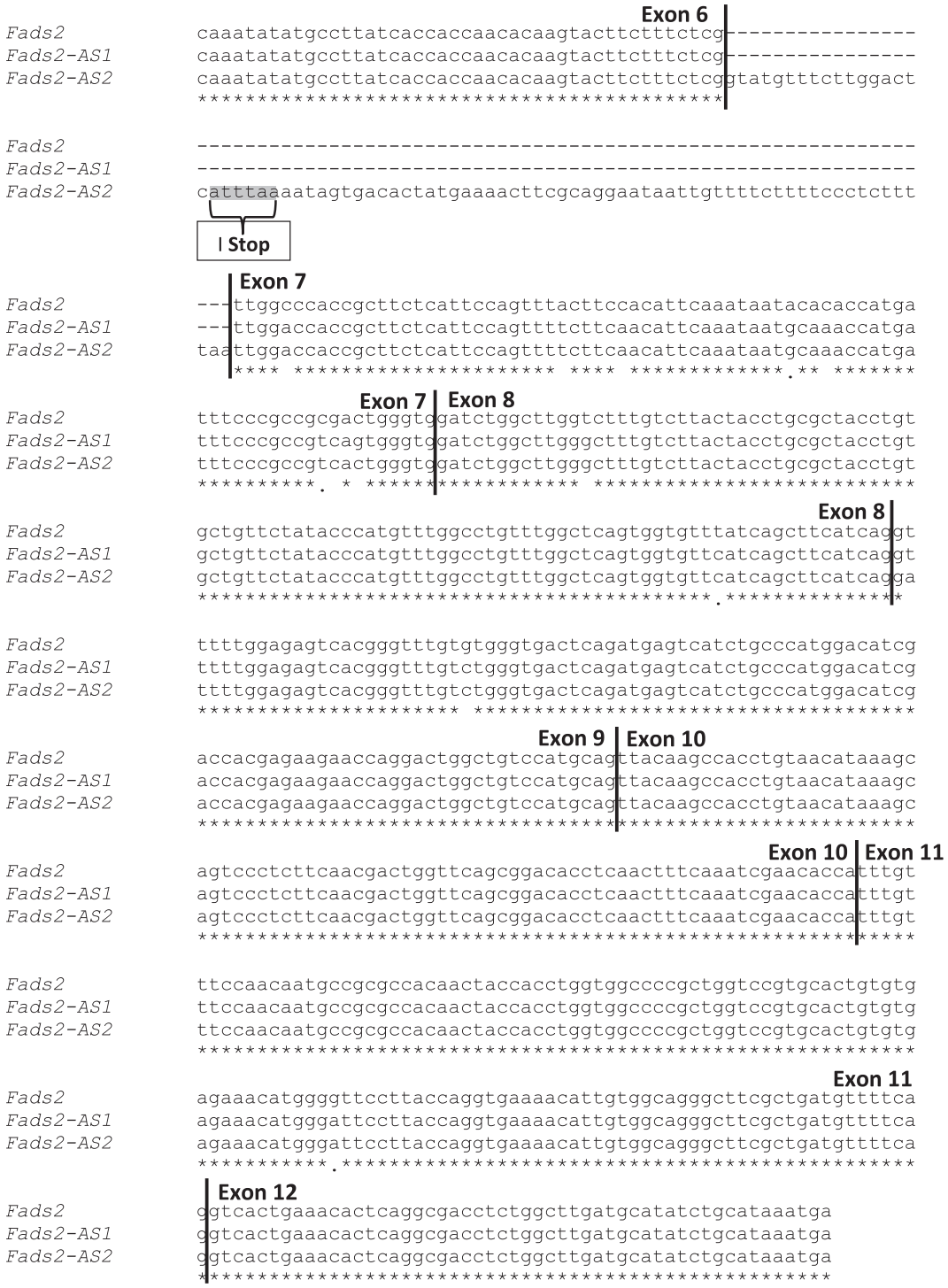


Fig. 3. Exon composition of Eurasian perch *fads2*, *fads2-AS1*, and *fads2-AS2* transcripts. Inter-exon junctions are shown by vertical black separations. Asterisks correspond to the nucleotides conserved between sequences. Retention of the nucleotide sequences between exon 3/exon 4 and exon 6/exon 7 inter-exon junctions is indicated in the alignment. Partial translation of the *fads2-AS1* and *fads2-AS2* transcripts is indicated in boxes to show the presence of the stop codon.

above was transferred into Pyrex tubes and lipids were extracted with 5 mL of chloroform/methanol/KCl 0.88% (2:2:1.8, v:v:v) (Biosolve, Valkenswaard, The Netherlands). After centrifugation, the supernatant was discarded and the sample was collected in a new Pyrex tube to be dried under nitrogen. The lipids were then methylated at 70 °C by the incubation of the sample with 0.1 M KOH in methanol for 1 h, followed by the addition of 1.2 M HCl in methanol and incubation during 15 min. Fatty acid methyl esters were then collected by adding hexane (Biosolve) and separated by gas–liquid chromatography. The GC Trace (Thermo Finnigan, Milan, Italy) was equipped with an RT2560 capillary column (100 m × 0.25 mm internal diameter, 0.2 μm film thickness) (Restek, Bellefonte, USA), an automatic injector and a flame ionization detector kept at a constant temperature of 255 °C. The carrier gas was H_2 and used at a constant pressure of 200 kPa. The temperature program was as follows: an initial temperature of 80 °C, which progressively increased at 25 °C/min up to 175 °C; a holding temperature of 175 °C during 25 min; an increase at 10 °C/min up to 205 °C; a holding temperature of 205 °C during 4 min; an increase at 10 °C/min up to 215 °C; a holding

temperature of 215 °C during 25 min; an increase at 10 °C/min up to 235 °C and a holding temperature of 235 °C during 10 min. A decrease at 20 °C/min was then imposed down to the initial temperature of 80 °C. Each peak was identified by comparison of retention times with pure methyl ester standards purchased from Larodan (Malmö, Sweden) and from Nu-Check Prep (Elysian, MN, USA). Data processing was operated with the ChromQuest software (version 5.0, Thermo Finnigan). Results were then expressed in g/100 g of total identified fatty acids.

2.7. Fads2 Eurasian perch tissue distribution

The liver, brain, intestine, kidney, muscle, gill, heart and spleen were sampled from 5 Eurasian perch juveniles fed a commercial diet containing fish ingredients (ME-1.8MP Presta, 55% protein and 15% lipid, Sketting, Stavanger, Norway). Total RNA was extracted from 50 to 100 mg of each sample tissue, with the EXTRACT'ALL (Eurobio), according to the manufacturer's recommendations. After quantification of nucleic acids with a spectrophotometer, 20 μg of total RNA from each sample was treated with the RTS DNAse kit to avoid gDNA contaminations. After this treatment, 1 μg of total RNA was reverse-transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). Relative *fads2*, *fads2-AS1* and *fads2-AS2* expressions were investigated by real-time quantitative RT-PCR using specific primers (Table 1). Specific amplification from the cDNA samples was performed using the iQ™SYBR® Green Supermix (Bio-Rad Laboratories). Thermal cycling and fluorescence detection were conducted in a StepOnePlus Real Time PCR System (Applied System) under the following conditions: 10 min of initial denaturation at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. After each run, amplification of single amplicon was confirmed by analyzing the melt curve and sequencing some of the PCR products.

3. Results

3.1. Cloning of fatty acid desaturase from Eurasian perch

Starting from degenerate primers designed on fish desaturase alignments, a partial transcript encoding a desaturase was amplified. The complete transcript was synthesized by 5′ and 3′ RACE RT-PCR. The cloning of the PCR product obtained from primers designed in the UTR regions allowed identifying a putative Eurasian perch desaturase transcript composed of 1338 bp and encoding a putative protein of 445 amino acids. As indicated in the alignment (Fig. 1), the putative cytochrome b_5-like domain (HPGG) and the three characteristic histidine boxes (H^{182}xxxH^{186}, H^{219}xxHH^{233} and Q^{384}xxHH^{388}) were conserved in this transcript. Phylogenetic analysis comparing the Eurasian perch amino acid sequence with those deduced from *fads1* and *fads2* cDNAs from fish and other vertebrates showed that Eurasian perch desaturase transcript clustered with the sequence deduced from *fads2* cDNA from *Epinephelus coioides*, *D. labrax* and *S. aurata*, all being carnivorous marine fish. Moreover, the cluster with desaturases from freshwater species was less strong (Fig. 2). Amino acid sequence pairwise comparison of Eurasian perch FADS2 with FADS2 of sea bream, rainbow trout, zebrafish and human revealed an identity of 87%, 77%, 69% and 65% respectively.

In addition to this desaturase transcript, the cloning of the PCR products obtained from the primers designed in the UTR regions allowed identifying two alternative RNA splicing variants, named *fads2-AS1* and *fads2-AS2*. The *fads2-AS1* was characterized by an insertion of 76 nt between the exon 3 and the exon 4, while the *fads2-AS2* variant was characterized by an insertion of 79 nt in the junction between exons 6 and 7 (Fig. 3). The retention of these nucleotide sequences between inter-exon junctions induced a modification of the ORF. Consequently, a stop codon appeared prematurely in the putative amino acid sequences deduced from these alternative splicing transcripts.

3.2. Hydropathy profiles

Hydropathy profiles of Eurasian perch FADS2 predict four TM-helices respectively among amino acids 135–155, 157–177, 263–283 and 300–330 (Fig. 4A). Front-end desaturase conserved patterns are also shown. The three histidine boxes are located in the cytoplasmic loop (H^{182}xxxH^{186} and H^{219}xxHH^{233}) or in the C-terminal extremity (Q^{384}xxHH^{388}). The N-terminal cytochrome b_5-like domain stretches from amino acid 36 to 78 and contains the heme-binding motif HPGG. Hydropathy profiles of several fish species and human FADS2 were compared (Fig. 4B). All sequences possess the four TM-helices located at the same position, except for rainbow trout, for which the *fads2* ORF is shifted by 9 amino acids as compared to the other fishes (10 if compared to human). The C^{135}…W^{155} and T^{157}…G^{177} ones form the first transmembrane domain, and the last two each constitute a single TM domain (three in total).

3.3. Tissue distribution of desaturase transcripts

The relative *fads2* gene expression in the liver, brain, intestine, kidney, muscle, gill, heart and spleen from Eurasian perch was investigated by the real-time quantitative RT-PCR method (Fig. 5). *Fads2* was mainly expressed in the liver, followed by the brain, anterior intestine, kidney and muscle, while detection of this transcript was very weak in the gill, heart and spleen. *Fads2-AS1* and *fads-AS2* alternative transcripts had globally the same relative gene expression patterns than the *fads2* transcript except that the maximal expression was observed in the brain instead of the liver.

3.4. Functional characterization in yeast

The functional characterization of Eurasian perch FADS2 protein was performed by heterologous expression in yeast (*S. cerevisiae*). Its activity was assessed by incubation of recombinant yeasts with Δ6 (18:3n−3, 18:2n−6, 24:5n−3), Δ5 (20:4n−3, 20:3n−6) and Δ8 (20:3n−3) desaturase substrates (Fig. 6, Table 2). Recombinant yeast containing pYES2-FADS2 exhibited stearidonic acid (18:4n−3) and gamma-linoleic acid (18:3n−6) contents when their respective desaturation substrates, namely 18:3n−3 and 18:2n−6, were added to the liquid medium. The 18:3n−3 and 18:2n−6 conversion rates were estimated at about 35% and 7%, respectively. In addition, no EPA (20:5n−3) and ARA (20:4n−6) products were detected when eicosatetraenoic acid (20:4n−3) and dihomo-γ-linoleic acid (20:3n−6) were added to the liquid medium. Similarly, no Δ6-desaturation and Δ8-desaturation products were found when tetracosapentaenoic acid (24:5n−3) and eicosatrienoic acid (20:3n−3) were respectively added in the liquid medium.

4. Discussion

The characterization of molecular actors involved in LC-PUFA biosynthesis is an essential step in the understanding of variable endogenous LC-PUFA synthesis capacities among fish species. In a context of fish oil replacement by plant oils in aquafeeds, previous studies mainly focused on fish species of interest in aquaculture such as marine carnivorous species (Zheng et al., 2004; Tocher et al., 2006; Zheng et al., 2009; Yamamoto et al., 2010; Morais et al., 2012), salmonids (Hastings et al., 2004; Zheng et al., 2005) and freshwater species (Ren et al., 2012; Tanomman et al., 2013). According to these studies performed on the functional characterization of desaturases and elongases and their regulations when fish were fed a plant oil-based diet, it is suggested that freshwater species and salmonids exhibit higher abilities in LC-PUFA endogenous synthesis than marine carnivorous species. However, the endogenous capacity of freshwater carnivorous species like Eurasian perch to biosynthesize LC-PUFA is poorly documented. Thus, this work represents a first step

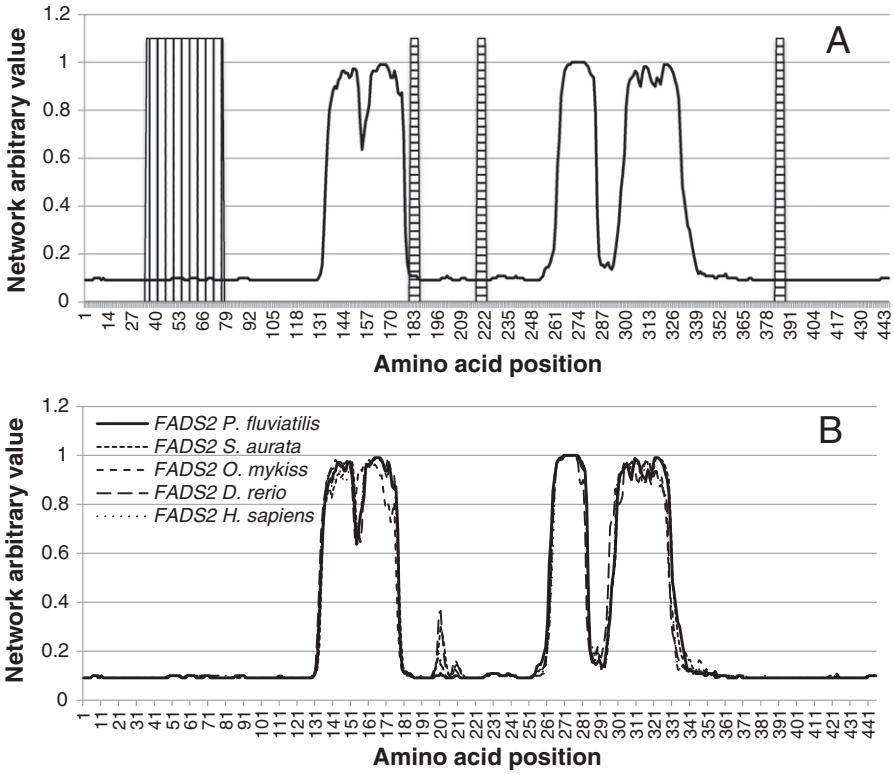


Fig. 4. (A) Hydropathy profile of Eurasian perch FADS2. Peaks indicate the transmembrane α-helices. The vertical and horizontal shaded boxes show the localisation of the N-terminal cytochrome b_5-like domain and the three conserved histidine boxes respectively. (B) Compared hydropathy profiles of some fish and human FADS2. The GenBank accession numbers of the aligned amino acid sequences are as follows: *Perca fluviatilis* (KM924433), *Sparus aurata* (GQ162822), *Oncorhynchus mykiss* (AF301910), *Danio rerio* (NP_571720), and *Homo sapiens* (NP_004256.1). In order not to shift the alignment, the $A^{14}KGDGVGPD^{22}$ amino acids of the *O. mykiss* sequence have been removed.

in the characterization of the endogenous potential of Eurasian perch to bioconvert PUFA into LC-PUFA.

The sequence analysis of the new cloned *fads2* cDNA reveals the presence of some characteristic features of this enzyme family (Hashimoto et al., 2008). The Eurasian perch *fads2* transcript is composed of three histidine boxes (HXXXH, HXXHH and QXXHH), a putative cytochrome b_5-like domain, and the heme-binding motif HPGG. As an endoplasmic reticulum membrane-bound enzyme, Δ6 desaturase is assumed to present two hydrophobic regions spanning twice through the ER membrane and consisting of two antiparallel transmembrane α-helices (Stukey et al., 1990; Shanklin et al., 1994). Hydropathy profiles of Eurasian perch FADS2 confirm this assumption, with four TM-helices respectively among amino acids 135–155, 157–177, 263–283 and 300–330 (Fig. 4A), which are conserved among all vertebrates (Fig. 4B).

The Eurasian perch FADS2 identified in this study encodes a protein of 445 amino acids, which is more similar to the sequences deduced for marine carnivorous species (*E. coioides*, *S. aurata* and *D. labrax*) than for freshwater species (*C. carpio*, *Oreochromis niloticus*) and salmonids (*S. salar*, *O. mykiss*). The functional characterization of the Eurasian perch FADS2 in yeast allowed detecting stearidonic acid (18:4n − 3) and gamma-linolenic acid (18:3n − 6), when the transgenic yeast was incubated with the substrates 18:3n − 3 and 18:2n − 6, respectively. These results confirm the Δ6 enzymatic activity of the Eurasian perch FADS2. In addition, a comparison of the 18:2n − 6 and 18:3n − 3 desaturation rates seems to indicate a higher affinity of Eurasian perch FADS2 toward the n − 3 substrate. Similar results were deduced from the functional characterization of *D. rerio* bifunctional FADS1/FADS2 (Hastings et al., 2001), and of the FADS2 from *O. mykiss*, *S. aurata*, *Psetta maxima* and *C. carpio* (Zheng et al., 2004). This higher affinity of FADS2 toward n − 3 fatty acid substrates may be a consequence of the higher n − 3 LC-PUFA requirements of carnivorous fish species as compared with those of the n − 6 series (Xu et al., 2001; Xu and Kestemont, 2002; Regost et al., 2003; Geay et al., 2010). Functional characterization of Eurasian perch FADS2 did not allow detecting tetracosahexaenoic acid (24:6n − 3) when the tetracosapentaenoic acid (24:5n − 3) substrate was added to the liquid medium. This absence of desaturation product may result in a lower enzymatic activity of the FADS2 toward the 24:5n − 3 substrate, in comparison to the C_{18} substrates.

Recently, it was shown that an alternative pathway to the "Sprecher pathway" involving an elongation and a desaturation step allows the production of EPA. Monroig et al. (2011, 2013) demonstrated the FADS2 implication in the Δ8 desaturation of eicosatrienoic acid (20:3n − 3) into eicosatetraenoic acid (20:4n − 3). As suggested by these authors, this alternative pathway seems to be more active in marine fish species such as meagre (*Argyrosomus regius*), cobia, gilthead sea bream and turbot than in freshwater species. This observation may explain the absence of 20:4n − 3 product detected from Eurasian perch FADS2 when recombinant yeast was incubated with the 20:3n − 3 substrate. In addition to this alternative pathway, a shorter pathway including a Δ4 desaturation reaction to convert 22:5n − 3 into 22:6n − 3 has been identified in FADS2 of the white-spotted spinefoot (Li et al., 2010), the Senegalese sole (Morais et al., 2012), the pike silverside (Fonseca-Madrigal et al., 2014) and the striped snakehead (Kuah et al., 2015). However, the Δ4 desaturation ability of the Eurasian perch FADS2 has not been analyzed in the present study and need further investigation.

Some of the fish FADS2 characterized so far display a low ability to desaturate 20:4n − 3 and 20:3n − 6 at the Δ5 position, with conversion rates from 0.2% in rainbow trout, sea bream or turbot (Zheng et al., 2004) to 2.3% in Atlantic salmon (Zheng et al., 2005) or cobia (Zheng et al., 2009). In our study, no Δ5 desaturase activity was recorded in transgenic yeasts containing the Eurasian perch FADS2 and incubated with the 20:4n − 3 and 20:3n − 6 substrates. Similar results were obtained from carnivorous fish species such as Atlantic cod (Tocher et al., 2006) and European sea bass (Santigosa et al., 2011). However, as described by Henrotte et al. (2011), [1-^{14}C]20:5n − 3 was detected when hepatocytes from Eurasian perch were incubated with the

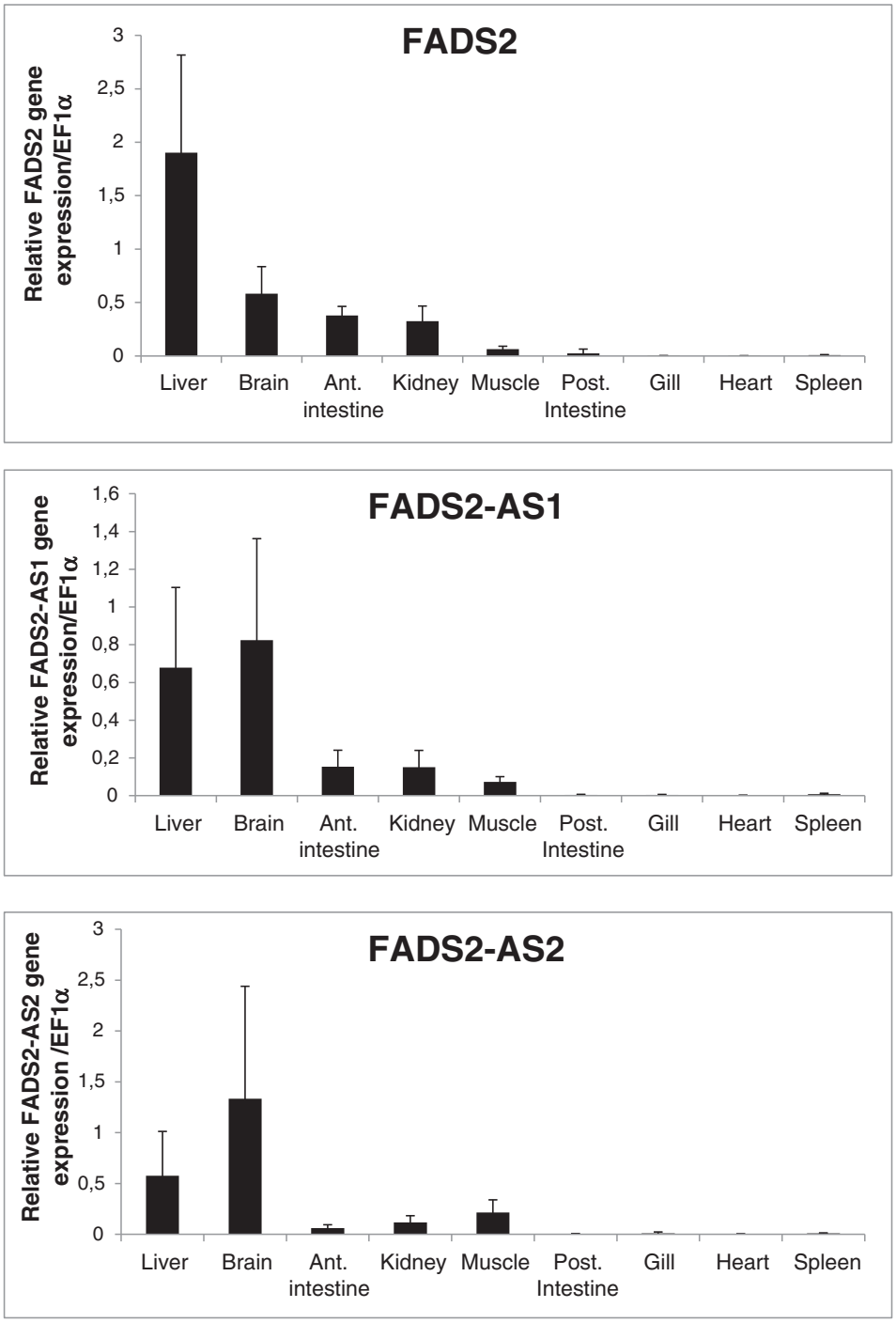


Fig. 5. Relative expression of *fads2*, *fads2-AS1*, and *fads2-AS2* in the liver, brain, anterior intestine, kidney, muscle, posterior intestine, gill, heart and spleen from Eurasian perch fed a fish based diet. Relative gene expression was determined by quantitative real-time RT-PCR after normalization with the expression of the EF1α housekeeping gene.

[1-^{14}C]18:3n − 3 substrate, suggesting a Δ5 desaturation activity. This Δ5 enzymatic reaction may be carried out by a FADS2 protein encoded by another *fads2* gene, resulting from the genome duplication. Indeed, as explained by Castro et al. (2012), a loss of the *fads1* gene was observed in teleost fish and the existence of the Δ5 desaturation reaction in fish is mainly a consequence of independent mutations into *fads2* genes. For example, in Atlantic salmon, Δ5 activity is performed by a *fads2-like* gene, which has up to 95% amino acid homology with another *fads2* gene, resulting from salmonid-specific duplication. In the present work, the cloning of the PCR products obtained from the primers designed in the UTR regions allowed the identification of another putative *fads2* transcript encoding a protein of 445 amino acids (Appendix A), with 96% amino acid homology with the functional Eurasian perch FADS2 described in this work. However, no desaturation products were detected in our experimental conditions when yeasts were transformed with this second *fads2* transcript and were incubated with 18:3n − 3, 18:3n − 6, 20:3n − 3, 20:4n − 3 and 24:5n − 3 substrates (not shown). In addition to these Eurasian perch *fads2* transcripts, two alternative splicing transcripts were identified in this study. Interestingly, the strong nucleotide homology of these alternative splicing transcripts with the putative Eurasian perch *fads2* transcript mentioned above may confirm this biological existence (Appendix B).

The retention of nucleotide sequences in inter-exons regions of the *fads2-AS1* and *fads2-AS2* did not make it possible to obtain functional

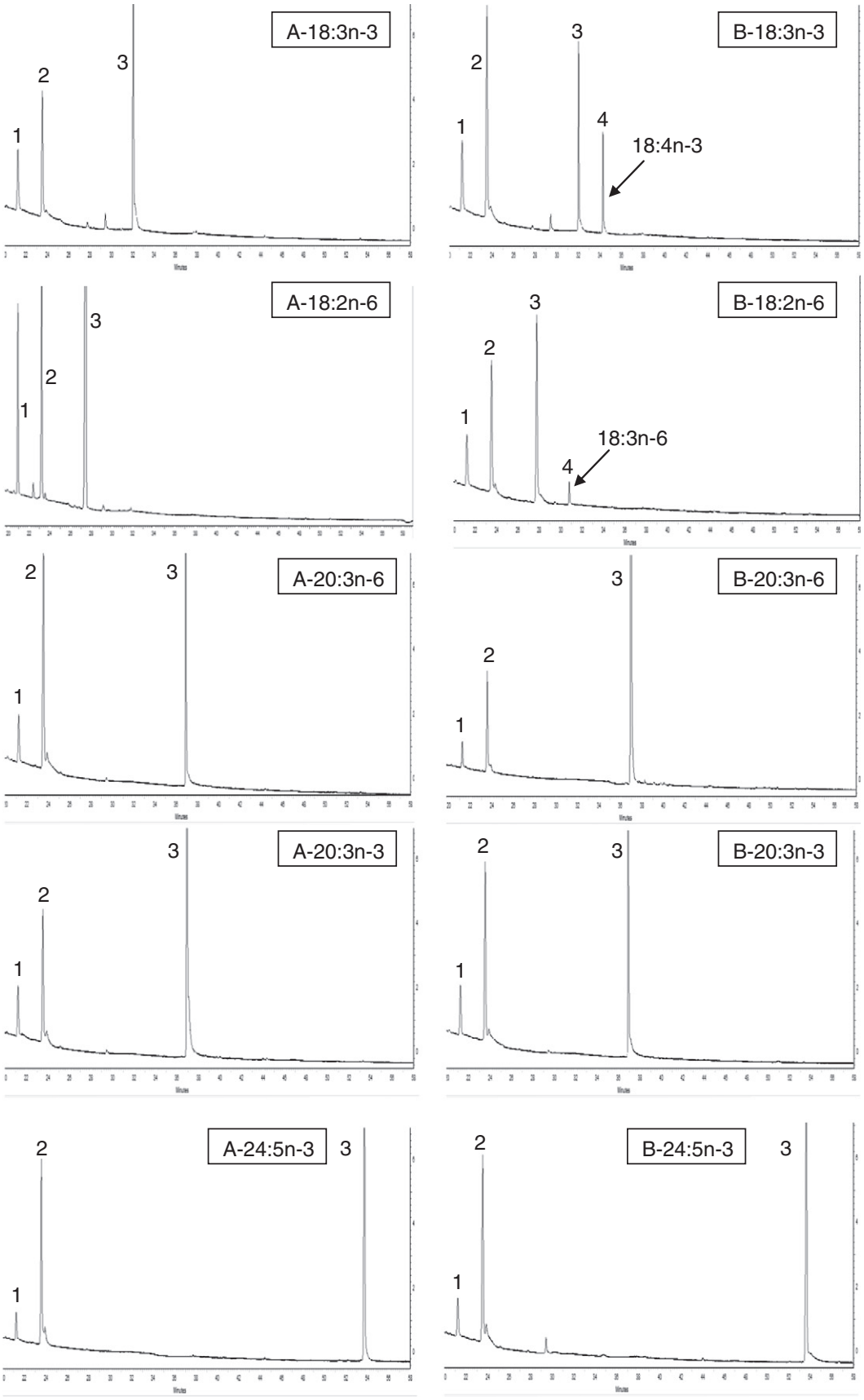


Fig. 6. Identification of fatty acid desaturation products in yeast transformed with pYES2 vector without insert (A) or containing the putative Eurasian perch *fads2* cDNA (B). The first two peaks in each graph are the endogenous 18:0 (1) and 18:1n−9 (with 18:1n−7 as shoulder) (2) fatty acids of *S. cerevisiae*. The next peak (3) in each panel is the exogenously added substrate fatty acid whereas the last peak (4) is the desaturated fatty acid product. The horizontal axis is the retention time.

Table 2
Evaluation of desaturation activities through incubation of *fads2* transformed or control yeast cells with different fatty acid substrates. Results are expressed in % of total detected fatty acids in the cells after 2 days of incubation with 18:3n−3 (0.5 mM), 18:2n−6 (0.5 mM), 20:3n−6 (0.5 mM), 20:3n−3 (0.5 mM), 20:4n−3 (0.5 mM) and 24:5n−3 (1 mM).

Clone tested	FA substrate tested	Enzymatic activity	FA substrate area	FA product area	Conversion (%)
pYES2-empty	18:3n−3	Δ6 activity	6,577,868	ND	–
	18:2n−6	Δ6 activity	3,583,988	ND	–
	20:3n−6	Δ5 activity	4,442,076	ND	–
	20:3n−3	Δ8 activity	5,251,772	ND	–
	20:4n−3	Δ5 activity	3,046,824	ND	–
	24:5n−3	Δ6 activity	5,634,182	ND	–
pYES2-FADS2	18:3n−3	Δ6 activity	2,538,775	1,388,934	35
	18:2n−6	ΔΔ6 activity	3,915,734	301,274	7
	20:3n−6	Δ5 activity	6,206,658	ND	–
	20:3n−3	Δ8 activity	6,573,263	ND	–
	20:4n−3	Δ5 activity	3,120,774	ND	–
	24:5n−3	Δ6 activity	7,321,140	ND	–

proteins. However, their roles in the regulation of the LC-PUFA biosynthesis could not be excluded. Indeed, as described by Lu et al. (2010), alternative splicings are relatively abundant in teleost fish like *D. rerio*, *Oryzias latipes*, *Gasterosteus aculeatus* and *Takifugu rubripes* and contribute as a major mechanism to increase diversity of gene products. Recently, five *fads2* alternative splicings were also identified in European sea bass (Gonzáles-Rovira et al., 2009; Santigosa et al., 2011) but their roles in LC-PUFA metabolism have never been investigated yet.

The expression of *fads2* Eurasian perch is stronger in the liver, followed by the brain and intestine, as compared to the other tissues. This high *fads2* gene expression level recorded in the liver may reflect a key role of this tissue in lipid metabolism in Eurasian perch. Similar *fads2* gene expression patterns were observed in freshwater species like common carp (Ren et al., 2012), rabbitfish (Li et al., 2008) and salmonids including Atlantic salmon (Zheng et al., 2005; Monroig et al., 2010) and rainbow trout (Seiliez et al., 2001), as well as in the herbivorous euryhaline spotted scat (*Scatophagus argus*) (Xie et al., 2014). At the opposite, studies performed on marine fish species showed a stronger *fads2* gene expression in the brain compared to the liver and intestine (Tocher et al., 2006; Zheng et al., 2009; Santigosa et al., 2011).

In summary, we have reported the cloning, the tissue distribution and the functional characterization of one Eurasian perch FADS2 protein and the identification of two alternative splicings. The *fads2* transcript, mainly expressed in the liver, encoded a functional protein able to desaturate n−3 and n−6 C_{18} substrates. However, further investigations should be performed to characterize the regulation of the LC-PUFA biosynthetic pathway, including FADS2, when Eurasian perch juveniles are fed diets composed with plant oils and deprived in LC-PUFA. In addition, complementary analysis would be necessary to investigate the role of the alternative splicings in LC-PUFA metabolism.

Acknowledgments

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Appendix A

Amino acid comparison between the functional Eurasian perch FADS2 described in this study and the putative Eurasian perch FADS2 mentioned in the Discussion section. The heme-binding motif "HPGG" of the cytochrome b_5-domain is underlined while the two transmembrane domains, including the four TM-helices, are shaded. The three histidine-rich regions are framed. The two highly conserved histidine positions are in bold. Asterisks correspond to the nucleotides conserved between sequences.

```
Funct.FADS2          MGGGGQLTDAEEPISGRAAGVYTWEEVQSHCNQNDQWLVIDRKVYNITQWTKRHPGGIRV
Put.  FADS2          MGGGGQLTEPEEPISGRAAGVYTWEEVQSHCNRNDQWLVIDRKVYNITQWAKRHPGGIRV
                     ********:.**********************:****************:*********

Funct.FADS2          ISHYAGENATEAFTAFHPDLKFVKKFLKPLQIGELAATEPSQDRNKNATIIQDFHTLRAQ
Put.  FADS2          ISHYAGEDATEAFTAFHPDLKFVQKFLKPLQIGELAATEPSQDRNKNATIIQDFHTLRAQ
                     *******:***************:************************************

Funct.FADS2          VESEGLFRARPLFFCLHLGHILLLEALAWLIIWVWGTSWTLTFLSAIMLATAQLQAGWLQ
Put.  FADS2          VESEGLFRARPLFFCLHLGHILLLEALAWLIIWVWGTSWTLTFLSAVILATTQLQAGWLQ
                     ********************************************::***:********

Funct.FADS2          HDFGHLSVFKKSSWNHLLHKFVIGHLKGASANWWNHRHFQHHAKPNIFSKDPDVNMLHIF
Put.  FADS2          HDFGHLSVFKKSSWNHLLHKFVIGHLKGASANWWNHRHFQHHAKPNIFSKDPDVNMLHVF
                     ************************************************************:*

Funct.FADS2          VVGETQPVEYGIKKIKYMPYHHQHKYFFLVGPPLLIPVFFNIQIMQTMISRRQWVDLAWA
Put.  FADS2          VVGETQPVEYGIKKIKYMPYHHQHKYFFLVGPPLLIPVYFHIQIIHTMISRRDWVDLAWS
                     ************************************:*:***::******:******:

Funct.FADS2          LSYYLRYLCCSIPMFGLFGSVVFISFIRILESHGFVWVTQMSHLPMDIDHEKNQDWLSMQ
Put.  FADS2          LSYYLRYLCCSIPMFGLFGSVVFISFIRFLESHGFVWVTQMSHLPMDIDHEKNQDWLSMQ
                     ****************************:*****************************

Funct.FADS2          LQATCNIKQPLFNDWFSGHLNHQIEHHLFPTMPRHNYHLVAPLVRALCEKHGIPYQVKTL
Put.  FADS2          LQATCNIKQSLFNDWFSGHLNHQIEHHLFPTMPRHNYHLVAPLVRALCEKHGVPYQVKTL
                     *********.*****************************************:********

Funct.FADS2          WQGFADVFRSLKHSGDLWLDAYLHK
Put.  FADS2          WQGFADVFRSLKHSGDLWLDAYLHK
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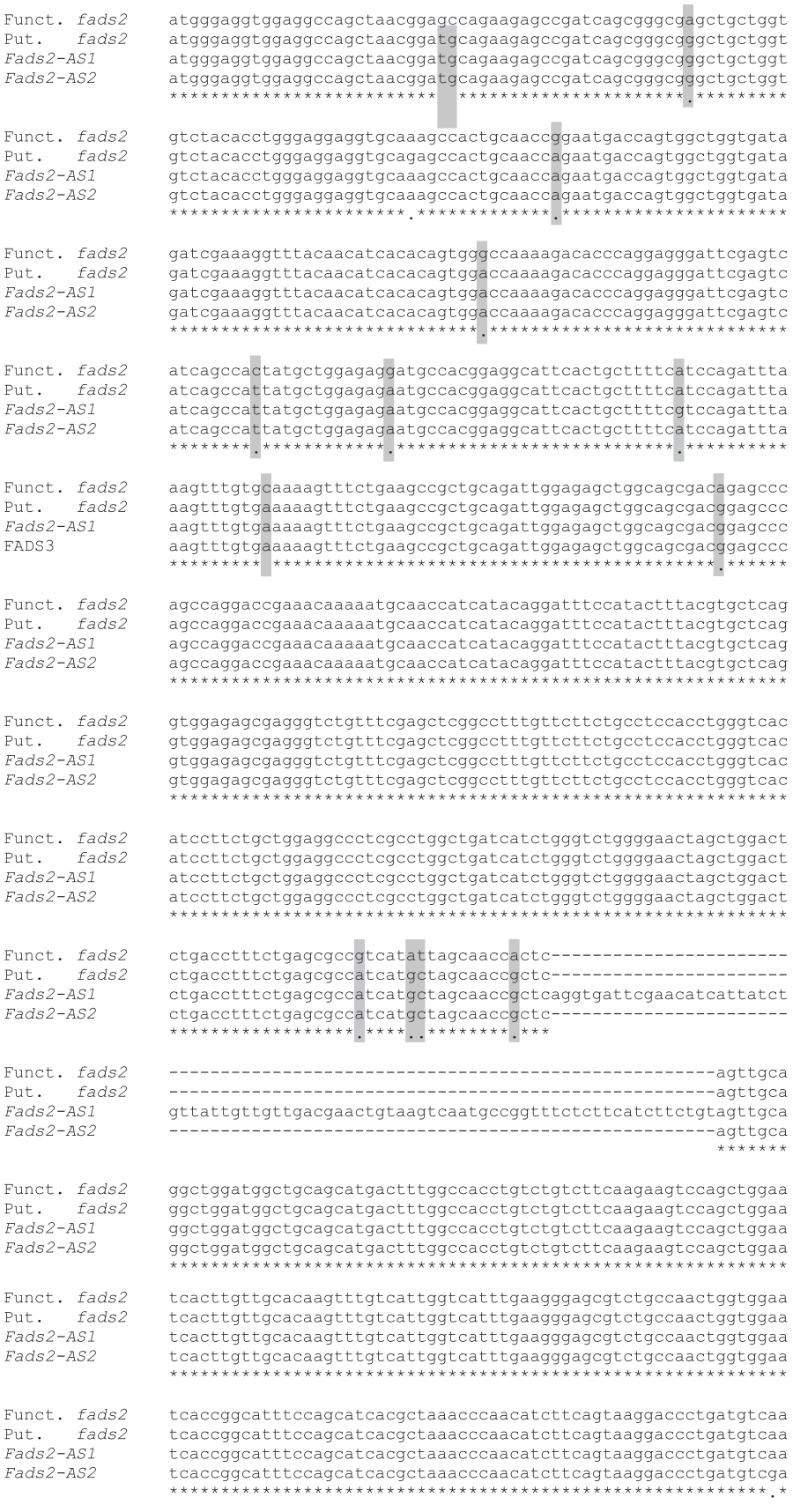
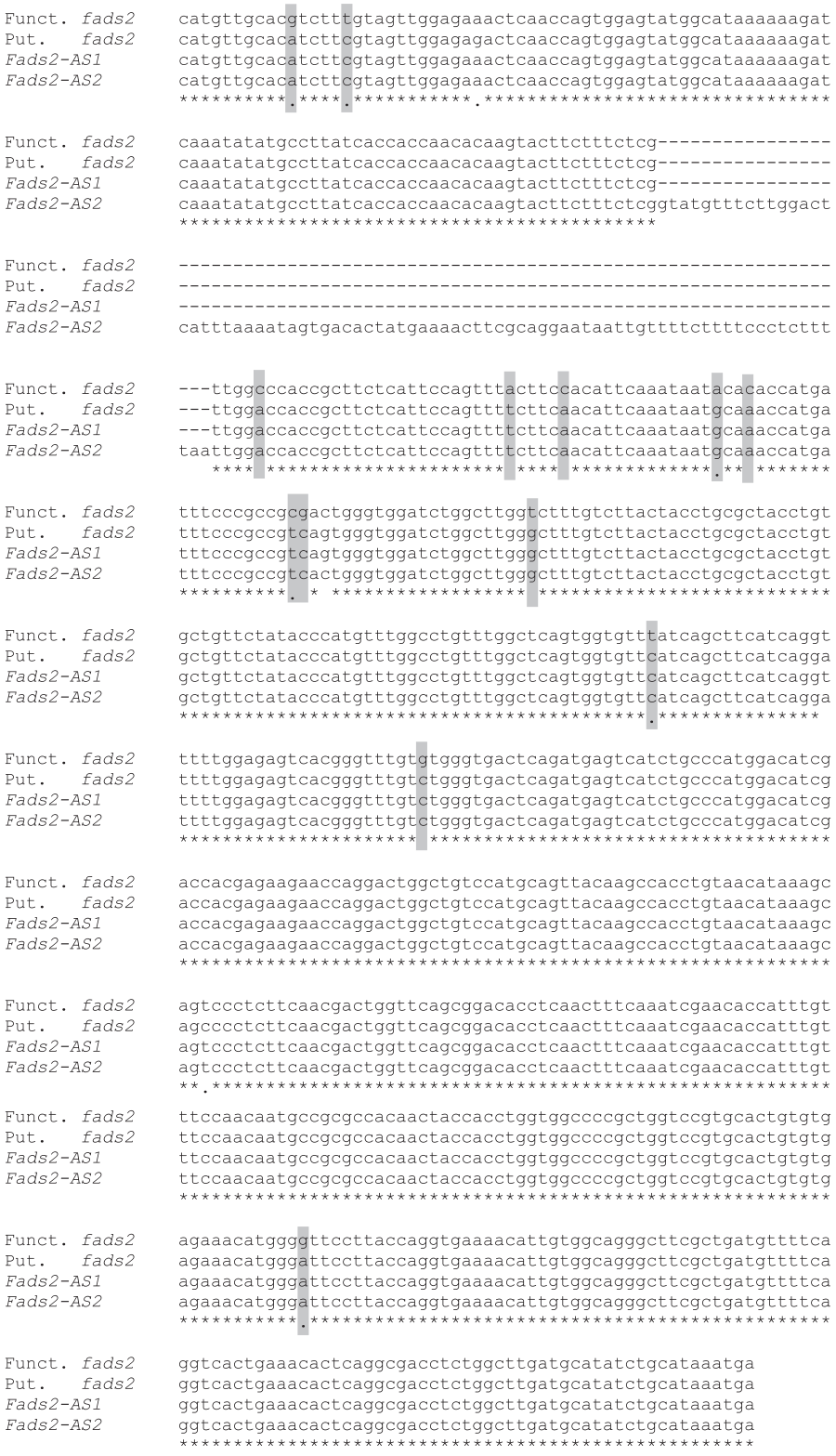
Appendix B

Nucleotide alignment of the functional Eurasian perch *fads2* transcript described in this study with the other putative Eurasian perch *fads2* transcript and the *fads2-AS1* and *fads2-AS2* transcripts. Pairwise nucleotide comparison allowed the identification of the specific homology of some nucleotides between the putative Eurasian perch *fads2* and the *fads2-AS1* and *fads2-AS2* transcripts (indicated in shaded).





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