Fatty acid desaturase 2 (*FADS2*) insertion/deletion polymorphism impact on muscle fatty acid profile in European grayling (*Thymallus thymallus*)

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Abstract

An insertion (In)/deletion (Del) polymorphism in the fatty acid desaturase 2 (*FADS2*) gene, which codes for Δ6-desaturase, was for the first time observed in fish. The polymorphism is located in the seventh intron of *FADS2* and the insertion consists of eleven repeats of CTGT (44 bp) with an allelic frequency, for the insertion, of 39 %. The polymorphism was associated with a modulation in Δ6-desaturase activity as significant effects on the ratio of EPA or DHA to their precursors were found ($P < 0.001$). A different distribution of SFA, MUFA and PUFA among the In/In, In/Del and Del/Del groups was also detected in fish fillet. The results suggest that genetic selection for this marker might improve the ability of European grayling to utilise dietary *n*-3 long-chain PUFA precursors, as Δ6-desaturase is the rate-limiting enzyme in the production of EPA and DHA from α-linolenic acid.

Key words: Δ6-Desaturase: EPA: DHA: Thymallidae: Mutations: Fish

Fish have the ability to elongate and/or desaturate fatty acid precursors to synthesise long-chain (LC) PUFA[1], but the efficiency of this metabolic pathway varies among fish species. In contrast to marine finfish species where the ability of synthesising LC-PUFA from precursors is limited and controversial[2,3], freshwater fish seem to be able to maintain a certain degree of Δ6-, Δ5-desaturase and elongase activities to produce arachidonic acid, EPA and DHA if their precursors linoleic and α-linolenic acids (ALA) are present in their diet. Fatty acid desaturase 2 (*FADS2*), also called Δ6-desaturase, is the rate-limiting enzyme in the production of EPA and DHA from ALA[4] even if two other enzymes (*FADS1*/Δ5-desaturase and elongase) are involved in this biosynthetic pathway. *FADS2* is a membrane-bound protein with amino-terminal cytochrome *b5* domains carrying haem-binding motifs, two-membrane-spanning domains and three His-box motifs[5]. *FADS2* converts ALA (18 : 3*n*-3) into stearidonic acid (18 : 4*n*-3) and linoleic acid (18 : 2*n*-6) into γ-linolenic acid (18 : 3*n*-6), which are the first step in the pathway of *n*-3 and *n*-6 LC-PUFA synthesis, respectively.

Δ6- and Δ5-Desaturases are of major interest in the field of fish nutrition, especially with regard to the possibility of dietary linoleic and ALA to fulfil the requirement of teleosts for the essential LC-PUFA of the *n*-3 and *n*-6 series. Until recently, LC-PUFA requirements have been satisfied by lipids from fishery-derived products, but such LC-PUFA sources have reached their limit of sustainability[6,7]. The ability to convert linoleic and α-linolenic fatty acids, abundant in alternative lipid sources such as vegetable oils, into LC-PUFA is well established in some species, such as rainbow trout[8–12], but has been poorly investigated in other freshwater species, including European grayling[13]. Individual variability in the capacity of fish to retain or synthesise *n*-3 LC-PUFA when fed vegetable oil diets has been shown in Atlantic salmon[14]. Moreover, Leaver *et al.*[15] demonstrated that flesh *n*-3 LC-PUFA composition is a highly heritable trait ($h^2 = 0.77$) in salmon. However, in their study, Morais *et al.*[16] showed that the hepatic expression of Δ6-desaturase was not responsible for the variations observed in the levels of *n*-3 LC-PUFA in four families of Atlantic salmon. Therefore, these authors have suggested that the observed variability might originate from the presence of different alleles of fatty acyl desaturases encoding proteins with altered biological activity or specificity.

Recent findings have outlined that genetic variation in *FADS1* and *FADS2* gene clusters is associated with altered desaturase activity in human subjects[17]. Moreover, *FADS2* polymorphism has been shown to modulate the activity of Δ6-desaturase in human subjects[18], Japanese quails[19] and pigs[20]; however, the association with desaturase activity has not yet been examined in fish. Therefore, *FADS2* is a candidate gene for increasing EPA and DHA synthesis from ALA in fish.

Abbreviations: ALA, α-linolenic acid; *FADS2*, fatty acid desaturase 2; LC-PUFA, long-chain PUFA.

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Thus, the present study aimed to study the Δ6-desaturase polymorphism and its impact on the muscle fatty acid profile in European grayling (*Thymallus thymallus* L., 1758), chosen as a cold freshwater fish model. This fish species has a natural distribution covering vast parts of Europe, from France and England eastwards to the Ural Mountains and from Scandinavia southwards to the Balkan region and Northern Italy[21]. Actually, there is a growing interest for this species in fisheries management and conservation plans (European Dir. Habitat 92/43/CEE) and, recently, also in aquaculture.

Materials and methods

Animal and rearing conditions

For the present study, 100 specimens of European grayling (initial body weight 12·1 (SD 0·1) g) were obtained from the hatchery Ribiška Druzina (Tolmin, Slovenija). Fish originated from a single stock were obtained by mating one 4-year-old female and two males. They were stocked in a 500-litre fibreglass tank in the experimental flow-through facilities of the Department of Food Science at the University of Udine (Italy) supplied with well water (water temperature 13·7 ± 0·22°C; dissolved oxygen 8·17 (SD 0·8) mg/l; pH 7·49 ± 0·21; total ammonia nitrogen 0·08 (SD 0·01) mg/l; photoperiod 12 h light–12 h dark). After stocking, fish were hand-fed a commercial diet for trout (feed formula: 42 % crude protein, 25 % crude lipid on dry feed, Dibaq ATX 5HMDI; Dibaq Italia) in two daily meals (09.00 and 16.00 hours) until the first feed item was refused, over 64 d. At the end of the trial, fish were weighed (final weight 31·8 (SD 2·5) g) under moderate anaesthesia (5 mg benzocaine/l alcohol solution) and twenty-seven specimens were killed with excess of the anaesthetic (30 mg benzocaine/l alcohol solution) to obtain the fillet. Handling of the fish was done according to the guidelines of the European Union Directive (2010/63/EU) on the protection of animals used for scientific purposes.

Molecular genetic marker evaluation

DNA was extracted from muscle samples (twenty-seven animals) using a standard DNA extraction method (GenElute Mammalian Genomic DNA Miniprep Kit; Sigma-Aldrich). A primer set (forward: AAAGAAGTTGAAGTACATGCCCTA; reverse: AGTTCCGTTGTGAAAACATGG) was designed in order to amplify a fragment of the *FADS2* gene based on the

Salmo salar sequence (GenBank accession no. AY736067.2). The forward primer was designed in *S. salar* exon 7 and the reverse one in exon 8. PCR conditions consisted of an initial step at 94°C for 3 min followed by forty cycles of 94°C for 30 s, annealing temperature (60°C) for 30 s and 72°C for 30 s. The final extension step consisted of 5 min at 72°C. PCR products were separated on a 2·5 % agarose gel in 1 × TBE buffer by electrophoresis. Separated fragments on agarose gels were sized by referencing with a 100 bp DNA ladder (Fig. 1). The bands were cut and DNA extracted with a gel extraction kit (Zymoclean Gel DNA Recovery Kit; Zymo Research). PCR products were sequenced from the gel extract using an ABI automated DNA sequencer (Applied Biosystems).

Sequence comparison

The sequence of European grayling (*T. thymallus*) was compared with the sequence of Atlantic salmon (*S. salar*, GenBank accession no. AY736067.2), cod (*Gadus morhua*, Ensembl ENSGMOG00000017746) and zebrafish (*Danio rerio*, Ensembl ENSDARG00000019532) using BLAST (bl2seq) from NCBI.

Lipid analysis

Muscle lipids were extracted with a chloroform–methanol (2:1, v/v) solution containing 0·01 % butylated hydroxytoluene according to Folch *et al.*[22].

A 5 % solution of HCl in methanol was used to prepare fatty acid methyl esters according to Christie[23]. Fatty acid methyl esters were separated and quantified by high-resolution GC (TRACE GC2000; Fisher Scientific SAS) using a 30 m × 0·32 mm (inner diameter) Omegawax™ Capillary GC Column (Supelco) with flame ionisation detection at 250°C. The carrier gas (H₂) flow rate was 1 ml/min, and samples (1 μl) were directly injected in a split ratio of 1:30. The oven temperature was programmed from 180°C for 6 min, then at 3°C/min until 225°C and held for 10 min. The chromatograms were acquired and processed using ChromQuest integration software (ThermoQuest Italia Limited) and individual methyl esters were identified by comparison with known standards obtained from Supelco, Inc. Repeated injections of the standard solution were carried out to test the analytical precision. All solvents and reagents were of analytical grade and were purchased from Sigma-Aldrich, Inc. Data on individual
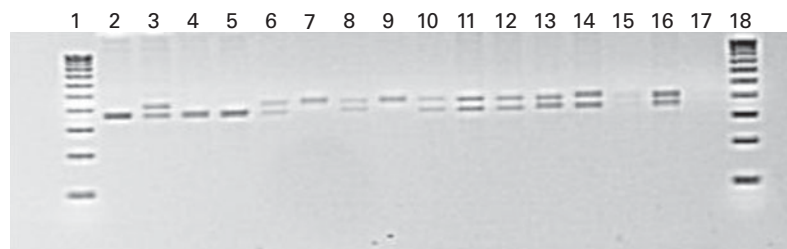

Fig. 1. PCR products of fatty acid desaturase 2. First and last lanes: molecular weight (100 bp/band); second, fourth and fifth lanes: homozygous for the deletion; seventh and ninth lanes: homozygous for the insertion; other lanes: heterozygous.

fatty acid composition are expressed as a percentage of total identified fatty acids.

Data analysis

Insertion/deletion associations with fatty acid proportions were analysed using the general linear mixed model procedure of SPSS version 17 (SPSS, Inc.) with a model that included insertion/deletion as a fixed effect. Significant differences were declared when the marker genotype effect was a significant source of variation in ANOVA, and the P value for the difference between the least-squares means for each marker genotype was less than 0·05. If appropriate, the post hoc least significant difference test was applied to compare the means.

Results

Sequencing revealed an insertion/deletion polymorphism in the seventh intron of FADS2. The insertion consists of eleven repeats of CTGT (44 bp); indeed, in this intronic region, the motif CTGT is repeated either forty times (insertion allele) or twenty-nine times (deletion allele). The allelic frequency was 39 % for the insertion (obtained by adding half of the heterozygous frequency to the homozygous frequency) and 61 % for the deletion.

The sequence for the deletion variant of the amplified fragment of FADS2 of European grayling is reported in Fig. 2 along with Atlantic salmon (S. salar), cod (G. morhua) and zebrafish (D. rerio) sequences. With respect to the exons, the intronic region was more dissimilar between salmon and grayling and the repeated motif was not present in S. salar. The grayling sequence of the exonic region was quite similar to that of D. rerio and G. morhua, while the intronic region was completely dissimilar.

The total lipid content of the fish fillets and the fatty acid composition associated with the observed polymorphism are presented in Table 1.

The FADS2 polymorphism is associated with clear differences in the incidence of specific fatty acid ratio along the desaturation pathway that almost reached significance in the case of 18:4n-3 to 18:3n-3 ($P=0.088$), but resulted highly significant in the case of 20:5n-3 to 18:3n-3 ($P=0.001$) and 22:6n-3 to 18:3n-3 ($P=0.001$). Such a clear effect of the polymorphism was not observed in the ratio of n-6 fatty acids, although the activity of Δ6-desaturase seemed to be lower in the Insertion/Insertion fish. The mutation is also associated with significant differences in palmitoleic acid, ALA, stearidonic acid and DHA percentages as well as SFA and MUFA.
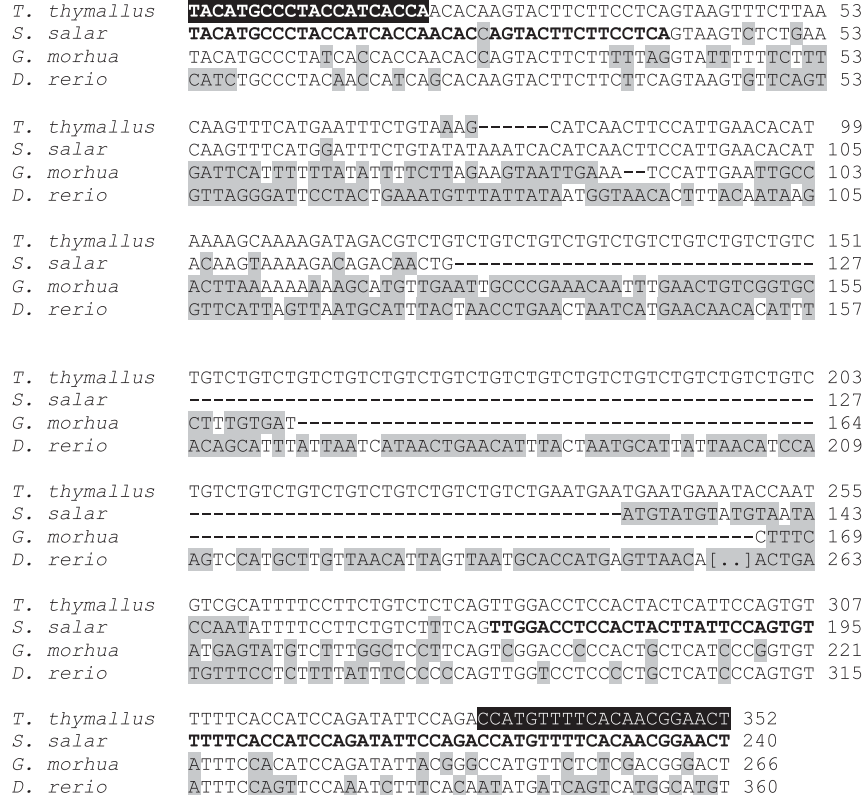

Fig. 2. Comparison of the nucleotide sequence of fatty acid desaturase 2 (FADS2) from Thymallus thymallus (deletion variant) with that of FADS2 from Salmo salar, Gadus morhua and Danio rerio. Sequences were aligned using Blast (bl2seq from the NCBI). White letters, black background: primers used; bold: salmon exons; grey background: sequence differences from T. thymallus.

Table 1. Genotypic least-squares means for fatty acid desaturase 2 on total lipid content (g/100 g muscle) and fatty acid concentrations (% total fatty acids)

(Least-squares mean values with their standard errors)

Fatty acid percentage	In/In LSM	SE	In/Del LSM	SE	Del/Del LSM	SE	P
10 : 0	0.13^a	0.11	0.40^b	0.05	0.55^b	0.07	0.03
12 : 0	3.07^a	1.98	7.83^b	0.89	9.53^b	1.15	0.03
14 : 0	3.33	1.72	5.15	0.77	6.77	1.00	NS
16 : 0	14.3	0.75	14.1	0.34	13.6	0.43	NS
16 : 1	3.21^{a,b}	0.67	2.73^a	0.30	1.68^b	0.39	0.065
18 : 0	3.42	0.24	3.53	0.11	3.77	0.14	NS
18 : 1n-9	20.8	1.70	19.4	0.76	17.8	0.98	NS
18 : 1n-7	3.12	0.96	2.83	0.44	1.98	0.57	NS
18 : 2n-6	11.5	0.91	12.7	0.41	12.3	0.53	NS
18 : 3n-6	0.42	0.11	0.37	0.05	0.40	0.06	NS
18 : 3n-3	0.60^a	0.95	2.19^a	0.43	3.72^b	0.55	0.02
18 : 4n-3	0.53^a	0.18	0.68^a	0.08	1.08^b	0.11	0.01
20 : 0	0.11	0.04	0.13	0.02	0.09	0.03	NS
20 : 1n-9	4.05	0.80	2.98	0.36	2.13	0.46	NS
20 : 4n-6	1.11^a	0.11	0.85^b	0.05	0.95^{a,b}	0.06	0.08
20 : 4n-3	0.44	0.07	0.49	0.03	0.55	0.04	NS
20 : 5n-3	2.84	0.25	2.22	0.11	2.33	0.15	NS
22 : 6n-3	17.2^a	1.43	13.3^b	0.64	13.0^b	0.82	0.05
18 : 4n-3 to 18 : 3n-3	0.87^a	0.18	0.48^{a,b}	0.08	0.39^b	0.10	0.088
20 : 5n-3 to 18 : 3n-3	5.27^a	0.87	1.74^b	0.40	1.02^b	0.50	0.001
22 : 6n-3 to 18 : 3n-3	31.1^a	4.84	10.4^b	2.24	5.6^b	2.79	0.001
18 : 3 n-6 to 18 : 2n-6	0.04	0.01	0.03	0.01	0.03	0.01	NS
20 : 4 n-6 to 18 : 2n-6	0.10^a	0.01	0.07^b	0.01	0.08^{a,b}	0.09	0.080
SFA	26.4^a	3.16	32.8^{a,b}	1.42	35.9^b	1.83	0.047
MUFA	34.5^a	3.15	30.4^{a,b}	1.41	25.7^b	1.82	0.043
n-6 PUFA	14.8	1.01	16.0	0.45	15.4	0.59	NS
n-3 PUFA	23.2	1.63	19.0	0.73	22.2	0.94	NS
Genotypic frequency (%)	11		56		33		
Fat content (g/100 g)	2.36	0.32	2.76	0.14	2.60	0.19	NS

In, insertion; Del, deletion.
^{a,b} Mean values within a row with unlike superscript letters were significantly different (P<0.05).

Discussion

In the present study, we revealed the presence of a polymorphism in the *FADS2* gene of *T. thymallus*. The repeated motif where we observed the insertion/deletion polymorphism was not present in *S. salar* nor in *G. morhua* or in *D. rerio* sequences, suggesting that if these species present polymorphisms in the *FADS2* gene, they will be different.

The effect of the *FADS2* polymorphism on Δ6-desaturase activity was first observed in human studies[24] where an association with the EPA:ALA ratio was also outlined. Moreover, in European adolescents, one minor allele of the *FADS1–FADS2* gene cluster was associated with higher Δ6-desaturase activity levels[25]. More recently, *FADS2* polymorphisms have also been observed in other mammals, such as pigs[20], where the authors have also shown a modulation of Δ6-desaturase activity. The insertion/deletion polymorphism in the seventh intron of the *FADS2* gene of *T. thymallus* observed in the present study affected DHA and ALA proportions in fillet lipids, which is in agreement with studies on human subjects showing that SNP in the *FADS1–FADS2* gene cluster reduces EPA concentrations and increases ALA concentrations in plasma phospholipids[17,26], but also in breast milk[27] and adipose tissue[28]. Several other studies in human subjects have shown the associations between mutations in the *FADS1–FADS2* gene cluster and fatty acid composition (reviewed in Merino *et al.*[18]). Consistently, Khang *et al.*[19] observed significant associations between the *FADS2* polymorphism and the DHA content in the egg yolk of Japanese quail.

Also in the present study, the ratio of products:precursors was used to assess the activity of Δ6-desaturase, as already proposed by Merino *et al.*[17]. The insertion of the repeated motif resulted in an increase of Δ6-desaturase activity in fatty acid biosynthetic pathways for n-3 PUFA. However, the polymorphism does not seem to affect the activity of the enzyme on n-6 PUFA. This might result from a different activity of the enzyme on the different precursors (n-3 or n-6), as it has been observed in dogs[29] and Murray cod[30]. Moreover, Thanuthong *et al.*[31] showed that, in the trout, the Δ6-desaturase has a 3.2-fold higher affinity towards 18 : 3n-3 over 18 : 2n-6. Despite a relatively limited number of animals analysed in the present study, these preliminary results suggest that the genetic selection of European grayling for this marker might improve its ability to utilise dietary LC-PUFA precursors, as *FADS2* is the rate-limiting enzyme in the production of EPA and DHA from ALA.

As nutritionists have recommended the consumption of fish for its beneficial properties due to the high content in LC-PUFA, namely EPA and DHA, which have a variety of important physiological functions[32–36], a similar approach applied to other finfish species of aquaculture interest may, by using marker-assisted selection, allow an improvement in the utilisation of dietary vegetable oils and meals, rich in *n*-3 precursors, and also provide human consumers a healthier fish product.

Acknowledgements

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