A Complete Inventory of Long-Chain Polyunsaturated Fatty Acids Biosynthesis Pathway Enzymes in the Miniaturized Cyprinid *Paedocypris Micromegethes*

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Abstract

The capacity for long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis activity in a species depends on the enzymatic activities of fatty acyl desaturase (Fads) and elongation of very long-chain fatty acid (Elovl). The miniaturized fish *Paedocypris micromegethes* is a developmentally truncated cyprinid living in highly acidic water conditions in tropical peat swamps. The capacity for LC-PUFA biosynthesis in this species, which has a reduced genome size, is unknown. A high-quality *de novo* transcriptome assembly enabled the identification of a putative Fads2 and four Elovl. The Fads2 was verified as a *P. micromegethes* Fads2 ortholog with *in vitro* $\Delta 5$ and $\Delta 6$ activities. The Elovl sequences were established as an Elovl5, Elovl2, and two Elovl4 paralogs, namely Elovl4a and Elovl4b. These Elovl enzymes, mainly Elovl5 and Elovl2, fulfill the necessary C18, C20, and C22 PUFA elongation steps for LC-PUFA biosynthesis. Collectively, these results validate the presence of a complete repertoire of LC-PUFA biosynthesis enzymes in a tropical miniaturized freshwater fish.

Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs), consisting of eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3), and arachidonic acid (ARA; 20:4n-6), are required for proper cellular membrane structure, signaling, gene regulation, and energy (Jump 2002). LC-PUFAs can be synthesized from C18 PUFA precursors *de novo* through the concerted action of enzymes known as fatty acyl desaturases (Fads) and elongation of very long-chain fatty acid (Elovl) proteins (Sprecher 1981). Fads catalytic activity involves introducing a double bond at a specific position of the fatty acyl backbone while the elongation of the fatty acyl chain with two carbon units is by Elovl catalyzing the rate-limiting condensation step (Jakobsson et al. 2006). In the conventional biosynthesis pathway, linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6) are converted to EPA and ARA, respectively, through two possible paths, either utilizing a $\Delta 6$ or $\Delta 8$ desaturation route (Park et al. 2009). Subsequent conversion of EPA to DHA could occur through the 'Sprecher' pathway where EPA is first converted to 24:5n-3, followed by a $\Delta 6$ desaturation and finally a β -oxidation step (Sprecher et al. 1995), or through a more direct route, which involves the elongation of EPA to docosapentaenoic acid (DPA, 22:5n-3), followed by a direct $\Delta 4$ desaturation to DHA (Li et al. 2010). To date, two Fads with different desaturation roles within the LC-PUFA biosynthesis pathway, namely Fads1 and Fads2, have been identified in vertebrates (Castro et al. 2012). As for Elovl, four Elovl families, namely Elovl2, 4, 5, and 8 have been identified to have PUFA elongation activities (Jakobsson et al. 2006; Sun et al. 2021).

Terrestrial consumers rely almost exclusively on the export of LC-PUFAs from various aquatic ecosystems (Gladyshev et al. 2009). This is due to the existence of various producers and primary consumers with *de novo* LC-PUFA biosynthesis capacities within the aquatic food web (Kabeya et al. 2018; Twining et al. 2021). Teleost also possesses different capacity for LC-PUFA biosynthesis, depending of the catalog of Fads and Elovl within the species. Findings so far have proposed the influence of species habitat, trophic level, evolutionary history, and taxonomy position on the LC-PUFA biosynthesis capacity (Garrido et al. 2019; Matsushita et al. 2020; Xie et al. 2021). Multiple works on the Fads/Elovl enzymes in species from

key phylogenetic lineages within the chordates have shown the contribution of whole genome duplication, incidences of neo-functionalizations, sub-functionalizations and gene loss in diversifying the number and function of Fads and Elovl in different species (Castro et al. 2016; Monroig et al. 2016; Xie et al. 2021).

The order Cypriniformes is represented by over 3,000 species, with many members of this order being major aquaculture food and ornamental fish species. Cyprinids have a widespread distribution, with natural freshwater populations in all continents except Oceania, Antarctic and South America (Berra 2001). While the major carp species are large-sized, cyprinids also include a group of dwarf-like fish, which underwent a dramatic reduction of body size, with a maturation size of ≤ 20 mm standard length (Weitzman and Vari 1988). This event, known as miniaturization, is intriguingly common in cyprinids, with over 20 species in South, Southeast Asia and Africa (Kottelat et al. 2006; Ruber et al. 2007). An extreme example is found in the genus *Paedocypris*, represented by *P. progenetica*, *P. micromegethes* and *P. carbunculus*, which are recognized as the world's smallest fish and vertebrate (Britz and Kottelat 2008; Kottelat et al. 2006; Ruber et al. 2007). *Paedocypris* is confined to Southeast Asia peat swamp forests, inhabiting the highly fragmented waterlogged swamps with highly acidic and tannin-rich waters low in nutrients and dissolved oxygen (Kottelat et al. 2006).

To date, several cyprinid species have been validated to have all the necessary Fads and Elovl for LC-PUFA biosynthesis, including the zebrafish (Agaba et al. 2004; Hastings et al. 2001; Monroig et al. 2010a) silver barb (Janaranjani et al. 2018; Janaranjani and Shu-Chien 2020), tench (Garrido et al. 2020) and giant mahseer (Sam et al. 2021). The draft genome assemblies of *P. micromegethes* and *P. carbunculus* have reported reduced genome size (Malmstrøm et al. 2018). In addition, very little is known about the production and transfer of PUFA in peat swamp waters. We report here the cloning and functional characterization of all the hypothetically necessary Fads and Elovl required for a functional LC-PUFA biosynthesis pathway in *P. micromegethes*. Our results suggest the retaining of a complete LC-PUFA biosynthesis pathway in this species, which most likely ensure successful colonization of the peat swamp environment.

Materials And Methods

Sample collection, total RNA extraction and sequencing

Adult *P. micromegethes* was obtained from a peat swamp forest in Matang, Sarawak, Malaysia (01° 34.78067' N, 110° 16.04233' E) from December-January 2019 under the permit 63/2018 under the reference of (114)JHS/NCCD/600-7/2/107. Collected samples were preserved in RNAlater® (Thermo Scientific, USA) and stored at -80°C before extraction. All experimental procedures were performed in accordance with the USM Institutional Animal Care and Use Committee guidelines under the approval code USM/IACUC/2019/(117)(988). The RNA extraction was carried out using RNeasy Mini Kit (Qiagen, USA) with DNase treatment, according to the manufacturer instruction. The quantity of RNA was measured using the Qubit® RNA BR Assay Kit on Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies).

RNA integrity was validated by Agilent 2100 Bioanalyzer Nano kit (Santa Clara, CA). Total RNA obtained from the three biological replicates was sent for library construction, followed by RNA sequencing using the HiSeq XTM Ten platform (Illumina, USA) with paired-end 150 bp.

De novo assembly, filtering and annotation of RNA-seq data

Nearly 235 million raw reads were generated from the three independent libraries. The paired-end data was processed for removal of adapter, low-quality sequences (quality score < 5; reads < 36bp) using TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was used to assess sequence quality. *De novo* transcriptome assembly was done with Trinity (Grabherr et al. 2011) software with parameters ‘–SS_lib_type RF –seqType fq –left <input_file> –right <input_file> –max_memory 500G –CPU 48 > run.log 2>&1’. A perl script, inside the Trinity package (TrinityStats.pl) was used to compute the assembly statistic. To obtain the non-redundant transcripts, clustering analysis was performed for redundancy reduction using the CD-Hit program v4.8.1 (Li and Godzik 2006) with an identity threshold of 90%. Benchmarking Universal Single-Copy Orthologs (BUSCOs) was applied to assess the transcriptome completeness using the Actinopterygii dataset (Kriventseva et al. 2015). Coding regions of the non-redundant assembled transcripts were predicted with the TransDecoder v5.5.0 (<https://github.com/TransDecoder/TransDecoder>) pipeline using a minimum cut-off of 200 amino acids (aa) and open reading frames (ORFs). Functional annotation of the generated sequences was performed through BLASTp homology search on Genbank Nr, SwissProt, KEGG, KOG, and Pfam databases with E-value cut-off of $1 \cdot 10^{-5}$. The BLAST results against the Nr database were processed through Blast2go to retrieve the Gene Ontology (GO) annotation (Conesa et al. 2005). KEGG pathway analysis was conducted using the online KEGG Automatic Annotation Server (KAAS) <https://www.genome.jp/kegg/kaas/> with the bi-directional best hit (BBH) method for KEGG orthology (KO) assignment.

Identification and phylogenetic analysis of Fads and Elovl

The putative sequences of known LC-PUFA biosynthesis Fads and Elovl from cyprinid species (*fads2*, *elovl5*, *elovl2*, *elovl4a*, and *elovl4b*) were identified and extracted from the transcriptome assembly through tblastn search with Fads and Elovl sequences as queries. Online InterProScan tool was used to infer the protein domains (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>). A putative full-length Fads transcript was mined utilizing two Pfam domains namely N-terminal *Cyt-b5* (PF00173) and *FA_desaturase* (PF00487), and three histidine motifs (HXXXH, HXXXHH and QXXXHH). For Elovl orthologs, full-length transcripts consisting of a Pfam domain *ELO* (PF01151) and a histidine motif (HXXXHH) were discovered from the assembly. Multiple sequence alignment of the discovered protein sequences with orthologs from various vertebrates was done using Clustal Omega program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

For phylogenetic analysis, protein sequences were aligned with MAFFT v7.475 using the L-INS-i method. The alignment was then used to determine the best-fit substitution model using ModelFinder based on

Akaike information criterion (AIC). Maximum likelihood (ML) tree were generated for *Fads* and *Elovl5* respectively, using the IQ-TREE v1.6.12 (Nguyen et al. 2014) software with JTT+G4+I substitution model and 1,000 bootstraps. The consensus trees of the ML phylogenies were visualized through FigTree v1.4.2.

cDNA cloning of the *fads2*, *elovl5*, *elovl2*, *elovl4a*, and *elovl4b*

Total RNA was extracted from three adult fishes as described in 2.1. Subsequently, cDNA was synthesized from 3µg of the total RNA with Oligo d(T)₂₀ primer using high performance SuperScript IV Reverse Transcription kit (Invitrogen). Gene-specific primers for amplification of the *fads2*, *elovl5*, *elovl2*, *elovl4a*, and *elovl4b* sequences were designed based on the ORF sequences generated from the TransDecoder (Table S1). PCR was performed using KAPA HiFi HotStart polymerase (KAPA Biosystems, USA) with the following settings: 95°C for 3 min, 35 cycles of 98°C for 20 s, 60°C for 30 s and 72°C for 1 min, and 72°C for 1 min. The final PCR products were purified and digested with corresponding restriction enzymes and cloned into yeast expression vector pYES2 (Invitrogen, USA).

Functional characterization of the *fads2*, *elovl5*, *elovl2*, *elovl4a*, and *elovl4b*

Purified plasmid constructs pYES2_*fads2*, pYES2_*elovl5*, pYES2_*elovl2*, pYES2_*elovl4a*, and pYES2_*elovl4b*, together with an empty pYES2_ without ORF were transformed into the yeast *Saccharomyces cerevisiae* (strain INVSc1), respectively. Transformation and culture of yeast were as previously (Kuah et al. 2015). For characterization of *fads2* Δ desaturation capacity, transgenic yeast expressing the ORF was incubated in the presence of the following fatty acid (FA) substrates: 18:3n-3, 18:2n-6, 24:5n-3, and 24:4n-6 for Δ 6, 20:3n-3 and 20:2n-6 for Δ 8, 20:4n-3 and 20:3n-6 for Δ 5 and 22:5n-3 and 22:4n-6 for Δ 4. For *elovl*, 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 substrates were assayed. In addition, C24:0 and C22:6n-3 substrates were also assessed for *elovl4a* and *elovl4b*. The final FA concentration added to the yeast culture were 0.5 mM (C18), 0.75 mM (C20), 1.0 mM (C22), and 1.25 mM (C24), respectively. Each assay was repeated thrice with replicate recombinants.

Fatty acid analysis

After two days of incubation with FA substrates (30°C, 250 rpm), yeast was harvested, followed by total of lipid extraction in chloroform/methanol (2:1, v/v). Fatty acid methyl ester (FAME) was extracted and purified as described previously (Kuah et al. 2016). The derived FAME was characterized in a gas chromatography-mass spectrometry (GC-MS) QP2010 (Shimadzu, USA), equipped with a Supelco 2380 high-polarity fused-silica capillary column (30 m length, 0.25 mm inner diameter, 0.20 µm film thickness: SGE, USA). Helium was applied as carrier gas, with the oven temperature maintained at 80°C, followed by an increase to 260°C at a rate of 2°C/min. The synthesized FA products were identified and confirmed based on retention time and the mass spectrometry NIST08s mass library. The desaturation or elongation conversion efficiency was calculated as [individual product area / (all products areas + substrate area)] · 100.

The FA composition of whole body *P. micromegethes* was determined after total lipid extraction and FAME extraction as mentioned above. The FA composition was calculated as [individual peak area/total peak area] x 100. All results are represented as mean $\pm$ SEM from the three independent samples.

Result

De novo transcriptome assembly and annotation

From the *de novo* transcriptome assembly, a total of 210,210 transcripts with a mean length of 1,021 bp and N50 of 2,350 bp were obtained. The assembly statistics of the *P. micromegethes* are presented in Table 1. Analysis of the BUSCOs (Benchmarking Universal Single Copy Orthologs) against Actinopterygii gene set on the non-redundant transcripts revealed a high percentage of complete orthologues (93.4%), either as single or duplicated sequences (Fig. S1a). The protein coding sequences (cds) within the transcripts were predicted using TransDecoder. Four ORF categories, which are complete ORF (44,083), 5' partial ORF (10,602), 3' partial ORF (3,975), and internal ORF (2,326) were obtained (Fig. S1b). Based on the homology search, 87.98%, 82.72%, 86.82%, 81.64%, and 74.63% of the peptide sequences showed significant matched to the Nr, SwissProt, KEGG, KOG, and Pfam databases, respectively (Fig. S1c). Another 71.11% of sequences were assigned with GO term consisting of three independent categories: biological process, molecular and cellular functions (Fig. S2). The highest *P. micromegethes* transcripts assigned into the biological process category belongs to cellular process, biological regulation, and response to stimulus. As for molecular function category, the top assigned transcripts were grouped into catalytic, transcription regulator and molecular transducer activities while for cellular function category, highest transcripts were assigned to cellular anatomical entity and protein-containing complex. KEGG Automatic Annotation Server (KAAS) assigned KO IDs to 22,003 of the transcripts, with 8,673 assigned as complete, with involvement in 403 pathways (Table S2).

Table 1
General statistics for the *de novo* assembly Trinity of *P. micromegethes*

Assembly characteristics	Value
Before filtering	235,289,694
Raw reads (PE)	
Total assembled bases (bp)	518,275,680
Total assembled contigs	388,294
Average contig length (bp)	1,334
Median contig length (bp)	599
Contig N50 length (bp)	2,715
Contig N90 length (bp)	483
GC content (%)	48.07
After filtering*	214,789,660
Total assembled bases (bp)	210,210
Total assembled contigs	1,021
Average contig length (bp)	413
Median contig length (bp)	2,350
Contig N50 length (bp)	337
Contig N90 length (bp)	46.90
GC content (%)	
* Redundant transcripts were eliminated using CD-HIT.	

Sequence and phylogenetic analyses of the Fads and Elovl enzymes in *P. micromegethes*

A total of five selected transcripts, corresponding to the Fads and Elovl involved in LC-PUFA biosynthesis were mined from the *P. micromegethes* transcriptome. This includes a putative *fads2* and four *elovls*, including the *elovl5*, *elovl2* and two *elovl4* paralogs, *elovl4a* and *elovl4b* (Table 2). The ORFs length of the *P. micromegethes fads2*, *elovl5*, *elovl2*, *elovl4a*, and *elovl4b* transcripts are 1,335 bp, 810 bp, 849 bp, 933 bp, and 903 bp, respectively, encoding the protein sequences of 445 aa, 270 aa, 283 aa, 311 aa, and 301 aa.

Table 2

List mined putative Fads and Elovl transcripts involved in the biosynthesis of polyunsaturated fatty acids for *P. micromegethes*

Annotated complete sequence_ID	Sequence description
TRINITY_DN36236_c1_g1_i1.p1	Delta-6 desaturase (Fads2)
TRINITY_DN38107_c1_g5_i10.p1	Elongation of very long chain fatty acids 5 (Elovl5)
TRINITY_DN33464_c1_g2_i2.p1	Elongation of very long chain fatty acids 2 (Elovl2)
TRINITY_DN30298_c0_g2_i1.p1	Elongation of very long chain fatty acids 4a (Elovl4a)
TRINITY_DN36298_c3_g2_i1.p1	Elongation of very long chain fatty acids 4b (Elovl4b)

The BLASTp searches showed that the *P. micromegethes* Fads2 is highly identical (>85%) with functionally characterized orthologs from cyprinids, including *Tinca tinca* (QIA97820), *Tor tambroides* (AZL94116), *Danio rerio* (Q9DEX7), and *Barbonymus gonionotus* (AXF92413). Alignment of the *P. micromegethes* putative Fads2 with six functional characterized Fads protein sequences from *Homo sapiens*, *Clarias gariepinus*, *B. gonionotus*, *D. rerio*, *Siganus canaliculatus* ($\Delta 4$), and *S. canaliculatus* ($\Delta 6/\Delta 5$) showed typical microsomal fatty acyl front-end Fads sequence features (Fig. 1). This includes a N-terminal cytochrome *b₅*-like domain, a heme binding motif HPGG, three conserved histidine boxes (HXXXH, HXXHH and QXXHH), and four putative transmembrane domains. A region (FQFQ) speculated to be important for the regioselectivity of Fads2 is also present. The tree topology of Fads protein sequences showed two well-supported distinct clades, Fads1 and Fads2, with the *P. micromegethes* ortholog placed within the latter group (Fig. 2). Within the Fads2 clade, the various Cypriniformes members are grouped together with orthologs from other Otomorpha fish, including Siluriformes and Characiformes. The other Fads2 clade is made of a branch containing Salmoniformes and a larger branch comprises of Fads2 from various Acantomorphan.

As for Elovls, BLASTp analysis on Elovl5 matched this sequence (79.0-77.6%) with functionally characterized Elovl5 of other cyprinids including *T. tambroides*, *B. gonionotus* and *D. rerio*. Likewise, the *P. micromegethes* Elovl2 is highly identical with *B. gonionotus*, *T. tinca* and *T. tambroides* (86.8-84.8%). The *P. micromegethes* Elovl4a and Elovl4b were 92.0-81.5% similar with several teleost Elovl4 elongases. The alignment of all the above elongases (Fig. 3) showed typical characteristics of a microsomal fatty acyl elongase: four conserved motifs (KXXEXXDT, QXXFLHXXHH, NXXXHXXMYXYY, and TXXQXX), seven putative transmembrane domains, and a histidine rich box (HXXHH). The putative endoplasmic reticulum (ER) retrieval signal with KXRXX in Elovl5, KXKRX in Elovl2 and R(K)XKXX in Elovl4 were also detected at the carboxyl terminal. The ML tree topology showed the Elovl2 and Elovl5 orthologs sharing a common ancestor whereas all Elovl4 orthologs are in a separate cluster (Fig. 4). The Elovl4a and Elovl4b isoforms are separated into two respective clades.

Functional characterization of *P. micromegethes* Fads2 desaturase and Elovl elongases

The FA composition of the *P. micromegethes fads2* ORF-inserted *S. cerevisiae* yeast indicates $\Delta 6$ desaturation activity towards C18 PUFA substrates (Table 3). A low and probably negligible $\Delta 8$ activity towards 20:3n-3 was detected. Furthermore, *P. micromegethes fads2* was able to catalyze the biosynthesis of EPA and ARA through $\Delta 5$ desaturation towards C20:4n-3 and C20:3n-6 substrates, respectively. Meanwhile, a single peak corresponding to the non-methylene interrupted (NMI) product ($\Delta^{5,11,14,17}$ 20:4) was also detected. Incubation of the transformed yeast with either of the C22 PUFA substrates did not yield any products, which implies a lack of $\Delta 4$ desaturation. Incubation with C24:4n-6 or C24:5n-3 resulted in their respective desaturation products, which suggest a role in this Fads2 in the 'Sprecher' pathway for the biosynthesis of DHA. Overall, the results indicate that the *P. micromegethes* Fads is a Fads2 with bifunctional $\Delta 6/\Delta 5$ activities.

Table 3

Functional characterization of *P. micromegethes* Fads2 through heterologous expression of the respective transcript ORF in yeast (*Saccharomyces cerevisiae*), followed by incubation with PUFA substrate to assay the desaturation rate leading to PUFA product

PUFA Substrate	PUFA Product	Conversion rate (%)	Activity
Fads2			
18:3n-3	18:4n-3	26.8 $\pm$ 1.7	$\Delta 6$
18:2n-6	18:3n-6	13.8 $\pm$ 0.4	$\Delta 6$
20:3n-3*	20:4n-3	0.9 $\pm$ 0.1	$\Delta 8$
20:2n-6	20:3n-6	0	$\Delta 8$
20:3n-3 ^a	$\Delta^{5,11,14,17}$ 20:4	4.8 $\pm$ 0.5	$\Delta 5$
20:4n-3	20:5n-3	18.6 $\pm$ 1.0	$\Delta 5$
20:3n-6	20:4n-6	7.34 $\pm$ 1.0	$\Delta 5$
22:5n-3	22:6n-3	0	$\Delta 4$
22:4n-6	22:5n-6	0	$\Delta 4$
24:5n-3	24:6n-3	7.5 $\pm$ 0.6	$\Delta 6$
24:4n-6	24:5n-6	10.0 $\pm$ 2.0	$\Delta 6$
Results are expressed as the percentage of the PUFA substrate converted to PUFA product with the following calculation: [individual product area / (all products area + substrate area)] $\times$ 100. Value is presented as the mean $\pm$ SEM (n = 3).			
*Conversion of 20:3n-3 involved step wise reactions of $\Delta 8$ desaturation, followed by a $\Delta 5$ desaturation of 20:4n-3 to EPA.			
** 20:3n-3 was also converted to non-methylene interrupted fatty acid, $\Delta^{5,11,14,17}$ 20:4.			
ND = not detected; PUFA= Polyunsaturated fatty acid			

Transgenic yeast harboring the ORF of *P. micromegethes elovl5* was able to elongate C18 and C20 PUFA substrates, with highest activities C18:4n-3 and C18:3n-6 substrates. There was no elongation of C22 substrates, implying the *P. micromegethes* Elov15 is a C18-C20 PUFA elongase (Table 4). In comparison, the *P. micromegethes elovl2* ORF catalyzed the elongation of all tested C18, C20 and C22 PUFA substrates. Notably, this ortholog exhibits elongation of 22:5n-3, and 22:4n-6 substrates to C₂₄ product, with the former a significant activity in the production of DHA. As for Elov14, both the *P. micromegethes* Elov14 paralogs exhibited elongation capacity toward the saturated fatty acid (SFA) C24:0, with detection of very-long-chain saturated fatty acid (VLC-SFA) elongated products up to C30:0 (Table 5). There was also elongation of C18 and C20 PUFA substrates, and low elongation of C22 PUFA. Between the two isoforms, Elov14a displayed higher elongation activities towards C18 PUFA. In contrast, the Elov14b showed higher activities towards the C20 substrates, with further elongation to C36 PUFA substrates. All chromatograms of the yeast FA profiles are presented in supplementary section (Fig. S3-S7).

Table 4

Functional characterization of *P. micromegethes* Elovl5 and Elovl2 through heterologous expression of the respective transcript ORF in yeast (*Saccharomyces cerevisiae*), followed by incubation with PUFA substrate to assay the elongation rate leading to PUFA product

PUFA substrate	PUFA product	Conversion rate (%)			Activity
		Pyes2 vector	Elovl5	Elovl2	
18:3n-3	20:3n-3	1.6 ± 0.0	6.8 ± 0.1	18.2 ± 0.8	C18 → 20
	22:3n-3	ND	ND	2.9 ± 0.1	C20 → 22
	24:3n-3	ND	ND	ND	C22 → 24
	Total	1.6 ± 0.0	6.8 ± 0.1	21.1 ± 0.9	
18:2n-6	20:2n-6	1.3 ± 0.3	2.3 ± 0.0	7.2 ± 0.6	C18 → 20
	22:2n-6	1.3 ± 0.1	0.6 ± 0.1	1.6 ± 0.0	C20 → 22
	24:2n-6	ND	ND	ND	C22 → 24
	Total	2.6 ± 0.4	2.8 ± 0.1	8.8 ± 0.6	
18:4n-3	20:4n-3	1.8 ± 0.1	39.4 ± 3.0	17.3 ± 0.4	C18 → 20
	22:4n-3	ND	0.3 ± 0.0	8.6 ± 0.3	C20 → 22
	24:4n-3	ND	ND	1.8 ± 0.1	C22 → 24
	Total	1.8 ± 0.1	39.7 ± 3.0	27.8 ± 0.6	
18:3n-6	20:3n-6	1.4 ± 0.1	32.0 ± 2.2	15.8 ± 0.7	C18 → 20
	22:3n-6	ND	ND	7.0 ± 0.4	C20 → 22
	24:3n-6	ND	ND	2.8 ± 0.4	C22 → 24
	Total	1.4 ± 0.1	32.0 ± 2.2	25.5 ± 1.0	
20:5n-3	22:5n-3	ND	12.5 ± 2.6	9.2 ± 0.4	C20 → 22
	24:5n-3	ND	ND	35.3 ± 1.1	C22 → 24
	Total		12.5 ± 2.6	44.5 ± 1.4	
20:4n-6	22:4n-6	ND	4.4 ± 0.7	3.4 ± 0.2	C20 → 22

Results are expressed as the percentage of the fatty acid substrate converted to elongated fatty acid products with the following calculation: [individual product area / (all products area + substrate area)] × 100. Value is presented as the mean ± SEM (n = 3).

Total conversion is calculated based on summation of all of the elongated products.

ND = not detected; PUFA= Polyunsaturated fatty acid

PUFA substrate	PUFA product		Conversion rate (%)		Activity
	24:4n-6	ND	ND	20.4 ± 1.8	C22 → 24
	Total		4.4 ± 0.7	23.8 ± 2.0	
22:5n-3	24:5n-3	ND	ND	10.8 ± 0.5	C22 → 24
22:4n-6	24:4n-6	ND	ND	8.4 ± 0.5	C22 → 24
Results are expressed as the percentage of the fatty acid substrate converted to elongated fatty acid products with the following calculation: [individual product area / (all products area + substrate area)] × 100. Value is presented as the mean ± SEM (n = 3).					
Total conversion is calculated based on summation of all of the elongated products.					
ND = not detected; PUFA= Polyunsaturated fatty acid					

Table 5

Functional characterization of *P. micromegethes* Elovl4a and Elovl4b through heterologous expression of the respective transcript ORF in yeast (*Saccharomyces cerevisiae*), followed by incubation with SFA/PUFA substrate to assay the elongation rate leading to SFA/PUFA product

Substrate	Product	Conversion rate (%)			Activity
		Pyes2 vector	Elovl4a	Elovl4b	
SFA					
24:0	26:0	3.25 ± 0.7	5.2 ± 0.3	3.05 ± 0.3	C24 → 26
	28:0	ND	2.8 ± 0.1	1.6 ± 0.3	C26 → 28
	30:0	ND	1.9 ± 0.1	0.4 ± 0.1	C28 → 30
	32:0	ND	ND	ND	C30 → 32
PUFA					
18:3n-3	20:3n-3	1.3 ± 0.3	15.1 ± 0.8	8.3 ± 0.1	C18 → 20
	22:3n-3	ND	1.7 ± 0.1	1.0 ± 0.1	C20 → 22
	24:3n-3	ND	ND	ND	C22 → 24
	Total	1.3 ± 0.3	16.9 ± 0.8	9.3 ± 0.2	
18:2n-6	20:2n-6	1.2 ± 0.0	14.8 ± 0.9	5.8 ± 0.2	C18 → 20
	22:2n-6	1.4 ± 0.0	1.8 ± 0.3	1.5 ± 0.0	C20 → 22
	24:2n-6	ND	ND	ND	C22 → 24
	Total	2.6 ± 0.0	16.5 ± 1.0	7.3 ± 0.3	
18:4n-3	20:4n-3	1.4 ± 0.3	5.5 ± 0.3	4.0 ± 0.2	C18 → 20
	22:4n-3	ND	0.6 ± 0.1	0.5 ± 0.0	C20 → 22
	24:4n-3	ND	ND	ND	C22 → 24
	Total	1.4 ± 0.3	6.0 ± 0.3	4.5 ± 0.2	
18:3n-6	20:3n-6	ND	7.2 ± 0.7	5.0 ± 0.1	C18 → 20
	22:3n-6	ND	1.2 ± 0.4	0.7 ± 0.1	C20 → 22
	24:3n-6	ND	ND	ND	C22 → 24
Results are expressed as the percentage of the fatty acid substrate converted to elongated fatty acid products with the following calculation: [individual product area / (all products area + substrate area)] × 100. Value is presented as the mean ± SEM (n = 3).					
Total conversion is calculated based on summation of all of the elongated products.					
ND = not detected; SFA= Saturated fatty acid; PUFA= Polyunsaturated fatty acid					

Substrate	Product	Conversion rate (%)		Activity	
	Total		8.3 ± 0.3	5.7 ± 0.2	
20:5n-3	22:5n-3	ND	3.9 ± 0.2	5.7 ± 0.8	C20 → 22
	24:5n-3	ND	0.5 ± 0.1	1.2 ± 0.2	C22 → 24
	26:5n-3	ND	ND	0.2 ± 0.0	C24 → 26
	28:5n-3	ND	ND	0.1 ± 0.0	C26 → 28
	30:5n-3	ND	ND	0.2 ± 0.0	C28 → 30
	32:5n-3	ND	ND	1.1 ± 0.1	C30 → 32
	Total		4.4 ± 0.3	8.3 ± 1.1	
20:4n-6	22:4n-6	ND	4.4 ± 0.2	10.5 ± 2.0	C20 → 22
	24:4n-6	ND	0.7 ± 0.1	2.8 ± 0.5	C22 → 24
	26:4n-6	ND	0.6 ± 0.1	0.6 ± 0.1	C24 → 26
	28:4n-6	ND	ND	0.3 ± 0.1	C26 → 28
	30:4n-6	ND	ND	2.4 ± 0.3	C28 → 30
	32:4n-6	ND	ND	3.8 ± 0.5	C30 → 32
	Total		5.1 ± 0.3	20.4 ± 2.8	
22:5n-3	24:5n-3	ND	1.5 ± 0.1	3.1 ± 0.1	C22 → 24
	26:5n-3	ND	ND	0.2 ± 0.1	C24 → 26
	28:5n-3	ND	ND	0.2 ± 0.1	C26 → 28
	30:5n-3	ND	ND	0.5 ± 0.1	C28 → 30
	32:5n-3	ND	ND	2.2 ± 0.2	C30 → 32
	Total		1.5 ± 0.1	6.1 ± 0.2	
22:4n-6	24:4n-6	ND	1.7 ± 0.2	2.4 ± 0.4	C22 → 24
	26:4n-6	ND	ND	0.3 ± 0.1	C24 → 26
	28:4n-6	ND	ND	0.2 ± 0.0	C26 → 28

Results are expressed as the percentage of the fatty acid substrate converted to elongated fatty acid products with the following calculation: [individual product area / (all products area + substrate area)] × 100. Value is presented as the mean ± SEM (n = 3).

Total conversion is calculated based on summation of all of the elongated products.

ND = not detected; SFA= Saturated fatty acid; PUFA= Polyunsaturated fatty acid

Substrate	Product	Conversion rate (%)			Activity
	30:4n-6	ND	ND	1.6 ± 0.3	C28 →30
	32:4n-6	ND	ND	2.6 ± 0.5	C30 →32
	Total		1.7 ± 0.2	7.1 ± 1.1	
22:6n-3	24:6n-3	ND	ND	ND	C22 →24
Results are expressed as the percentage of the fatty acid substrate converted to elongated fatty acid products with the following calculation: [individual product area / (all products area + substrate area)] × 100. Value is presented as the mean ± SEM (n = 3).					
Total conversion is calculated based on summation of all of the elongated products.					
ND = not detected; SFA= Saturated fatty acid; PUFA= Polyunsaturated fatty acid					

Fatty acid composition of *P. micromegethes*

The FA composition of *P. micromegethes* whole body showed a higher proportion of SFAs (Table 6). Among these, C16:0 is the predominant FA, while for monounsaturated fatty acids (MUFAs), C18:1n-7 was the most abundance. As for PUFA, the proportion of the total n-6 PUFA is twice higher than the n-3 PUFA, with significant contribution from LA and ARA. Within the n-3 PUFA, the percentage of DHA is also higher than EPA.

Table 6
Whole body fatty acid composition of *P. micromegethes*

Fatty acid	Total fatty acid detected (%)
C14:0	2.0 ± 0.1
C15:0	1.6 ± 0.2
C16:0	29.8 ± 1.3
C17:0	3.0 ± 0.4
C18:0	16.9 ± 0.6
Σ SFAs	53.4 ± 1.7
C14:1	1.3 ± 0.1
C15:1	0.7 ± 0.1
C16:1	3.8 ± 0.4
C17:1	0.5 ± 0.0
C18:1n-9	0.9 ± 0.3
C18:1n-7	15.8 ± 0.3
C20:1n-9	0.4 ± 0.0
Σ MUFAs	23.4 ± 0.5
C18:3n-3 (ALA)	0.4 ± 0.1
C18:4n-3	0.2 ± 0.0
C20:3n-3	-
C20:4n-3	0.2 ± 0.0
C20:5n-3 (EPA)	0.3 ± 0.0
C22:5n-3	0.3 ± 0.0
C22:6n-3 (DHA)	4.7 ± 0.8
C18:2n-6 (LA)	8.0 ± 0.6
C18:3n-6	0.4 ± 0.0
C20:3n-6	0.5 ± 0.1
C20:4n-6 (ARA)	6.3 ± 0.7
SFA = Saturated fatty acid; MUFA = Monounsaturated fatty acid; PUFA = Polyunsaturated fatty acid; ALA = α-Linolenic acid; EPA = Eicosapentaenoic acid; DHA = Docosahexaenoic acid; LA = Linoleic acid; ARA = Arachidonic acid. Values are presented as the Mean ± SEM of triplicate measurement.	

Fatty acid	Total fatty acid detected (%)
Σ PUFAs	21.4 $\pm$ 2.0
Σ n-3	6.2 $\pm$ 0.8
Σ n-6	15.2 $\pm$ 1.2
n-3/n-6	0.4 $\pm$ 0.0
SFA = Saturated fatty acid; MUFA = Monounsaturated fatty acid; PUFA = Polyunsaturated fatty acid; ALA = α -Linolenic acid; EPA = Eicosapentaenoic acid; DHA = Docosahexaenoic acid; LA = Linoleic acid; ARA = Arachidonic acid. Values are presented as the Mean $\pm$ SEM of triplicate measurement.	

Discussion

Studies in different fish species have shown diverse combinations of Fads and Elovl orthologs, coupled with differences in substrate specificity and activity rate for both enzymes (Xie et al. 2021). The repertoire of LC-PUFA biosynthesizing Fads and Elovl in teleost was initially linked to species habitat, with species living in the LC-PUFA rich marine habitat displaying a loss of biosynthesis activities, presumably due the lack of evolutionary pressure to retain the biosynthesis pathway (Tocher et al. 2006; Zheng et al. 2009). The presence of multiple Fad and Elovl isoforms in Atlantic salmon was also linked to the transition from marine to freshwater lifestyle in this species (Monroig et al. 2010b; Morais et al. 2009; Zheng et al. 2005). Subsequently, the LC-PUFA biosynthesis capacity was hypothesized to be associated with the species trophic level (Li et al. 2010; Morais et al. 2012) and taxonomy position (Garrido et al. 2019).

The miniaturized cyprinid *P. micromegethes*, with its reduced smaller genome size and adaptation to peat swamp habitat offer insights into the relationship between an extreme habitat niche and biosynthesis of LC-PUFA (Malmstrøm et al. 2018). The use of RNA-seq data has become a cost-effective and useful approach for functional genomics research in species with or without a reference genome. *De novo* transcriptome assembly has been applied in a terrestrial crustacean species to mine for Fads or Elovl orthologs (Ting et al. 2021). Through the *P. micromegethes* transcriptome assembly, a single Fads2 and four Elovl sequences were putatively identified. This cluster is consistent with the known orthologs in various Cyprinidae representatives (Agaba et al. 2004; Garrido et al. 2020; Hastings et al. 2001; Janaranjani et al. 2018; Janaranjani and Shu-Chien 2020; Monroig et al. 2010a; Sam et al. 2021).

The *P. micromegethes* Fads2 transcript encompassed all typical characteristics of a front-end desaturase. The presence of a cytochrome b₅-like domain at the N-terminal end enables the electron-transfer process during the desaturation reaction and is obligatory for the catalytic activities of all front-end desaturases (Napier et al. 2003; Schenkman and Jansson 2003). Located within this domain is a HPGG motif, which forms the heme-binding core of the protein (Sayanova et al. 1999). Additionally, the sequence also consists of three conserved histidine boxes (HxxxH, HxxxHH and QxxHH) pivotal for Fads catalytic activities (Hashimoto et al. 2008; Shanklin et al. 1994). Among front-end desaturases, the substitution of histidine 'H' with glutamine 'Q' in the third histidine box QxxHH is essential for $\Delta 6$

desaturase activity and conserved among all teleost Fads2 (Castro et al. 2016; Sayanova et al. 2001). The four amino acid residues FQFQ situated between the second and third histidine boxes of the putative *P. micromegethes* Fads2 sequence has also been reported in other cyprinid fish (Garrido et al. 2020; Hastings et al. 2001; Janaranjani et al. 2018; Ren et al. 2013; Sam et al. 2021). This region was previously postulated to influence the Fads selectivity towards $\Delta 6/\Delta 5$ or $\Delta 4$ substrates (Lim et al. 2014).

Phylogenetic tree built with Fads1 and Fads2 amino acid sequences from various orthologs placed the *P. micromegethes* Fads within the Fads2 clade, clustered within other Cypriniformes orthologs. This clade shares a similar ancestor with Fads2 from Siluriformes and Characiformes fish. Another larger Fads2 clade consist of orthologs from a diverse taxonomy group, including the ancient Ostoglossomorpha, Salmoniformes and Acanthomorpha. The Fads1 ortholog is found in Agnathans, Chondrichthyes, non-teleost ray-finned fish and early teleost groups such as *Anguilla japonica*. Several works have established the $\Delta 5$ function of these Fads1 orthologs, which complement the $\Delta 6$ activity of the Fads2 (Castro et al. 2016; Lopes-Marques et al. 2018). After the 3R whole genome duplication (3R WGD), the loss of Fads1 occurred in most surviving teleost, which was subsequently compensated by the sub-functionalization of Fads2 to acquire the required $\Delta 5$ function (Lopes-Marques et al. 2017). Taken together, the sequence alignment and phylogenetic analysis suggest the isolation of a Fads2 from *P. micromegethes*.

All the four *P. micromegethes* Elovl sequences possess the characteristic features of an Elovl elongase, namely four conserved motifs (KXXEXXDT, QXXFLHXXHH, NXXXHXXMYXYY, and TXXQXX), seven transmembrane domains, a histidine rich box (HXXHH) and a predicted ER retrieval signal with lysine (K) or arginine (R) residues located near the carboxyl terminus (Jakobsson et al. 2006). The highly conserved HXXHH box is responsible for the electron transfer process during elongation (Leonard et al. 2004). Phylogenetic analysis of the four isolated Elovl transcripts placed these sequences to the respective Elovl5, Elovl2, Elovl4a, and Elovl4b clades. In early pre-teleost lineages such as lampreys and chondrichthyans, separate orthologues of Elovl5 and Elovl2 can be observed, as result of genome duplications in vertebrate ancestry (Monroig et al. 2016). Similarly, both these families are also present in early teleost groups, such as Anguilliformes and Otomorpha (Ferraz et al. 2019; Garrido et al. 2020; Janaranjani and Shu-Chien 2020; Machado et al. 2018; Oboh et al. 2016; Sam et al. 2021; Xu et al. 2020). The salmonids is the last teleost group retaining Elovl2 as this ortholog is missing in Acanthomorphans (Morais et al. 2009). Both the putative *P. micromegethes* Elovl4 sequences were placed in two separate clades comprising of the Elovl4a and Elovl4b, respectively. The existence of two Elovl4 paralogues in modern teleost groups are speculated to be the consequence of teleost specific 3R WGD.

Functional characterization of the *P. micromegethes* Fads2 revealed a Fads2 with bifunctional $\Delta 6/\Delta 5$ activities. This recapitulates the activities reported in zebrafish (Hastings et al. 2001), tench (Garrido et al. 2020), silver barb (Janaranjani et al. 2018) and mahseer (Sam et al. 2021). The $\Delta 6/\Delta 5$ steps fulfil all the required desaturation steps for the biosynthesis of EPA and ARA. Additionally, the $\Delta 6$ desaturation of 24:5n-3 was also present, implying the utilization of the 'Sprecher' route for the conversion of EPA to DHA, which is typical of cyprinids. The $\Delta 5$ activity, while present in Fads2 of numerous freshwater teleosts and several marine species, are absent in most of the carnivorous marine teleost studied so far (Mohd-Yusof

et al. 2010; Morais et al. 2011; Zheng et al. 2009). This impairment could ostensibly be the result of the LC-PUFA rich prey these species consumed (Tocher et al. 2006). Additionally, there was no prominent $\Delta 8$ or $\Delta 4$ activities in the *P. micromegethes* Fads2. In mammals and teleost, the $\Delta 8$ step offers the possibility of an alternate direction for EPA synthesis besides the conventional $\Delta 6$ route (Monroig et al. 2011a; Park et al. 2009). While some bifunctional $\Delta 6/\Delta 5$ Fads2 or $\Delta 6$ Fads2 were reported to have $\Delta 8$ activity, overall findings seemed to suggest marine teleost conspicuously utilizing the $\Delta 8$ pathway as compared to freshwater or diadromous fish (Monroig et al. 2011a). The presence of $\Delta 4$ Fads in the more recent teleost lineages suggest the $\Delta 4$ pathway to be comparatively recent in teleost evolution and hence, missing in earlier lineages such as cyprinids (Obloh et al. 2017a).

The *P. micromegethes* Elovl5 showed elongation capacity towards C18 and C20 PUFAs, without any activity towards C22 substrates. This preference towards C18-C20 PUFA substrates is similar with most of the Elovl5 orthologs (Monroig et al. 2016). Between the C18 substrates, *P. micromegethes* Elovl5 exhibited substantially higher activities towards C18:4n-3 or C18:3n-6, as compared to the ALA or LA. This befits the $\Delta 6$ desaturation of ALA or LA as the first step of the biosynthesis pathway, as compared to the $\Delta 8$ path (Monroig et al. 2011a). The lack of elongation activity towards C22 PUFA substrates is commonly reported in teleost Elovl5 orthologs, except for orthologs from several marine species (Monroig et al. 2012; Soo et al. 2020; Xie et al. 2016). The *P. micromegethes* Elovl2 appears to be an elongase with wide range of substrate specificity. More importantly, this Elovl2 could elongate C22 PUFA substrates to their respective C24 PUFA substrates. Since the first discovery of Elovl4 in zebrafish, subsequent findings have showed its capacity for VLC-PUFA elongation (Ferraz et al. 2020; Monroig et al. 2010a; Monroig et al. 2012; Monroig et al. 2011b; Obloh et al. 2017b). While freshwater fish species such as cyprinids could potentially utilize Elovl4 for C22 PUFA elongation, Elovl4 is postulated to compensate for the loss of Elovl2 in marine species (Betancor et al. 2020; Luo et al. 2021). Between the two isoforms, the *P. micromegethes* Elovl4a displayed higher elongation activities towards C18 PUFA while the Elovl4b showed higher activities towards the C20 substrates. Majority of the studied teleost species also reported higher rate of C20 and C22 PUFA elongation with Elovl4b (Ferraz et al. 2020; Monroig et al. 2010a; Morais et al. 2020). In contrast, the African catfish Elovl4a showed higher activities among the two paralogs (Obloh et al. 2017b). The elongation of C24:0 by *P. micromegethes* Elovl4 is consistent with SFA elongation activities observed in both freshwater and marine species (Ferraz et al. 2020; Janaranjani and Shu-Chien 2020; Morais et al. 2020). It is worth mentioning that a new Elovl8 family, was recently described in teleost, and was shown to play a role in elongation of PUFA substrates (Li et al. 2020; Sun et al. 2021). Although hits matching Elovl8 were found in *P. micromegethes*, these sequences were not further studied as all the steps required for LC-PUFA biosynthesis are already fulfilled by the above described Elovl families.

Taken together, the functional characterization of *P. micromegethes* Fads, Elovl5, Elovl2, and Elovl4 showed that *P. micromegethes* possess a complete LC-PUFA biosynthesis pathway. The detection of intermediates PUFA such as C18:4n-3, C20:4n-3 and C22:5n-3 in *P. micromegethes* whole body also suggest biosynthesis activities *in vivo*. Like other cyprinids, *P. micromegethes* whole body also contain higher proportion of DHA than EPA (Jaya-Ram et al. 2018). While the biosynthesis of EPA and ARA

follows the conventional $\Delta 6$ route, the conversion of EPA to DHA utilizes the 'Sprecher' route (Fig. 5). This route is conserved in all studied otomorphans, except for a marine clupeid species which possess a Fads2 devoid of $\Delta 5$ capacity (Ferraz et al. 2019; Garrido et al. 2020; Janaranjani et al. 2018; Janaranjani and Shu-Chien 2020; Machado et al. 2018; Oboh et al. 2016; Sam et al. 2021). This finding supports the suggestion that the phylogeny position of a fish species takes preeminence over trophic level as a factor influencing the LC-PUFA biosynthesis capacity of a species (Galindo et al. 2021; Garrido et al. 2019). Beside these species, the Atlantic salmon and rainbow trout also utilized a similar route for the biosynthesis of EPA and DHA (Abdul Hamid et al. 2016; Hastings et al. 2004; Monroig et al. 2010b; Oboh et al. 2017a). Therefore, despite having a relatively reduced genome size as compared to other cyprinids, the LC-PUFA biosynthesis capacity in *P. micromegethes* remain intact (Malmstrøm et al. 2018). The retaining of this pathway indicates the importance of LC-PUFA biosynthesis for life in peat swamps. Due to the extreme acidic condition and poor light penetration, peat swamp water bodies are characterized by low plankton abundance and limited availability of nutrients (Helmizuryani et al. 2020; Yule and Gomez 2009).

Conclusions

A Fads2 and Elovl5, Elovl2 and Elovl4 orthologs were mined from a high quality *de novo* transcriptome assembly of a miniaturized cyprinid, *P. micromegethes*. Using yeast-based heterologous expression approach, the functional characterization of each orthologs were studied. Collectively, results showed that *P. micromegethes* possess all the necessary Fads and Elovl for the biosynthesis of LC-PUFA from C18 PUFA substrates.

Declarations

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Statements and Declarations

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Data Availability This current research was registered in NCBI database under BioProjet ID PRJNA737281. The datasets generated during and/or analyzed during this current research were deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP323968.

Code availability Not applicable.

Ethics approval This study was performed in accordance with the USM Institutional Animal Care and Use Committee guidelines under the approval code USM/IACUC/2019/(117)(988).

Consent to participate Not applicable.

Consent for publication All the authors have approved the final manuscript.

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Figures

colons, respectively. The cytochrome *b5*-like domain is underlined with a dotted line and the four putative transmembrane domains are underlined with solid lines. The heme-binding motif (HPGG) is highlighted in blue. The three conserved histidine boxes (HXXXH, HXXHH and QXXHH) are illustrated and highlighted with green, whereas regions involved in substrate specificity and regioselectivity are highlighted in yellow

Figure 2

Maximum likelihood phylogenetic analysis of the deduced amino acid sequence of the *P. micromegethes* Fads2 with Fads1 and Fads2 orthologs from other vertebrate species rooted with invertebrate group. Node values indicate bootstrap values. The *P. micromegethes* sequence is indicated in bold

```
HaeElov12 -----MEHLKAFCEINAFLENNFGPSRERVRGFMGLDSVLPFFLTWMLLSI 49
PaiKlov12 -----MEHFETVRLINSIVGLFGERDRVRGMLLDQVETPLGLGLGSDV 49
DreElov12 -----MEVEIIEGLANVVDGLFGERDVRGMLLDQVETPLGLGLGSDV 49
TaeElov12 -----MEHLKIDGLANSIVGLFGERDVRGMLLDQVETPLGLGLGSDV 49
HaeElov15 -----MEHPICLSITPTALLGPRDTRVRGMLLDQVETPLGLGLGSDV 46
PaiKlov15 -----MEHISFQNTWIDNNQPRDLAVTQWFLLOVTPFTFWGLLV 46
DreElov15 -----METFERRVNSVIDNNQPRDLAVTQWFLLOVTPFTFWGLLV 46
TaeElov15 -----MEGFRQNTWIDNNQPRDLAVTQWFLLOVTPFTFWGLLV 46
DreElov18a -----MYVFSSTWQLGILVERILENGERTDGLVYSFLVGGIPLCYLNNY 50
PaiKlov18a -----MYVGSSTWQLGILVERILENGERTDGLVYSFLVGGIPLCYLNNY 50
ScaElov18a -----MEALTMQGLQLFPGILNGERTDGLVYSFLVGGIPLCYLNNY 48
ScaElov18b -----MASAMEVLSVTSVLNGERTDGLVYSFLVGGIPLCYLNNY 46
DreElov18b -----MEASQGLSNNQVINGERTDGLVYSFLVGGIPLCYLNNY 46
PaiKlov18b -----MEGTQKLESLANNILNGERTDGLVYSFLVGGIPLCYLNNY 46
HaeElov14  MGLDSEFGVLRVSTALNGVTFVFWTWIADKRVENPLDQWPLDSTLVLLFP 60
PaiKlov14a -----MEYIKHINQTLQTFWMLTIADKRVENPLDQWPLDSTLVLLFP 49
CpaElov14a -----MEYVNLVNDCTFTFWMLTIADKRVENPLDQWPLDSTLVLLFP 49
DreElov14a -----MEIIGHINQTVFWMLTIADKRVENPLDQWPLDSTLVLLFP 49
HaeElov14a -----MEYIKHINQTFWMLTIADKRVENPLDQWPLDSTLVLLFP 49
CpaElov14b -----METVLRLINDTAEFTWMLTIADKRVENPLDQWPLDSTLVLLFP 49
PaiKlov14b -----MEAVVHNDGILEFTWMLTIADKRVENPLDQWPLDSTLVLLFP 49
DreElov14b -----MEYVHNDGILEFTWMLTIADKRVENPLDQWPLDSTLVLLFP 49
HgoElov14b -----METVHNDGILEFTWMLTIADKRVENPLDQWPLDSTLVLLFP 49
      * * * : : *
HaeElov12  WLQFTYNNRPAISLNGILITVNLGTELSANGVLELITWNGVNLQCGSLR-AGEA 108
PaiKlov12  WLQFTYNNRPAISLNGILITVNLGTELSANGVLELITWNGVNLQCGSLR-AGEA 108
DreElov12  WLQFTYNNRPAISLNGILITVNLGTELSANGVLELITWNGVNLQCGSLR-AGEA 108
TaeElov12  WLQFTYNNRPAISLNGILITVNLGTELSANGVLELITWNGVNLQCGSLR-AGEA 108
HaeElov15  WLQFTYNNRPAISLNGILITVNLGTELSANGVLELITWNGVNLQCGSLR-AGEA 105
PaiKlov15  WLQFTYNNRPAISLNGILITVNLGTELSANGVLELITWNGVNLQCGSLR-AGEA 105
DreElov15  WLQFTYNNRPAISLNGILITVNLGTELSANGVLELITWNGVNLQCGSLR-AGEA 105
HaeElov15  WLQFTYNNRPAISLNGILITVNLGTELSANGVLELITWNGVNLQCGSLR-AGEA 105
```

Figure 3

Comparison of the deduced amino acid (aa) sequence of the newly cloned elongases (Elovl5) of the *P. micromegethes* (Elovl5-UBB58396, Elovl2-UBB58397, Elovl4a-UBB58398, and Elovl4b-UBB58399) and orthologues from *Homo sapiens* (Elovl5-NP_068586, Elovl2-NP_060240, and Elovl4-NP_073563), *Clarias gariepinus* (Elovl4a-ASY01350 and Elovl4b-ASY01351), *Danio rerio* (Elovl5-AAN77156, Elovl2-NP_001035452, Elovl4a-NP_957090, Elovl4b-NP_956266, Elovl8a-QBZ96188, and Elovl8b-NP_001191453), *Barbonymus gonionotus* (Elovl5-AXG50646, Elovl2-AZN23179, Elovl4-QEE04393), *Misgurnus anguillicaudatus* (Elovl4a-QFR04622), *Siganus canaliculatus* (Elovl8a-QMU95576 and Elovl8b-QMU95577), and *Tor tambroides* (Elovl5-QJU12162 and Elovl2-QJU12161) through alignment using Clustal Omega. Identical residues are marked with asterisk, whereas strongly and weakly similar residues are indicated with full stops and colons, respectively. The four conserved motifs (KXXEXXDT, QXXFLHXXHH, NXXXHXXMYXYY, and TXXQXX) are illustrated and highlighted with green, whereas the seven putative transmembrane domains are underlined with bold lines. The conserved histidine box (HXXHH) is solid black framed. The putative endoplasmic reticulum (ER) retrieval signal located at the carboxyl terminus are highlighted in yellow

Figure 4

Phylogenetic tree inferred from deduced amino acid sequence of the *P. micromegethes* Elovl5, Elovl2, Elovl4a, and Elovl4b with orthologs from other vertebrate species using maximum likelihood analysis. Node values indicate bootstrap values. The *P. micromegethes* sequences are indicated in bold

Figure 5

Proposed biosynthetic pathways of LC-PUFA (C20-24) and VLC-PUFA (>C24) from linolenic (18:3n-3) and linoleic (18:2n-6) acids in *P. micromegethes* by *in vitro* characterization of Fads2 desaturase ('Dx' coloured in orange) and Elovl elongases ('Elox' coloured in green) capacities using yeast. Elongation reactions of VLC-PUFA catalyzed by Elovl4 enzymes are indicated using blue arrow. 'Sprecher' pathway involved in the biosynthesis of docosahexaenoic acid (DHA 22:6n-3) from 22:5n-3 is highlighted with yellow background

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