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Methyl-end desaturases determine the capability for de novo biosynthesis of polyunsaturated fatty acids in bivalves

Naoki Kabeya [a,*], Marc Ramos-Llorens [b], Yo Nakano [c], André Gomes-dos-Santos [d], Amílcar Teixeira [e], Megumu Fujibayashi [f], Juan G. Haro [b], Juan C. Navarro [b], L. Filipe C. Castro [d,g], Yutaka Haga [a], Óscar Monroig [b]

[a] Department of Marine Biosciences, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato, Tokyo 108-8477, Japan
[b] Instituto de Acuicultura de Torre de la Sal (IATS), CSIC, 12595 Ribera de Cabanes, Castellón, Spain
[c] Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato, Tokyo 108-8477, Japan
[d] CIIMAR/CIMAR – Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, S/N, P 4450-208 Matosinhos, Portugal
[e] CIMO, LA SusTEC, Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[f] Faculty of Engineering, Kyushu University, 774, Motooka, Nishi-Ku, Fukuoka 819-0395, Japan
[g] Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal

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ABSTRACT

Recent studies have shown that many invertebrate species possess methyl-end desaturases (herein referred to as 'ωx'), enabling biosynthesis of polyunsaturated fatty acids (PUFA). However, the phylogenetic distribution of these enzymes across the animal kingdom remains puzzling, possibly due to horizontal gene transfer (HGT) and/or independent large-scale gene loss in certain invertebrate lineages. In molluscs, ωx genes have been identified in various cephalopods and gastropods but remain barely explored in bivalves. The increasing availability of genomic and transcriptomic resources enables a comprehensive exploration of the ωx gene repertoire in bivalves. To elucidate the distribution of ωx in bivalves, we conducted a broad homology search across existing genome and transcriptome assemblies, followed by functional characterisation of ωx in lineage representative species. Our results revealed no ωx-like sequences in any of the 65 Pteriomorphia species, suggesting gene loss in this clade. However, ωx-like sequences were found in Protobranchia, Palaeoheterodonta and Imparidentia. We analysed ωx from *Solemya pusilla* (Protobranchia), *Lanceolaria oxyrhyncha* and *Margaritifera margaritifera* (Palaeoheterodonta), and *Ruditapes philippinarum* and *Tridacna crocea* (Imparidentia). Except for *M. margaritifera*, which had two ωx genes, each species had a single ωx gene. Functional analysis showed Δ15Δ17Δ19 desaturase activity in the *R. philippinarum* and *T. crocea* ωx, while the L. *oxyrhyncha* ωx exhibited Δ15Δ17 activity but not Δ19. Both ωx from *M. margaritifera* showed no detectable activity in yeast. Interestingly, the *S. pusilla* ωx exhibited Δ12 desaturase activity. These findings highlight the diversity of ωx desaturation capabilities in bivalves, with significant gene loss in Pteriomorphia.

1. Introduction

Omega-3 (ω3 or n-3) long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3), are well-documented for their beneficial effects on human health, including the prevention of cardiovascular and coronary heart diseases, and the enhancement of immune responses [1,2]. These compounds are primarily produced by marine microorganisms including bacteria, microalgae and heterotrophic protists, and are subsequently accumulated in higher trophic level organisms such as fish. Climate change and other global events leading to habitat pollution and destruction are expected to reduce the primary production of ω3 LC-PUFA by microbes, ultimately impacting marine ecosystems and the availability of health-promoting nutrients for humans [3–5]. Invertebrates have been recently suggested to contribute to the primary production of ω3 LC-PUFA at a global scale since they possess key enzymatic capacity for the de novo synthesis of polyunsaturated fatty acids (PUFA) and, from them, LC-PUFA including EPA and DHA [6].

* Corresponding author at: Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato, Tokyo, Japan.
E-mail address: naoki.kabeya@kaiyodai.ac.jp (N. Kabeya).

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The biosynthesis of LC-PUFA in animals requires the coordinated action of fatty acyl desaturases and elongation of very long-chain fatty acid (Elovl) proteins as depicted in Fig. 1A. Briefly, a Δ9 desaturase first introduces a double bond into stearic acid (18:0) to produce the monounsaturated fatty acid (MUFA) oleic acid (18:1ω9). The de novo synthesis of PUFA from 18:1ω9 requires the action of methyl-end desaturases (also known as "ωx desaturases"), which introduce double bonds (unsaturations) between a pre-existing one and the methyl group (-CH_3) located at one end of the fatty acids. Methyl-end desaturases convert oleic acid (18:1ω9) into the PUFA linoleic acid (18:2ω6) (Δ12 desaturation), the latter being subsequently converted to α-linolenic acid (18:3ω3) (Δ15 desaturation) (Fig. 1A). Similar to the Δ15 desaturation activity above, other ωx desaturases are also capable of converting ω6 PUFA into corresponding ω3 PUFA products through Δ17 and Δ19 desaturations [6,7]. Additionally to ωx desaturases, further reactions involved in the LC-PUFA biosynthetic pathways are mediated by front-end desaturases, other fatty acyl desaturases like ωx desaturases, which introduce a new double bond between a pre-existing one and the other (-COOH, carboxyl group) end of the fatty acid. Along with desaturases, Elovl enzymes are responsible for the initial condensation reaction in the fatty acyl chain elongation pathway [6,46], and their action results in the extension of the substrate acyl chain by two carbons (Fig. 1A). A key feature distinguishing the LC-PUFA biosynthetic ability between vertebrates and invertebrates is that only the latter possess ωx desaturases [8]. However, with few exceptions [8–10], an accurate delineation of the ωx desaturase gene distribution is still lacking for the vast majority of invertebrate lineages.

The distribution of ωx desaturase genes within the animal kingdom is notably perplexing, partly due to unusual evolutionary events such as horizontal gene transfer (HGT) and large-scale gene loss [8,11]. Indeed, ωx desaturases have been identified exclusively in Cnidaria and several Protostomia phyla, including Nematoda, Arthropoda, Rotifera, Annelida and Mollusca, but are absent in Deuterostomia such as Echinodermata [8]. The pattern of presence or absence of ωx desaturase genes remains puzzling, even within the same phylum. For instance, in Nematoda, ωx desaturase genes (*fat-1* and *fat-2*) appear to be absent in species belonging to Clade I and II according to [12]. Species in Clades III and IV possess only *fat-2*, whereas both *fat-1* and *fat-2* are present solely in Clade V species, including *Caenorhabditis elegans* [9]. Arthropoda also exhibits a sporadic occurrence currently demonstrated in some specific copepod groups [13,14] and the whitefly *Bemisia tabaci* [11]. Within Mollusca, ωx desaturase genes are present in Cephalopoda and Gastropoda [8,31], although their occurrence in other classes remains largely unknown. Bivalves, a group of commercially important molluscs that includes clams, oysters, mussels, and scallops etc., have received little attention regarding their ωx desaturase gene complement, despite the fact that many of these species are important food items and sources of essential nutrients, including LC-PUFA [8,15]. In silico searches of ωx desaturase-like sequences across all currently available genomes and transcriptomes from bivalves revealed their absence in species with high-quality genomic resources such as Pacific oyster *Magallana* (formally known as *Crassostrea*) *gigas*, and presence in the freshwater mussel *Elliptio complanata* [8]. Moreover, a recent study reported that the Chinese razor clam, *Sinonovacula constricta*, possesses two ωx desaturases, albeit no activity was detected [15]. While somewhat fragmentary, these findings suggest that the repertoire of ωx desaturases in Bivalvia, and, consequently, their capacity for de novo biosynthesis of PUFA and ω3 LC-PUFA, is diverse. However, the specific drivers behind this diversity remain unclear.

In addition to biosynthesis, the fatty acid composition of bivalves and hence their nutritional value as sources of physiologically important LC-PUFA for higher trophic level organisms including the human being, is highly influenced by the diet [e.g. [16–18]]. Considering the expected decrease of dietary input of ω3 LC-PUFA linked to global events alluded to above, it is important to gain insight into the capacity for endogenous production of these essential compounds in bivalves to fully understand their potential contribution as ω3 LC-PUFA sources in aquatic ecosystems and as a mariculture product. In the present study, we aimed to

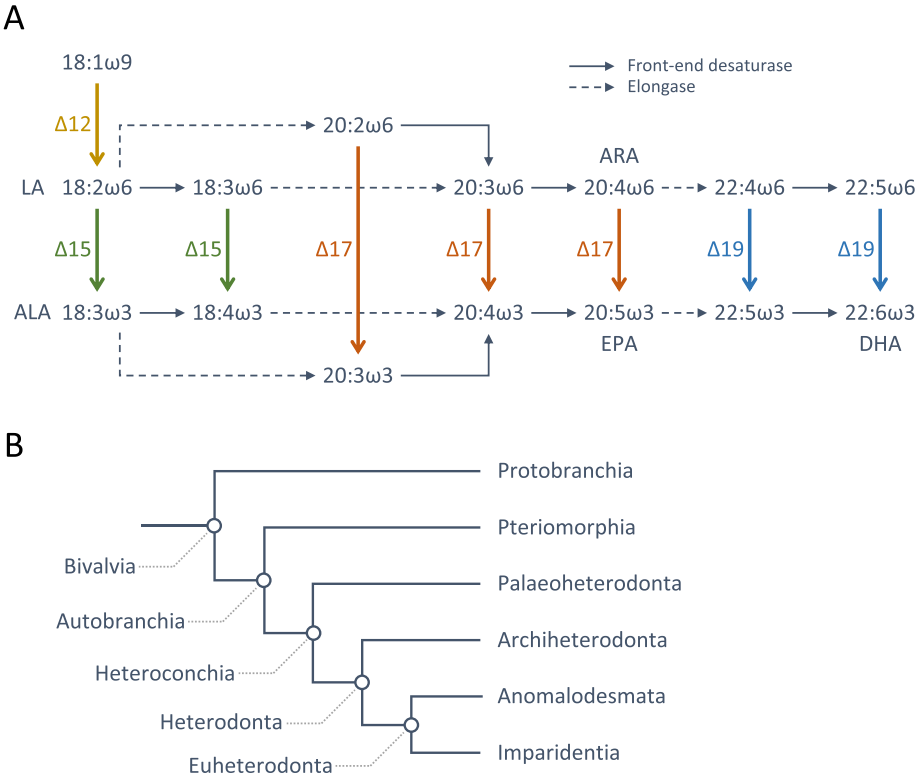

Fig. 1. (A) General biosynthetic pathways for polyunsaturated fatty acids and long-chain polyunsaturated fatty acids. ARA; arachidonic acid, EPA; eicosapentaenoic acid and DHA; docosahexaenoic acid. (B) Phylogenetic relationships of extant bivalves based on Lemer et al. [21].

elucidate the distribution and enzymatic function (regioselectivity) of ωx desaturases among the class Bivalvia. To achieve this goal, we conducted a systematic search for ωx desaturase-like sequences in the existing genomic and transcriptomic assemblies of bivalves. Subsequently, the phylogenetic relationships and copy number variations of the ωx desaturase sequences were analysed across bivalves. Additionally, ωx desaturases isolated from several representative species from major bivalve taxa, namely Protobranchia, Plaeoheterodonta and Imparidentia [19–21], were functionally characterised using a yeast heterologous expression system.

2. Materials and methods

2.1. Search for ωx desaturase-like sequences from bivalve genomes and transcriptomes

In order to clarify the overall distribution of ωx desaturase in bivalves, we conducted a comprehensive retrieval of ωx desaturase-like sequences using homology searches. A deduced amino acid (aa) sequence of an ωx desaturase-like transcript from *Elliptio complanata* (GAHW01017207) [8] was employed as query. Initially, *blastp* and *tblastn* searches were performed against the non-redundant protein sequence (nr) and transcriptome shotgun assembly (TSA) databases, respectively (searches performed in April/2024). Both databases were restricted to Clade 3 animals [8], namely Rotifera (taxid:10190), Lophotrochozoa (taxid:1206795) and Copepoda (taxid:6830). The retrieved ωx desaturase-like sequences were subsequently utilised in the phylogenetic analysis described below. Following this search, we observed that ωx desaturase-like sequences were not retrieved from any Pteriomorphia bivalves, suggesting the absence of this gene in this group. To confirm this observation, we further examined the whole-genome shotgun contigs (WGS) of Pteriomorphia species, verifying the bona fide absence of this gene in Pteriomorphia. The genome and transcriptome datasets of the searched Pteriomorphia species are listed in Supplementary Table 1. In contrast, some species from other bivalve groups, such as Palaeoheterodonta and Imparidentia, appeared to possess more than one ωx desaturase-like sequence. To investigate copy number variation of ωx desaturase in these groups, *tblastn* searches were also conducted against the WGS database and GenBank reference genomes, regardless of whether sequences were annotated or unannotated. The resulting copy number variations are summarised in Supplementary Table 2.

2.2. Phylogenetic analysis

The full-length open reading frame (ORF) of the ωx desaturase-like sequences was annotated within the TSA sequences described above, using the "Find Open Reading Frames" tool in CLC Main Workbench 23 (Qiagen, Aarhus, Denmark). Following the translation of the ORF into deduced aa sequences, only those containing three histidine boxes (H-X-X-X-H, H-X-X-H-H, and H-X-X-H-H), which are typically conserved within ωx desaturases [8], were selected for the phylogenetic analysis. A multiple sequence alignment (MSA) was subsequently generated using the aa sequences deduced from ωx desaturases retrieved from the nr database and from the TSA sequences mentioned above, along with the deduced aa sequences from representative species, namely Protobranchia (*Solemya pusilla*), Palaeoheterodonta (*Lanceolaria oxyrhyncha* and *Margaritifera margaritifera*) and Imparidentia (*Ruditapes philippinarum* and *Tridacna crocea*) (see below). The alignment was performed using MAFFT v7.511 with *E*-INS-i criteria [22]. The obtained MSA was then cleaned by TrimAl v1.4.rev15 to remove columns containing gaps in >95 % of the sequences [23]. Subsequently, the best-fit aa substitution model was estimated as LG + I + G4 by ModelTest-NG v0.1.6. [24] and then the maximum likelihood phylogenetic inference was carried out by RAxML-NG v.1.2.0 with *–all* option and automatic bootstrapping (autoMRE, cutoff = 0.03) [25]. The resulting tree was visualised by Interactive Tree of Life (iTOL) v6 [26].

2.3. RNA extraction and cDNA synthesis from representative bivalve species

Based on the phylogenetics and ωx desaturase gene distribution, we carried out functional characterisation assays in a selection of ωx desaturase-like sequences from representative species within those three taxonomic groups, namely *S. pusilla* from Protobranchia, *L. oxyrhyncha* and *M. margaritifera* from Palaeoheterodonta, and *R. philippinarum* and *T. crocea* from Imparidentia. *R. philippinarum* was purchased from a local fish market (Tokyo, Japan) and cultured *T. crocea* was purchased from an aquarium shop (Charm Co., Ltd., Gunma, Japan). *L. oxyrhyncha*, *M. margaritifera* and *S. pusilla* were collected from Biwa Lake (Shiga, Japan), Tuela River (Bragança, Portugal) and Shimoda Marine Research Center, University of Tsukuba (Shizuoka, Japan), respectively. All samples, except for *M. margaritifera*, were processed using a freeze crusher (μT-48, TAITEC Corporation, Saitama, Japan) following shucking. Total RNA was extracted from approximately 100 mg of freeze-crushed tissue using the RNeasy Plus Universal Mini Kit (QIAGEN Corporation). The extracted RNA was subsequently reverse transcribed into cDNA using SuperScript™ IV Reverse Transcriptase (Thermo Fisher Scientific) with both random primers and an oligo(dT) primer in a 3:1 (mol) ratio, following the manufacturer's instructions. For *M. margaritifera*, 50 mg of hepatopancreas and mantle tissues preserved in RNAlater were homogenised using the FastPrep-24™ 5G (MP Biomedicals, USA). Total RNA was then extracted from the hepatopancreas and mantle tissues using RNeasy Plus Universal Mini Kit (Qiagen Corporation) following the manufacturer's instructions. Subsequently, 1 μg of total RNA from a 1:1 mixture of hepatopancreas and mantle total RNA extracts was reverse transcribed into cDNA using Moloney Murine Leukaemia Virus Reverse Transcriptase (Promega, USA) with random primers and oligo(dT) primers in a 3:1 (mol) ratio (Promega, USA), following the manufacturer's instructions.

2.4. Isolation of bivalve ωx desaturase sequences for functional characterisation in yeast

Full-length ORF sequences of the bivalve ωx desaturases were isolated following different strategies. For L. *oxyrhyncha* and *S. pusilla*, species lacking existing genomic or transcriptomic data, RNA-seq analyses followed by de novo transcriptome assembly (GIKEN bio Ltd.) were carried out to identify the sequences of interest, which enabled primer design for subsequent polymerase chain reaction (PCR) (Supplementary Table 3). RNA-seq for *S. pusilla* and L. *oxyrhyncha* generated 45,904,354 and 17,497,454 paired-end reads, respectively, with 92.2 % and 92.5 % of sequences achieving a Q30 score or higher. Transcriptome assembly yielded 316,536 and 304,252 sequences for *S. pusilla* and *L. oxyrhyncha*, respectively. For *M. margaritifera*, the genomic and transcriptomic assemblies, along with the raw RNA-seq data [27,28], were initially examined to identify ωx desaturase-like sequences. Briefly, we verified the presence of two copies of ωx desaturase-like sequences, within the latest version of the annotated genome (Gene Ids: MarG00000025895 and MarG0000002589 in the original annotations doi:https://doi.org/10.6084/m9.figshare.22048250.v2). Subsequently, using raw RNA-seq data available for the species (from kidney and gill), partial transcripts were obtained by blast search (blast-n v.2.11.0), suggesting expression of both genes. Partial amplification of these genes from cDNA via PCR was also explored. However, despite repeated efforts, the full-length ORF of the two *M. margaritifera* ωx desaturase-like sequences could not be amplified by PCR. As a result, the full-length ORF were synthesised as synthetic genes by Genscript Biotech (Leiden, The Netherlands). PCR primers for the *R. philippinarum* and *T. crocea* ωx desaturases were designed based on the sequences retrieved from the existing reference genome assemblies GCA_026571515.2 and GCA_943736015.1, respectively. Each primer contained the restriction

enzyme recognition sites required for the ligation of each ωx desaturase ORF sequence into the yeast expression vector (pYES2, Thermo Fisher Scientific) (Supplementary Table 3). PCR were performed with PrimeSTAR Max DNA Polymerase (TaKaRa Bio Inc.) following the manufacturer's instructions, using cDNA as a template. The resulting amplicons were purified (NucleoSpin® Gel and PCR Clean-up, TaKaRa Bio Inc.), digested with the corresponding restriction enzymes (TaKaRa Bio Inc.), and then ligated into similarly digested pYES2 with T4 DNA ligase (Promega). For *M. margaritifera* ωx desaturases, the synthesised full-length ORF sequences included the necessary restriction enzyme recognition sites for ligation into pYES2 (Supplementary Table 3). The synthetic genes were digested with the appropriate restriction enzymes (New England Biolabs, Ipswich, MA, USA) and ligated into pYES2 as described above. Subsequently, an aliquot of the ligation reaction was transformed into *Escherichia coli* competent cells (ECOS Competent *E. coli* DH5α, Nippon Gene), except for the *M. margaritifera* gene which was transformed into *E. coli* Top10 competent cells (Thermo Fisher Scientific). Plasmids were extracted from positive transformants using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich) and sequenced to confirm the ORF sequence. The resulting plasmid constructs pYES2-Spωx, pYES2-Loωx, pYES2-Mmωx1, pYES2-Mmωx2, pYES2-Rpωx and pYES2-Tcωx were transformed into competent *S. cerevisiae* INv*Sc*1 cells (Thermo Fisher Scientific) using the *S.c.* EasyComp® Transformation kit (Thermo Fisher Scientific).

2.5. Functional characterisation of bivalve ωx desaturases

The yeast transformation with the above pYES2 constructs and subsequent culture were carried out following the method described previously [29]. Since previous studies have demonstrated that ωx desaturases have diverse substrate specificities enabling them to desaturate both MUFA like 18:1ω9 and PUFA substrates, we conducted two different assays as previously described in [8]. In order to determine the ωx desaturase activity towards MUFA, the transgenic yeast expressing each ωx desaturase were grown ($n = 3$) in the absence of exogenously added substrates since the yeast *S. cerevisiae* contain prominent peaks of MUFA including 18:1ω9. Their fatty acid profiles were then compared to those of control yeast transformed with empty pYES2 (n = 3). In order to determine the ωx desaturase activity towards PUFA substrates, 18:2ω6, 18:3ω6, 20:2ω6, 20:3ω6, 20:3ω6, 22:4ω6 and 22:5ω6 were added to the culture medium at 0.5 mM (C_{18}), 0.75 mM (C_{20}) and 1.0 mM (C_{22}). After 48 h incubation, yeast were harvested, and their total lipids were extracted for further preparation of fatty acid methyl esters (FAME) following the method described previously [14].

2.6. Fatty acid analysis by gas chromatography

A gas-chromatograph (Nexis GC-2030, Shimadzu Corporation) equipped with flame ionisation detector (FID) and capillary column (FAMEWAX, 30 m × 0.25 mm, i.d. × 0.25 μm, Restek) was used to analyse the fatty acid composition of yeast. Hydrogen was used as the carrier gas and the temperature conditions were 250 °C for sample injection and 280 °C for sample detection. The initial column temperature, 50 °C for 1 min, was raised to 190 °C at a rate of 40 °C/min, and then from 190 °C to 240 °C at a rate of 4 °C/min, followed by a final holding at 240 °C for 3 min. Qualitative determination of fatty acids was based on retention times obtained from commercial FAME standards (Qualmix fish S, Larodan AB, Solna, Sweden and Supelco 37 Component FAME Mix, Merck KGaA, Darmstadt, Germany). From the results, the conversion of each desaturase enzyme towards the exogenously supplied PUFA was calculated according to the following formula: [product area/(product area + substrate area)] × 100.

2.7. Statistical analysis

The assays aiming to determine the ability of ωx desaturases from representative bivalves to desaturate the yeast endogenous MUFA were conducted in triplicate. After confirming no statistically significant difference in variances using the F-test, Student's *t*-test ($P \leq 0.05$ indicating significance) was employed to compare the fatty acid profiles of control yeast and yeast expressing the corresponding bivalve ωx desaturase. Fatty acid contents were expressed as mean percentages of total fatty acids ± standard error of the mean (s.e.m.). All statistical analyses were performed using Microsoft Excel for Mac (version 16.92).

3. Results

3.1. Distribution of ωx desaturase in bivalves

The *tblastn* search of the nr database and TSA database, including TSA retrieved from 84 bivalve species and the hybrid *Magallana gigas* x *M. sikamea*, resulted in the identification of 20 full-ORF of ωx desaturase-like sequences from 18 bivalve species. These included one from Protobranchia (*Solemya velum*), four from Palaeoheterodonta and 13 from Imparidentia (Supplementary Table 4). Additionally, the full-length ORF of ωx desaturase sequences were obtained from two ad hoc transcriptome assemblies prepared in the present study (*S. pusilla* and L. *oxyrhyncha*), as well as from publicly available databases at NCBI. These sequences were isolated by PCR from all the representative species except *M. margaritifera*, where two ωx desaturase-like sequences were obtained as synthetic genes. In total, 25 ωx desaturase-like sequences from 22 bivalve species were used for the subsequent phylogenetic analysis described below. The lengths of full-length ORF sequences and the deduced aa sequences for all bivalve species are provided in Supplementary Table 4. All deduced aa sequences exhibited three conserved histidine boxes, H-D-C-G/C-H, H-K/R-H/L/N-H-H and H-Q-I/V-H-H (Supplementary Table 4), a characteristic pattern of Lophotrochozoa ωx desaturases [8]. Although mRNA expression could not be confirmed, a search of existing WGS databases from Palaeoheterodonta and Imparidentia species demonstrated that most Imparidentia species possessed a single copy of the ωx desaturase-like sequence in their genome, whereas the copy number appeared to increase in Palaeoheterodonta species (Order Unionida) (Supplementary Table 2). As mentioned above, no ωx desaturase-like sequences were identified in species belonging to Pteriomorphia, despite an extensive search against both TSA and WGS databases covering 65 species from six orders (Arcoida, Limoida, Mytilida, Ostreida, Pectinida and Pterioida) and the hybrid *Magallana gigas* x *M. sikamea* (Supplementary Table 1).

3.2. Maximum likelihood phylogenetic analysis

The maximum likelihood phylogenetic analysis (ML) demonstrated that Bivalvia ωx desaturases did not form a single monophyletic clade, but were divided into two major clades (Fig. 2). However, the sister groups of these clades could not be determined due to the low resolution of the tree topology. One clade contained ωx desaturases from two Protobranchia species, namely *S. pusilla* and *S. velum* with high bootstrap support (100 %), whilst the other clade contained the remaining sequences from Palaeoheterodonta and Imparidentia species. However, the bootstrap value (63 %) did not sufficiently support the monophyly of this clade. Within the latter clade, all seven ωx desaturases from six Palaeoheterodonta species clustered together with high bootstrap support (100 %) (Fig. 2 and Supplementary Fig. 1). Interestingly, although all remaining ωx desaturases were isolated from the superorder Imparidentia, they did not form a single monophyletic clade (Fig. 2). In particular, ωx desaturases from four species belonging to the order Lucinida, namely *Phacoides pectinatus* (GGWH01118026), *Loripes orbiculatus* (GJRD01018908), *Thyasira* sp. (GKMQ01001931 and GKMQ01007741) and *Thyasira tokunagai* (GKOU01014166), were divided into two clades (Fig. 2). The ωx desaturases from Neoheterodontei clustered together with relatively high bootstrap support (88 %), and each of the orders within Neoheterodontei, namely Myida

and Venerida, formed distinct monophyletic clades with 84 % and 100 % bootstrap values, respectively (Fig. 2 and Supplementary Fig. 1).

3.3. Functions of the bivalve ωx desaturases

In order to determine the activity of bivalve ωx desaturases towards MUFA, we compared the fatty acid profiles of transgenic yeast expressing each ωx desaturase with those of control yeast transformed with the empty pYES2 vector. Control yeast displayed the typical yeast endogenous fatty acids, namely 16:0, 16:1ω7, 18:0 and 18:1ω9. However, an additional peak corresponding to 18:2ω6 was detected in the yeast expressing the *S. pusilla* ωx desaturase, indicating that this enzyme possesses Δ12 desaturase activity (Table 1, Fig. 3). No other ωx desaturases assayed in the present study exhibited Δ12 desaturase activity

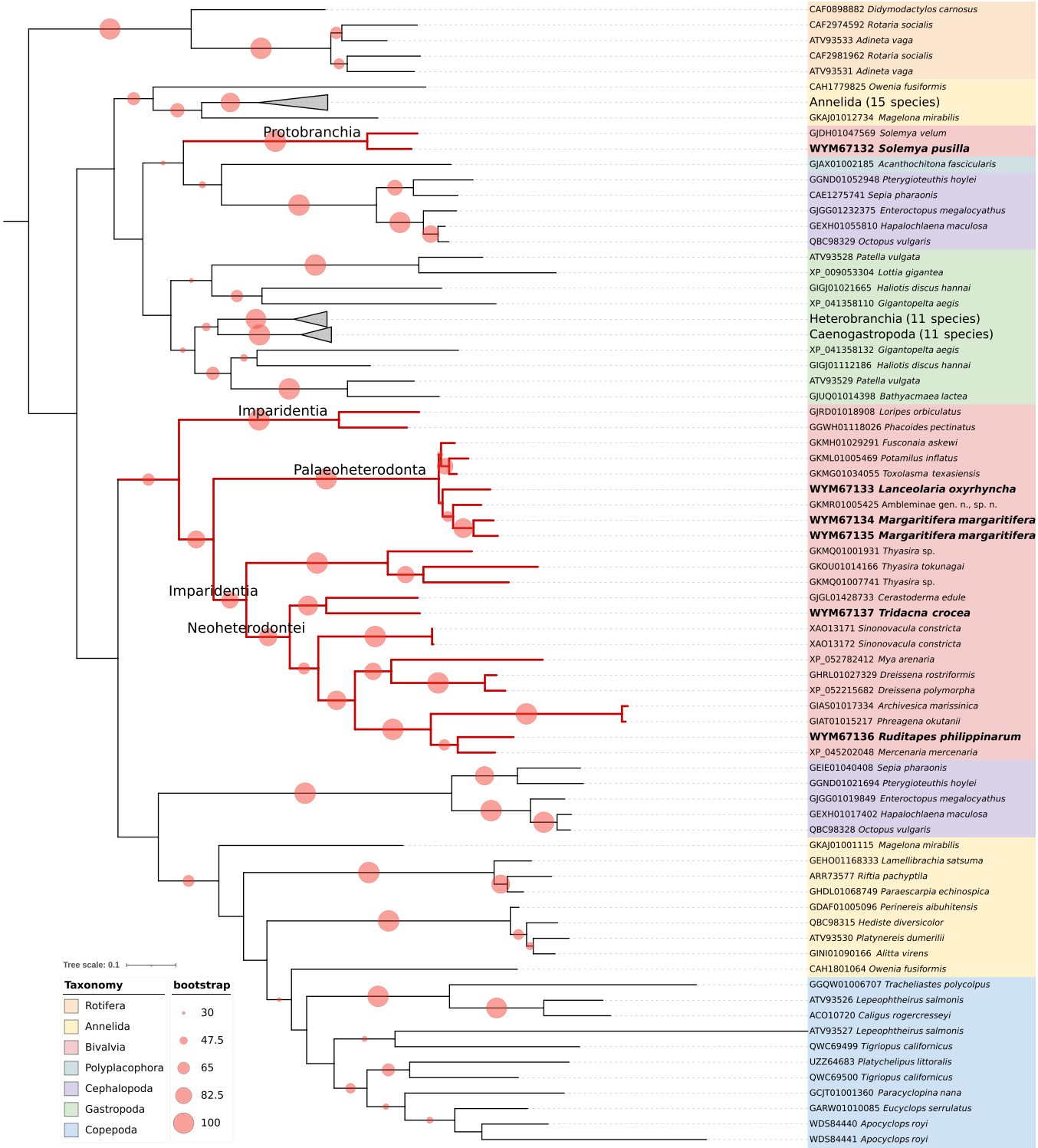

Fig. 2. Maximum likelihood phylogenetic tree of ωx desaturase amino acid sequences isolated from Clade 3 animals [8]. The branches representing bivalve sequences are highlighted in red. NCBI accession numbers are given before the scientific names at each leaf. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparison of fatty acid profiles from the transgenic yeast expressing *S. pusilla* ωx desaturase with control yeast transformed with the empty pYES2 vector. The results are presented as area percentage of total fatty acids in each sample (mean ± s.e.m., $n = 3$). n.d., not detected. Asterisks (*) indicate statistically significant differences (Student's *t*-test, $P \leq 0.05$).

	Control	*S. pusilla*
18:0	7.8 ± 0.2	8.2 ± 0.1
18:1ω9	25.1 ± 0.2	23.7 ± 0.1*
18:2ω6	n.d	2.3 ± 0.0
18:3ω3	n.d	n.d

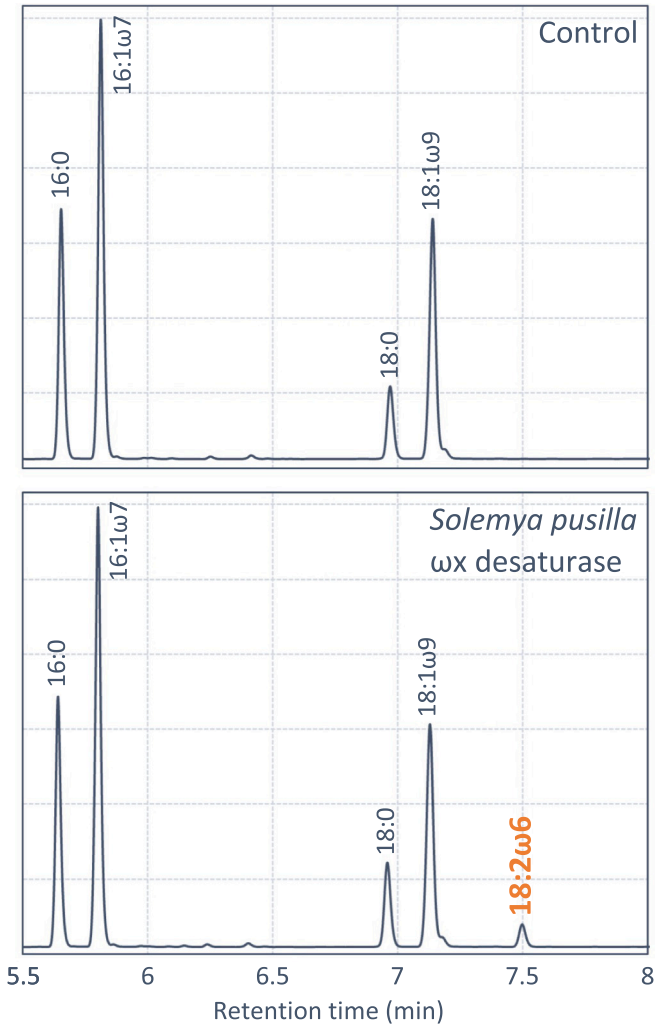

Fig. 3. Δ12 desaturase activity detected in an ωx desaturase isolated from *Solemya pusilla*. The upper panel shows a GC chromatogram of FAME prepared from control yeast transformed with an empty pYES2 vector. The lower panel shows a GC chromatogram of FAME prepared from transgenic yeast expressing the *S. pusilla* ωx desaturase. The yeast endogenous fatty acids, 16:0, 16:1ω7, 18:0 and 18:1ω9, are indicated.

(data not shown). We further determined the capacity of the bivalve ωx desaturases to convert ω6 PUFA into ω3 PUFA products (Table 2). The transgenic yeast expressing the ωx desaturases from L. *oxyrhyncha*, *R. philippinarum* and *T. crocea*, showed additional peaks corresponding to ω3 PUFA desaturation products when supplemented with a variety of ω6 PUFA substrates (Table 2, Fig. 4). Interestingly, unlike the ωx desaturases from *R. philippinarum* and *T. crocea*, which exhibited Δ15Δ17Δ19 desaturase activities, only Δ15 and Δ17 desaturase activities but not Δ19, were detected in the ωx desaturase from L. *oxyrhyncha* (Table 2). Additionally, the two ωx desaturases from *M. margaritifera* and the ωx desaturases from *S. pusilla* showed no detectable activity towards any of the tested ω6 substrates or the yeast endogenous fatty acids (Table 2).

4. Discussion

Our comprehensive examination of ωx desaturase-like sequences within Bivalvia revealed that this gene type is most likely absent in one of the major groups of bivalves, Pteriomorphia, which includes oysters, scallops and mussels (Supplementary Table 2). Pteriomorphia is believed to have diverged from the common ancestor of all Autobranchia species, encompassing nearly all extant bivalves, with the exception of Protobranchia, which diverged earlier [19–21]. Given the presence of ωx desaturases in Protobranchia and several recent-diverging bivalve groups, such as Palaeoheterodonta and Imparidentia, it is likely that this gene was lost in the common ancestor of all Pteriomorphia species. In the present study, we were unable to confirm the presence of full-length ωx desaturase-like sequences in two major bivalve groups, namely Archiheterodonta and Anomalodesmata, due to the lack of available genomic or transcriptomic assemblies in the NCBI database. Nevertheless, it remains plausible that species within these groups also possess ωx desaturases, given that they belong to Heteroconchia, where Palaeoheterodonta and Imparidentia represent the earliest and most recently diverging groups, respectively [19–21].

The results from the present study revealed that many bivalve species possess one single ωx desaturase gene, as confirmed through *blast* searches of both annotated and unannotated bivalve genome assemblies, particularly among Imparidentia species (Supplementary Table 3). Notably, two or even three ωx desaturase genes were detected in five out of eight Palaeoheterodonta species, in contrast to only three out of 48 Imparidentia species (Supplementary Table 3). Gene duplication is generally associated with the functional diversification and novelty, following neofunctionalisation or subfunctionalisation [30]. Indeed, duplicated ωx desaturases isolated from several animal lineages (e.g., corals, cephalopods, gastropods, annelids, copepods) tend to exhibit complementary desaturase activities, facilitating the completion of various ωx desaturation pathways within canonical PUFA and LC-PUFA biosynthetic pathways (Fig. 1A). For instance, one ωx desaturase has Δ12 desaturase activity, while the other exhibits ω3 desaturase activity, which refers to ωx desaturases that convert multiple ω6 PUFA substrates into the corresponding ω3 PUFA products through Δ15, Δ17 and Δ19 desaturase activities [8,14,31,32]. It is, therefore, intriguing that both ωx desaturases from *M. margaritifera* showed no detectable activity in the yeast heterologous expression system used in the present study. Nevertheless, sequence analysis did not reveal any abnormalities in their conserved histidine box motifs. In fact, both ωx desaturases exhibited typical histidine box sequences, as observed in other bivalve ωx desaturases (Supplementary Table 4), and their phylogenetic positions were consistent with their taxonomic identities, showing no evidence of unusual branch lengths (Fig. 2). Although there remains the possibility that these enzymes possess desaturase activity towards other fatty acids that were not tested here, similar results were also reported in a recent study on *S. constricta*, where two paralogous ωx desaturases also showed no detectable activity using the same yeast expression system, despite the evolutionary distance between *S. constricta* and *M. margaritifera* [15]. Furthermore, a high ratio of n-3 to n-6 PUFA in the diet is preferable for *M. margaritifera* growth, consistent with its inability to alter the n-3/n-6 ratio [33]. In contrast, an ωx desaturase isolated from L. *oxyrincha*, which is closely related to *M. margaritifera*, showed ω3 desaturase activity towards several C_{18} and C_{20} ω6 PUFA substrates (Δ15 and Δ17 desaturations, respectively). However, it remains unclear whether L. *oxyrincha* possesses one single ωx desaturase gene or multiple, due to the lack of available genomic resources. Consequently, further research is required to elucidate the relationship between ωx desaturase gene

Table 2
Substrate conversions of the transgenic yeast expressing the ωx desaturase isolated from bivalves. The results are presented as the percentage of the fatty acid substrate converted into the corresponding desaturated product.

		Conversion (%)					
Substrate	Product	*S.pusilla*	*L. oxyrhyncha*	*M. margaritifera*	*R. philippinarum*	*T. crocea*	Activity
18:2ω6	18:3ω3	n.d.	9.5	n.d./n.d.	4.5	15.9	Δ15
18:3ω6	18:4ω3	n.d.	2.8	n.d./n.d.	3.6	13.0	Δ15
20:2ω6	20:3ω3	n.d.	3.1	n.d./n.d.	9.7	33.6	Δ17
20:3ω6	20:4ω3	n.d.	2.7	n.d./n.d.	5.6	29.3	Δ17
20:4ω6	20:5ω3	n.d.	4.9	n.d./n.d.	8.9	34.2	Δ17
22:4ω6	22:5ω3	n.d.	n.d.	n.d./n.d.	2.4	15.9	Δ19
22:5ω6	22:6ω3	n.d.	n.d.	n.d./n.d.	1.3	7.7	Δ19

duplication and functional diversification within Palaeoheterodonta species.

The ωx desaturases identified in Protobranchia are distantly related to those found in other bivalves, although the sequences analysed in the present study are limited to two species within the same genus (*S. velum* and *S. pusilla*). This finding corresponds with the observation that the regioselectivity of the *S. pusilla* ωx desaturase, which exhibits Δ12 activity, differs from that of the ωx desaturases identified in the other three bivalves examined. However, due to the lack of available genomic resources for Protobranchia species, it remains uncertain whether *S. pusilla* harbours additional ωx desaturase genes. Species within Protobranchia, such as *Solemya* spp., are notable for their highly reduced or entirely absent digestive systems, relying predominantly on symbiotic bacteria located within their gills for nutrition [47]. Consequently, they ingest minimal quantities of food directly [34]. Interestingly, arachidonic acid (ARA, 20:4ω6), a physiologically relevant ω6 LC-PUFA, is the most abundant LC-PUFA in *S. velum*, whereas EPA and DHA, typically prominent LC-PUFA in marine bivalves, are present at significantly lower levels [35]. Given that bacterial symbionts appear not to provide any PUFA to the host [35], the ARA detected in *S. velum* is highly likely to be synthesised endogenously. Although further studies are needed to elucidate the complete PUFA biosynthetic pathway in *Solemya* species, it is plausible that the ωx desaturase with Δ12 desaturase activity plays a crucial role in the production of ARA, as its initial precursor is 18:2ω6, a product of Δ12 desaturation from 18:1ω9 (Fig. 1A).

The ωx desaturases from the two Imparidentia species, *R. philippinarum* and *T. crocea*, were capable of Δ15, Δ17 and Δ19 desaturation activities, demonstrating their function as ω3 desaturases that enable the biosynthesis of multiple ω3 PUFA from ω6 PUFA substrates (Fig. 1A). Since their genomic assemblies do not reveal additional ωx desaturase-like sequences, these appear to be the sole ωx desaturases in both species. Given the commercial importance of *R. philippinarum*, several studies have demonstrated its ability to convert dietary C_{18} or C_{20} PUFA into longer, more unsaturated PUFA when fed on LC-PUFA-free or C_{22} PUFA-free diets [36–38]. Environmental stresses, such as cold temperatures, also appear to stimulate endogenous LC-PUFA biosynthesis [39]. However, none of these feeding trial studies has conclusively demonstrated the significance of the desaturation pathways catalysed by ω3 desaturases capable of Δ15, Δ17 and Δ19 desaturations, most likely due to the near impossibility of preparing a diet containing exclusively ω6 PUFA as the sole PUFA component. Moreover, in *T. crocea* and other *Tridacna* species, symbionts like *Symbiodinium* spp. and *Cladocopium* spp. (from the family Symbiodiniaceae) seem to provide specific lipids and fatty acids to the host [40–42], adding further complexity to understanding endogenous PUFA biosynthesis in these species. To fully elucidate the biological significance of the ω6 to ω3 PUFA pathway in these species, future studies may benefit from "in vivo" tracing experiments using stable isotope- or radioisotope-labelled fatty acids, as well as artificial manipulation of ωx desaturase gene expression through transgenesis or gene knockout/knockdown techniques [e.g. [43–45]].

In conclusion, the present study highlights the diversification of enzymatic capabilities in the ωx desaturation steps within the PUFA and LC-PUFA biosynthetic pathways across Bivalvia, driven by large-scale gene loss in Pteriomorphia and functional diversification of ωx desaturase in several other lineages. De novo ω6 PUFA biosynthesis was demonstrated in the Protobranchia species *S. pusilla*, whose ωx desaturase capable of Δ12 desaturation of the MUFA precursor 18:1ω9. Among the two Palaeoheterodonta species studied, the L. *oxyrhyncha* ωx desaturase is an ω3 desaturase capable of Δ15 and Δ17 desaturations towards multiple C_{18} and C_{20} ω6 PUFA substrates, respectively. Intriguingly the two ωx desaturases identified in *M. margaritifera* did not show any detectable activity towards any of the substrates tested. In contrast, both *R. philippinarum* and *T. crocea* possess ω3 desaturases with Δ15, Δ17, and Δ19 desaturation capabilities, enabling the biosynthesis of a broad range of ω3 PUFA from corresponding ω6 PUFA substrates. These functional differences may reflect species-specific ecological contexts, such as variation in trophic levels, symbiotic associations, or the environmental availability of substrate fatty acids, although further studies are required to confirm such associations. Nonetheless, the absence of ωx desaturase capable of Δ12 desaturation in these species precludes them from synthesising 18:2ω6 from 18:1ω9, which enables the de novo biosynthesis of ω6 PUFA. This comparative analysis of ωx desaturases across bivalve lineages uncovers lineage-specific gene loss, copy number variation and functional diversification, deepening our understanding of the evolutionary dynamics of PUFA biosynthesis in bivalves. These findings provide valuable insight into the role of bivalves as producers of physiologically important PUFA within aquatic ecosystems, and underscore their potential as a source of PUFA for human consumption.

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CRediT authorship contribution statement

Naoki Kabeya: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Marc Ramos-Llorens:** Writing – review & editing, Investigation. **Yo Nakano:** Writing – review & editing, Resources, Investigation. **André Gomes-dos-Santos:** Writing – review & editing, Resources, Investigation. **Amílcar Teixeira:** Writing – review & editing, Resources. **Megumu Fujibayashi:** Writing – review & editing, Resources, Funding acquisition. **Juan G. Haro:** Writing – review & editing, Investigation. **Juan C. Navarro:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition. **L. Filipe C. Castro:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Yutaka Haga:** Writing – review & editing, Supervision, Resources. **Óscar Monroig:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Ethical approval

No ethical evaluation was required to accomplish the goals of this

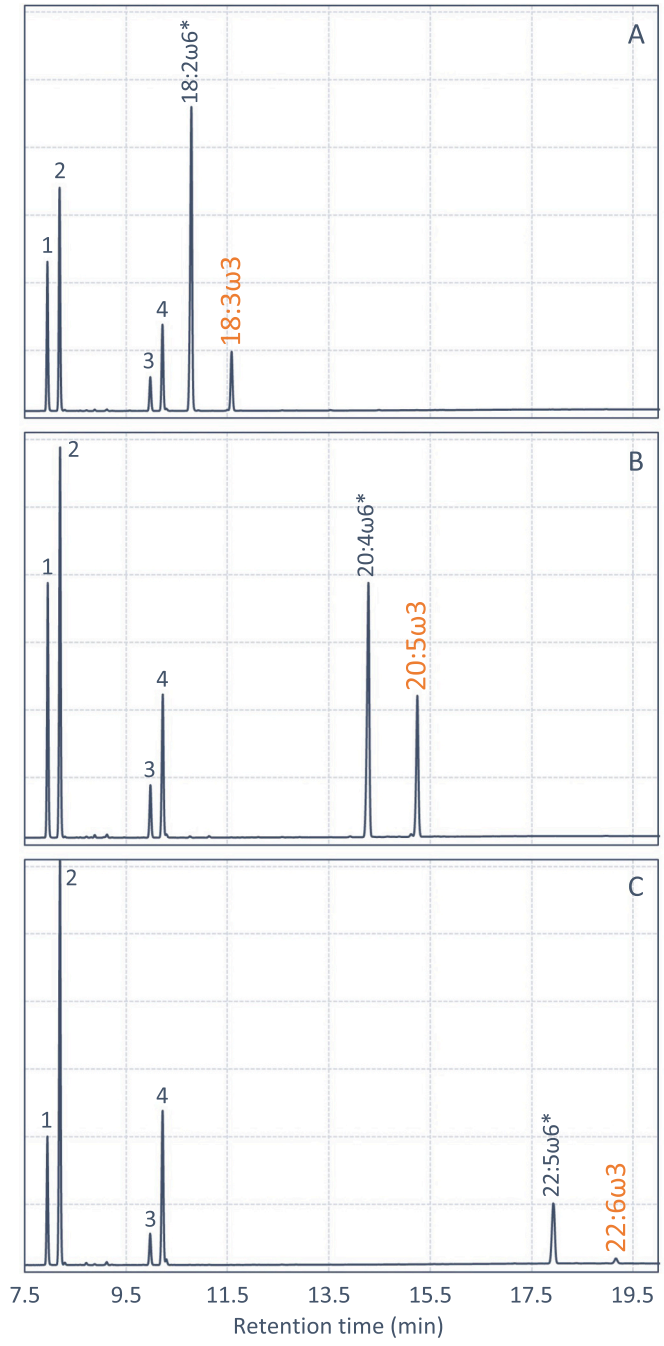

Fig. 4. Δ15Δ17Δ19 desaturase activities towards multiple ω6 PUFA substrates detected in an ωx desaturase isolated from *Tridacna crocea*. GC chromatograms of FAME prepared from transgenic yeast expressing the *T. crocea* ωx desaturase grown in the presence of 18:2ω6 (A), 20:4ω6 (B) and 22:5ω6 (C), as indicated by an asterisk (*). Resulting desaturation products are indicated in orange. Peaks 1-4 represent the yeast endogenous fatty acids, namely 16:0, 16:1ω7, 18:0 and 18:1ω9, respectively.

study because experimental work was conducted with an unregulated invertebrate species except *M. margaritifera*. Nevertheless, all experiments conformed to the guidelines for the care and use of laboratory animals of the Tokyo University of Marine Science and Technology. Regarding *M. margaritifera*, the described work was approved by the CIIMAR Ethical Committee and CIIMAR Managing Animal Welfare Body (ORBEA), according to the European Union Directive 2010/63/EU. This study complies with the International Union for Conservation of Nature (IUCN), the Convention on Biological Diversity, and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. Permits for fieldwork and collection of samples were acquired from the local coauthor Amílcar Teixeira.

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Declaration of competing interest

The authors declare there is no competing interests.

Data availability

All isolated ωx desaturase sequences in the present study were registered into the NCBI GenBank with the accession numbers PP552780 to PP552785. The RNA-seq data for *Solemya pusilla* and *Lanceolaria oxyrhyncha* have been deposited under the NCBI BioProject PRJNA1213110.

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