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ORIGINAL ARTICLE

Chemistry and Biochemistry

Capability of DHA biosynthesis in a marine teleost, Pacific saury *Cololabis saira*: functional characterization of two paralogous Fads2 desaturases and Elovl5 elongase

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Abstract

The Pacific saury *Cololabis saira* is an important fishery resource in the Far East, where it is appreciated for its high lipid content, which includes long-chain polyunsaturated fatty acids (LC-PUFAs) such as docosahexaenoic acid (DHA). Although it has been hypothesized that these fatty acids are derived from their prey items, this study focused on evaluating the capacity of saury for endogenous production of LC-PUFAs. To describe its LC-PUFA biosynthetic pathway, we characterized fatty acid desaturase 2 (Fads2) and elongation of very long-chain fatty acid protein 5 (Elovl5) in Pacific saury. Two paralogous *fads2* genes, namely *fads2a* and *fads2b*, were isolated from the saury and their recombinant protein showed Δ6Δ8 and Δ4Δ5 desaturase activities, respectively. Meanwhile, saury Elovl5 had elongase activity toward C_{18} and C_{20} PUFA. These three enzyme genes were expressed in the brain and liver, although *fads2a* was absent in the latter. Our results suggest that the saury has sufficient enzymatic functions, particularly in its brain, for DHA biosynthesis through the Δ4 pathway even from α-linolenic acid. This information provides novel insights into the origin of LC-PUFAs in Pacific saury and the future perspective for its potential as a source of such vital fatty acids for human consumption.

Keywords Pacific saury · Desaturase · Fads2 · Elongase · Elovl5 · Polyunsaturated fatty acid · Docosahexaenoic acid · Biosynthesis

Introduction

Fish and seafood are now widely accepted as important components of a healthy diet for humans (Tacon and Metian 2013; Tilami and Sampels 2018). Their beneficial effects can be particularly accounted to their unique lipid contents characterized by the abundance of eicosapentaenoic acid (EPA; 20:5n–3) and docosahexaenoic acid (DHA; 22:6n–3)—the members of the family of n–3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFAs) (Tur et al. 2012). These fatty acids play a wide range of essential physiological roles as components of the cellular biomembrane and as precursors of bioactive derivatives that provide various health benefits (Russell and Bürgin-Maunder 2012; Zárate et al. 2017).

In vertebrates, n–3 LC-PUFAs can be converted endogenously from α–linolenic acid (ALA; 18:3n–3), through the LC-PUFA biosynthetic pathway that is catalyzed by two types of key enzymes, namely fatty acid desaturases (Fads) and elongation of very long-chain fatty acids (Elovl) proteins (commonly referred to as "elongases") (Fig. 1; Castro et al. 2016). The desaturases introduce a double bond in the acyl chain and are called Δ6, Δ8, Δ5, and Δ4 desaturases, depending on the specific position of the double bond. Meanwhile, elongases are rate-limiting enzymes that catalyze the addition of two carbon units to the carboxyl terminus of the chain. ALA, which is a dietary essential fatty

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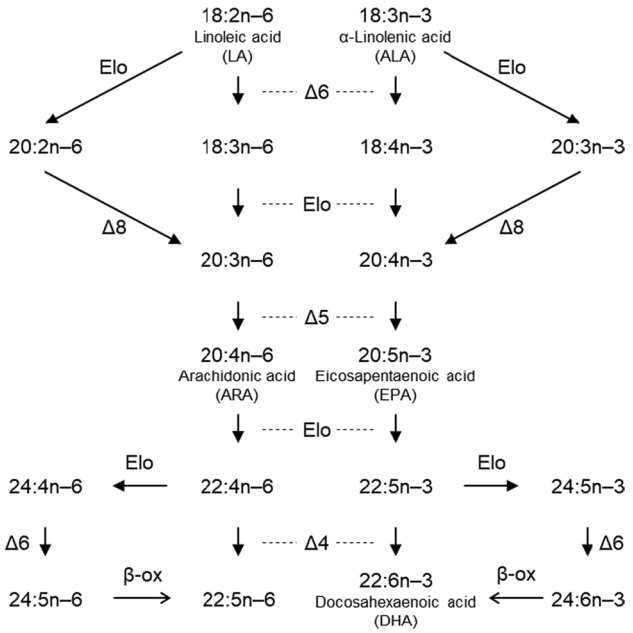


Fig. 1 LC-PUFA biosynthetic pathway in vertebrates. Reactions catalyzed by desaturase are indicated by "Δx" according to the position to which the double bond is introduced. "Elo" and "β-ox" indicate chain elongation catalyzed by elongase and chain shortening by beta-oxidation, respectively

acid found even in many land plants, is converted to EPA by two alternative routes (Fig. 1), namely the Δ6 pathway (Δ6 desaturation–elongation–Δ5 desaturation) and the Δ8 pathway (elongation–Δ8 desaturation–Δ5 desaturation). Subsequently, DHA is biosynthesized from EPA via two alternative pathways (Fig. 1): the Sprecher pathway (elongation–elongation–Δ6 desaturation–β-oxidation) or the Δ4 pathway (elongation–Δ4 desaturation).

Most of the marine Acanthopterygii species, which include numerous marine food fish that provide substantial EPA and DHA for human consumption, are not able to produce n–3 LC-PUFAs due to their incomplete array of genes for LC-PUFA biosynthesis. It has been shown that Acanthopterygii lost *fads1* and *elovl2* through evolution, whose orthologues encode Δ5 desaturase and elongase that preferentially act toward C_{22} substrate, respectively (Morais et al. 2009; Castro et al. 2012; Monroig et al. 2022). Notably, only *fads2* and *elovl5* encoding Δ6Δ8 desaturase and elongase preferentially acting toward C_{18} and C_{20} substrates, respectively, are the genes retained by most marine Acanthopterygii (Monroig et al. 2022). Thus, they are not capable of LC-PUFA biosynthesis and strictly require preformed LC-PUFAs in their diet for normal development and growth (Tocher 2010).

The Pacific saury *Cololabis saira* (Acanthopterygii; Beloniformes; Belonidae) is an epipelagic planktivorous fish distributed from the subarctic to subtropical zones of the North Pacific Ocean (Fuji et al. 2019). It is a commercially important food fish in Asia–Pacific countries and is particularly appreciated in Japan as an autumnal treat. During this season, the saury, with its bountiful lipid content (sometimes over 30% of its edible parts) (Chiba prefecture 2015), migrates through the Oyashio waters along northeastern Japan (Oyaizu et al. 2022). According to the Standard Tables of Food Composition in Japan, 100 g of fillets of Pacific saury contain 25.6 g of lipids that include 7% (= 1.79 g) and 10% (= 2.56 g) of EPA and DHA, respectively (Ministry of Education, Culture, Sports, Science and Technology of Japan 2020). These levels are sufficient to meet the recommended daily intake of EPA + DHA (200–500 mg/day) advised by global health authorities to obtain their health-protective effects, with just one or two pieces of sashimi (about 10–20 g of its muscle). Some part of saury fatty acids is thought to be derived from its prey; however, this study focused on investigating the unknown endogenous capability of this species for LC-PUFA biosynthesis. To explore whether the saury, like other marine Acanthopterygii, lacks the capability for LC-PUFA biosynthesis, we cloned putative *fads2* and *elovl5* genes from Pacific saury and functionally characterized them by the yeast heterologous expression system. Here, we discuss the elucidated LC-PUFA biosynthetic pathway of the Pacific saury, and we present insights into the origin of EPA and DHA in this species, as well as the future perspective of the saury as a source of these vital fatty acids for human well-being.

Materials and methods

Molecular cloning of putative *fads2* and *elovl5* cDNAs from Pacific saury

Individuals of Pacific saury were purchased at the Tsukiji Fish Market (Tokyo, Japan) or caught in Tateyama Bay (Chiba, Japan). Total RNA was isolated from the brains and livers of the sauries using ISOGEN reagent (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions, and trace genomic DNA was eliminated by RQ1 RNase-Free DNase (Promega, WI, USA). First-strand complementary DNA (cDNA) was synthesized from 5 μg of total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, IL, USA) with the Oligo (dT) primer following the manufacturer's instructions. All primers used in this study are summarized in Online Resource 1. The first fragment amplifications of *fads2*-like and *elovl5*-like cDNA were carried out using TaKaRa Ex *Taq* polymerase (Takara Bio, Shiga, Japan) with degenerate primers that were designed to anneal highly conserved regions of *fads2* and *elovl5* orthologs of several fish species. All PCR conditions employed in this

study are summarized in Online Resource 2. The resulting products were analyzed on a 0.7% agarose gel, purified with FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan) and cloned using the pGEM T-Easy Vector System I (Promega). The clones were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, MA, USA) and an ABI PRISM 3100-Avant Genetic Analyzer (Thermo Fisher) following the manufacturer's instructions. To amplify 5′ and 3′ ends of the cDNA, rapid amplifications of cDNA ends (RACE) were performed by a GeneRacer Kit (Thermo Fisher) in accordance with the manufacturer's instructions using gene-specific primers designed for RACE PCR. The resulting sequences were assembled to produce the full open reading frame (ORF) for each gene using CLC Main Workbench 7.7.2 (QIAGEN, Venlo, The Netherlands). The cDNA containing the full ORF was amplified using gene-specific primers annealing to the 5′ and 3′ untranslated regions and PrimeSTAR Max DNA polymerase (Takara Bio). The subsequent purification, cloning, and sequencing of the ORF-containing amplicons were performed as described above, except for the addition of 3′ adenosine overhangs to the PCR products by TaKaRa *Taq* polymerase (Takara Bio) for ligating into the T-vector.

Sequence and phylogenetic analysis of putative two *fads2* and *elovl5*

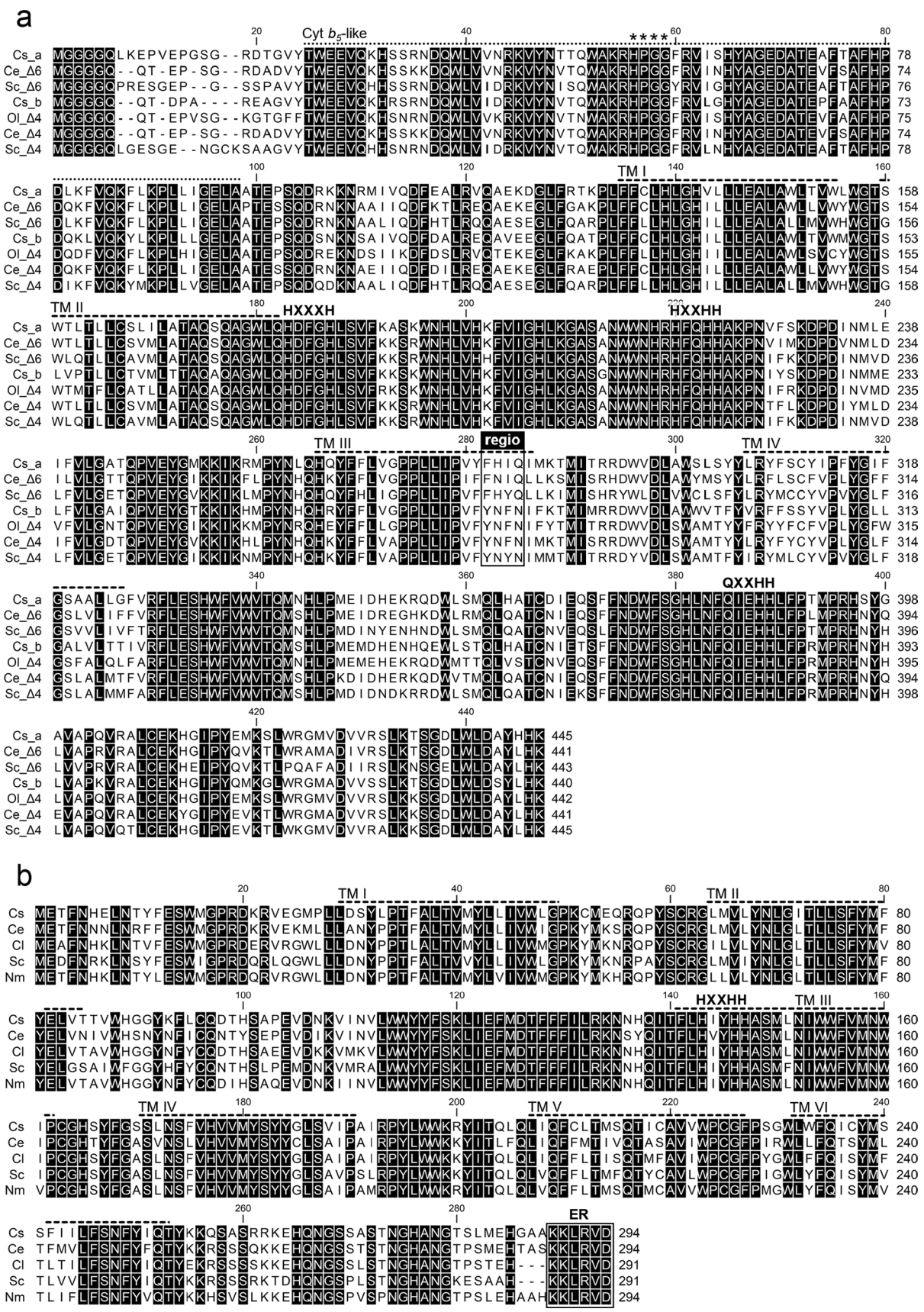
The deduced amino acid (aa) sequences of fish Fads1, Fads2, Elovl2, and Elovl5 orthologs were obtained from the NCBI and Ensembl databases by referring to Monroig et al. 2022. For sequence analysis, several Fads2 or Elovl5 sequences were aligned with the putative orthologs from the Pacific saury using CLC Main Workbench. The Conserved Domain Database of the NCBI (Lu et al. 2020) was used to search for the conserved cytochrome b_5-like domain of Fads2. Transmembrane topologies of the enzymes were predicted by the TOPCONS webserver (Tsirigos et al. 2015). For phylogenetic analyses, the Fads or Elovl enzymes were aligned by MAFFT (v7.222; strategy, L-INS-i; Katoh and Standley 2013) and trimmed by trimAl (option, automated1; Capella-Gutiérrez et al. 2009). The resulting alignments consisted of 396 and 256 aa for Fads and Elovl, respectively. The maximum likelihood phylogenies of these enzymes were calculated by the PhyML 3.0 webserver (Guindon et al. 2010). Both enzymes were analyzed using JTT + G + I + F model as selected by Smart Model Selection in PhyML with Akaike's information criterion and 1000 repeats of standard bootstrap analysis (Lefort et al. 2017). The resulting trees were visualized by FigTree v1.4.3 available at http://tree.bio.ed.ac.uk/software/figtree/.

Functional characterization of two Fads2 and Elovl5 by heterologous expression in yeast

The cDNAs corresponding to the two *fads2* and *elovl5* ORFs of the saury were amplified by PrimeSTAR Max DNA polymerase using primers containing *Hin*dIII and *Xba*I restriction enzyme sites (Online Resource 1, 2). The PCR products were digested by *Hin*dIII and *Xba*I (Takara Bio) after gel purification and then cloned into similarly digested yeast expression vector pYES2 (Thermo Fisher). Transformation and culture of yeast *Saccharomyces cerevisiae* INV*Sc*1 (Thermo Fisher) were performed by following the methods described by Li et al. 2010. The following fatty acids (Larodan Fine Chemicals, Malmo, Sweden) were used as substrates: 18:2n–6, 18:3n–3, 20:2n–6, 20:3n–3, 20:3n–6, 20:4n–3, 22:4n–6, and 22:5n–3 for the yeast transformed with *fads2*; and 18:2n–6, 18:3n–3, 18:3n–6, 18:4n–3, 20:4n–6, 20:5n–3, 22:4n–6, and 22:5n–3 for the yeast transformed with *elovl5*. After 48 h of culture at 30 °C in the presence of each substrate fatty acid, the yeast cells were collected, washed twice with ice-cold Hank's balanced salt solution, and then lyophilized (FDU-1200 lyophilizer; Tokyo Rikakikai, Tokyo, Japan). Δ6 activities toward 24:5n–3 and 24:4n–6 of Fads2 were analyzed by following the co-transformation method described by Oboh et al. 2017. Briefly, the yeast cells were co-transformed with pYES2 and p415TEF vectors carrying the *fads2* and zebrafish *Danio rerio elovl2* ORFs, respectively. The co-transformed yeast cells were grown with 22:5n–3 or 22:4n–6 during the first 24 h to produce 24:4n–6 or 24:5n–3 by the Elovl2 expressed constitutively under the regulation of the *TEF1* promoter. Then, 2% galactose was added to induce the expression of *fads2* connected to the *GAL1* promoter, and the cultures were continued for another 48 h to allow catalysis of consecutive desaturations.

Fatty acid analysis of the transgenic yeast by gas chromatography

Fatty acid methyl esters (FAMEs) were prepared using the Fatty Acid Methylation Kit (Nacalai Tesque, Kyoto, Japan) and purified using the Methylated Fatty Acid Purification Kit (Nacalai Tesque) following the manufacturer's instructions. The FAMEs were analyzed using a gas chromatograph (GC-2025; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a silica capillary column (L × I.D. 30 m × 0.32 mm, df 0.25 μm, SUPELCOWAX 10: Merck, Darmstadt, Germany) as previously described (Kabeya et al. 2015). The conversion ratios indicating each of the enzymatic activities were calculated as the proportion of fatty acid substrate converted to products by following formula: [product area/(product area + substrate area)] × 100 (%).


a
Cyt b_5-like
TM I
TM II
HXXXH
HXXHH
TM III
regio
TM IV
QXXHH
Cs_a
Ce_Δ6
Sc_Δ6
Cs_b
Ol_Δ4
Ce_Δ4
Sc_Δ4
b
TM I
TM II
HXXHH
TM III
TM IV
TM V
TM VI
ER
Cs
Ce
Cl
Sc
Nm

◂**Fig. 2** Deduced amino acid alignments of Fads2 (**a**) and Elovl5 (**b**) from Pacific saury. The cytochrome b_5-like domain with a heme-binding motif (HPGG, labeled by asterisks) and predicted transmembrane regions are indicated by dots and dashed lines, respectively. The regioselectivity motif (regio) in Fads2 and endoplasmic reticulum retrieval signal (ER) in Elovl5 are boxed. The histidine boxes of Fads2 (HXXXH, HXXHH, QXXHH) and Elovl5 (HXXHH) are annotated. The known major activities of Fads2 are also shown following the abbreviated scientific name: Cs, *Cololabis saira*; Ce, *Chirostoma estor*; Sc, *Siganus canaliculatus*; Ol, *Oryzias latipes*; Cl, *Chelon labrosus*

Tissue expression analysis of putative *fads2* and *elovl5* genes by RT–PCR

Gene-specific primers for *fads2* and *elovl5* cDNA were designed to cross the exon boundary predicted from the genomic *fads2* (NCBI Gene ID, 101156814) and *elovl5* (101168981) structures in medaka *Oryzias latipes*, thus avoiding amplification of residual genomic DNA. Universal primers for Beloniformes *actb2* (used as an internal control) were also designed based on the sequences from medaka (100049433), freshwater garfish *Xenentdon cancila* (retrieved from CM026214.1), and mirrorwing flyingfish *Hirundichthys speculiger* (JAGFBZ010000555.1). Brain and liver cDNA were used as templates for RT–PCR amplification with TaKaRa Ex *Taq* polymerase. The primer sequences and PCR conditions are presented in Online Resources 1 and 2, respectively. Aliquots of PCR products were analyzed by electrophoresis on a 1.0% agarose gel. The products were sequenced as described above to confirm the specificity of the primers.

Results

Sequence and phylogenetic analysis of two Fads2 and Elovl5 of Pacific saury

Clones of the two putative *fads2* (*fads2a* and *fads2b*) included ORFs of 1335 and 1320 bp encoding 445 and 440 aa, respectively (GenBank accession LC744974 and LC744975, Fig. 2a). The deduced aa sequences exhibited 77.8% identity and contained all the features of front-end desaturases, including a cytochrome b_5-like domain with heme-binding motif (HPGG) at the N-terminus (Gostincar et al. 2010) and three histidine boxes (HXXXH, HXXHH, and QXXHH; Fig. 2a; Hashimoto et al. 2008). The sequences contained four transmembrane regions that are characteristic of membrane-bound proteins (Fig. 2a; Meesapyodsuk and Qiu 2012). The four aa residues comprising regioselectivity motifs of the Fads2 enzyme (Lim et al. 2014) differ between Fads2a and Fads2b, which have the sequences FHIQ and YNFN, respectively (Fig. 2a). Phylogenetic analysis of the Fads enzymes shows that the two putative Fads2 enzymes of Pacific saury are grouped with Fads2 of other Teleostei with a strong bootstrap support of 98% (Fig. 3a). Notably, the two Fads2 from saury do not comprise a single clade in the present phylogeny (Fig. 3a). All Fads2 enzymes are strongly separated from the Fads1 from mammals and cartilaginous fish, with a bootstrap value of 100% (Fig. 3a).

A clone of putative *elovl5* cDNA harbored an ORF consisting of 882 bp and encoding 294 aa (LC744976). The ORF has conserved features of the Elovl family, i.e., a histidine box (HXXHH), six transmembrane regions, and endoplasmic reticulum retrieval signal with lysine and arginine residues at the C-terminus (Fig. 2b; Jakobsson et al. 2006). The phylogenetic tree of Elovl enzymes shows that the putative Elovl5 of Pacific saury grouping with Euteleostei Elovl5 sequences with a high bootstrap value of 86% (Fig. 3b). Elovl5 and Elovl2 clusters were consistently separated in the bootstrap analysis (Fig. 3b).

Functional characterization of Pacific saury Fads2a, Fads2b, and Elovl5

The Pacific saury Fads2a, Fads2b, and Elovl5 were functionally characterized by heterologous expression in the yeast cultured in the presence of various fatty acid substrates. Fads2a showed Δ6 desaturase activity toward 18:2n–6 and 18:3n–3 (ALA) to produce 18:3n–6 and 18:4n–3, respectively, while Fads2b did not (Table 1; Fig. 4a, b). In addition, Fads2a was more effective at Δ8 desaturation of 20:2n–6 and 20:3n–3 to 20:3n–6 and 20:4n–3, respectively, compared with Fads2b, which converted less of the n–3 substrate (Table 1; Fig. 4c, d). Both carried out relatively low conversions of Δ5 desaturation, although Fads2b was more efficient than Fads2a, which could only utilize 20:4n–3 as a substrate (Table 1; Fig. 4e, f). Fads2b showed Δ4 desaturase activity toward 22:4n–6 and 22:5n–3 to produce 22:5n–6 and 22:6n–3 (DHA), respectively, whereas Fads2a did not (Table 1; Fig. 4g, h). Furthermore, only Fads2a exhibited Δ6 desaturase activity toward 24:4n–6 and 24:5n–3 (Table 1; Fig. 4i, j).

$C_{20–22}$ products (20:2n–6 to 22:2n–6, 20:3n–3 to 22:3n–3, 20:3n–6 to 22:3n–6, and 20:4n–3 to 22:4n–3) were observed in the transgenic yeast expressing Pacific saury Elovl5 and cultured with C_{18} substrates (18:2n–6, 18:3n–3, 18:3n–6, and 18:4n–3; Table 2; Fig. 5a, b). The transgenic yeast also converted C_{20} substrates (20:4n–6 and 20:5n–3) to C_{22} products (22:4n–6 and 22:5n–3), consistent with the above result on C_{18} substrates (Table 2; Fig. 5c). We detected no C_{24} product even in the presence of readily used substrates, such as 22:4n–6 and 22:5n–3 (Table 2; Fig. 5d). Finally, the two Fads2 and Elovl5 of Pacific saury consistently converted n–3 fatty acids more efficiently than the n–6 (Tables 1 and 2).

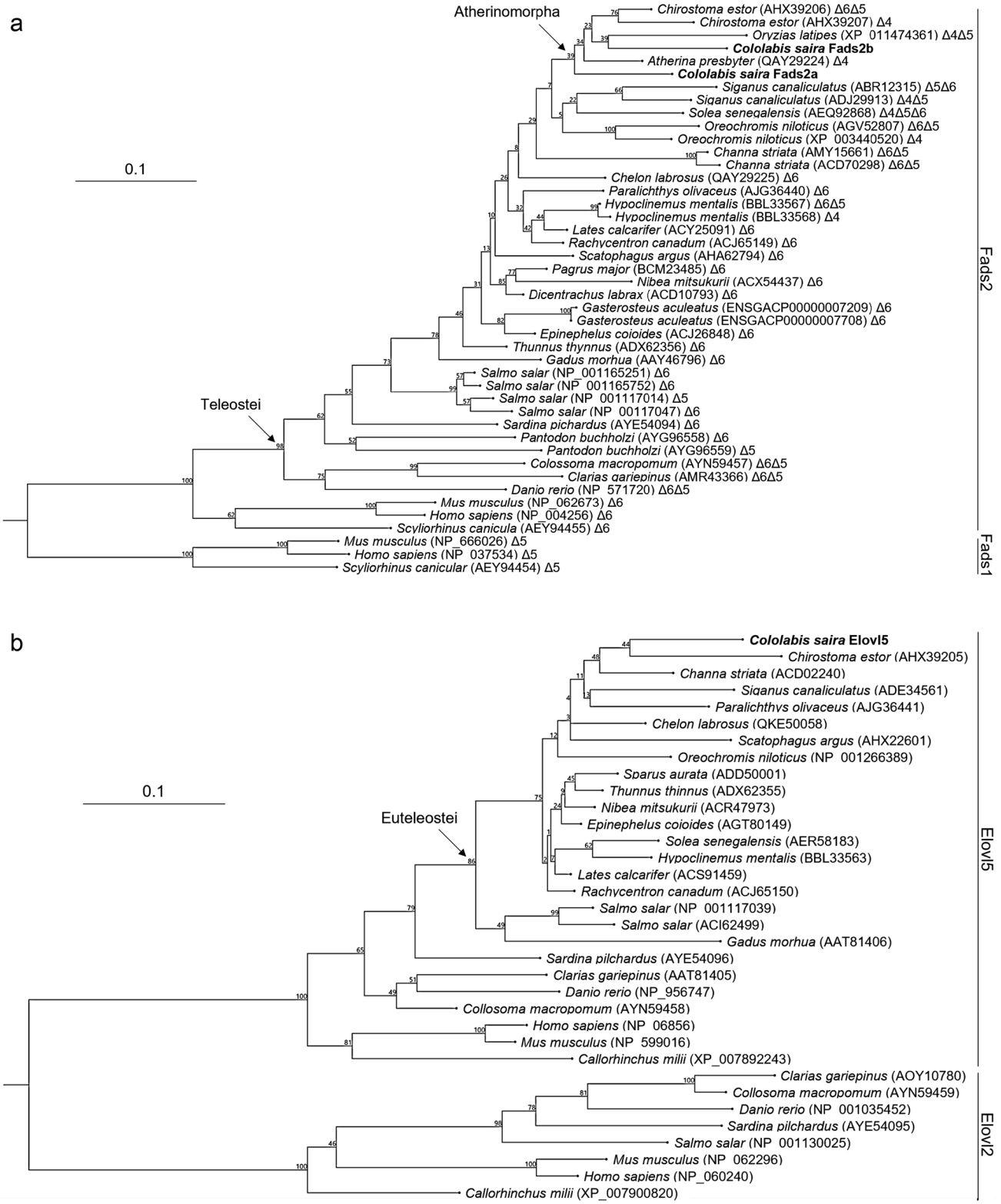


Fig. 3 Maximum likelihood phylogeny of Fads (**a**) and Elovl (**b**) sequences. Accession numbers of each sequence are shown and, for Fads enzymes, followed by its known activities summarized by Monroig et al (2022). The clade for each ortholog is indicated by a vertical line with the gene name. Numbers at each node represent bootstrap values

Table 1 Substrate conversions in yeast transformed with Pacific saury Fads2a or Fads2b

Substrate	Product	Conversion (%)		Activity
		Fads2a	Fads2b	
18:2n–6	18:3n–6	18.3	n.d	Δ6
18:3n–3	18:4n–3	48.6	n.d	Δ6
20:2n–6	20:3n–6	27.3	n.d	Δ8
20:3n–3	20:4n–3	39.7	1.8	Δ8
20:3n–6	20:4n–6	n.d	1.1	Δ5
20:4n–3	20:5n–3	1.1	3.6	Δ5
22:4n–6	22:5n–6	n.d	3.0	Δ4
22:5n–3	22:6n–3	n.d	9.8	Δ4
Co-expressed with *Danio rerio* Elovl2				
18:2n–6	18:3n–6	11.0	n.d	Δ6
18:3n–3	18:4n–3	21.0	n.d	Δ6
24:4n–6	24:5n–6	19.4	n.d	Δ6
24:5n–3	24:6n–3	27.5	n.d	Δ6

Conversion percentages were calculated by the following formula: [product area/(product area + substrate area)] × 100

n.d. not detected

Tissue expression analysis of Pacific saury *fads2a*, *fads2b*, and *elovl5*

RT–PCR conducted using brain and liver cDNA revealed that saury *fads2a* was expressed only in the brain, whereas *fads2b* was expressed in both brain and liver, with higher expression levels in the brain (Fig. 6). Transcripts of *elovl5* were also amplified in both tissues, with a slightly higher expression level in the liver (Fig. 6).

Discussion

The high levels of EPA and DHA in lipid-rich Pacific saury makes this fish an important source of these bioactive fatty acids for humans. To understand the source of such fatty acids in this species, and especially to verify the possibility of endogenous production, we examined its LC-PUFA biosynthetic pathway based on functional evidence from three newly cloned genes, *fads2a*, *fads2b*, and *elovl5*, which encode the enzymes involved in LC-PUFA biosynthesis. The deduced amino acid sequences of the two Fads2 and Elovl5 contained the conserved features of front-end desaturases and Elovl proteins, respectively; and they clustered phylogenetically with their respective Fads2 and Elovl5 orthologs of vertebrates. These results indicate that the two proteins play a role in LC-PUFA biosynthesis, although the substrate specificity of the two paralogous Fads2 is presumed to be different because of substitutions in the regioselectivity motif. The results of functional analyses in yeast indicated that the prominent activities of Fads2a were Δ6 and Δ8 desaturations; in contrast, Fads2b performed Δ4 desaturation. Although both Fads2a and Fads2b perform Δ5 desaturation, the former utilized only n–3 substrate when expressed by yeast. Pacific saury Elovl5 converted C_{18} (18:2n–6, 18:3n–3, 18:3n–6, and 18:4n–3) and C_{20} (20:4n–6 and 20:5n–3) substrates to products that can function as substrates of Δ8 desaturation (20:2n–6 and 20:3n–3), Δ5 desaturation (20:3n–6 and 20:4n–3), and Δ4 desaturation (22:4n–6 and 22:5n–3). However, Elovl5 did not convert C_{22} substrates (22:4n–6 and 22:5n–3) to C_{24} products (24:4n–6 and 24:5n–3), even though Fads2a performed Δ6 desaturation in the Sprecher pathway. These results indicate that sequential reactions involving the above three proteins can biosynthesize LC-PUFA through the Δ6 and Δ8 pathways, followed by the Δ4 pathway (Fig. 7). We therefore conclude that, in contrast to the traditional viewpoint, Pacific saury has a potential to biosynthesize EPA and DHA from ALA as well as arachidonic acid from linoleic acid, especially in the brain, where these three genes were expressed.

This is the first report of a marine planktivorous fish species possessing Fads2 with Δ4 desaturase activity and a set of enzymatic functions sufficient to achieve DHA biosynthesis from ALA. In the subtropical areas of the northwestern Pacific, larvae of Pacific saury feed on copepod nauplii and several tiny copepod species (Odate 1977; Fuji et al. 2019). They migrate northward to a transitional zone on the Oyashio and Kuroshio current, where the saury shows outstanding growth and massive energy storage for the following spawning migration to subtropical areas, relying on abundant zooplankton such as *Neocalanus* copepods and the North Pacific krill *Euphausia pacifica* (Sugisaki and Kurita 2004; Miyamoto et al. 2020). Due to the substantial amounts of bioactive n–3 LC-PUFAs in the aforementioned important prey items (Saito and Kotani 2000; Kusumoto et al. 2004; Yamada et al. 2016, 2017), Pacific saury would not experience depletion of preformed EPA and DHA throughout their entire life as a zooplankton feeder, although the capability of endogenous production is available.

Marine fish were widely considered to have lost the enzymes for the LC-PUFA biosynthetic pathway because of the relaxed selective pressure from its habitat, where n–3 LC-PUFAs are bountiful, or because they occupy a high trophic level in such an environment (Tocher 2010; Castro et al. 2016). Given that these conditions can also apply in the case of saury, it was only natural to predict that this species has also lost the enzymes. However, recent extensive research on a wide range of teleosts and their relatives has indicated the need to review this simplistic concept. The studies in the relevant literature suggest that the capability of LC-PUFA biosynthesis of each fish species is influenced by its phylogenetic position, which determines the inherent gene array of the enzymes through genetic events, such as

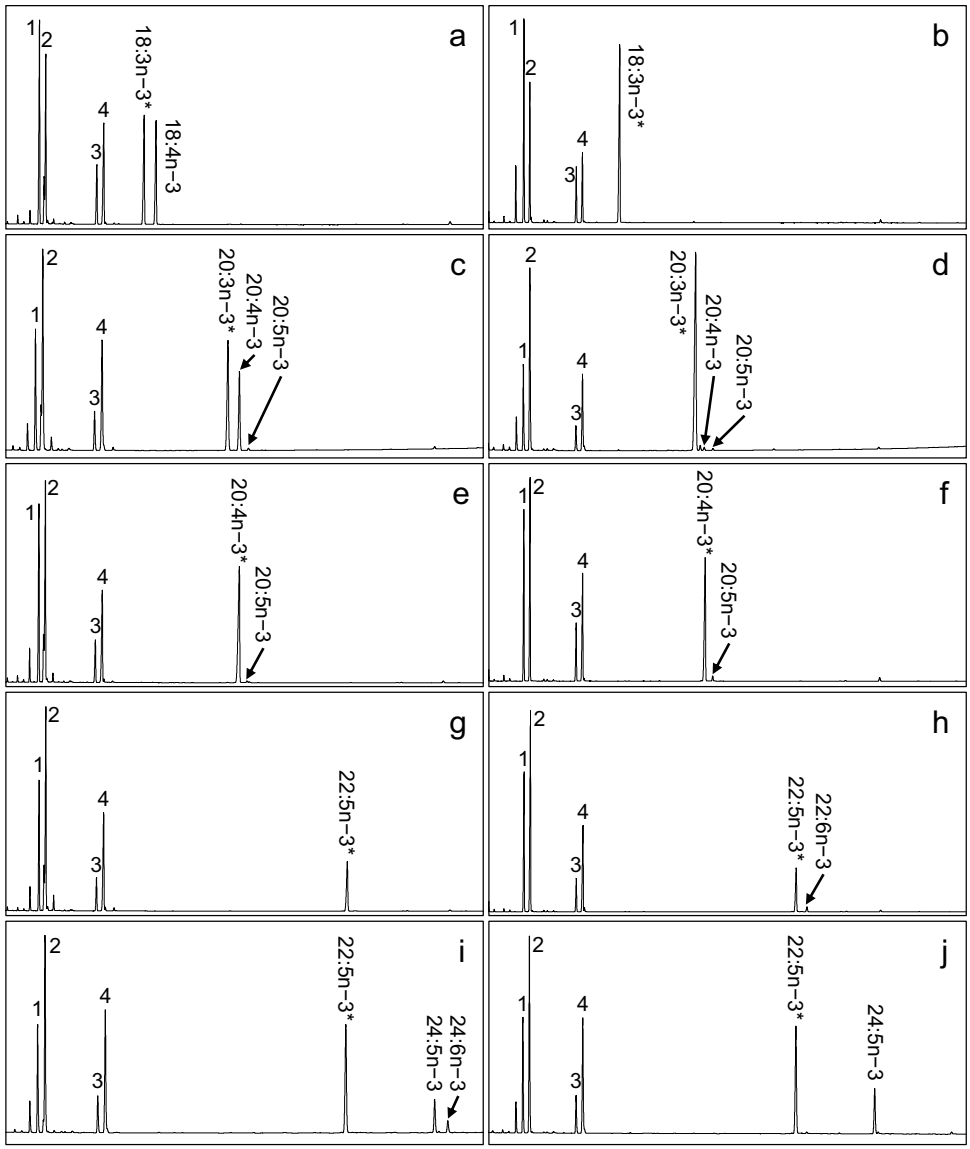


Fig. 4 Gas chromatographic fatty acid profiles of yeast transformed with pYES2 carrying Pacific saury *fads2a* (**a**, **c**, **e**, **g**) or *fads2b* (**b**, **d**, **f**, **h**); and those of co-transformed yeast with zebrafish *elovl2* in p415TEF and *fads2a* (**i**) or *fads2b* (**j**) in pYES2. The yeast were grown in the presence of exogenously added fatty acid substrate indicated by asterisk, namely 18:3n–3 (**a**, **b**), 20:3n–3 (**c**, **d**), 20:4n–3 (**e**, **f**), 22:5n–3 (**g**–**j**). Horizontal and vertical axes represent retention time and FID signal intensity, respectively. The first four major peaks are the yeast endogenous fatty acids, namely 16:0 (1), 16:1 isomers (2), 18:0 (3), and 18:1n–9 (4)

loss, duplication, and functional diversification of the genes, and these events occur in each lineage (Monroig et al. 2018; Garrido et al. 2019; Galindo et al. 2021).

Vertebrate Fads2 with Δ4 desaturase activity and the complete LC-PUFA biosynthetic pathway with another Fads2 with Δ5Δ6 activities have been first reported in rabbitfish *Siganus canaliculatus* (Li et al. 2010). Subsequently, these features have also been reported in several fish species within Acanthopterygii, such as pike silverside *Chirostoma estor*, a member of Atheriniformes (Fonseca-Madrigal et al. 2014; Monroig et al. 2022). Oboh et al. (2017) demonstrated multiple emergences of Fads2 with the Δ4 regioselectivity motif (YXXN) in Acanthopterygii by in silico screening; and they functionally characterized one of the three Fads2 paralogs as a Δ4Δ5 desaturase in medaka *Oryzias latipes*, which belongs to Beloniformes. Our phylogenetic analysis revealed a single cluster of species within Beloniformes (Pacific saury and medaka) and Atheriniformes (pike silverside and sand smelt *Atherina presbyter*), in which Δ4 Fads2 has been characterized (Garrido et al. 2019), suggesting that the superorder Atherinomorphae (from which these two orders descended) has undergone diversification events, such as gene duplication and subsequent neo-/subfunctionalization, particularly on *fads2*. The discovery of an enzyme set that enables LC-PUFA biosynthesis in Pacific saury—a species of marine fish that feeds on n–3 PUFA-rich prey—supports the recent shift in the concept of LC-PUFA biosynthesis in marine teleost.

The evolutionary consequences of retaining the enzyme complement in LC-PUFA biosynthesis are unclear; however, it would guarantee the endogenous availability and homeostasis of the bioactive fatty acids in Pacific saury. During the feeding period, flesh lipid content of Pacific saury increases dramatically from 3% to > 15% owing to its high feeding

Table 2 Substrate conversions in yeast transformed with Pacific saury Elovl5

Substrate	Product	Conversion (%)	Activity
18:2n–6	20:2n–6	22.1	$C_{18\rightarrow20}$
	22:2n–6	2.1	$C_{20\rightarrow22}$
	Total	24.2	
18:3n–3	20:3n–3	43.0	$C_{18\rightarrow20}$
	22:3n–3	3.8	$C_{20\rightarrow22}$
	total	46.8	
18:3n–6	20:3n–6	57.3	$C_{18\rightarrow20}$
	22:3n–6	9.6	$C_{20\rightarrow22}$
	Total	66.9	
18:4n–3	20:4n–3	52.0	$C_{18\rightarrow20}$
	22:4n–3	26.3	$C_{20\rightarrow22}$
	Total	78.3	
20:4n–6	22:4n–6	55.3	$C_{20\rightarrow22}$
20:5n–3	22:5n–3	60.2	$C_{20\rightarrow22}$
22:4n–6	22:4n–6	n.d	$C_{22\rightarrow24}$
24:5n–3	24:6n–3	n.d	$C_{22\rightarrow24}$

Conversion percentages were calculated by the following formula: [product area/(product area + substrate area)] × 100

n.d. not detected

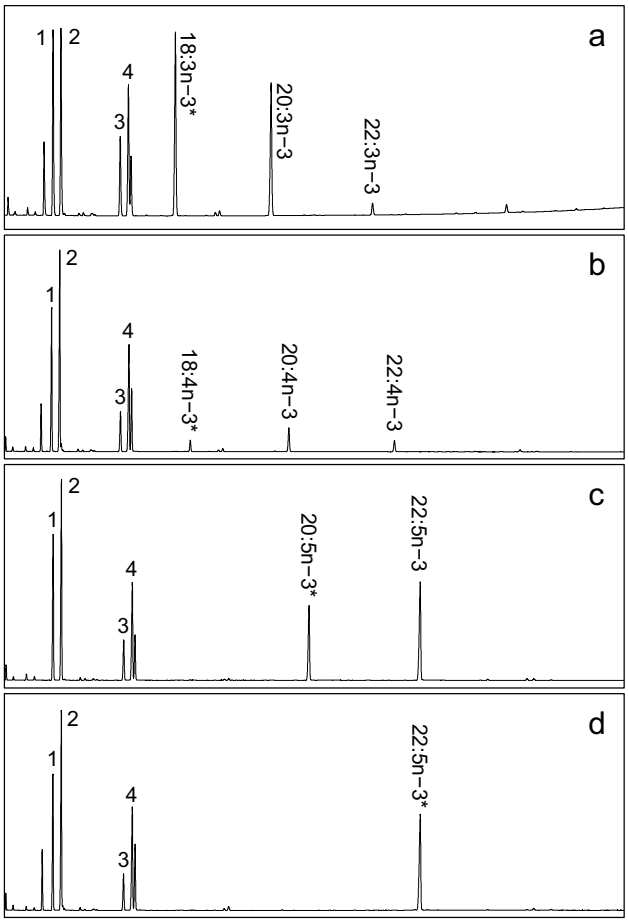


Fig. 5 Gas chromatographic fatty acid profiles of yeast transformed with Pacific saury *elovl5* in pYES2. The yeasts were grown in the presence of exogenously added fatty acid substrate indicated by asterisk, namely 18:3n–3 (**a**), 18:4n–3 (**b**), 20:5n–3 (**c**), and 22:5n–3 (**d**). Horizontal and vertical axes represent retention time and FID signal intensity, respectively. The first four major peaks are the yeast endogenous fatty acids, namely 16:0 (1), 16:1 isomers (2), 18:0 (3), and 18:1n–9 (4)

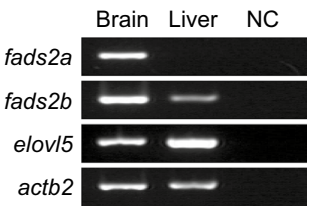


Fig. 6 Expression pattern of *fads2a*, *fads2b*, and *elovl5* in the brain and liver of Pacific saury analyzed by RT–PCR. The gene *actb2* (LC744977) was used as an internal control. NC, negative control

activity on the abundant prey (Ota et al. 1980; Sugisaki and Kurita 2004). One of the important preys of saury during this period is the copepod *Neocalanus cristatus*, with a DHA/EPA ratio of 0.84 in wax ester, accounting for most of its lipid content, particularly in the copepodite stage found at depths shallower than 250 m, where the Pacific saury hunts (Yamada et al. 2016; Miyamoto et al. 2020). A lower DHA/EPA ratio of < 0.3 has also been reported in *Neocalanus* copepodites at depths shallower than 400 m (Saito and Kotani 2000). The Pacific saury's other important prey is the North Pacific krill, with DHA/EPA ratios of 0.79 ± 0.26 and 0.72 ± 0.40, respectively, in total lipid, according to Kusumoto et al. 2004 and Yamada et al. 2017. Thus, the DHA/EPA ratios of major prey items of the Pacific saury tend to have values less than one, reflecting the low DHA/EPA ratio (0.63 on average) in neutral lipids of the saury tissues with a total lipid content of 21.1%, which occurs during the feeding period (Hara et al. 1982). In contrast, polar lipids during the same period have an average DHA/EPA ratio of 3.08, which is similar to that of exhausted saury during the spawning period, with a total lipid content of 1.6% (DHA/EPA = 2.88 on average; Hara et al. 1982). Higher DHA/EPA ratios in polar lipids regardless of the migratory stage suggest a possible contribution of endogenous DHA biosynthesis from EPA to maintain an appropriate balance of fatty acid composition in this lipid class, which is for biostructural purposes. The selective accumulation and retention of DHA could also contribute to the relatively high DHA/EPA ratios in polar lipids (Glencross 2009). Here, we have confirmed the expression of *elovl5* and *fads2b* in Pacific saury, indicating the functionality of the Δ4 pathway, which contributes to DHA biosynthesis from the high levels of dietary EPA in

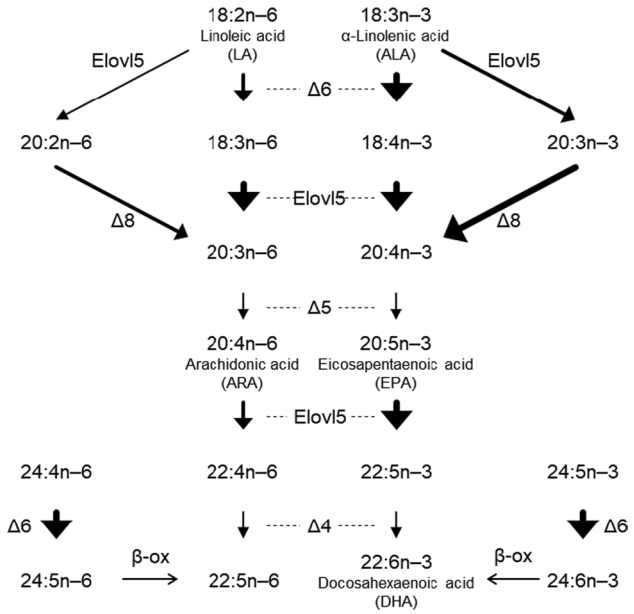


Fig. 7 The reconstructed LC-PUFA biosynthetic pathway of Pacific saury based on the activities of recombinant Fads2a, Fads2b, and Elovl5 enzymes expressed in a yeast expression system. The thickness of arrows reflects the conversion ratio for each step although comparison among the steps and estimation of in vivo efficiency are still difficult: for desaturations represented by the sum of Fads2a and Fads2b conversions, thick lines indicate higher than 30%; medium lines indicate higher than 15% and lesser than or equal to 30%; fine lines indicate lesser than 15%. For elongation by Elovl5, thick lines indicate higher than 60%; medium lines indicate higher than 30% and lesser than or equal to 60%; fine lines indicate higher than 0% and lesser than or equal to 30%; null lines indicate "not detected"

the liver, where dietary fatty acids are delivered after intestinal absorption and circulation throughout the body (Tocher 2003).

In the Sprecher pathway in Pacific saury, Fads2a can perform Δ6 desaturation of C_{24} fatty acids, but Elovl5 cannot provide the C_{24} products. The key enzyme for elongation of C_{22} fatty acids is encoded by *elovl2* in vertebrates; however, this gene has been lost in the Acanthopterygian genome (Castro et al. 2016; Monroig et al. 2022). Recently, another elongase, Elovl4, which plays a pivotal role in the biosynthesis of very long-chain ($> C_{24}$) fatty acids, was considered to compensate for the absence of Elovl2 by elongating C_{22} substrates to C_{24} products in Acanthomorpha species (Monroig et al. 2018, 2022). The two paralogs of *elovl4*, namely *elovl4a* and *elovl4b*, are widely distributed in the teleost lineage and can be found in medaka (XM_023964398.1 and XM_004083728.4), a close relative of Pacific saury. Analyses of the tissue expression pattern of these genes in several species of Acanthopterygii have demonstrated that *elovl4a* and *elovl4b* are primarily expressed in the brain and eye, respectively, whereas neither is expressed at high levels in the liver (Xue et al. 2014; Jin et al. 2017; Morais et al. 2020; Luo et al. 2021). Thus, we hypothesize that Pacific saury Fads2a is expressed in the brain but not in the liver because only low levels of C_{24} substrates are produced by Elovl4s in the liver, while Elovl4a is able to provide more of the substrate in the brain. On-site DHA biosynthesis through the Sprecher pathway driven by Fads2a and Elovl4a would contribute to an adequate supply of DHA, which is essential for the proper function of the neural tissue (Mourente 2003). However, the presence, function, and expression pattern of Elovl4s in Pacific saury remain to be investigated. Furthermore, the ratio of Δ6 activity toward 24:5n–3 to that toward 18:3n–3 in the co-expression analysis of Pacific saury Fads2a was 1.31, comparable to that of Fads2 of zebrafish (1.33), which biosynthesize DHA through the Sprecher pathway (Oboh et al. 2017). Overall, there is evidence to suggest the possibility of DHA biosynthesis via the Sprecher pathway, in addition to the Δ4 pathway, in the brain of Pacific saury.

Pacific saury is an essential fisheries resource for the people of several countries in the Far East. However, stock biomass of the saury has been decreasing since the mid-2000s, reaching a historically low level in 2020 and remaining low in recent years (North Pacific Fisheries Commission 2022). Although both environmental factors and fishing may drive the abundance levels of Pacific saury (Yatsu et al. 2020), the current stock of this fish has been determined to be overfished and subject to overfishing (North Pacific Fisheries Commission 2022). Thus, shifting to the sustainable use of the saury is required, and to this end, the North Pacific Fisheries Commission agreed to limit the total allowable catch of this species toward proper management of the stocks since 2019 (North Pacific Fisheries Commission 2019, North Pacific Fisheries Commission 2023). To compensate for some extent of our consumption, saury aquaculture may be an option, despite the associated difficulties owing to the success in spawning parental fish raised from natural eggs attached to drifting algae and eggs obtained over generations in aquariums (Tsuzaki 2000a, b, 2001a, b; Nakaya et al. 2010). Saury aquaculture is potentially beneficial as it has the full range of enzymes for DHA biosynthesis, even those using ALA, a substrate found in vegetable oils that is used as a substitute for fish oil for the sustainable formulation of aquafeed. In this respect, it will be important to elucidate the in vivo efficiency of Δ5 desaturation driven by the two Fads2 enzymes, which showed relatively low Δ5 activities in yeast. This can be accomplished by analyses such as tracer experiments and feeding trials with live organs and seedlings, respectively, of Pacific saury.

Recent predictions have claimed that global warming influences the lipid content and composition of fatty acid, especially EPA and DHA, in algae, which are the principal suppliers of these unique compounds (Kang 2011; Gladyshev et al. 2013; Hixson and Arts 2016). Hixson and Arts

(2016) reported a potential decrease in global EPA and DHA production of phytoplankton by 8.2% and 27.8%, respectively, if the water temperature rises by 2.5 °C. Most marine teleosts without the capability of LC-PUFA biosynthesis will be impacted by such nutritional changes due to their heavy dependence on preformed DHA that is transmitted over aquatic food webs. Based on the above-mentioned study, Colombo et al. (2020) estimated that DHA availability from marine fish for human consumption will decrease by 10% to over 50% in 2100, depending on humanity's efforts to mitigate climate change. Nonetheless, due to the metabolic capability of the saury, it could present robustness in biological performances and become a reliable source of LC-PUFAs in an environment with low primary LC-PUFA production. Furthermore, based on the above phylogenetic position concept, the relative lineages of the saury may offer a treasure trove of marine fish with substantial LC-PUFA biosynthetic capabilities. Future studies should aim to clarify their characteristics and potential uses, whether in the wild or under cultivation, to secure the human dietary requirement for LC-PUFA in the changing world.

In conclusion, the present study demonstrated that Pacific saury possesses two paralogous *fads2*, namely *fads2a* and *fads2b*, encoding Δ6Δ8 and Δ4Δ5 desaturases, respectively; and *elovl5*, encoding elongase, which catalyzes the elongation of C_{18} and C_{20} PUFAs. The Fads2a has Δ6 desaturation activity toward C_{24} substrates in addition to those of C_{18}. The activities of these three enzymes can complete DHA biosynthesis from ALA via the Δ4 pathway. These enzymes were expressed in the brain, whereas *fads2b* and *elovl5* were expressed in the liver. Although the saury are epipelagic zooplanktivores that feed on LC-PUFA-rich items, the results of our study indicate that a certain proportion of their high LC-PUFA content, particularly DHA, can be synthesized by themselves via concerted reactions of their enzymes. Our finding supports the concept that phylogenetic position highly influences LC-PUFA biosynthetic capability of fish species. Furthermore, the results indicate that saury may have the potential to be a reliable DHA source for human consumption even in the uncertain future environment.

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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