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Title: Functional characterization reveals a diverse array of metazoan fatty acid biosynthesis genes

Running Head: fatty acid biosynthesis in metazoans

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Abstract

Long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFAs) are physiologically important fatty acids for most animals, including humans. Although most LC-PUFA production occurs in aquatic primary producers such as microalgae, recent research indicates the ability of certain groups of (mainly marine) invertebrates for endogenous LC-PUFA biosynthesis and/or bioconversion from dietary precursors. The genetic pathways for and mechanisms behind LC-PUFA biosynthesis remain unknown in many invertebrates to date, especially in non-model species. However, the numerous genomic and transcriptomic resources currently available can contribute to our knowledge of the LC-PUFA biosynthetic capabilities of metazoans. Within our previously generated transcriptome of the benthic harpacticoid copepod *Platychelipus littoralis*, we detected expression of one methyl-end desaturase, one front-end desaturase, and seven elongases, key enzymes responsible for LC-PUFA biosynthesis. To demonstrate their functionality, we characterized eight of them using heterologous expression in yeast. The *P. littoralis* methyl-end desaturase has Δ15/17/19 desaturation activity, enabling biosynthesis of α-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid (DHA) from 18:2n-6, 20:4n-6 and 22:5n-6, respectively. Its front-end desaturase has Δ4 desaturation activity from 22:5n-3 to DHA, implying that *P. littoralis* has multiple pathways to produce this physiologically important fatty acid. All studied *P. littoralis* elongases possess varying degrees of elongation activity for saturated and unsaturated fatty acids, producing aliphatic hydrocarbon chains with lengths of up to 30 carbons. Our investigation revealed a functionally diverse range of fatty acid biosynthesis genes in copepods, which highlights the need to scrutinize the role that primary consumers could perform in providing essential nutrients to upper trophic levels.

Keywords: harpacticoids copepods, polyunsaturated fatty acid biosynthesis, functional characterization, heterologous expression

1. Introduction

Long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFAs), including arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are key nutritional components that are particularly abundant in marine ecosystems but are also vital for the functioning of freshwater and terrestrial ecosystems (Bell & Tocher, 2009; Závorka et al., 2022). In animals specifically, LC-PUFAs play important roles in energy storage, lipid membrane structures, signalling pathways (as precursors of eicosanoids) and gene regulation (Bazinet & Layé, 2014; Tocher, 2015). While widely distributed, LC-PUFAs are particularly abundant in aquatic ecosystems, especially in marine waters (Colombo et al., 2017). Due to global climate change and rising water temperatures, the production of LC-PUFAs in phytoplankton and other microalgae is expected to decrease significantly (Hixson & Arts, 2016; Holm et al., 2022). This predicted temperature-related LC-PUFA reduction occurs due to changes in the lipid composition of cell membranes through a process known as homeoviscous adaptation (Sinensky, 1974). Moreover, changes in phytoplankton community composition and declining net primary production could further impair global LC-PUFA availability (Bi et al., 2021; Kwiatkowski et al., 2020). This will consequently have an impact on organisms at higher trophic levels via trophic cascading (Colombo et al., 2020).

The increasing availability of genomic data on multiple invertebrate taxa facilitates the investigation of the LC-PUFA biosynthetic pathways at a molecular level by characterization of its key biosynthesizing enzymes (Fig. 1) (Monroig et al., 2022). Stearoyl-CoA-desaturase (*scd*), also known as Δ9 desaturase, is present in all eukaryotes and enables the first desaturation from stearic acid (18:0) to oleic acid (OA, 18:1n-9). It was long assumed that endogenous further *de novo* LC-PUFA biosynthesis is restricted to (micro-)algae, bacteria and heterotrophic protists, and that metazoans need to cover their LC-PUFA needs through their diet. In aquatic habitats microalgae supposedly synthesize the major LC-PUFAs, which are then transferred to higher trophic levels via first-order consumers. However, this assumption

has been challenged in recent years by the discovery of a particular type of enzyme termed "methyl-end desaturases" in numerous groups of (mainly marine) invertebrates such as cnidarians, nematodes, arthropods, annelids and molluscs (Kabeya et al., 2018; Malcicka et al., 2018; Zhou et al., 2008). Methyl-end desaturases, previously reported mostly in plants, algae and microbes, introduce a double bond between the pre-existing one and the methyl-end of the carbon chain. They are required for *de novo* biosynthesis of the C_{18} PUFAs linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) from OA. Hence, these enzymes allow a consumer to not only depend upon exogenously (i.e. via their diet) supplied precursors required to biosynthesize LC-PUFAs (Monroig et al., 2022). The ecological implications of the occurrence of methyl-end desaturases in invertebrates have been largely disregarded so far, and many trophic ecology studies using FAs as trophic markers still assume that metazoans lack the capacity to endogenously produce LA and ALA, an issue also addressed by Galloway & Budge (2020).

The conversion of LA and ALA into ARA and EPA, respectively, occurs by the sequential reaction of front-end desaturases and elongases. While front-end desaturases introduce a double bond between the pre-existing one and the front-end of the carbon chain, elongases are the critical catalysing enzymes in the two-carbon chain elongation process (Bell & Tocher, 2009). Two possible pathways enabling ARA and EPA biosynthesis are known to exist, which are the so-called "Δ6 pathway" (Δ6 desaturation – elongation – Δ5 desaturation) and the "Δ8 pathway" (elongation – Δ8 desaturation – Δ5 desaturation) (Fig. 1). Similarly, the production of DHA from EPA can be performed via two pathways, namely the "Sprecher pathway" which involves two consecutive elongations and a Δ6 desaturation toward 24:6n-3 followed by a β-oxidation to DHA, and the "Δ4 pathway" involving only one elongation from EPA and one Δ4 desaturation (Fig. 1). The presence and activity of each of the enzymes determine an organism's capacity for LC-PUFA biosynthesis from endogenously produced or dietary obtained FAs (Monroig et al., 2022; Monroig & Kabeya, 2018).

Copepods are a globally distributed and highly abundant group of crustaceans that perform pivotal ecological functions at the basis of aquatic food webs (George et al., 2020). They are primary consumers of microalgae and are important prey for higher trophic levels, such as early life-cycle stages of fish (Gee, 1987) to which they provide essential nutrients including LC-PUFAs. Indeed, copepods generally have high levels of LC-PUFAs, particularly DHA, but whether these LC-PUFAs have an exclusive dietary origin or are, to some extent, produced endogenously remains unclear. Dietary studies in which copepods were fed diets lacking LC-PUFAs, or were given diets or specific FAs labelled with stable isotopes, suggest that multiple copepod species have the capacity for endogenous production of LC-PUFAs including DHA from ALA and/or possibly even OA (Arndt & Sommer, 2014; Caramujo et al., 2008; De Troch et al., 2012; Desvilettes et al., 1997; Farkas et al., 1981; Moreno et al., 1979; Nanton & Castell, 1998, 1999; Nielsen et al., 2020; Titocci & Fink, 2022; Werbrouck et al., 2017). While similar evidence is scarce for other aquatic primary consumers, their potential ability for endogenous LC-PUFA biosynthesis and conversion raises questions about their role and importance in aquatic food webs, especially in the context of future environmental changes.

Recent extensive revisions of the repertoire and functions of desaturases and elongases involved in LC-PUFA biosynthesis of invertebrates illustrate the remarkably high diversity in comparison to vertebrates (Monroig et al., 2022; Monroig & Kabeya, 2018). Among copepods, our knowledge on the LC-PUFA biosynthesis pathways remains fragmentary as only a subset of genes has been scrutinized in few species (Kabeya et al., 2018, 2021). Heterologous expression in a model system such as the yeast *Saccharomyces cerevisiae* is an ideal benchmark method to verify the function of a candidate gene (Monroig et al., 2022). This method can eliminate the possibility of the gene being a defunct pseudogene, and/or that the observed LC-PUFA biosynthesis is performed by microbial activity (Kabeya et al., 2021). While LC-PUFA biosynthesis genes have been identified in harpacticoid, cyclopoid and siphonostomatoid copepods (Amparyup et al., 2022; Boyen et al., 2020; Kabeya et al., 2018, 2021; M.-C. Lee, Choi, Kim, et al., 2020; M.-C. Lee, Choi, Park, et al., 2020; Nielsen et al., 2019), functional characterization has only been performed on genes from the parasitic siphonostomatoid

Lepeophtheirus salmonis (methyl-end desaturases) and the rocky intertidal harpacticoid *Tigriopus californicus* (methyl-end desaturases, front-end desaturases and elongases) (Kabeya et al., 2018, 2021).

While phylogenetic analyses suggest that LC-PUFA elongase and desaturase activity might be common across a diverse range of metazoan taxa, we lack direct empirical evidence to generalize this finding even among closely related species. Therefore, the aim of this work was to perform a phylogenetic exploration of crustacean (mainly copepod) methyl-end desaturases, front-end desaturases and elongases, and clone and functionally characterize a total of eight desaturases and elongases of the benthic harpacticoid copepod *Platychelipus littoralis* (Brady, 1880), using heterologous expression in yeast. The transcriptome of *P. littoralis* has been generated in an earlier study (Boyen et al., 2020). While often disregarded due to their small size and subsequent difficulty to collect, study and cultivate in laboratory environments, harpacticoid copepods play a pivotal role at the algae-animal interface of benthic food webs and thus the overall functioning of marine sediment communities (Hicks & Coull, 1983). Knowing their LC-PUFA biosynthesis capacity will allow us to better understand the role of not only *P. littoralis* but potentially benthic copepods in general as LC-PUFA providers within the marine ecosystem. *Platychelipus littoralis* was found to have a temperature-mediated capacity for endogenous LC-PUFA biosynthesis (Boyen et al., 2020; Werbrouck et al., 2016, 2017), yet functional molecular evidence is still lacking. Detailed knowledge of the metabolic pathways of LC-PUFA biosynthesis will allow the use of copepods as model organisms to study the effects of global warming on LC-PUFA-mediated food web interactions.

2. Methods

a. Protein identification and phylogenetic analysis

To perform the phylogenetic analysis, sequences from various crustacean species were retrieved from NCBI GenBank through BLAST (tblastn), using, as queries, the sequences of the functionally characterized *T. californicus* desaturases and elongases (Kabeya et al., 2018, 2021). Sequences were

only selected when they contained the full-length open reading frame (ORF) and their predicted protein sequences contained specific features according to Hashimoto et al. (2008). Briefly, front-end desaturases had to contain three diagnostic histidine boxes (H-box) "HXXXH", "HXXXHH" and "QXXHH" and a heme binding motif (HPGG) in the cytochrome b5 domain. Putative desaturase sequences with the third box "HXXHH" instead of "QXXHH" were not regarded as front-end desaturases based on evidence collected from other crustaceans suggesting these enzymes lack fatty acyl desaturation capacity (Monroig et al., 2022; Monroig & Kabeya, 2018). Methyl-end desaturases had to contain the three H-boxes "HXXXH", "HXXHH" and "HXXHH" and had to lack the cytochrome b5 domain. Fatty acid elongases had to contain the H-box "HXXHH" or "QXXHH" (Boyen et al., 2020; Hashimoto et al., 2008; Kabeya et al., 2021). For *P. littoralis* specifically, sequences were retrieved from the previously assembled transcriptome (NCBI BioProject PRJNA575120). The phylogenetic analysis was completed with the addition of sequences of functionally characterized genes from *T. californicus*, *L. salmonis*, *Platynereis dumerilli*, *Hediste diversicolor, Leishmania major* (Kabeya et al., 2018, 2020, 2021; Tripodi et al., 2006), as well as human sequences. The deduced protein sequences were aligned using MAFFT v. 7.490 (Katoh & Standley, 2013) using the E-INS-i method. For each gene family, a maximum-likelihood phylogenetic tree was built using RAxML v. 8.2.4 (Stamatakis, 2014) with a GAMMA model of rate heterogeneity, automatic selection of the best protein substitution model (MTZOA for methyl-end desaturases, LG for front-end desaturases and elongases), and 100 bootstrap replicates. The final trees were rooted with an outgroup (*P. dumerilli* and *H. diversicolor* for the methyl-end desaturases and *L. major* for the front-end desaturases) or using midpoint rooting (for the elongases). Trees were visualized and edited with FigTree v. 1.4.3 (http://tree.bio.ed.ac.uk/soft- ware/figtree).

b. Plasmid construction and transformation in yeast

Platychelipus littoralis adult specimens were collected from the top sediment layer of the Paulina intertidal mudflat (Westerscheldt estuary, The Netherlands; 51°21' N, 3°43' E) (Boyen et al., 2020). Total RNA was extracted from 50 pooled individuals using the RNeasy Plus Micro Kit (QIAGEN)

following a modified protocol (Boyen et al., 2020). Total RNA quality and quantity were assessed by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and 2100 Bioanalyzer (Agilent Technologies). cDNA was synthesized using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with dsDNase treatment to remove potential genomic DNA contamination. The full-length ORF sequences of one methyl-end desaturase, one front-end desaturase and six elongases were amplified by PCR from *P. littoralis* cDNA using high-fidelity Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific) and primers containing restriction enzyme sites (Table 1). RestrictionMapper (www.restrictionmapper.org) was used to select restriction enzymes which would not cut internally within the corresponding ORF sequence. The calculation of the annealing temperature and GC content (ThermoFisher Scientific Tm Calculator) was done only using the primer sequence part specific to the DNA fragment to be amplified. All PCR runs consisted of an initial denaturation step of 30 s at 98 °C, 35 cycles of 10 s at 98 °C, 30 s at the sequence-specific amplification temperature (Table 1) and 30 s at 72 °C, ending with a final extension step of 10 min at 72 °C. The PCR products were purified with Purelink PCR Purification Kit (Thermo Fisher Scientific) and subsequently digested with the corresponding restriction enzymes (New England Biolabs) (Table 1). The restricted ORF fragments were ligated (T4 DNA ligase, Promega) into a similarly restricted pYES2 yeast expression vector and transformed into One Shot TOP10F´ chemically competent *E. coli* cells. Positive transformant colonies were grown overnight in LB broth containing ampicillin (50 µg/ml). Plasmids were purified with PureLink™ HiPure Plasmid Miniprep Kit, sequenced using T7 forward and CYC1 reverse primers (Macrogen Europe, The Netherlands) and compared with the original sequences from the transcriptome assembly. Concentrations of raw and restricted PCR products, ligated vectors and purified plasmids were all quantified using Qubit 2.0 dsDNA BR Assay Kit (Invitrogen).

c. Functional characterization of *P. littoralis* desaturase and elongase genes

The plasmid constructs containing the ORF sequences of the *P. littoralis* desaturases and elongases were independently transformed into *S. cerevisiae* competent cells (strain INVSc1) using the *S.c.*

EasyComp yeast transformation kit (Invitrogen). The recombinant yeast cells were grown on *S. cerevisiae* minimal medium minus uracil (hereafter referred to as "SCMM^{-ura}") agar plates for 3 d at 30 °C, the optimal temperature for growth of *S. cerivisiae*. One individual colony per gene was individually grown in SCMM^{-ura} broth for 48 h at 30 °C to produce a bulk culture with an OD_{600} of 8-10. Subsequently, an appropriate volume of the yeast bulk cultures was diluted to an OD_{600} of 0.4 in 5 ml of SCMM^{-ura} broth contained in a 250 ml Erlenmeyer flask. Each putative PUFA substrate was assayed in independent flasks. The Erlenmeyer flasks were incubated for 4 h at 30 °C under constant shaking (250 rpm) until they reached an OD_{600} of approximately 1. At that point, cultures were supplemented with 25% galactose to induce transgene expression, and one of the putative PUFA substrates as follows. For the methyl-end desaturase, the exogenously supplied PUFA substrates were 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6. For the front-end desaturase, the PUFA substrates were 18:3n-3, 18:2n-6, 20:3n-3, 20:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 and 22:4n-6. For the elongases, the PUFA substrates included 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 and 22:4n-6. All PUFA substrates were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA), except 18:4n-3 from Larodan AB (Solna, Sweden) and 20:4n-3 from Cayman Chemicals (Ann Arbor, MI, USA). Each PUFA substrate was supplemented as sodium salts at concentrations of 0.5 mM (C_{18}), 0.75 mM (C_{20}) and 1.0 mM (C_{22}) as uptake efficiency decreases with increasing carbon chain length (Zheng et al., 2009). In addition, we wanted to determine the capacity of the *P. littoralis* methyl-end desaturase and elongases to utilize the yeast endogenous saturated and monounsaturated FAs as substrates. For this, transgenic yeast expressing either the methyl-end desaturase or one of the six elongases were grown in triplicate Erlenmeyer flasks supplemented with 2% galactose but without exogenously added FA substrates, in parallel with a control treatment consisting of yeast transformed with an empty pYES2 vector (n=3). Yeast cultures were incubated again for 48 h at 30 °C under constant shaking (250 rpm), harvested by centrifugation (2 min, 2000 rpm) and washed twice with double distilled H_2O. Yeast pellets were subsequently homogenized in 8:4:3 (v/v/v) chloroform:methanol:saline solution (0.88%

KCl) containing 0.01% (w/v) butylated hydroxytoluene (BHT, Sigma-Aldrich) as antioxidant, and stored at -20 °C under anaerobic conditions for a minimum of 24 h prior to FA analysis.

d. Fatty acid analysis

Total lipids were extracted from the homogenized yeast samples with 8:4:3 (v/v/v) chloroform:methanol:saline solution (0.88% KCl) according to the Folch method (Folch et al., 1957). Fatty acid methyl esters (FAMEs) were prepared through acid-catalyzed transesterification and subsequently purified by thin-layer chromatography. FAMEs from the desaturase functional assays were analyzed using a Thermo Trace GC Ultra (Thermo Electron Corporation, Waltham, MA, USA) equipped with a fused silica 30 m × 0.25 mm open tubular column (Tracer, TR-WAX (film thickness 0.25 µm); Teknokroma, Spain), coupled to a flame ionization detector. Identification was carried out by comparing the retention times with those from commercial FAME standards. Further confirmation of peaks and analysis of FAMEs from the elongase assays was carried out using an Agilent 6850 GC equipped with a mass spectrometry detector (5975 Series) and a 30 m × 0.25 mm open tubular column (Tracer, DB5-MS (film thickness 0.25 µm); Teknokroma, Spain), and comparing the spectra against those from the NIST library. The conversion efficiency of all assayed *P. littoralis* enzymes toward the exogenously supplied PUFA substrates was calculated as: (all product areas/(all product areas + substrate area)) × 100 (Kabeya et al., 2021).

e. Statistical analysis

The assays aiming to determine the ability of the *P. littoralis* methyl-end desaturase and elongases toward the yeast endogenous saturated and monounsaturated FAs were run in replicates (n=3) and the FA contents were expressed as mean percentages ± standard deviation. Homogeneity of variances was checked using Levene's test. The FA profiles of control yeast and yeast expressing the methyl-end desaturase and the elongases were compared, and differences were statistically tested using Student's t-test for the methyl-end desaturase and Dunnett's multiple comparisons test for the elongases with

$p \leq 0.05$ indicating statistical significance. All statistical analyses were conducted in R v.4.0.2 (R Core Team, 2020).

3. Results

a. Protein identification and phylogenetic inference

We constructed three phylogenetic trees of the metazoan methyl-end desaturases, front-end desaturases and elongases respectively (Fig. 2). For each gene family, we found multiple subclades containing one or more sequences from different species. Each tree contains multiple well supported clades, however certain clades are not strongly supported. For *P. littoralis* specifically, we identified one putative methyl-end desaturase, one putative front-end desaturase and seven putative elongases. The *P. littoralis* methyl-end desaturase (ON075828) has the three specific H-boxes and lacks a cytochrome b5 domain. It clusters together with other harpacticoid sequences, including the functionally characterized *T. californicus* methyl-end desaturase "*ωx2*" with Δ15/Δ17/Δ19 activities (Fig. 2a). The front-end desaturase (ON075829) remained unnoticed in the initial phylogenetic analysis since its Pfam domain *Cyt-b5* (PF00173) had an E-score of 6.1E-05 and therefore did not pass the criteria at that time (Boyen et al., 2020). It was placed in the copepod-specific clade previously identified (Kabeya et al., 2021), with its closest functionally characterized sequence being the *T. californicus* Δ4 desaturase "*Fed2*" (Fig. 2b). In addition, we found a front-end desaturase in a transcriptome of the decapod *Eurypanopeus depressus* (GFJG01059607), which clustered inside the copepod-specific clade and contained the correct third histidine box "QIEHH" as opposed to previously identified decapod front-end desaturase sequences. One *P. littoralis* elongase (ON075836) aligned closely with the vertebrate elovl3/elovl6 subfamily, which is specifically known to elongate saturated FAs, and was therefore not included in the subsequent functional characterization. Another *P. littoralis* elongase (ON075835) formed a clade with the human and *T. californicus* elovl4 sequences. The five other *P. littoralis* elongase sequences all belonged within the Pancrustacea-specific elovl1/7-like clade

identified earlier (Boyen et al., 2020; Kabeya et al., 2021) (Fig. 2c). Therefore, we subsequently labelled them elovl1a-e. *Platychelipus littoralis* elovl1a, elovl1b and elovl1e closely aligned with the functionally characterized *T. californicus* "*elo1*", "*elo5*" and "*elo2*" respectively, as well as the corresponding *Tigriopus japonicus* sequences. *Platychelipus littoralis* elovl1d does not have a direct relationship with any other copepod elongases, though it aligned most closely with the *P. littoralis* elovl1a/*T. californicus* "*elo1*" clade. The *P. littoralis* elovl1c did not match with any functionally characterized *T. californicus* elongase, but did align with elongase sequences from *L. salmonis, Caligus rogercresseyi* and *Caligus clemensi* (all siphonostomatoids). Remarkably, all sequences from the two subclades containing *P. littoralis* elovl1c (ON075832) and elovl1e (ON075834) contained a histidine box "QXXHH" instead of the typical "HXXHH" observed in other FA elongases.

b. Functional characterization

The functions of all putative desaturases and elongases identified from *P. littoralis,* except for elovl3/6, were characterized in yeast by heterologous expression of the corresponding coding region and growing in the presence of potential PUFA substrates. The FA profiles of the transgenic yeast expressing the *P. littoralis* methyl-end desaturase and grown in the presence of exogenously added C_{18}, C_{20} and C_{22} n-6 PUFA substrates showed n-3 desaturation products denoting that this enzyme has Δ15, Δ17 and Δ19 desaturation capacity, respectively (Table 2). No Δ12 desaturation capacity was detected for the *P. littoralis* methyl-end desaturase, since levels of the Δ12 desaturation product 18:2n-6 were not statistically different when compared with yeast transformed with the empty pYES2 vector (Student's t-test, $p>0.05$) (Supp. Table 1).

Functional characterization assays of the *P. littoralis* front-end desaturase showed this enzyme has Δ4 desaturation capacity since transgenic yeast expressing its coding region were able to convert 22:5n-3 and 22:4n-6 into 22:6n-3 and 22:5n-6, respectively (Table 3). No activity toward 18:3n-3, 18:2n-6, 20:3n-3, 20:2n-6, 20:4n-3 and 20:3n-6 was detected, confirming that the *P. littoralis* front-end desaturase does not have Δ5, Δ6 or Δ8 desaturation capacities (Table 3).

The capacity of the *P. littoralis* elongases to act toward saturated FAs was assessed by comparing the FA profiles of yeast transformed with the empty vector with those of yeast each expressing one of the six elongases under study (Fig. 3, Supp. Table 2). Yeast expressing *P. littoralis* elovl4 showed a significant increase of 20:0 and production of 28:0 and 30:0 (Dunnett's test, $p<0.05$), while levels of other FAs were not different from those of the control yeast. Yeast expressing *P. littoralis* elovl1a showed significantly lower levels of 16:0, 17:0, 18:0 and 20:0 and higher levels of 22:0, 24:0 and 26:0 compared to the control yeast. Expression of *P. littoralis* elovl1d resulted in significantly reduced levels of 16:0 and 18:0 and increased levels of 26:0, 28:0 and 30:0, while expression of *P. littoralis* elovl1e resulted in significantly reduced levels of 16:0 and increased levels of 26:0. *Platychelipus littoralis* elovl1b and elovl1c did not show any capacity for elongation of yeast endogenous FAs (Dunnett's test, $p>0.05$) (Fig. 3, Supp. Table 2).

The activities of the *P. littoralis* elongases toward PUFA substrates were assessed by growing transgenic yeast expressing each elongase in the presence of exogenously added PUFA substrates. *Platychelipus littoralis* elovl4 was able to elongate all of the supplied substrates, with additional elongation of the product 24:5n-3 toward 26:5n-3 (Table 4). *Platychelipus littoralis* elovl1a was able to elongate all of the supplied substrates except 18:2n-6 (Table 4). The *P. littoralis* elovl1b had a relatively low elongation capacity toward C_{18} substrates and particularly high elongation capacity toward C_{20} substrates, with up to 84.7% and 57.2% conversion of 20:5n-3 (EPA) and 20:4n-6 (ARA) toward 22:5n-3 and 22:5n-3, respectively, yet no detectable activity toward C_{22} substrates (Table 4). Similarly, *P. littoralis* elovl1c and elovl1e had elongation capacity of C_{18} and C_{20} substrates, but no detectable activity toward C_{22} substrates (Table 4). While *P. littoralis* elovl1d was able to elongate C_{18} and C_{20} substrates to some extent, it was found to have particularly high elongation capacity of C_{22} substrates, enabling the production of polyenes up to C_{28} via stepwise elongations from exogenously added substrates (Table 4).

4. Discussion

In this study, we identified nine *P. littoralis* desaturases and elongases and demonstrated the functions of eight of them. We found that these enzymes exhibited highly diverse enzymatic capacities enabling biosynthesis of not only LC-PUFAs but also very long-chain FAs (up to C_{30}). We found one *P. littoralis* methyl-end desaturase with a multifunctional Δ15, Δ17 and Δ19 desaturation capacity similar to the *T. californicus* "*ωx2*" and *L. salmonis* "*ω3*" orthologs (Kabeya et al., 2018, 2021). Additionally, we found that the *P. littoralis* methyl-end desaturase is able to convert both 22:4n-6 and 22:5n-6 into 22:5n-3 and 22:6n-3 (DHA) respectively. This contrasts with the *T. californicus* "*ωx2*" methyl-end desaturase, which is only able to desaturate 22:4n-6 but not 22:5n-6 (Kabeya et al., 2021). While *T. californicus, T. japonicus* and *L. salmonis* have been found to possess two methyl-end desaturases (Kabeya et al., 2018, 2021), other copepods such as *C. rogercresseyi, Eucyclops serrulatus, Paracyclopina nana, Tigriopus kingsejongensis* and now also the benthic harpacticoid *P. littoralis* seem to possess only one (Fig. 2). While improved transcriptomic resources from the latter species might reveal a second methyl-end desaturase, a broader investigation including genomic data from more copepod species could disclose whether one or two methyl-end desaturases is the dominating trait among copepods. If assumed that *P. littoralis* only contains one sole methyl-end desaturase lacking Δ12 desaturation capacity, this implies that *P. littoralis* is not able to convert OA into LA and therefore does not have the capacity for complete *de novo* LC-PUFA biosynthesis from endogenously produced saturated and monounsaturated FAs. Such capacity has been reported to exist in specific species of Cnidaria, Mollusca, Annelida, Rotifera and Arthropoda, including the copepods *L. salmonis* and *T. californicus* (Kabeya et al., 2018, 2020, 2021). On the other hand, our results show that the *P. littoralis* methyl-end desaturase has Δ17 and Δ19 desaturation capacity, including a capacity for biosynthesis of EPA and DHA from ARA and 22:5n-6, respectively. This Δ17 and Δ19 desaturation of omega-6 FAs could be an

important alternative pathway toward omega-3 LC-PUFAs, enabling their production from an increased variety of dietary precursors.

The copepod front-end desaturase gene family is distinct from other metazoan front-end desaturase clades (Kabeya et al., 2021; M.-C. Lee, Choi, Kim, et al., 2020; Nielsen et al., 2019). It is more closely related to protists and algae such as *Leishmania major*, which was previously hypothesized to be a result of horizontal gene transfer (Kabeya et al., 2021). We found that the *P. littoralis* front-end desaturase had a single Δ4 desaturation capacity enabling the production of 22:5n-6 and, more importantly, DHA. This is the second Δ4 desaturase found in harpacticoids, further supporting the hypothesis that they use the so-called "Δ4 pathway" as opposed to the Sprecher pathway to synthesize DHA (Kabeya et al., 2021). Functional characterization of more front-end desaturases will allow us to verify the potential universal presence of the Δ4 pathway in other copepod orders. The *P. littoralis* Δ4 desaturase is phylogenetically placed in the previously discovered copepod-specific clade, within a subclade that also contains the *T. californicus* Δ4 desaturase. The four other *T. californicus* front-end desaturases all cluster within a second subclade mostly containing harpacticoid species (*Tigriopus* and *Tisbe*), further speculating that more *P. littoralis* front-end desaturases with Δ5, Δ6 or Δ8 desaturation capacity remain to be discovered. While our phylogenetic inference of the front-end desaturases is similar to Nielsen et al. (2019), the phylogenetic tree of Kabeya et al. (2021) does not separate the *T. californicus* Δ4 desaturase "*Fed2*" from the other sequences. Therefore, a more substantial phylogenetic analysis involving even more putative copepod sequences coupled with additional functional characterization of front-end desaturases from other copepod species is essential to clarify the evolution and diversification of this gene family, including its potential origin from horizontal gene transfer (Kabeya et al., 2021).

The phylogenetic analysis of the *P. littoralis* elongases indicated extensive gene differentiation prior to copepod species differentiation. A large expansion of the elongase gene family was found in the harpacticoid genus *Tigriopus* as well (Kabeya et al., 2021; M.-C. Lee, Choi, Kim, et al., 2020). Two *P.*

littoralis elongases each clustered with the known "vertebrate" elovl3/6 and elovl4 clades, with *P. littoralis* elovl4 having a rather general elongation capacity similar to the *T. californicus* ortholog. Five *P. littoralis* elongases (elovl1a-e) clustered within the recently discovered Pancrustacea-specific elovl1/7-like clade (Ribes-Navarro et al., 2021), and the detected strong elongation capacities of the tested elongases further emphasize the importance of this clade. The results from *P. littoralis* elovl1c and elovl1e as well as *T. californicus* "elo2" show that elongases with a "QXXHH" histidine box instead of the usual "HXXHH" histidine box can still exhibit elongation capacities. While elovl1c and elovl1d do not have *T. californicus* orthologs, *P. littoralis* elovl1b and elovl1e mirrored the C_{18} and C_{20} elongation capacities of their functionally characterized *T. californicus* orthologs "elo5" and "elo2" (Kabeya et al., 2021). Furthermore, while *P. littoralis* elovl1a matched its *T. californicus* ortholog "elo1" in its ability to elongate all C_{18} and C_{20} substrates except 18:2n-6, it was additionally able to elongate C_{22} substrates toward C_{24} products. We found a combination of general (elovl4) and specific (elovl1b and elovl1d) elongases, illustrating a large functional diversity. We acknowledge that cautious interpretation of these data is warranted as heterologous expression of single genes in a yeast system - while being a very robust system to establish the substrate specificities of the assayed enzymes - does not necessarily mirror the extent to which that enzyme is active in a more complex multicellular *in vivo* scenario where numerous competing enzymes, regulatory mechanisms (e.g. via transcription factors or epigenetic signals) and environmental drivers interact (Monroig et al., 2022; Xie et al., 2021).

Overall, our results show that gene family expansion can lead to an improved elongation capacity. The gene copy number increase of the elovl1/7-like elongase family found in *P. littoralis* and other harpacticoids can be considered an important evolutionary response enabling them to synthesize their well-documented high levels of LC-PUFAs, e.g. when compared to calanoids (Twining et al., 2020). Additionally, having multiple gene copies could lead to certain copies becoming tissue- or development stage-specific, or acquiring substrate-specific enzymatic functions, as seen in *P. littoralis* elovl1a (selective elongation of omega-3 instead of omega-6 substrates), and previously demonstrated in vertebrates (Ishikawa et al., 2019). Understanding the gene family diversity of these harpacticoids will

help us to better understand the adaptions of copepods within their nutritional landscape. This could be an important driver of evolutionary divergence and copepod diversity, as observed in other species (Ishikawa et al., 2019, 2021, 2022; Twining et al., 2021).

While PUFA elongases have been successfully functionally characterized in other crustacean lineages such as decapods, branchiopods and amphipods (Mah et al., 2019; Ribes-Navarro et al., 2021; Sun et al., 2020; Ting et al., 2020), the occurrence and functionality of methyl-end and front-end desaturases in these lineages remains highly questionable (Chen et al., 2017; Kabeya et al., 2021; Lin et al., 2017; Monroig & Kabeya, 2018; Nielsen et al., 2019; Ting et al., 2021; Wu et al., 2018; Yang et al., 2013). In our phylogenetic study, we detected a putative front-end desaturase in a transcriptome of the decapod *Eurypanopeus depressus* clustering together with the *T. californicus* Δ5 front-end desaturase "*Fed5*" with high bootstrap support (86%). Importantly, the *E. depressus* putative front-end desaturase identified in the present study has all correct signatures including the three H-boxes "HXXXH", "HXXXHH" and "QXXHH" and a heme binding motif (HPGG) in the cytochrome b5 domain (Hashimoto et al., 2008). Assuming this was not due to contamination during RNA sequencing, the *E. depressus* putative front-end desaturase could be the first report of this type of LC-PUFA biosynthesizing enzymes in decapods. Further functional assays will be required to test this hypothesis.

5. Concluding remarks

The present study demonstrates that the benthic copepod *P. littoralis* has the genes for biosynthesis of EPA from ARA (using its Δ15/Δ17/Δ19 methyl-end desaturase), as well as the synthesis of DHA from either EPA (using its elovl1b elongase and its Δ4 front-end desaturase) or 22:5n-6 (using its methyl-end desaturase). However, due to the lack of a Δ12 methyl-end desaturase and Δ5, Δ6 or Δ8 front-end desaturases, we could not confirm the capacity for full *de novo* endogenous LC-PUFA synthesis from MUFAs or short-chain PUFAs, as found in *T. californicus* (Kabeya et al., 2021). Since *P. littoralis* was shown to synthesize DHA from stable-isotope labelled diets containing high amounts of ALA and no

LC-PUFAs (Werbrouck et al., 2017), at least enzymes with Δ5, Δ6 or Δ8 desaturation activity should theoretically be present but remain yet undetected.

Thus, copepods such as *P. littoralis* and *T. californicus* could play an important role as LC-PUFA producers in marine and estuarine food webs. Endogenous biosynthesis of EPA and DHA by primary consumers - even when synthesized from other LC-PUFAs such as ARA as evidenced here - has large-scale implications for global food webs. In aquatic ecosystems, where LC-PUFA production by microalgae is expected to decrease due to climate change, LC-PUFA production by primary consumers could potentially still provide secondary and tertiary consumers with their required LC-PUFA levels (Závorka et al., 2021). Future research should examine a number of impacts and consequences resulting from this observation. First, this biosynthetic capacity in benthic and intertidal harpacticoids is unlikely to be representative for other copepod orders, such as pelagic calanoids, freshwater cyclopoids or parasitic siphonostomatoids, or even other primary consumers. Therefore, absolute quantities of LC-PUFA production in different taxa should be calculated and an assessment should be made whether this could significantly contribute to overall LC-PUFA biomass worldwide. Second, in a warming ocean, copepods not only face declining LC-PUFA from their diet, but also face climate change effects directly. Direct negative effects of ocean warming on LC-PUFA content and production have been demonstrated in *P. littoralis* (Boyen et al., 2020; Sahota et al., 2022; Werbrouck et al., 2017) and other primary consumers (M.-C. Lee et al., 2022; S.-H. Lee et al., 2017; Masclaux et al., 2012). These impacts should be considered, as they can severely limit the consumer's biosynthesis ability (when present) to make up for a reduced dietary LC-PUFA provision due to climate change. Third, endogenous LC-PUFA synthesis means facing higher metabolic costs, and the potentially associated reduced fitness should be accounted for as well. Finally, studies using FAs as biomarkers should integrate consumer FA metabolism into their considerations. For instance, Jardine et al. (2020) calculated FA regression equations and used those to correct for trophic modification. Updated knowledge on specific

conversion capacities of certain species as well as controlled feeding experiments can further improve future models.

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8. Data Accessibility and Benefit-Sharing

Data Accessibility: All isolated sequences from *P. littoralis* in the present study were deposited at NCBI GenBank with the accession numbers ON075828 to ON075835.

Benefit-Sharing: There are no benefits outlined in the Nagoya protocol associated with this study to report.

9. Author Contributions

J.B., N.K., Ó.M, J.C.N. and M.D.T conceptualized the study. J.B., N.K. and Ó.M. and P.I.H. performed the phylogenetic analysis. J.B. and A.R. constructed the plasmids. J.B., A.R.-N., O.M. and J.C.N. performed the functional assays and fatty acid analysis. J.B. performed the statistical analysis and prepared the initial version of the manuscript. All authors assisted in the interpretation of the results and the revision of the manuscript.

10. Tables

Table 1 original transcript contig (above) and coding sequence (below) NCBI accession numbers, restriction enzymes, sequences and annealing temperatures (T_A) of each gene. Restriction sites are underlined.

Gene	Accession numbers	Enzyme	Primer name	Primer sequence (5'-3')	T_A (°C)
ωx	GHXK01184360	*Hin*dIII	plwxHF	CCCAAGCTTAAAATGTCGTCTAGAAGAAG	56.0
	ON075828	*Xho*I	plwxXR	CCGCTCGAGTCACTTAGACTTTGTATCGC	
fad	GHXK01205503	*Sac*I	plfadSF	CCCGAGCTCACCATGGATCCCTCAATAGA	59.8
	ON075829	*Xho*I	plfadXR	CCGCTCGAGTTATGACAGAAGCTTGTTGAAG	
elovl1a	GHXK01177303	*Hin*dIII	ple1aHF	CCCAAGCTTAAGATGAACGTCGTTTCTGAAAAATGG	64.5
	ON075830	*Xho*I	ple1aXR	CCGCTCGAGTCAATGTTGCTTTTTGTCGCTAGA	
elovl1b	GHXK01255463	*Sac*I	ple1bSF	CCCGAGCTCACCATGGCCACTCAGAA	61.1
	ON075831	*Xho*I	ple1bXR	CCGCTCGAGTCAATTTTCTTTTTTTGCAGCAGA	
elovl1c	GHXK01260983	*Sac*I	ple1cSF	CCCGAGCTCAAAATGAGTGAAACATTTTTGGACGG	61.9
	ON075832	*Xho*I	ple1cXR	CCGCTCGAGTTATGTACTTTTCTTCTTTTTCTGGTTG	
elovl1d	GHXK01228992	*Hin*dIII	ple1dHF	CCCAAGCTTAACATGCTGGATGTGTTAGTC	57.9
	ON075833	*Xho*I	ple1dXR	CCGCTCGAGTTATGTCACTTTTTTCTTGGAG	
elovl1e	GHXK01223266	*Hin*dIII	ple1eHF	CCCAAGCTTAGAATGACCAAGTCAGTGATCCC	65.1
	ON075834	*Xba*I	ple1eXbR	CCGTCTAGATTAGTCCAATTTTGTTGCATTTAAATGCC	
elovl4	GHXK01149108	*Hin*dIII	ple4HF	CCCAAGCTTACAATGGTTAGTGAAAATTTATATTCC	59.4
	ON075835	*Xho*I	ple4XR	CCGCTCGAGTCATTTCTTCTTTTTCTGAACAAC	

Table 2 Substrate conversions of the transgenic yeast expressing the *P. littoralis* methyl-end desaturase. The results are presented as a percentage of the fatty acid (FA) substrate converted into the corresponding desaturated product.

FA substrate	Product	Conversion (%)	Activity
18:2n-6	18:3n-3	29.6	Δ15
18:3n-6	18:4n-3	25.7	Δ15
20:2n-6	20:3n-3	10.7	Δ17
20:3n-6	20:4n-3	13.7	Δ17
20:4n-6	20:5n-3	57.0	Δ17
22:4n-6	22:5n-3	13.4	Δ19
22:5n-6	22:6n-3	7.4	Δ19

Table 3 Substrate conversions of the transgenic yeast expressing the *P. littoralis* front-end desaturase. The results are presented as a percentage of the fatty acid (FA) substrate converted into the corresponding desaturated product.

FA substrate	Product	Conversion (%)	Activity
18:3n-3	18:4n-3	-	Δ6
18:2n-6	18:3n-6	-	Δ6
20:3n-3	20:4n-3	-	Δ8
20:2n-6	20:3n-6	-	Δ8
20:4n-3	20:5n-3	-	Δ5
20:3n-6	20:4n-6	-	Δ5
22:5n-3	22:6n-3	7.8	Δ4
22:4n-6	22:5n-6	7.3	Δ4

- : not detected (<0.1%).

Table 4 Substrate conversions of the transgenic yeast expressing the *P. littoralis* elongases. The results are presented as a percentage of the fatty acid (FA) substrate converted into the corresponding elongated product.

FA substrate	Product	Conversion (%)						Activity
		elovl4	elovl1a	elovl1b	elovl1c	elovl1d	elovl1e	
18:3n-3	20:3n-3	5.9	0.5	2.2	14.2	1.2	13.2	$C_{18} \rightarrow C_{20}$
	22:3n-3	-	2.6	-	0.2	6.6	0.1	$C_{20} \rightarrow C_{22}$
18:2n-6	20:2n-6	2.2	-	0.3	3.0	0.3	1.6	$C_{18} \rightarrow C_{20}$
	22.2n-6	-	-	-	-	9.2	3.0	$C_{20} \rightarrow C_{22}$
18:4n-3	20:4n-3	3.7	2.9	1.5	11.0	3.7	10.6	$C_{18} \rightarrow C_{20}$
	22:4n-3	2.0	1.2	-	-	3.1	0.8	$C_{20} \rightarrow C_{22}$
	24:4n-3	-	-	-	-	12.1	-	$C_{22} \rightarrow C_{24}$
18:3n-6	20:3n-6	2.5	2.9	1.0	6.4	4.9	4.7	$C_{18} \rightarrow C_{20}$
	22:3n-6	3.0	1.0	0.8	0.5	2.7	0.5	$C_{20} \rightarrow C_{22}$
	24:4n-3	-	-	-	-	40.9	-	$C_{22} \rightarrow C_{24}$
20:5n-3	22:5n-3	8.3	4.6	84.7	0.7	4.3	5.2	$C_{20} \rightarrow C_{22}$
	24:5n-3	4.4	3.3	-	-	20.4	-	$C_{22} \rightarrow C_{24}$
20:4n-6	22:4n-6	6.0	2.3	57.2	0.1	4.0	0.4	$C_{20} \rightarrow C_{22}$
	24:4n-6	4.1	4.0	-	-	55.6	-	$C_{22} \rightarrow C_{24}$
	26:4n-6	-	-	-	-	57.5	-	$C_{24} \rightarrow C_{26}$
	28:4n-6	-	-	-	-	9.3	-	$C_{26} \rightarrow C_{28}$
22:5n-3	24:5n-3	3.5	2.4	-	-	14.5	-	$C_{22} \rightarrow C_{24}$
	26:5n-3	8.3	-	-	-	18.7	-	$C_{24} \rightarrow C_{26}$
	28:5n-3	-	-	-	-	9.9	-	$C_{26} \rightarrow C_{28}$
22:4n-6	24:4n-6	1.7	0.9	-	-	18.7	-	$C_{22} \rightarrow C_{24}$
	26:4n-6	-	-	-	-	58.8	-	$C_{24} \rightarrow C_{26}$
	28:4n-6	-	-	-	-	13.1	-	$C_{26} \rightarrow C_{28}$

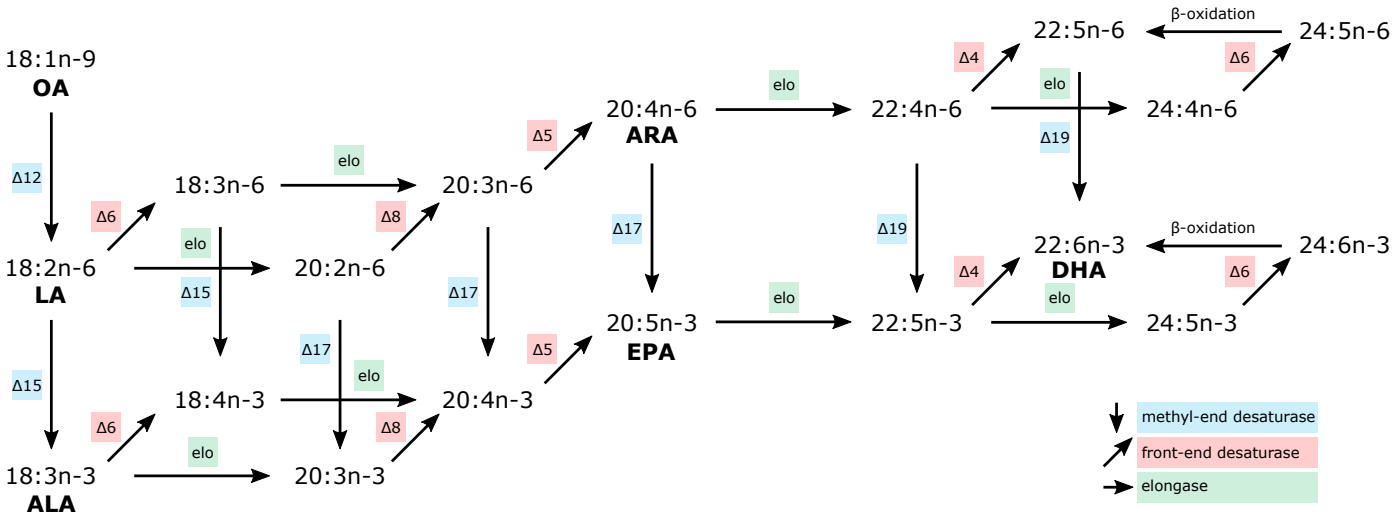
- : not detected (<0.1%).

11. Figure captions

Figure 1 Theoretical polyunsaturated fatty acid biosynthesis pathway in metazoans. Methyl-end desaturase reactions in blue (vertical arrows), front-end desaturase reactions in red (diagonal arrows), and elongase reactions in green (horizontal arrows). Desaturase reactions are further specified by "Δy", with "y" referring to the location of insertion of the double bond counting from the methyl-end of the carbon chain. β-oxidation reactions are also shown (horizontal reverse arrows) but not included in this study. OA: oleic acid; LA: linoleic acid; ALA: α-linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid. Adapted from Monroig et al. (2022).

Figure 2 Maximum-likelihood phylogenetic trees of (a) methyl-end desaturases (blue), (b) front-end desaturases (red), and (c) elongases (green). Values below branches show bootstrap support after 100 RAxML iterations. *P. littoralis* sequences identified in this study are highlighted in bold. The different functions (when characterized) of each gene are indicated in unique colour shades, and are represented as desaturation activities ("Δ") for the methyl-end (a) and front-end (b) desaturases, and elongated PUFA substrates for the elongases (c).

Figure 3 Heatmap illustrating mean endogenous saturated fatty acid levels of the transgenic yeast expressing the *P. littoralis* elongases as well as the control yeast (n=3). Fatty acids percentages (%) were scaled to z-scores per fatty acid, with blue indicating lower and red indicating higher than average percentages. Data used to generate this heatmap can be found in Supplementary Table 2.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ALA
Δ6
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elo
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Δ8
20:3n-6
Δ5
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ARA
elo
22:4n-6
Δ4
22:5n-6
β-oxidation
24:5n-6
Δ6
elo
24:4n-6
Δ19
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Δ5
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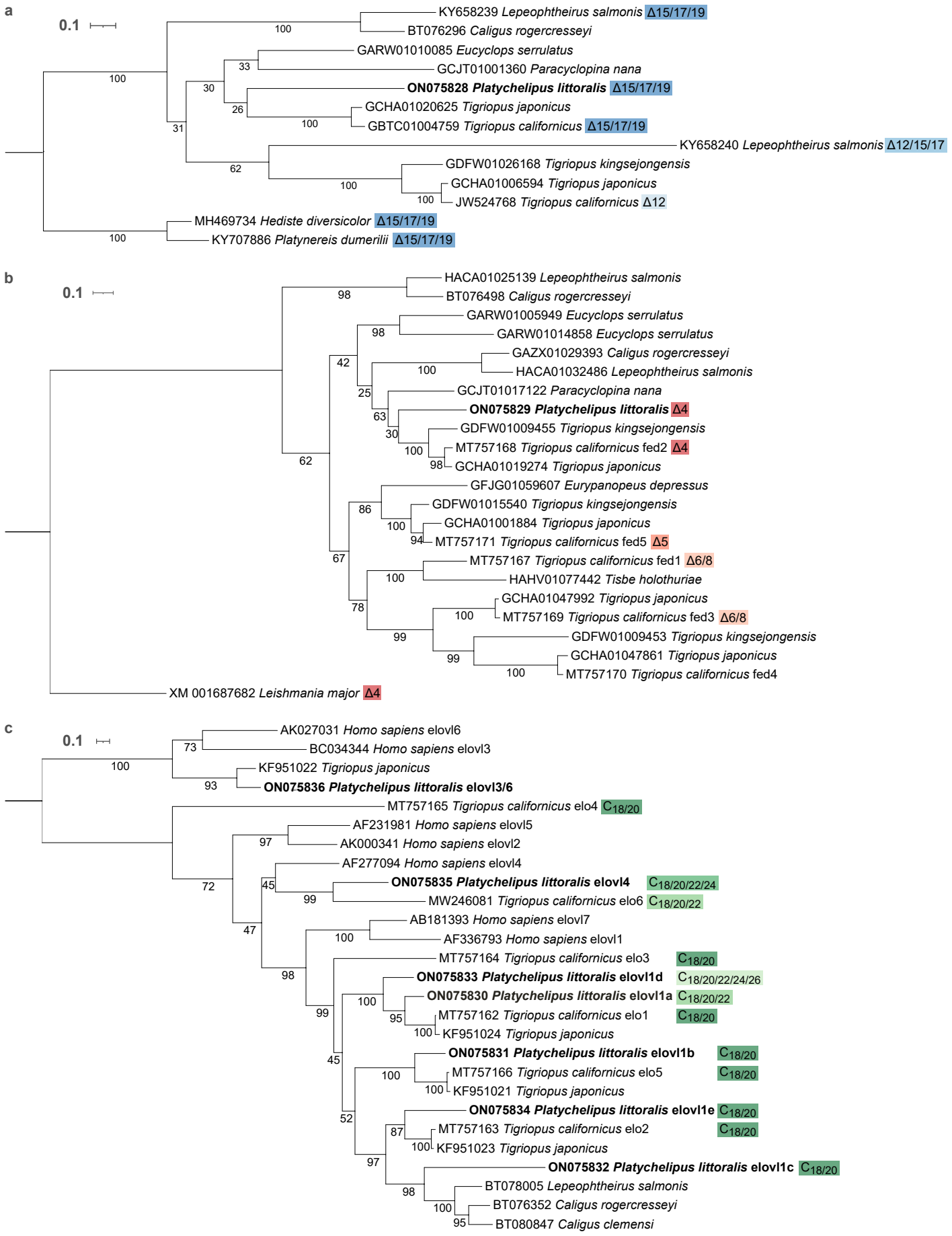
front-end desaturase
elongase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GCJT01001360 Paracyclopina nana
ON075828 Platychelipus littoralis Δ15/17/19
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GBTC01004759 Tigriopus californicus Δ15/17/19
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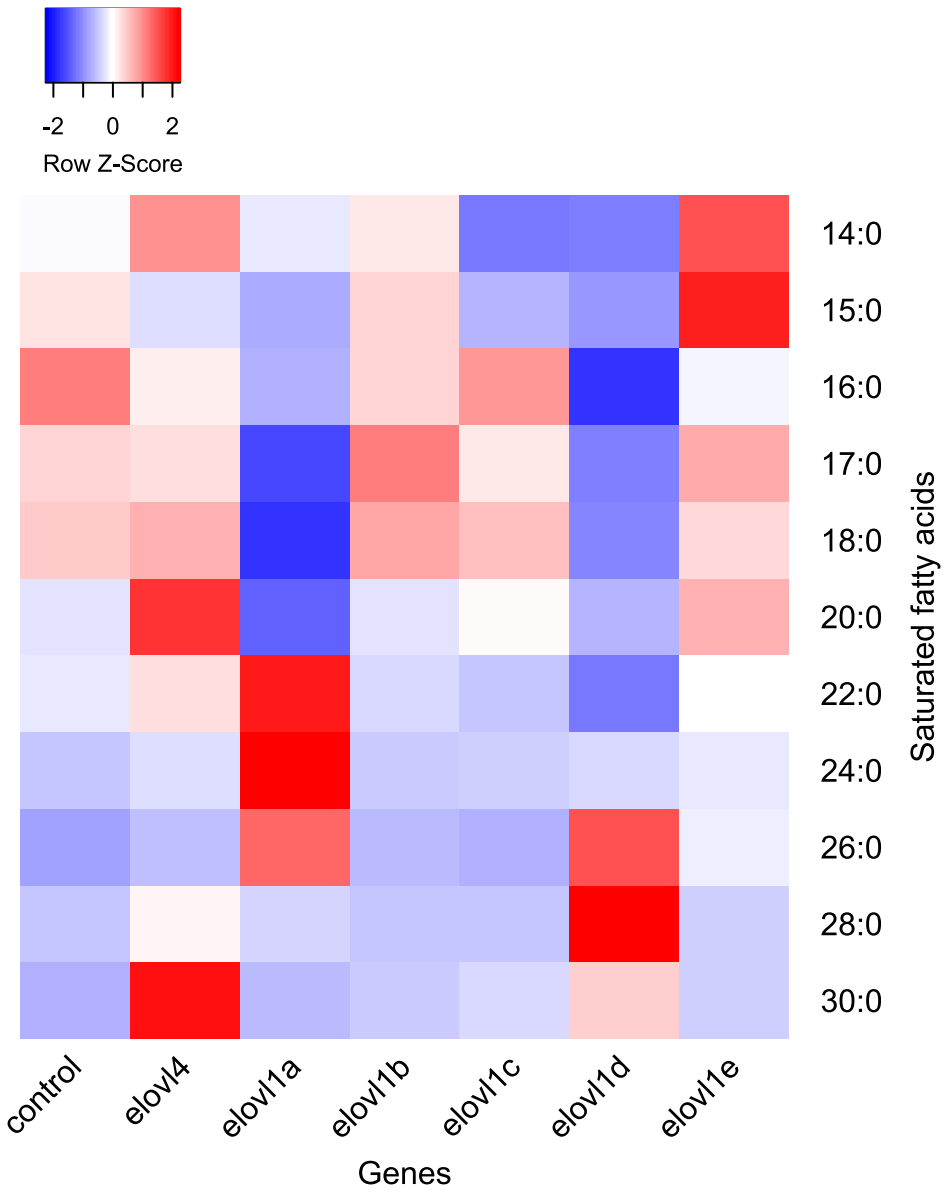
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elovl1c
elovl1d
elovl1e
Genes