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1 **Title**

2 Biosynthesis of long-chain polyunsaturated fatty acids in the African catfish *Clarias*
3 *gariiepinus*: Molecular cloning and functional characterisation of fatty acyl desaturase (*fads2*)
4 and elongase (*elovl2*) cDNAs

5

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20

21 **Abstract**

22 Fish differ in their capacity for endogenous synthesis of long-chain (C₂₀₋₂₄) polyunsaturated
23 fatty acids (LC-PUFA) from dietary C₁₈ precursors (α -linolenic and linoleic acids).
24 Understanding this capacity is of benefit to fish feed formulation. This, together with the
25 importance of fish as the primary source of omega-3 LC-PUFA in the human diet has
26 necessitated the rigorous study of the biochemical and molecular mechanisms involved in the
27 LC-PUFA biosynthesis pathway in fish species. Studies have shown the potential of a species
28 for LC-PUFA biosynthesis is associated with the complement and function of fatty acyl
29 desaturase (*fads*) and elongase of very long chain fatty acid (*elovl*) gene it possesses. The
30 present study therefore aimed to investigate these genes in the African catfish (*Clarias*
31 *garipepinus*), the most commercially important farmed fish species in Sub-Saharan Africa. A
32 *fads2* and an *elovl2* cDNA were cloned containing open reading frames (ORF) of 1338 base
33 pair (bp) and 864 bp specifying proteins of 445 and 287 amino acids, respectively. Functional
34 characterisation by heterologous expression in yeast showed that the Fads2 was bifunctional
35 with $\Delta 5\Delta 6$ activities catalysing the desaturation of both 18:3n-3 and 20:4n-3 and their
36 corresponding n-6 fatty acids, 18:2n-6 and 20:3n-6. The Elov12 showed activity towards C₁₈,
37 C₂₀ and C₂₂ PUFA with highest activity towards C₂₀ and C₂₂ PUFA. Tissue expression
38 analysis showed a typical freshwater species expression pattern; higher expression in the liver
39 compared to brain and all other tissues with the exception of *elovl5* which showed highest
40 expression in the intestine. Consistent with feeding studies of typical freshwater fish species
41 that show their essential fatty acid requirement can be satisfied by dietary C₁₈ PUFA, the
42 present study confirms that the LC-PUFA biosynthesis pathway is active in the African
43 catfish *C. garipepinus*.

44

45

46 **Keywords**

47 Biosynthesis; *Clarias gariepinus*; elongase; essential fatty acids; desaturase; long-chain

48 polyunsaturated fatty acids

49

50 **Introduction**

51 Fish, like all vertebrates, are dependent on dietary sources of polyunsaturated fatty acids
52 (PUFA) such as α -linolenic (ALA, 18:3n-3) and linoleic (LA, 18:2n-6) acids as they lack the
53 Δ 12 and Δ 15 desaturases required for the synthesis of LA and ALA from oleic acid (18:1n-9)
54 (Tocher, 2010, 2015; Tocher and Glencross, 2015). However, whereas the C₁₈ PUFA, ALA
55 and LA, can satisfy essential fatty acid (EFA) requirements of some fish species, long-chain
56 (C₂₀₋₂₄) polyunsaturated fatty acids (LC-PUFA) including eicosapentaenoic acid (EPA, 20:5n-
57 3), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6), which play
58 physiologically important roles, are required in the diet to meet the EFA requirements of
59 other species. This reflects the differing ability of fish species to endogenously synthesise
60 LC-PUFA from C₁₈ precursors, associated with the complement of fatty acyl desaturases
61 (Fads) and elongation of very long-chain fatty acids (Elovl) enzymes they possess (Bell and
62 Tocher, 2009; Castro et al., 2016; Tocher, 2010). This has important implications with
63 regards to feed formulation for fish farming. Species with active and complete biosynthetic
64 pathways can convert C₁₈ PUFA contained in vegetable oils (VO) that are now common
65 ingredients in aquafeeds, to LC-PUFA, and thus are less dependent on the inclusion of fish
66 oil (FO) to supply LC-PUFA in their diets.

67 The LC-PUFA biosynthesis pathways involves successive desaturation and elongation of
68 the C₁₈ precursors catalysed by Fads and Elovl elongases (Castro et al., 2016; Monroig et al.,
69 2011a; Tocher, 2003; Vagner and Santigosa, 2011). Fads enzymes introduce double bonds
70 (unsaturations) at specific positions of the fatty acyl chain (Nakamura and Nara, 2004). It has
71 been shown that all *fads* so far studied in teleost genomes are paralogues of *fads2*, a gene
72 encoding an enzyme that typically acts as Δ 6 Fads in vertebrates, while *fads1*, another
73 vertebrate *fads* encoding an enzyme with Δ 5 activity, appears to be absent in teleosts (Castro
74 et al., 2012, 2016). While most fish Fads2 enzymes functionally characterised are typically

75 $\Delta 6$, others have been characterised as bifunctional $\Delta 6\Delta 5$ Fads2 (Fonseca-Madriral et al.,
76 2014; Hastings et al., 2001; Li et al., 2010; Tanomman et al., 2013) or monofunctional $\Delta 5$
77 Fads (Abdul Hamid et al., 2016; Hastings et al., 2005). In recent years, Fads2 with $\Delta 4$
78 activities have been found in a variety of teleost species (Fonseca-Madriral et al., 2014; Kuah
79 et al., 2015; Li et al., 2010; Morais et al., 2012). Furthermore, fish Fads2, as described in
80 mammals (Park et al., 2009), also display $\Delta 8$ activity, an activity that appeared to be
81 relatively higher in marine fish compared to freshwater fish species (Monroig et al., 2011a).

82 Elov1 enzymes catalyse the condensation step in the elongation pathway resulting in the
83 addition of a two-carbon unit to the pre-existing fatty acid (Guillou et al., 2010). Functional
84 characterisation of fish Elov12, Elov14 and Elov15, elongase enzymes that function in the LC-
85 PUFA biosynthesis pathway, show that they display somewhat overlapping activities (Castro
86 et al., 2016). Thus Elov15 generally elongate C_{18} and C_{20} PUFA, whereas Elov12 and Elov14
87 are more efficient towards C_{20} and C_{22} PUFA (Gregory and James, 2014; Monroig et al.,
88 2011a, 2011b, 2009; Morais et al., 2009). While both *elov15* and *elov14* genes are present in
89 teleost genomes (Monroig et al., 2016), *elov12* appears to be lost in Acanthopterygii, a
90 phylogenetic group that, with the exception of salmonids, includes the vast majority of the most
91 important farmed fish species (Castro et al., 2016). To the best of our knowledge, Elov12
92 cDNAs have been characterised only in Atlantic salmon (*Salmo salar*) (Morais et al., 2009),
93 zebrafish (*Danio rerio*) (Monroig et al., 2009) and rainbow trout (*Oncorhynchus mykiss*)
94 (Gregory and James, 2014).

95 Evidence indicates that the complement and functionalities of *fads* and *elov1* genes existing
96 in any teleost species has been shaped by evolutionary drivers leading to the retention,
97 subfunctionalisation or loss of these genes (Castro et al., 2016). Moreover, the habitat
98 (marine vs freshwater), and specifically the availability of LC-PUFA in food webs, has also
99 been implicated as influencing the LC-PUFA biosynthetic capability of fish (Bell and Tocher,

100 2009; Castro et al., 2016; Monroig et al., 2011b). Freshwater fish, having evolved on diets
101 low in LC-PUFA, are believed to have all the genes and/or enzymatic functionalities required
102 for endogenous LC-PUFA production (NRC, 2011; Tocher, 2015). Whereas, many marine
103 species have not retained all the genes and/or enzymatic functionalities required for
104 endogenous LC-PUFA production as a consequence of LC-PUFA being readily available in
105 their natural diets (NRC, 2011; Tocher, 2015). However, such dichotomy has been recently
106 seen as too simplistic and other factors including trophic level (Li et al., 2010) and trophic
107 ecology (Morais et al., 2015, 2012) also appear to influence LC-PUFA biosynthesis capacity
108 of fish species. Within an aquaculture nutrition context, investigations of the *fads* and *elovl*
109 gene repertoire involved in LC-PUFA biosynthesis, as well as the functions of the enzyme
110 they encode, are necessary to ascertain whether the EFA requirements of a species can be
111 satisfied by C₁₈ PUFA or whether dietary LC-PUFA are required.

112 The African catfish *Clarias gariepinus*, a freshwater species belonging to the family
113 Clariidae and order Siluriformes, is the most important aquaculture species in Sub-Saharan
114 Africa (FAO, 2012). Yet, neither its LC-PUFA biosynthetic pathway nor EFA requirement is
115 fully understood. Studies on *C. gariepinus* and other African catfishes suggest they can
116 effectively utilise C₁₈ PUFA contained in VO to cover their physiologically important LC-
117 PUFA requirements (Baker and Davies, 1996; Sotolu, 2010; Szabo et al., 2009). Intriguingly,
118 lower growth performance has been reported for *C. gariepinus* fed diets with FO compared to
119 those fed diets containing VO (Hoffman and Prinsloo, 1995; Ng et al., 2003) in contrast to
120 most fish species including those with full LC-PUFA biosynthetic capability like salmonids
121 (Sargent et al., 2002; Tocher and Glencross, 2015).

122 The aim of this study was to investigate the functions of the genes encoding putative Fads
123 and Elovl enzymes that account for the LC-PUFA biosynthetic capability of *C. gariepinus*
124 and thus understand the potential of this species to utilise diets containing VO and low

125 contents of LC-PUFA. Here, we report the cloning and functional characterisation of *fads2*
126 and *elovl2* genes from *C. gariiepinus*. We further investigated the mRNA tissue distribution of
127 the newly cloned genes, as well as that of the previously cloned *elovl5* (Agaba et al., 2005).

128

129 **Materials and Methods**

130 *Sample collection and RNA preparation*

131 Tissue samples were obtained from adult *C. gariiepinus* (~1.8 kg) raised in the tropical
132 aquarium of the Institute of Aquaculture, University of Stirling, UK, on standard salmonid
133 diets. Eight *C. gariiepinus* individuals were sacrificed with an overdose of benzocaine before
134 the collection of tissue samples including liver, intestine, pituitary, testis, ovary, skin, muscle,
135 gills, kidney, and brain. The samples were immediately preserved in an RNA stabilisation
136 buffer (3.6 M ammonium sulphate, 18 mM sodium citrate, 15 mM EDTA, pH 5.2) and stored
137 at -80 °C prior to extraction of total RNA following homogenisation in TRI Reagent[®]
138 (Sigma-Aldrich, USA). Purity and concentration of total RNA was assessed using the
139 NanoDrop[®] (Labtech International ND-1000 spectrophotometer) and integrity was assessed
140 on an agarose gel. First strand complementary DNA (cDNA) was synthesised using High
141 Capacity cDNA Reverse Transcription Kit (Applied Biosystems[™], USA) following the
142 manufacturer's instructions.

143 *Molecular cloning of *fads2* and *elovl2* cDNAs*

144 Amplification of partial fragments of the genes was achieved by polymerase chain reaction
145 (PCR) using a mixture of cDNA from eye, liver, intestine and brain as template and primers
146 FadCGF2F1 and FadCGF2R1 for *fads2*, and EloCGE2F1 and EloCGE2R1 for *elovl2* (Table
147 1). For clarity, it should be noted that the standard gene/protein nomenclature has been used
148 in this study (Castro et al., 2016). Following conventions accepted for zebrafish (*Danio*

149 *rerio*), proteins are termed with regular fonts (e.g. Fads2) whereas genes are italicised (e.g.
150 *fads2*). Primers used for amplification of the first fragment of target genes were designed on
151 conserved regions of fish orthologues of *fads2* and *elovl2* according to the following strategy.
152 For *fads2*, sequences from the broadhead catfish (*Clarias microcephalus*) (gb|KF006248.1|),
153 spot pangasius (*Pangasius larnaudii*) (gb|KC994461.1|), striped catfish (*Pangasianodon*
154 *hypophthalmus*) (gb|JX035811.1|) and *Clarias* hybrid (*C. macrocephalus* and *C. gariepinus*)
155 (gb|KC994463.1|) were aligned with the ClustalW tool (BioEdit v7.0.9, Tom Hall,
156 Department of Microbiology, North Carolina State University, USA) for degenerate primer
157 design. For *elovl2*, homologous sequences from *D. rerio* (gb|NM_001040362.1|), *S. salar*
158 (gb|NM_001136553.1|) and Mexican tetra (*Astyanax mexicanus*) (gb|XM_007260074.2|)
159 were retrieved from NCBI (<http://ncbi.nlm.nih.gov>), aligned (BioEdit) and conserved regions
160 used to retrieve expressed sequence tags (ESTs) from catfish species. Six Channel catfish
161 (*Ictalurus punctatus*) ESTs (GenBank accession numbers GH651976.1, GH651977.1,
162 FD328544.1, FD284236.1, FD284235.1 and BM438219.1) were obtained and aligned with
163 BioEdit. Subsequently, the consensus *elovl2*-like sequences from *I. punctatus*, and those from
164 *D. rerio*, *S. salar* and *A. mexicanus*, were aligned for degenerate primer design. PCR
165 conditions consisted of an initial denaturation step at 95 °C for 2 min, followed by 33 cycles
166 of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min 30
167 s, followed by a final extension at 72 °C for 7 min. The PCR fragments were purified using
168 the Illustra GFX PCR DNA/gel band purification kit (GE Healthcare, Little Chalfont, UK),
169 and sequenced (GATC Biotech Ltd., Konstanz, Germany). The primers used in this study and
170 their sequences are presented in Table 1.

171 In order to obtain full-length cDNA sequences, Rapid Amplification of cDNA Ends
172 (RACE) was performed with the FirstChoice[®] RLM-RACE RNA ligase mediated RACE kit
173 (Ambion[®], Life Technologies[™], USA). The 5' RACE outer primer and gene-specific primer

174 FadCGRF2R3 were used in a PCR using the 5' RACE cDNA as template (first round PCR)
175 for *fads2*. The resulting PCR product was then used as template for the second round PCR
176 with the 5' RACE inner primer and the gene-specific primer FadCGRF2R2. A similar
177 approach was followed to perform 3' RACE PCR, with primers FadCGRF2F1 and
178 FadCGRF2F2 used for first and second round PCR, respectively. For *elovl2*, the primers
179 CGRE2R3 and CGRE2R2 were designed and used for first and second round PCR,
180 respectively, for the 5' RACE PCR, while CGRE2F1 and CGRE2F2 were used for first and
181 second round PCR, respectively, for the 3' RACE PCR. The first fragments, 5' and 3' RACE
182 PCR fragments were then cloned into PCR 2.1 vector (TA cloning[®] kit, Invitrogen, Life
183 Technologies[™], USA) and sequenced as above. The full nucleotide sequences of the *fads2*
184 and *elovl2* cDNAs were obtained by aligning sequences of the first fragments, together with
185 those of the 5' and 3' RACE PCR positive products (BioEdit).

186

187 *Sequence and phylogenetic analysis*

188 The deduced amino acid (aa) sequences of the *C. gariepinus fads2* and *elovl2* cDNAs were
189 compared to corresponding orthologues from other vertebrate species and sequence identity
190 scores were calculated using the EMBOSS Needle Pairwise Sequence Alignment tool
191 (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Phylogenetic analysis of the deduced aa
192 sequences of *fads2* and *elovl2* cDNAs from *C. gariepinus* and those from a variety of species
193 across vertebrate lineages were carried out by constructing trees using the neighbour-joining
194 method (Saitou and Nei, 1987), with the MEGA 4.0 software
195 (www.megasoftware.net/mega4/mega.html). Confidence in the resulting tree branch topology
196 was measured by bootstrapping through 1,000 iterations.

197

198 *Functional characterisation of C. gariepinus fads2 and elovl2 by heterologous expression in*
199 *Saccharomyces cerevisiae*

200 PCR fragments corresponding to the open reading frame (ORF) of *C. gariepinus fads2* and
201 *elovl2* were amplified from a mixture of cDNA synthesised from liver, intestine, eye and
202 brain total RNA, using the high fidelity *Pfu* DNA polymerase (Promega, USA) with primers
203 containing *Bam*HI (forward) and *Xho*I (reverse) restriction sites (Table 1). PCR conditions
204 consisted of an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of
205 denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 3 min 30 s
206 followed by a final extension at 72 °C for 7 min. The DNA fragments obtained were purified
207 as above, digested with the appropriate restriction enzymes, and ligated into similarly
208 digested pYES2 yeast expression vector (Invitrogen).

209 Yeast competent cells InvSc1 (Invitrogen) were transformed with the plasmid constructs
210 pYES2-*fads2* (desaturase) or pYES-*elovl2* (elongase) or with empty vector (control) using
211 the S.c. EasyComp™ Transformation Kit (Invitrogen). Selection of yeast containing the
212 pYES2 constructs was performed on *S. cerevisiae* minimal medium minus uracil (SCMM^{-ura})
213 plates. One single yeast colony was grown in SCMM^{-ura} broth for 2 days at 30 °C, and
214 subsequently subcultured in individual Erlenmeyer flasks until an optical density measured at
215 a wavelength of 600nm (OD₆₀₀) reached 1, after which galactose (2 %, w/v) and a PUFA
216 substrate were added. For both genes, final concentration of substrates were 0.5 mM (C₁₈),
217 0.75 mM (C₂₀) and 1.0 mM (C₂₂) to compensate for differential uptake related to fatty acyl
218 chain (Zheng et al., 2009). For the *fads2*, Δ6 (18:3n-3 and 18:2n-6), Δ8 (20:3n-3 and 20:2n-
219 6), Δ5 (20:4n-3 and 20:3n-6), and Δ4 (22:5n-3 and 22:4n-6) Fads substrates were used. For
220 *elovl2*, substrates included C₁₈, (18:3n-3, 18:2n-6, 18:4n-3 and 18:3n-6), C₂₀ (20:5n-3 and
221 20:4n-6) and C₂₂ (22:5n-3 and 22:4n-6) PUFA. After 2 days, the yeasts were harvested,
222 washed and homogenised in chloroform/methanol (2:1, v/v) containing 0.01 % butylated

223 hydroxytoluene (BHT) and stored at -20 °C until further use. All FA substrates (> 98-99 %
224 pure) used for the functional characterisation assays, except for stearidonic acid (18:4n-3) and
225 eicosatetraenoic acid (20:4n-3), were obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA).
226 Stearidonic acid (> 99 % pure) and yeast culture reagents including galactose, nitrogen base,
227 raffinose, tergitol NP-40 and uracil dropout medium were obtained from Sigma-Aldrich
228 (UK). Eicosatetraenoic acid was purchased from Cayman Chemical Co. (Ann Arbor, USA).

229

230 *Fatty acid analysis of yeast*

231 Total lipids extracted according to Folch et al. (1957) from yeast samples were used to
232 prepare fatty acid methyl esters (FAME). FAME extraction, purification and analysis were
233 performed as described by Li et al. (2010). Substrate FA conversion was calculated as the
234 proportion of exogenously added FA substrate desaturated or elongated [all product peak
235 areas / (all product peak areas + substrate peak area)] x 100 (Monroig et al., 2016). GC-MS
236 was used to confirm double bond positions when necessary (Li et al., 2010).

237

238 *Gene expression analysis*

239 Expression of the newly cloned *fads2* and *elovl2* genes, as well as that of the previously
240 characterised elongase *elovl5* (Agaba et al., 2005), were determined by quantitative real-time
241 PCR (qPCR). Extraction of RNA from tissues and cDNA synthesis were carried out as
242 described above. qPCR amplifications were carried out in duplicate using Biometra
243 Thermocycler (Analytik Jena company, Germany) and Luminaris Color Hignreen qPCR
244 master mix (Thermo Scientific, CA, USA) following the manufacturer's instruction. The
245 qPCR was performed in a final volume of 20 µl containing 5 µl diluted (1/20) cDNA, 1 µl (10
246 µM) of each primer, 3 µl nuclease free water and 10 µl Luminaris Color Hignreen qPCR
247 master mix. The qPCR conditions were 50 °C for 2 min, 95 °C for 10 min followed by 35

248 cycles of denaturation at 95 °C for 15 s, annealing at 59 °C for 30 s and extension at 72 °C for
249 30 s. After the amplifications, a dissociation curve of 0.5 °C increments from 60 to 90 °C was
250 performed, enabling confirmation of a single product in each reaction. Negative controls (no
251 template control, NTC) containing no cDNA were systematically run. Absolute copy number
252 of the target and reference gene in each sample was calculated from the linear standard curve
253 constructed. Normalisation of each target gene was carried out by dividing the absolute copy
254 number of the candidate gene by the absolute copy number of the reference gene 28S rRNA
255 (gb|AF323692.1). In order to prepare solutions of known copy numbers, DNA concentration
256 linearised PCR 2.1 vectors containing a fragment of either candidate or reference genes was
257 determined, and their molecular weights were estimated as 660 g bp x length (bp) of the
258 plasmid constructs. Primers used for qPCR analysis are also presented in Table 1.

259

260 *Statistical analysis*

261 Tissue expression (qPCR) results were expressed as mean normalised ratios (\pm SE)
262 corresponding to the ratio between the copy numbers of the target genes (*fads2*, *elovl2* and
263 *elovl5*) and the copy numbers of the reference gene, 28S rRNA. Differences in gene
264 expression among tissues were analysed by one-way analysis of variance (ANOVA) followed
265 by Tukey's HSD test at a significance level of $P \leq 0.05$ (IBM SPSS Statistics 21).

266

267 **Results**

268 *Sequence and phylogenetic analysis*

269 *C. gariepinus Fads2* sequence was deposited in the GenBank database with the accession
270 number KU925904. The full length of the *C. gariepinus Fads2* was 1812 bp, comprising of a
271 5' untranslated region (UTR) of 162 bp, an ORF of 1338 bp encoding a putative protein of
272 445 aa, and a 3' UTR of 312 bp. The deduced *C. gariepinus Fads2* enzyme showed
273 distinctive structural features of fatty acyl desaturases including the three histidine boxes

274 HDFGH, HFQHH, and QIEHH (aa 181-185, 218-222 and 383-387, respectively) and
275 cytochrome b5-domain (aa 26-77) containing the heme binding motif HPGG (aa 54-57).
276 Pairwise aa sequence comparisons of *C. gariepinus* Fads2 with other Fads2-like proteins
277 showed highest identities with Fads from members of the catfish family such as *C.*
278 *macrocephalus* (97 %) and *P. hypophthalmus* (91.5 %). Comparisons with bifunctional
279 $\Delta 6\Delta 5$ Fads2 of *D. rerio* (gb|AF309556.1|) and *C. estor* (gb|AHX39207.1|), bifunctional $\Delta 5\Delta 4$
280 Fads2 of *C. striata* (gb|ACD70298.1|) and *S. canaliculatus* (gb|ADJ29913.1|) and $\Delta 4$ Fads2
281 of *S. senegalensis* (gb|AEQ92868.1|) and *C. estor* (gb|AHX39206.1|) showed identities
282 ranging from 65.2-70.2 %. Lowest identities were observed when *C. gariepinus* Fads was
283 compared to Fads1-like sequences from different vertebrate lineages. Phylogenesis of *C.*
284 *gariepinus* Fads with Fads from a variety of vertebrate species showed it clustered with all
285 other Fads2 in one group that was separate from the Fads1 group confirming that the newly
286 cloned *fads* was a *fads2* (Fig. 1). The *C. gariepinus* Fads2 clustered most closely with Fads2
287 from bony fish species (with the exception of the sarcopterygian, *L. chalumnae* which formed
288 a separate cluster with Fads2 from chondrichthyes (*C. milli* and *S. canicula*), mammalian (*H.*
289 *sapiens*, *M. musculus* and *B. taurus*) and avian species (*G. gallus*) (Fig. 1).

290

291 *C. gariepinus* Elov12 sequence was deposited in the GenBank database with the accession
292 number KU902414. The full-length cDNA sequence of *C. gariepinus elov12* was 1432 bp (5'
293 UTR 91 bp, ORF 864 bp, 3' UTR 477 bp) encoding a protein of 287 aa. Analysis of the
294 deduced aa sequence of *C. gariepinus* Elov12 revealed characteristic features of fatty acyl
295 elongases such as the highly conserved histidine box (HVYHH, aa 151-155) and the
296 carboxyl-terminal region, but the aa residues at the carboxyl terminus were KHKLQ, more
297 similar to the KXRXX found in Elov15 than to the KKXX in *H. sapiens* and *S. salar* Elov12
298 (Morais et al., 2009). Comparisons of *C. gariepinus* Elov12 with homologues from *A.*

299 *mexicanus* (gb|XP_007260136.1|), *S. salar* (gb|ACI62500.1|), *D. rerio*
300 (gb|XP_005162628.1|), *Clupea harengus* (gb|XP_012671565.1|), and *H. sapiens*
301 (gb|NP_060240.3|) showed identities of 81.7, 72.9, 72.7, 69.1 and 64.8 %, respectively. *C.*
302 *gariiepinus* Elov12 shared 52 % identity with *C. gariiepinus* Elov15. Phylogenetic analysis of
303 the Elov12 with members of the Elov1 family confirmed that the newly cloned elongase was
304 indeed an Elov12 elongase. Thus, the *C. gariiepinus* Elov12 clustered together with all the
305 Elov12 and more distantly from Elov15 sequences including that from *C. gariiepinus* (Agaba et
306 al., 2005) and even more distantly to Elov14 enzymes (Fig. 2).

307

308 *Functional characterisation of C. gariiepinus Fads2 and Elov12 in S. cerevisiae*

309 Consistent with previous studies (Hastings et al., 2001), control yeast transformed with the
310 empty pYES2 vector did not show any activity towards any of the PUFA substrates assayed
311 (data not shown). Functional characterisation by heterologous expression in yeast revealed
312 that the *C. gariiepinus* Fads2 had the ability to introduce double bonds at $\Delta 5$, $\Delta 6$ and $\Delta 8$
313 positions in the appropriate PUFA substrates (Fig. 3; Table 2). The FA composition of the
314 yeast transformed with pYES2-*fads2* showed peaks corresponding to the four main yeast
315 endogenous FA, namely 16:0, 16:1n-7, 18:0 and 18:1n-9, the exogenously added PUFA and
316 the corresponding PUFA product(s) (Fig. 3; Table 2). Thus, the C₁₈ PUFA substrates 18:3n-3
317 and 18:2n-6 were desaturated to 18:4n-3 (42 % conversion) and 18:3n-6 (23 %), respectively,
318 indicating the encoded protein had $\Delta 6$ Fads activity (Fig. 3A; Table 2). Moreover, the
319 transgenic yeast was able to desaturate 20:4n-3 and 20:3n-6 to 20:5n-3 (19 %) and 20:4n-6
320 (14 %), respectively, indicating the *C. gariiepinus* Fads2 also had $\Delta 5$ activity (Fig. 3C; Table
321 2), and thus these results confirm that this Fads2 from *C. gariiepinus* is a bifunctional $\Delta 6\Delta 5$
322 Fads. Additionally, the *C. gariiepinus* Fads2 showed $\Delta 8$ Fads activity as the yeast transformed
323 with pYES2-*fads2* were able to desaturate 20:3n-3 and 20:2n-6 to 20:4n-3 and 20:3n-6,

324 respectively (Fig. 3B and Table 2). No additional peaks were observed when yeast expressing
325 the *C. gariepinus fads2* were grown in the presence of 22:5n-3 and 22:4n-6 (Fig. 3D; Table
326 2).

327 The *C. gariepinus Elov12* showed the ability to elongate C₁₈₋₂₂ PUFA substrates (Fig. 4;
328 Table 3), with highest conversions towards the C₂₀ substrates 20:5n-3 (73.4 %) (Fig. 4B) and
329 20:4n-6 (56 %). Conversion of the C₂₂ substrate was 36.7 % for 22:5n-3 (Fig. 4C) and 9.7 %
330 for 22:4n-6 (Table 3). Elongations of C₁₈ PUFAs were generally lower compared to those for
331 C₂₀ and C₂₂ substrates. Stepwise elongations derived from further activity of the *C.*
332 *gariepinus Elov12* towards products of initial substrate elongation resulted in the production
333 of several polyenes up to 24 carbons (Fig. 4; Table 3).

334

335 *Tissue expression analysis of C. gariepinus fads2, elov12 and elov15*

336 Tissue distribution analysis of *C. gariepinus fads2, elov12* and *elov15* transcripts confirmed
337 that these genes were expressed in all tissues analysed (Fig. 5). Liver and brain were found to
338 contain the highest transcript levels of the *C. gariepinus fads2*, followed by pituitary,
339 intestine and kidney. Liver, brain and pituitary were also found to contain the highest
340 transcript levels of the *C. gariepinus elov12*. Generally, gonads including testis and ovary
341 showed the lowest transcript levels for both *fads2* and *elov12* (Fig. 5). Intestine and liver
342 exhibited the highest level of *elov15*, while the lowest expression levels were found in muscle.

343

344 **Discussion**

345 Elucidating the LC-PUFA biosynthesis pathway in farmed fish is crucial for formulating
346 diets that satisfy physiological requirements and thus ensure normal growth and development.
347 These studies are particularly relevant in the current scenario whereby FO are being replaced
348 by VO in aquafeed, the latter naturally devoid of essential LC-PUFA and thus potentially

349 compromising both health of the fish and nutritional value for human consumers (Monroig et
350 al., 2011b; Tocher and Glencross, 2015). Relevant to the present study, identification and
351 production of fish that can efficiently utilise VO-based diets due to their high capacity for
352 LC-PUFA biosynthesis is a valid strategy to expand aquaculture considering that marine
353 ingredients (FO and fish meal) will be increasingly limited in the future (Tocher, 2015). *C.*
354 *gariiepinus* feed and grow well on a variety of feed ingredients and are, therefore, a good
355 model for studying the endogenous capacity for LC-PUFA synthesis of freshwater fish.

356 Phylogenetic analysis of the *fads*-like desaturase cDNA isolated from *C. gariiepinus*,
357 together with the possession of all the main structural features common to the Fads2 protein
358 family confirmed it to be a Fads2. Sequence and phylogenetic analyses also showed that the
359 *C. gariiepinus* Fads2 shared highest aa sequence similarities with other catfish species, with
360 relatively low scores when compared with Fads from more distantly related fish lineages
361 (Betancur-R et al., 2013). Nevertheless, recent advances in functional analyses of fish Fads
362 have concluded that some Fads2 have acquired novel functions (subfunctionalisation) during
363 evolution and thus phylogeny of fish Fads2 does not necessarily correlate with their
364 functionalities (Castro et al., 2016). The herein reported functions of the *C. gariiepinus* Fads2
365 further confirm such a conclusion.

366 Functional characterisation demonstrated that the *C. gariiepinus* Fads2 is a bifunctional
367 $\Delta 6\Delta 5$ desaturase able to operate towards a range of substrates including n-3 (18:3n-3 and
368 20:4n-3) and n-6 (18:2n-6 and 20:3n-6) PUFA. Similar substrate specificities were previously
369 described in *D. rerio*, which represented the first ever report of dual $\Delta 6\Delta 5$ functionality in a
370 vertebrate Fads (Hastings et al., 2001). More recent studies have now shown that
371 bifunctionality appear to be a more common feature of fish Fads2 than originally thought.
372 Thus dual $\Delta 6\Delta 5$ Fads have been described in *S. canaliculatus* (Li et al., 2010), Nile tilapia
373 (*Oreochromis niloticus*) (Tanomman et al., 2013) and *C. estor* (Fonseca-Madrigal et al.,

374 2014). Interestingly, fish Fads2 with $\Delta 4$ capability reported in *S. canaliculatus* (Li et al.,
375 2010), *S. senegalensis* (Morais et al., 2012) and *C. striata* (Kuah et al., 2015) showed as well
376 some minor $\Delta 5$ activity and can thus be regarded as dual $\Delta 5\Delta 4$ Fads (Castro et al., 2016). In
377 contrast, other teleost Fads2 are single function $\Delta 6$ desaturases (González-Rovira et al., 2009;
378 Mohd-Yusof et al., 2010; Monroig et al., 2010; 2013; Zheng et al., 2009), in agreement with
379 Fads activities reported in mammalian FADS2 (Castro et al., 2016). Such substrate plasticity
380 exhibited amongst fish Fads2 is believed to be the result of a combination of multiple
381 evolutionary drivers including habitat, trophic level and ecology underlying the specific
382 phylogenetic position of each fish species (Castro et al., 2016, 2012; Li et al., 2010; Monroig
383 et al., 2011b). In contrast, Fads1, another “front-end” Fads encoding a $\Delta 5$ Fads in mammals
384 (Castro et al., 2012, 2016), appears to have been lost during evolution of teleost and is absent
385 in the vast majority of farmed fish species (Castro et al., 2016).

386 The *C. gariepinus* Fads2 also exhibited $\Delta 8$ desaturation capability, an intrinsic feature of
387 vertebrate Fads2 (Monroig et al., 2011a; Park et al., 2009). Although conversions in yeast
388 might quantitatively vary from those occurring *in vivo*, it appeared that the *C. gariepinus*
389 Fads2 had lower efficiency as $\Delta 8$ Fads than as $\Delta 6$ Fads, in agreement with the “ $\Delta 8$ pathway”
390 being regarded as a minor pathway compared to the more prominent $\Delta 6$ desaturation pathway
391 (Monroig et al., 2011a; Park et al., 2009). Interestingly, the $\Delta 8$ desaturation capabilities of *C.*
392 *gariepinus* Fads2 towards 20:3n-3 (12.9 %) was relatively high leading to lower $\Delta 6\Delta 8$ ratio
393 (3.26), a parameter used to compare $\Delta 8$ desaturation capability among fish Fads2 enzymes
394 (Monroig et al., 2011a). Thus, the $\Delta 6/\Delta 8$ ratio of *C. gariepinus* Fads2 is more similar to that
395 of marine species like gilthead seabream (*Sparus aurata*) (2.7) and turbot (*Psetta maxima*)
396 (4.2). Whereas it is notably lower than those of freshwater or salmonid Fads2 including *D.*
397 *rerio* (22.4) and *S. salar* (12 and 14.7 for Fad_b and Fad_c, respectively) (Monroig et al.,
398 2011a). These results suggest that the $\Delta 8$ pathway, while possibly not to such an extent as the

399 $\Delta 6$ pathway, can still contribute to the initial steps of LC-PUFA biosynthesis in *C.*
400 *gariepinus*. Note that $\Delta 8$ activity introduces the same double bond as $\Delta 6$ activity, after
401 elongation rather than before, and so a Fads having $\Delta 6\Delta 8$ activity is not regarded as
402 “bifunctional”.

403 The ability of the *C. gariepinus* Fads2 to desaturate a range of $\Delta 5$, $\Delta 6$ and $\Delta 8$ Fads
404 substrates from both n-3 and n-6 series clearly shows it is a multifunctional enzyme. This is
405 emphasised by the stepwise desaturation reactions that occurred when transgenic yeast
406 expressing the *C. gariepinus* Fads2 were grown in the presence of certain FA substrates such
407 as 20:3n-3 and 20:2n-6. *C. gariepinus* Fads2 enzyme activity toward 20:3n-3 led to the
408 production of either 20:4n-3 ($\Delta 8$ desaturation) that was subsequently desaturated to 20:5n-3
409 ($\Delta 5$ desaturation), or the non-methylene interrupted (NMI) FA products $\Delta^{5,11,14,17}20:4$ or
410 $\Delta^{6,11,14,17}20:4$ resulting from direct $\Delta 5$ or $\Delta 6$ desaturation, respectively. While the biological
411 significance of these pathways is difficult to determine, particularly for NMI FA biosynthesis,
412 the results further confirm that all the Fads capabilities ($\Delta 5$, $\Delta 6$ and $\Delta 8$) are present in the
413 characterised Fads2.

414 Moreover, we can further confirm that all the elongase activities required in the LC-PUFA
415 biosynthesis pathways also exist in *C. gariepinus*. Agaba et al. (2005) characterised an Elovl5
416 from *C. gariepinus* that, like the vast majority of fish Elovl5 investigated to date, showed C₁₈
417 and C₂₀ PUFA as preferred substrates, with markedly lower affinity towards C₂₂ substrates
418 (Castro et al., 2016). In contrast, the *C. gariepinus* Elovl2 showed higher elongation
419 efficiencies towards C₂₀ and C₂₂ PUFAs compared to C₁₈ substrates. Generally, these results
420 are consistent with the activities shown by the only three fish Elovl2 enzymes characterised
421 to date, i.e. *S. salar*, *D. rerio* and *O. mykiss* (Gregory and James, 2014; Monroig et al., 2009;
422 Morais et al., 2009). Although, similar to the human orthologue, the latter did not show any
423 activity on C₁₈ FA substrates (Leonard et al., 2002). The presence of Elovl2 and particularly

424 its ability to elongate C₂₂ PUFA to a greater extent compared to Elovl5 elongases has been
425 acknowledged as evidence supporting LC-PUFA biosynthetic capability in freshwater species
426 and salmonids (Morais et al., 2009). The plethora of genomic and transcriptomic sequences
427 currently available from a varied range of fish species and lineages strongly suggests that,
428 rather than the habitat (freshwater versus marine) of fish, it is the phylogeny of each species
429 that actually correlates with the presence or absence of Elovl2 within their genomes. Here we
430 show that marine species such as the Atlantic herring *Clupea harengus* (Fig. 2) possess a
431 putative Elovl2, whereas freshwater species including *O. niloticus* or medaka (*Oryzias*
432 *latipes*) appear to have lost Elovl2 from their genomes.

433 The functions of the herein reported Fads2 and Elovl2, together with the previously
434 characterised Elovl5 (Agaba et al., 2005), allow us to predict the biosynthetic pathways of
435 LC-PUFA in *C. gariepinus* (Fig. 6). Thus, the dual $\Delta 6\Delta 5$ Fads2 catalyses the initial
436 desaturation of 18:3n-3 and 18:2n-6 ($\Delta 6$ desaturation), as well as the desaturation of 20:4n-3
437 and 20:3n-6 ($\Delta 5$ desaturation). Although we cannot confirm whether the *C. gariepinus* Fads2
438 can desaturate 24:5n-3 and 24:4n-6 ($\Delta 6$ desaturation) required to synthesise 22:6n-3 and
439 22:5n-6, respectively, through the so-called Sprecher pathway (Sprecher et al., 2000). Such
440 ability of vertebrate Fads2 has been demonstrated in *O. mykiss*, *S. salar* and *D. rerio* (Bell
441 and Tocher, 2009; Buzzi *et al.*, 1996; Tocher et al., 2003). Further studies will aim to
442 elucidate whether the newly cloned Fads2 or other Fads potentially co-existing in the *C.*
443 *gariepinus* genome, have the ability to desaturate C₂₄ PUFA in position $\Delta 6$. The Elovl2 was
444 able to catalyse the elongation of C₁₈ (18:3n-3, 18:2n-6, 18:4n-3 and 18:3n-6), C₂₀ (20:5n-3
445 and 20:4n-6) and C₂₂ (22:5n-3 and 22:4n-6) PUFA. Its activity towards C₁₈ PUFA was
446 however very low compared to activity towards C₂₀ and C₂₂ PUFA. This, together with the
447 activity of Elovl5, which is high towards C₁₈ and C₂₀ PUFA (Agaba et al., 2005), confirm that

448 the activities required to catalyse all the elongation steps required for LC-PUFA synthesis are
449 present in *C. gariepinus*.

450 Expression analysis showed *fads2*, *elovl2* and *elovl5* were expressed in all tissues analysed.
451 Consistent with the vast majority of freshwater species studied, the tissue distribution patterns
452 of *C. gariepinus fads2* and *elovl2* mRNAs showed liver as a major metabolic site for LC-
453 PUFA biosynthesis. In contrast, marine fish species typically have brain as the tissue with
454 highest expression levels of LC-PUFA biosynthesis genes, with production of DHA from
455 EPA in brain being hypothesised as driving the retention of at least part of the LC-PUFA
456 biosynthetic pathway in species with high inputs of dietary LC-PUFA (Monroig et al.,
457 2011b). An exception to this pattern is represented by the Nile tilapia *fads2*, with highest
458 expression in the brain (Tanomman et al., 2013). *C. gariepinus fads2* expression in liver was
459 approximately four-fold greater than in intestine, in contrast to salmonid *fads2* that have been
460 reported to be most highly expressed in intestine (Zheng et al., 2005). The expression of
461 *elovl5* was also high in liver but was highest in the intestine.

462 In conclusion, we have successfully cloned and characterised *fads2* and *elovl2* genes that
463 encode enzymes with a broad range of substrate specificities from *C. gariepinus*. These two
464 enzymes, and the previously reported Elov15, enable the African catfish *C. gariepinus* to
465 carry out all the desaturation and elongation reactions required for endogenous LC-PUFA
466 synthesis from C₁₈ precursors, namely ALA and LA. These results strongly suggest that *C.*
467 *gariepinus* has the ability to effectively utilise VO rich in C₁₈ PUFA to satisfy essential LC-
468 PUFA requirements.

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625 Zheng, X., Ding, Z., Xu, Y., Monroig, O., Morais, S., Tocher, D.R., 2009. Physiological roles
626 of fatty acyl desaturase and elongase in marine fish: characterisation of cDNAs of fatty
627 acyl $\Delta 6$ desaturase and Elovl5 elongase of cobia (*Rachycentron canadum*). Aquaculture
628 290, 122–131.

629 **Figures**

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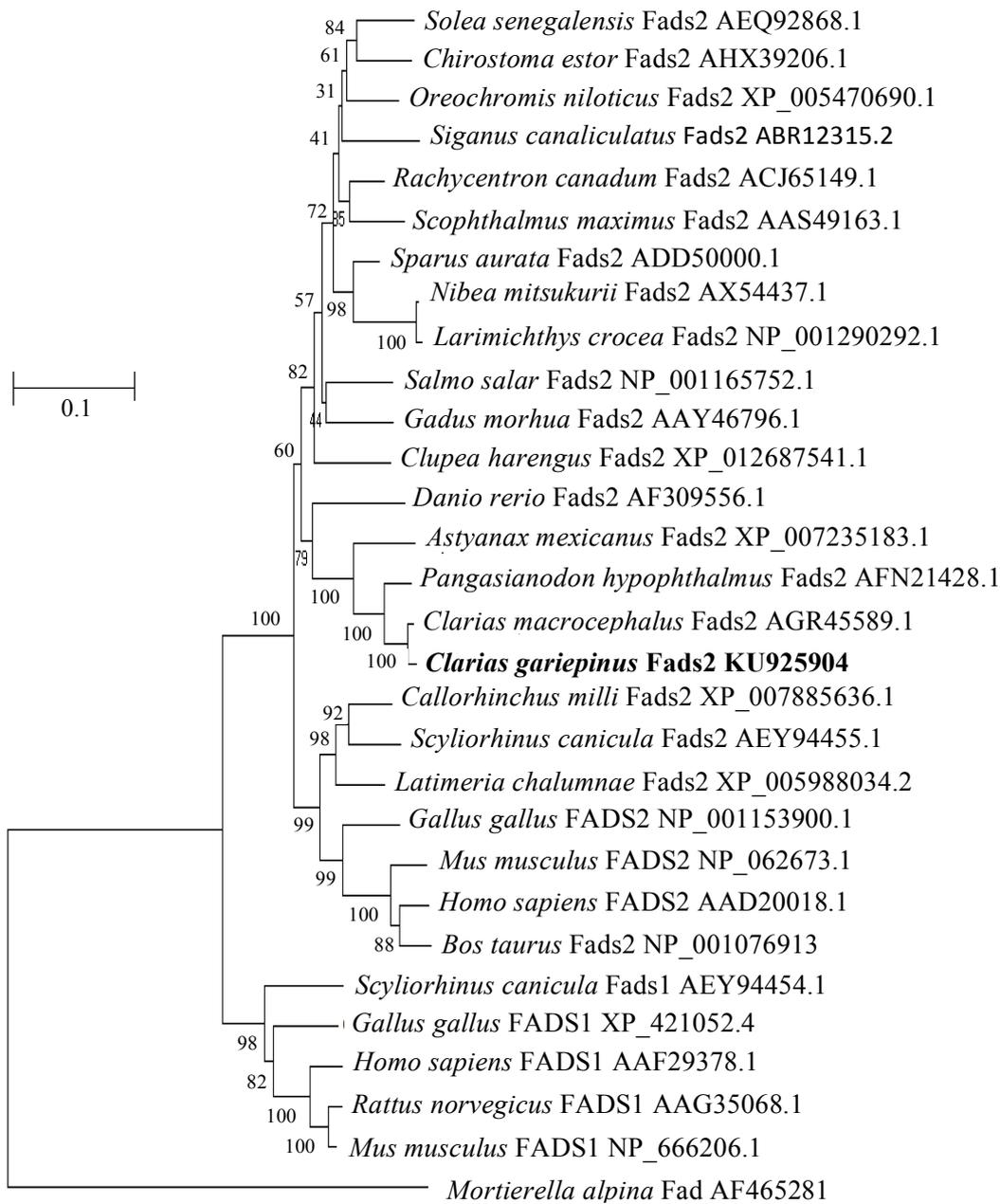
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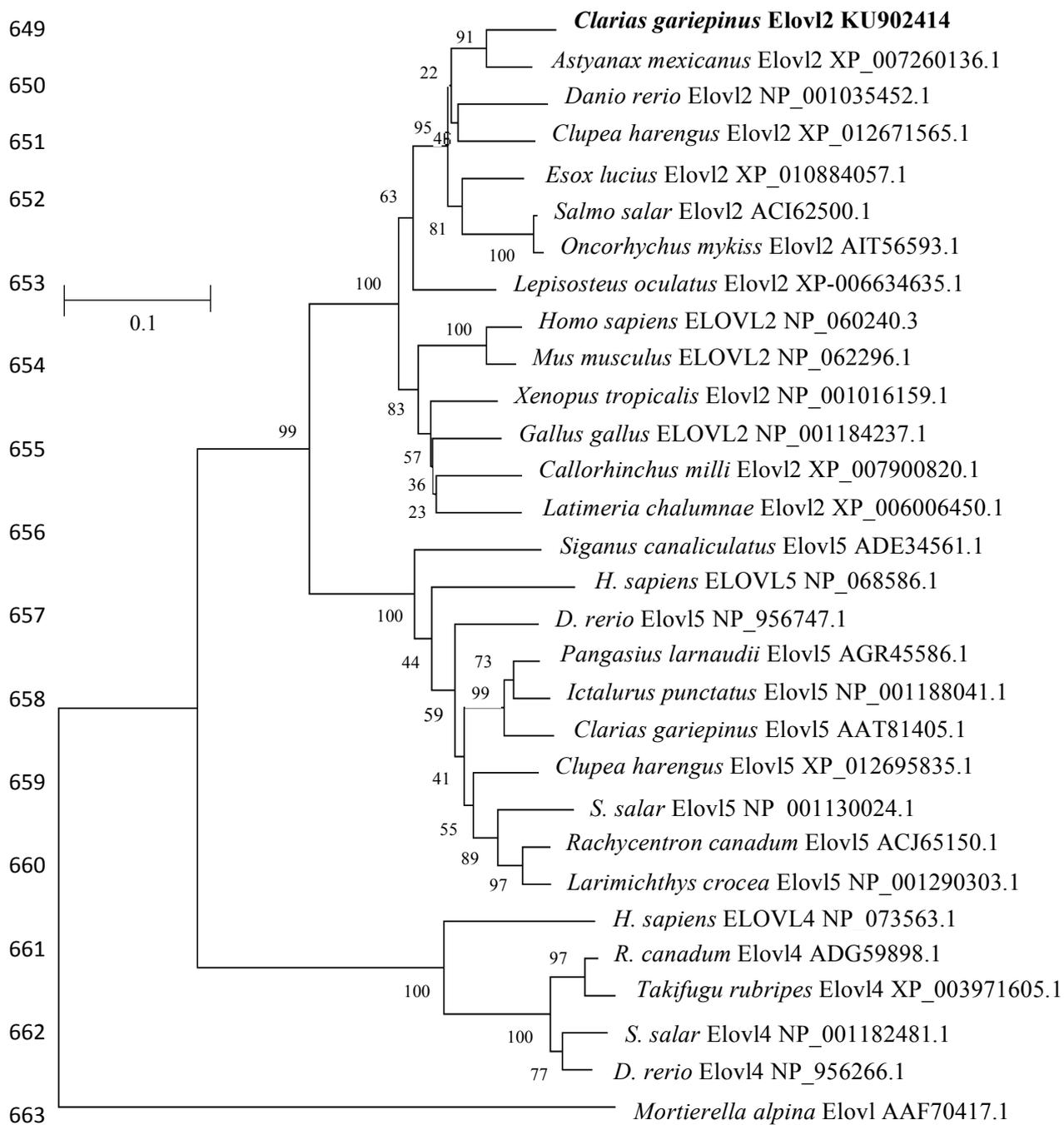
644 Fig. 1. Phylogenetic tree comparing the deduced amino acid sequence of *Clarias gariepinus*

645 *fads2* with Fads from a range of vertebrates. The tree was constructed using the neighbour-

646 joining method (Saitou and Nei, 1987) with the MEGA 4.0 software. The numbers represent

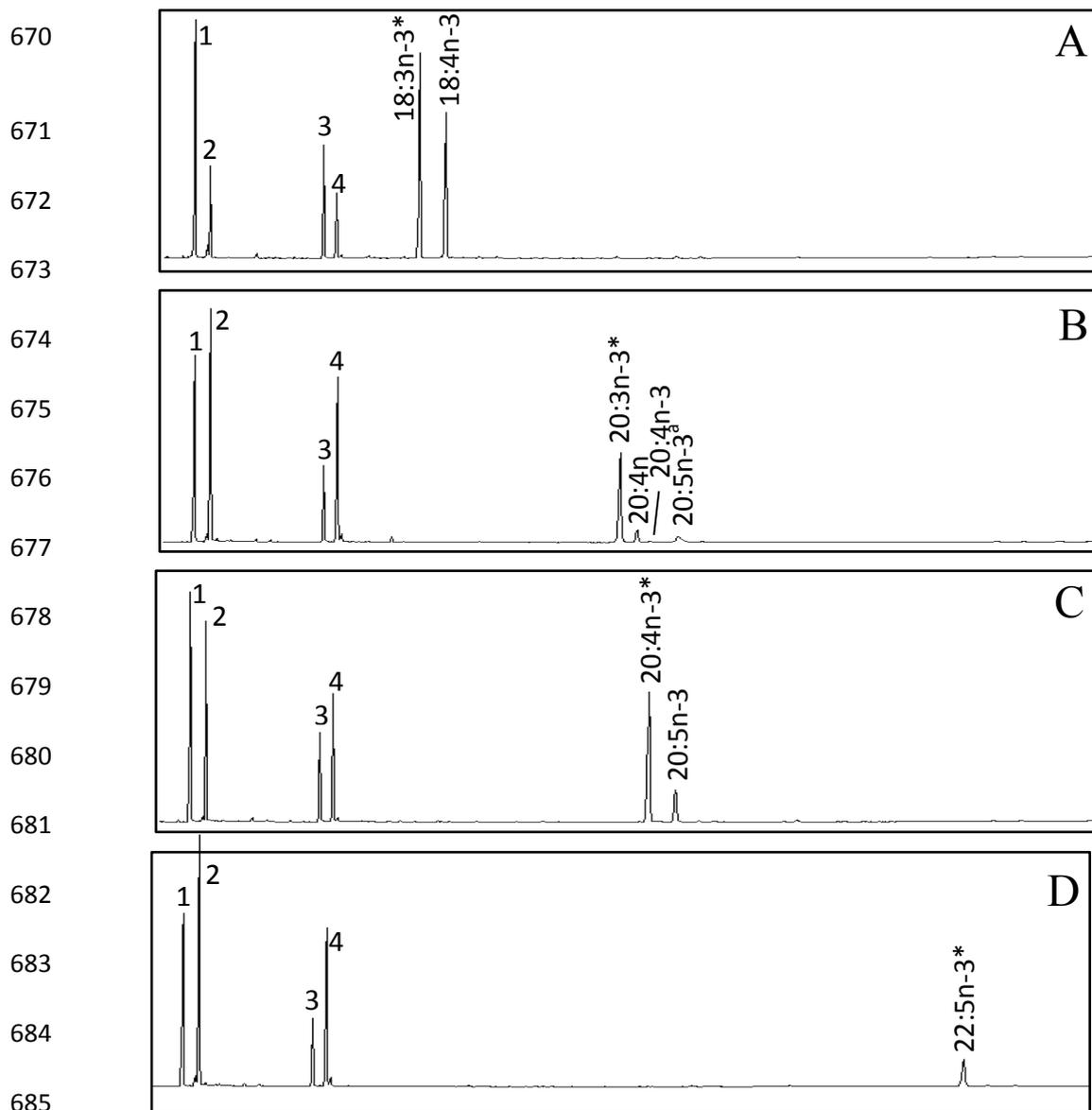
647 the frequency (%) with which the tree topology presented was replicated after 10,000

648 iterations.



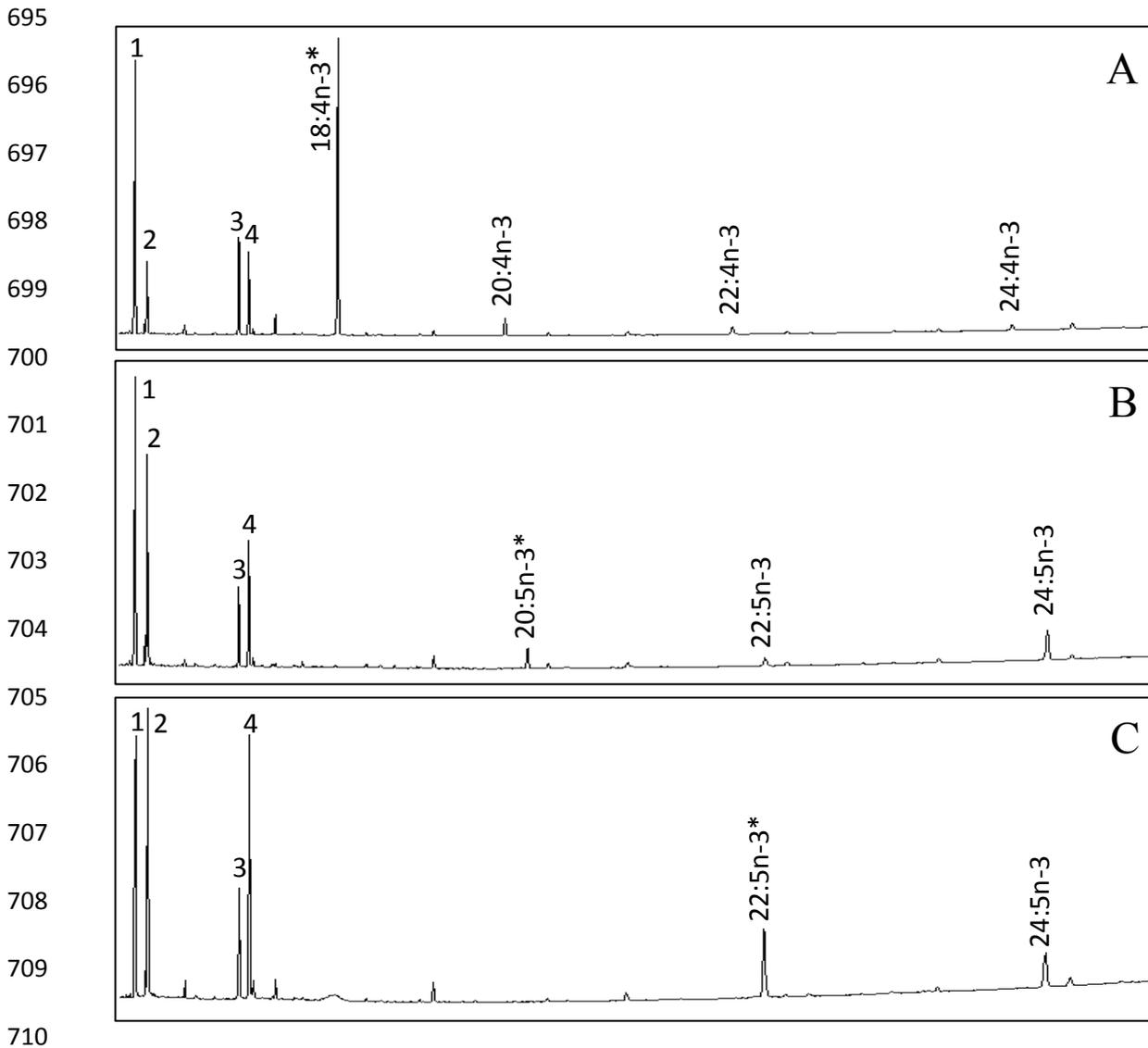
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665 Fig. 2. Phylogenetic tree comparing the deduced amino acid (aa) sequence of *Clarias*
 666 *gariepinus elovl2* with Elov2, Elov4 and Elov5 from a range of vertebrates. The tree was
 667 constructed using the neighbour-joining method (Saitou and Nei, 1987) with the MEGA 4.0
 668 software. The numbers represent the frequencies (%) with which the tree topology presented
 669 was replicated after 10,000 iterations.



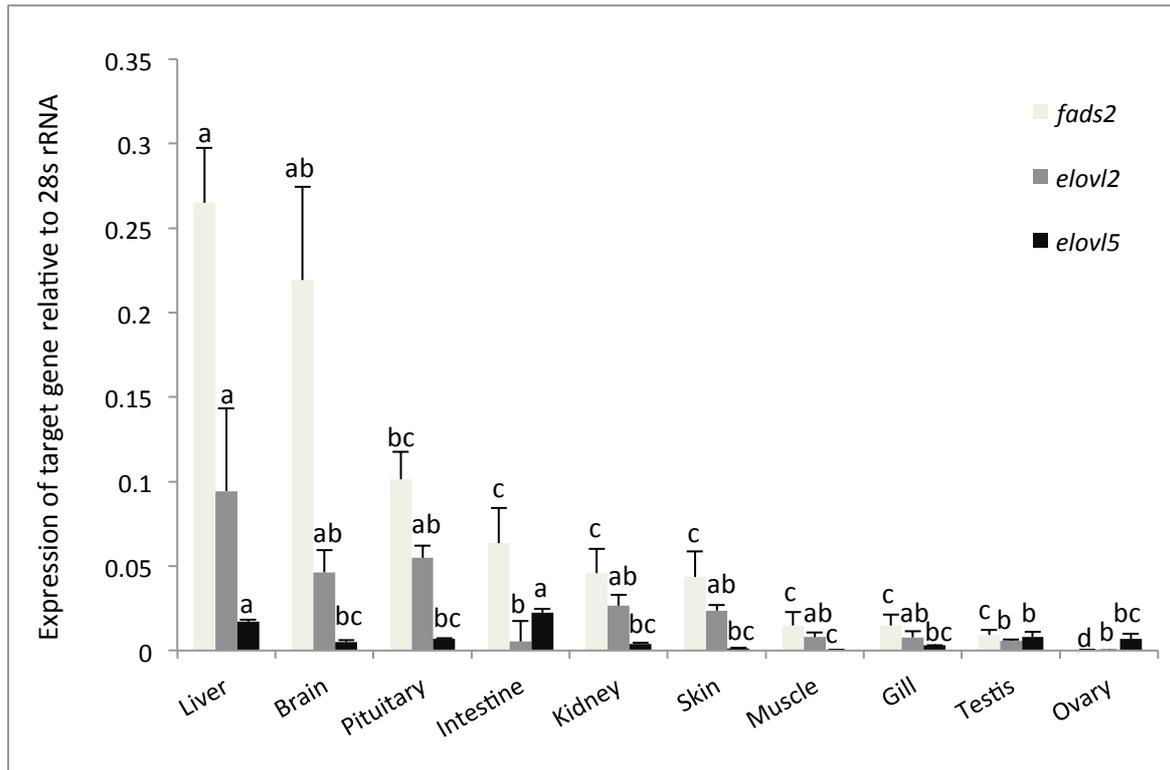
686 Fig. 3. Functional characterisation of the newly cloned *Clarias gariepinus* Fads2 in yeast
 687 (*Saccharomyces cerevisiae*). The fatty acid (FA) profiles of yeast transformed with pYES2
 688 containing the coding sequence of *fads2* were determined after the yeast were grown in the
 689 presence of one of the exogenously added substrates 18:3n-3 (A), 20:3n-3 (B), 20:4n-3 (C)
 690 and 22:5n-3 (D). Peaks 1-4 represent the *S. cerevisiae* endogenous FA, namely 16:0 (1), 16:1
 691 isomers (2), 18:0 (3) and 18:1n-9 (4). Additionally, peaks derived from exogenously added
 692 substrates (*) or desaturation products are indicated accordingly. The peak indicated as
 693 “20:4*” is a non-methylene interrupted FA ($\Delta^{6,11,14,17}$ 20:4 or $\Delta^{5,11,14,17}$ 20:4) (panel B).

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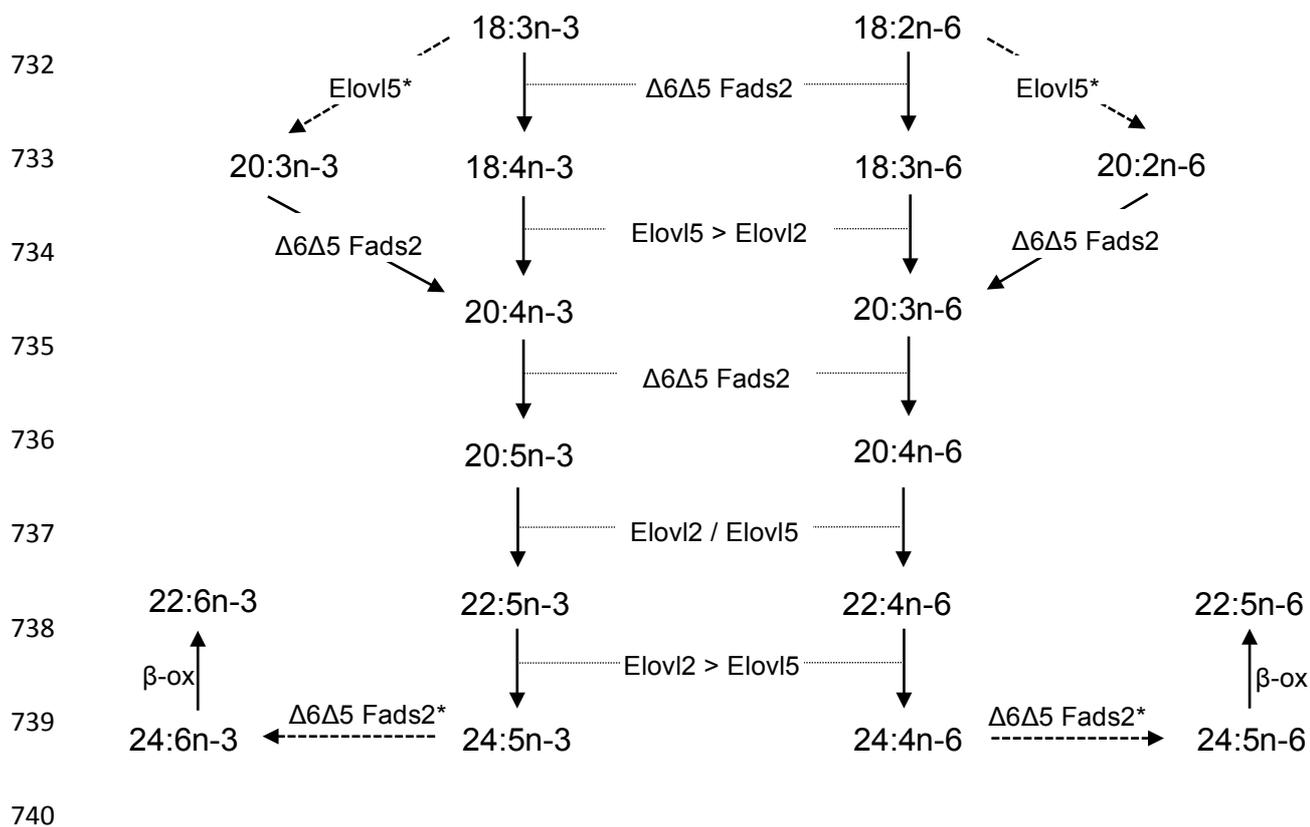
711 Fig. 4. Functional characterisation of the of the newly cloned *Clarias gariepinus* Elov12 in
 712 yeast (*Saccharomyces cerevisiae*). The fatty acid (FA) profiles of yeast transformed with
 713 pYES2 containing the coding sequence of *elov12* were determined after the yeast were grown
 714 in the presence of one of the exogenously added substrates 18:3n-3 (A), 18:4n-3 (B), 20:5n-3
 715 (C) and 22:5n-3 (D). Peaks 1-4 represent *S. cerevisiae* endogenous FAs namely 16:0 (1), 16:1
 716 (2), 18:0 (3) and 18:1n-9 (4). Additionally, peaks derived from exogenously added substrates
 717 (*) or elongation products are indicated accordingly.

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720 Fig. 5. Tissue distribution of *fads2*, *elovl2* and *elovl5* transcripts in *Clarias gariepinus*.
 721 Expression levels quantified for each transcript were normalised expression levels of the
 722 reference gene (28s rRNA) of the same tissue. The data are reported as mean values with
 723 their standard errors (n = 4). Within each target gene, different letters indicate statistically
 724 significant differences between expression levels (ANOVA and Tukey's HSD *post hoc* tests).

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741 Fig. 6. The biosynthesis pathway of long-chain polyunsaturated fatty acids ($\leq C_{24}$) from α -linolenic
 742 (18:3n-3) and linoleic (18:2n-6) acids in *Clarias gariepinus*. Enzymatic activities shown in the
 743 scheme are predicted from heterologous expression in yeast of the herein investigated $\Delta 6\Delta 5$ fatty acyl
 744 desaturase 2 ($\Delta 6\Delta 5$ Fads2) and Elov12 elongase, and the previously reported Elov15 (Abaga et al.,
 745 2005). β -ox, partial β -oxidation; Elov1, fatty acyl elongase; Fads, fatty acyl desaturase.

746 * Enzymatic activities not yet demonstrated in *C. gariepinus*.

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749 **Tables**

750 Table 1

751 Sequences of primers used for cDNA cloning and tissue expression analysis (qPCR) of
 752 *Clarias gariepinus fads2* and *elovl2*. Restriction sites *Bam*HI and *Xho*I are underlined.

Name	Direction	Sequence
<i>Initial cDNA cloning</i>		
FadCGF2F1	Forward	5'-ATGGGCGGCGGAGGACAC-3'
FadCGF2R1	Reverse	5'-GCATCTAGCCACAGCTCACC-3'
EloCGE2F1	Forward	5'-TACTTGGGACCAAAGTACATGA-3'
EloCGE2R1	Reverse	5'-AGATAGCGTTTCCACCACAG-3'
<i>5' RACE PCR</i>		
FadCGRF2R2	Reverse	5'-CGATCACAACCCACTGATCA-3'
FadCGRF2R3	Reverse	5'-CGTCCCTCCAGGATGTCTTTT-3'
EloCGRE2R3	Reverse	5'-AGCTTGCTGAAATAAGCTCCACT-3'
EloCGRE2R2	Reverse	5'-TGTAGAAGGACAGCATGGTGAC-3'
<i>3' RACE PCR</i>		
FadCGRF2F1	Forward	5'-CAGTCGCCATTCAACGATT-3'
FadCGRF2F2	Forward	5'-GAACACCATCTCTTTCCCATG-3'
EloCGRE2F1	Forward	5'-TTGTCCACCATTTCCTTCAATG-3'
EloCGRE2F2	Forward	5'-ACTGAACAGCTTCATCCATGTG-3'
<i>ORF cloning</i>		
FadCGF5UF1	Forward	5'-AGAGGAGCGCAGTGATGAG-3'
FadCGF3UR1	Reverse	5'-GTGGGAATTACAGAATTGTTATGG-3'
FadCGFVF	Forward	5'-CCC <u>G</u> GATCCAAGATGGGCGGCGGAGGAC-3'
FadCGFVR2	Reverse	5'-CCGCTCGAGT <u>T</u> ATTTGTGGAGGTATGCATC-3'
EloCGE2VF	Forward	5'-CCC <u>G</u> GATCCAACATGGATTTTATTGTGAAGAA-3'
EloCGE2VR	Reverse	5'-CCGCTCGAGT <u>C</u> ACTGCAGCTTATGTTTGGC-3'
EloCGE25UF	Forward	5'-CCAGTTACATTAAGAGGCACCG-3'
EloCGE23UR	Reverse	5'-AGATTAGTCAACATGAAAGGTGAA-3'
<i>Quantitative PCR</i>		
FadCGqF2F1	Forward	5'-TCCTATATGCTGGA <u>A</u> CTAATGTGG-3'
FadCGqF2R1	Reverse	5'-AGGATGTAACCAACAGCATGG-3'
EloCGqE2F1	Forward	5'-GCAGTACTCTGGGCATTTGTC-3'
EloCGqE2R1	Reverse	5'-GGGACATTGGCGAAAAAGTA-3'
EloCGqE5F1	Forward	5'-ACTCACAGTGGAGGAGAGC-3'
EloCGqE5R1	Reverse	5'-GGAATGGTGGTAAACGTGCA-3'
28SrRNAF1	Forward	5'-GTCCTTCTGATGGAGGCTCA-3'
28SrRNAR1	Reverse	5'-CGTGCCGGTATTTAGCCTTA-3'

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754 Table 2

755 Substrate conversions of *Saccharomyces cerevisiae* transformed with *Clarias gariepinus*
756 *fads2* coding region and grown in the presence of exogenously added substrate (18:3n-3,
757 18:2n-6, 20:3n-3, 20:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 or 22:4n-6). Conversions were
758 calculated according to the formula [individual product peak area / (all products peak areas +
759 substrate peak area)] × 100.

FA substrate	FA Product	Conversion (%)	Activity
18:3n-3	18:4n-3	42.0	Δ6
18:2n-6	18:3n-6	22.5	Δ6
20:3n-3	20:4n-3	12.9 ^a	Δ8
20:2n-6	20:3n-6	2.5 ^a	Δ8
20:4n-3	20:5n-3	18.7	Δ5
20:3n-6	20:4n-6	13.8	Δ5
22:5n-3	22:6n-3	Nd	Δ4
22:4n-6	22:5n-6	Nd	Δ4

760 ^a Conversions of Δ8 substrates (20:3n-3 and 20:2n-6) by Fads2 include stepwise reactions
761 due to multifunctional desaturation abilities. Thus, the conversion rates of 20:3n-3 and 20:2n-
762 6 include the Δ8 desaturation toward 20:4n-3 and 20:3n-6, respectively, and their subsequent
763 Δ5 desaturations to 20:5n-3 and 20:4n-6, respectively.

764 FA, Fatty acid; Nd, not detected.

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766 Table 3

767 Substrate conversions of *Saccharomyces cerevisiae* transformed with *Clarias gariepinus*
768 *elovl2* and grown in the presence of PUFA substrate exogenously added (18:3n-3, 18:2n-6,
769 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 or 22:4n-6). Conversions were calculated for
770 each stepwise elongation according to the formula [peak areas of first products and longer
771 chain products / (peak areas of all products with longer chain than substrate + substrate peak
772 area)] x 100.

FA substrate	FA Product	Conversion (%)
18:3n-3	20:3n-3	7.5
18:2n-6	20:2n-6	3.0
18:4n-3	20:4n-3	15.2
18:3n-6	20:3n-6	20.5
20:5n-3	22:5n-3	73.4
20:4n-6	22:4n-6	56.0
22:5n-3	24:5n-3	36.7
22:4n-6	24:4n-6	9.7

773 FA, fatty acid

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