Δ6 FATTY ACYL DESATURASE GENE IN A SOUTHERN BLUEFIN TUNA (*Thunnus maccoyii*) CELL LINE

Senni Juniawati Bunga[1]*, Kathy Schuller[2]
[1]Department of Food Crop and Horticulture, Kupang State Polytechnic of Agriculture, Kupang, Indonesia.
[2]Laboratory of Biological Sciences, Room 320, Flinders University, Adelaide, 5042, South Australia.
*e-mail : senny_pung@yahoo.com

ABSTRACT

Fish is the main source of ω-3 long chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:5ω-3), which have positive effects on human health and can be beneficial in human diet context. Studies involving fatty acyl desaturase (Fads) and elongase of very long chain fatty acids (Elovl) enzymes that convert C18 PUFA to C20/22 LC-PUFA have been performed in some fish species. However, very little is known about LC-PUFA biosynthesis in tuna species. This study investigated the Δ6 Fads gene performance in the SBT cell line. The Δ6 Fads nucleotide sequences from various fish species were identified and retrieved to compare them with the SBT Δ6 Fads nucleotide sequence. The Δ6 Fads gene was performed using real time PCR (RT-PCR) and then was compared it with β-actin gene performance as a reference housekeeping gene. By performing multiple sequence alignments and comparing the highly conserved regions among fish Δ6 Fads sequences, the SBT Δ6 Fads nucleotide sequence was determined to be ≥ 75% identical to the other fish Δ6 Fads sequences. The results showed that when the SBT Δ6 Fads and β-actin cDNAs were performed in a standard PCR system and the products were analysed by electrophoresing them on a 2% (w/v) agarose gel, the target genes that were obtained were similar to the expected sizes. The observed band size for the Δ6 Fads PCR product was 207 bp and for the β-actin PCR product was 98 bp. The presence of the observed bands indicated that the primer pairs that were designed and used were successfully amplified the target genes. The results of this study might provide relevant information to support further investigating of the desaturase and elongase gene expression that might contribute to a better understanding of ω-3 LC-PUFA biosynthesis in fish.

Key words: Δ6 Fads gene, Southern Bluefin tuna, cell line, ω-3 LC-PUFA

INTRODUCTION

Tunas are large and highly athletic predatory fish that are widespread in tropical and temperate seas and contain high concentration of omega-3 (ω-3) long chain-polyunsaturated fatty acids (LC-PUFA), especially eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3), in their flesh (Givens & Gibbs. 2006). These fatty acids are essential in the diets of marine fish such as seabreams and founders and probably also tunas, but tunas have not yet been investigated in this way (Tocher 2010). Oil-rich fish are unique sources in the human diet of the omega-3 LC-PUFA such as EPA and DHA (Givens & Gibss 2006), that have well-established beneficial effect in a number of inflammatory and pathological conditions, including cardiovascular and neurological diseases (Calder 2003). Through sufficient level of incorporation EPA and DHA influence the physical nature of cell membranes and membrane responses, cell signaling and gene expression in many different cell types (Calder & Yaqoob 2009).

In vertebrates, LC-PUFA can be biosynthesized from short-chain (C18) PUFA (Miller et al. 2008). There are two types of LC-PUFA, omega-3 (ω-3) and omega-6 (ω-6). The ω-3 LC-PUFA are biosynthesized from α-linolenic acid (ALA, 18:3ω-3) whereas the ω-6 LC-PUFA are biosynthesized from linoleic acid (LA, 18:2ω-6). ALA cannot be used to biosynthesize ω-3 PUFA. However, the enzymes used to biosynthesize ω-3 LC-PUFA and ω-6 LC-PUFA are the same. These enzymes are called Δ5 fatty acyl desaturase (Δ5Fads), Δ6 fatty acyl desaturase (Δ6Fads), fatty acyl elongase 2 (Elovl2) and fatty acyl elongase 5 (Elovl5).

The biochemical and molecular genetics regulation of LC-PUFA in various fish species has attracted increasing in recent years. For example, the LC-PUFA biosynthesis pathway has been studied in great detail in Atlantic salmon (Minghetti et al. 2011). These fish were shown to have multiple Fad proteins with the necessary Δ6 and Δ5 desaturase activities as well as, and Elovl proteins, including Elovl5 and Elovl2. Moreover, several studies have revealed that Atlantic salmon fads and elovl genes are up-regulated at the transcriptional level and LC-PUFA biosynthesis is increased in response to reduced dietary LC-PUFA (Leaver et al. 2008).

Compared with the wealth of information available for Atlantic salmon, very little is known about LC-PUFA biosynthesis in SBT or any other tuna species (Gregory et al. 2010). This is largely due to the fact that SBT cannot be bred in large numbers in captivity and wild-caught SBT are too commercially valuable to be used for traditional fish feeding trials. Therefore, in this study, a SBT cell line as an in vitro model of fatty acid metabolism in tuna was used. produce tuna with high yields of DHA. This is important because DHA has many human benefits and fish are the main source of DHA in the human diet (Calder & Yaqoob 2009).

The specific aims of this study were to culture the SBT cell line in the presence of 10% (v/v) of foetal bovine serum (FBS) and to determine *Δ6 fatty acyl desaturase* (*Δ6 fads*) gene in the SBT cell line. Overall, the results of this study will be useful for designing feeds for farmed SBT that

MATERIALS AND METHODS

Routine Culture/Subculture of the Adherent Cells

The SBT-E1 cell line used in this study was originally established from tissues of a southern bluefin tuna (SBT, *Thunnus maccoyii*) fingerling (Bain et al. 2013). Several aliquots of the cell line were revived from cryostorage in liquid nitrogen. For routine culture, the SBT-E1 cells were maintained in 25 cm^2 flasks containing complete medium which consisted of 5 ml of Leibovitz's L-15 medium (Gibco®) supplemented with 15 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.4 (Sigma), 1% (v/v) of penicillin-streptomycin-glutamine 100X, (Gibco®) and 10% (v/v) of foetal bovine serum (FBS) (Sigma).

The cultures were monitored daily and the medium was changed after 2-3 days. The cells were sub-cultured upon forming confluent monolayers. To do this, the medium was decanted and the cell monolayers were washed three times with 1X phosphate buffered saline (PBS) (Gibco®) before the addition of 1 ml of 1X Trypsin/EDTA (Gibco®). After approximately 5 minutes, the cells had detached from the flask surface. The detached cells then were resuspended in 4 ml of complete medium before being transferred to a new 75 cm^2 flask. The total volume of the complete medium in the new flask was made up to 15 mL and the subsequent sub-culturing time ranged from 2-5 days.

Harvesting the Cells

After 4 days, the medium was removed and the cell monolayer was washed twice with 2 mL of PBS and the cells were detached by adding 1 mL of 1X Trypsin/EDTA (Gibco®) solution. The cells then were suspended in L-15 medium containing 2% (v/v) FBS and centrifuged at 1,000 g for 5 min to pellet the cells. After centrifugation, the cell pellet was washed once with PBS, re-suspended in 1 ml of PBS and transferred to a 1.5 ml tube. The cells were centrifuged again at 10,000 g for 5 min and the PBS was decanted. Finally, the cell pellets were homogenised with RNA extraction buffer.

RNA Extraction from the SBT Cells

Purification of total RNA from the SBT cell pellets was performed using the RNeasy®Mini Kit (Qiagen) according to the manufacturer's protocol. The SBT cell pellets (containing ≤ 5 x 106 cells) were disrupted in a micro centrifuge tube containing 350 µl RLT buffer supplemented with 1% (v/v) β-mercaptoethanol and the homogenized lysate was transferred into a QIAshredderTM spin column (Qiagen) for 2 min at 10,000 g. After purification, the RNA concentration was checked by UV absorbance at 260 nm using a Thermo Scientific NanoDrop®1000 spectrophotometer.

First-Strand cDNA Synthesis from the Extracted RNA

First strand cDNA synthesis was performed using 5 µg of the total RNA together with the M-MuLV reverse transcriptase (RNAse H-) supplied by New England BioLabs® Inc. Briefly, 5 µg of the total RNA was mixed with 1 µl of oligo (dT) (50 µM) and 1 µl of 10 mM dNTP mix and then RNase-free water was added to make a total volume of 12 µl. The mixture was heated to 70°C for 5 min and then placed immediately on ice for at least 1 min.

After adding 4 µl of 5x first-strand buffer, 2 µl of 10X DTT (0.1 M), 1 µl of RNaseOUT inhibitor and 1 µl of SuperScript™ III RT (200 units/µl), the final volume of each reaction was adjusted to 20 µl. The reactions were then incubated at 42°C for 1 hour. Finally, to inactive the reverse transcriptase enzyme, the reactions were heated to 80°C for 5 min.

Sequence Alignment

Δ6 fatty acyl desaturase (Δ6Fads) nucleotide sequences from different fish species were aligned using the ClustalX2 program provided by EMBL-EBI service to determine the regions of conservation. The Δ6Fads nucleotide sequences from seven fish species then were imported into FASTA format from NCBI website (http://www.ncbi.nlm.nih.gov). All imported sequences were loaded and verified by doing complete alignment on the ClustalX2 tools. The alignment was saved into MSF format and finally, uploaded them into the GeneDoc software for getting publication quality images based on colour shading. This enabled the design of the forward and reverse primers for polymerase chain reaction (PCR).

Primer Design

PCR Primers for the Δ6Fads gene were designed by the author of this study according to the published SBT amino acid and nucleotide sequences accessed from the GenBank database (http://www.ncbi.nlm.nih.gov). The β-actin PCR primers were designed by Dr. Peter Bain from Flinders University. The Δ6Fads primers were designed based on a SBT Δ6Fads sequence (GenBank accession no.HM032095.1) with the aid of the Primer3 software tool (http://primer3.wi.mit.edu). The forward primer was designed in the original 5'→3' direction, while the reverse primer was designed in the reverse direction and complementary to the coding sequence.

To check the properties of the potential primers (G/C content, optimal annealing temperature, optimal primer length, the likelihood of the primers to form homo-dimers), the primer pairs were analyzed using IDT oligoAnalyzer (http://idtdna.com). Each primer was designed to be 20 nucleotides long with a GC content of at least 50%. The melting temperature (Tm) for the primers was designed to be between 50°C and 60°C.

Standard PCR

The primer pairs were tested to ensure that the expected PCR product was being amplified. The standard PCR reaction was done using GoTaq®DNA polymerase according to the protocol described in the manufacturer's instructions (Promega). The reaction was performed in a GeneAmp PCR system 2400 (Perkin Elmer). Amplification involved an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing between the primers and the template at 57°C for 30 seconds, and extension between the primers after

annealing to the template at 72°C for 1 min, followed by a final extension at 72°C for 5 min.

The PCR products from the PCR reaction mixture then were purified with the aid of the Promega Wizard® SV Gel and PCR Clean-Up System by using the centrifugation protocol, according to the manufacturer's instructions. Following the clean-up step, the PCR products were then quantified from their absorbance at 260 nm and stored at -20°C for further use.

Agarose Gel Electrophoresis

The extracted RNA and the PCR products from the PCR reaction mixture (before and after purification) were analyzed by electrophoresing them on a 2% (w/v) agarosegel. The gel was prepared by dissolving 0.6 g of analytical grade agarose in 30 mL of 1X TAE buffer using a microwave oven for melting the agarose. Three microliters of SYBR®Safe DNA gel stain (10,000X concentrate in DMSO) supplied by Invitrogen was added to the dissolved agarose gel and poured into the agarose gel apparatus. The gel was then immersed in 1X TAE buffer in the agarose gel electrophoresis tank.

RESULT

SBT-E1 Cell Line

The SBT-E1 cell line was established from tissues of a southern bluefin tuna (SBT, *Thunnus maccoyii*) fingerling (Bain et al. 2013). The cell line was used at passage number 48. Images of the SBT-E1 cells are shown in Figure1. Cells with epithelial-like morphology were visible 4 hours after retrieving them from liquid nitrogen storage (Figure1A). The cells formed a monolayer after incubating them for 2-5 days. Figure 1B shows epithelial-like morphology of the cells and the cells displayed greater than 80% confluence after incubation for more than 3 days (Figure1C & 1D).

Multiple Sequence Alignment

The nucleotide sequences of a number of fish Δ6Fads cDNAs are compared in Figure2. The SBT Δ6Fads nucleotide sequence was 100% identical to the Δ6Fads nucleotide sequence of the Northern Bluefin Tuna (NBT). Identical residues are shaded black, and similar residues are shaded grey. Dark grey shading indicates ≥75% identity and light grey indicates ≥50% identity. The red boxes indicate the annealing sites for the polymerase chain reaction (PCR) primers.

The primers were designed to exactly match the SBT and NBT sequences, because they were 100% identical to each other. The forward primer annealing site is indicated by the right arrow line and the reverse primer annealing site is indicated by the left arrow line. The blue boxes indicate highly conserved regions between all sequences.

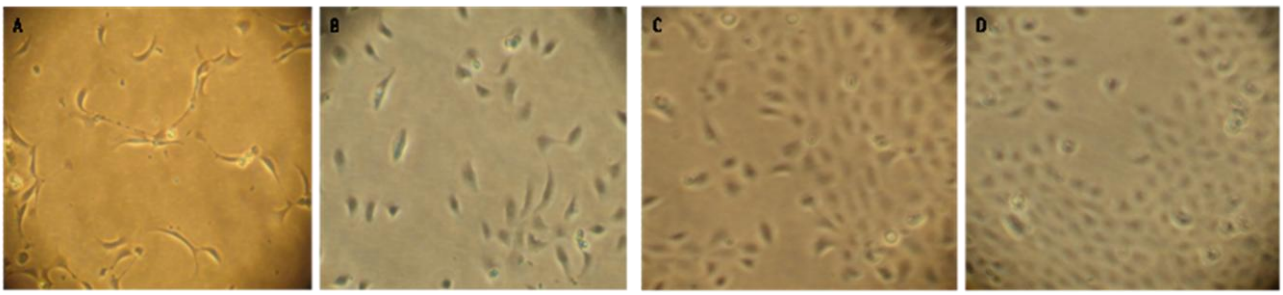

Figure 1. SBT E1 cell images after retrieving them from liquid nitrogen storage. The cells were cultured in Leibovitz's L-15 medium containing 10% FBS (v/v). The cell images were captured at 10X magnification using an Olympus CK2 microscope (Olympus Optical Co, LTD). (A) after 4 hours incubation, (B) after 1 day incubation, (C) after 3 days incubation, and (D) after 5 days incubation.

Identical residues are shaded black, and similar residues are shaded grey. Dark grey shading indicates ≥75% identity and light grey indicates ≥50% identity. The blue boxes indicate highly conserved regions between the sequences. The regions selected to design the forward and reverse primers used for PCR are in red boxes. The forward primer annealing site is indicated by a right arrow and the reverse primer annealing site is indicated by a left arrow.

Primer Design

There were two different primer pairs that were designed and used in this study. The primer pairs were designed to amplify SBT Δ6Fads and SBT β-actin cDNAs. Each primer pair was used for PCR amplification of cDNA templates. For Δ6Fads cDNAs, the primers were designed based on regions selected from a multiple sequence alignment of various fish Δ6Fads cDNAs (Figure2). The primers for the β-actin cDNA were designed previously by Dr. Peter Bain. The nucleotide sequences of the primers and PCR product sizes that were expected are shown in Table 1.

Standard PCR

Standard PCR for both the SBT Δ6Fads and β-actin cDNAs was performed using the same first strand cDNA template prepared from the SBT cells cultured in the presence of 10% FBS. The obtained PCR products were analysed using a 2% (w/v) agarose gel (Figure3). For each cDNA, a single PCR product was obtained with a size close to the expected size. The expected size for the Δ6Fads PCR product was 207 bp and for the β-actin PCR product it was 98 bp. The observed sizes (Figure3) were similar to the expected sizes (Table 1).

In addition, the optimal annealing temperatures for the Δ6Fads and β-actin PCR primers were 54.9°C and 53°C, respectively (Figure 4). However, the annealing temperature performed in the standard PCR was 57°C.

Table 1. Nucleotide sequences of the primer pairs used inqRT-PCR and GenBank accession number of the sequence used as for primer design and expected amplification product size.

Gene		Primer sequence	Accession no.	Product size (bp)
Δ6Fads	Forward	5′-ACTACCTTCGCTACTTCTGC-3′	HM032095	207
	Reverse	5′-AGTGGGACTGCTCGATATTG-3′		
β-actin	Forward	5′-TCCCTGGAG AAG AGCTACGA-3′	*	98
	Reverse	5′-AGGAAGGAAGGCTGGAAGAG-3′		

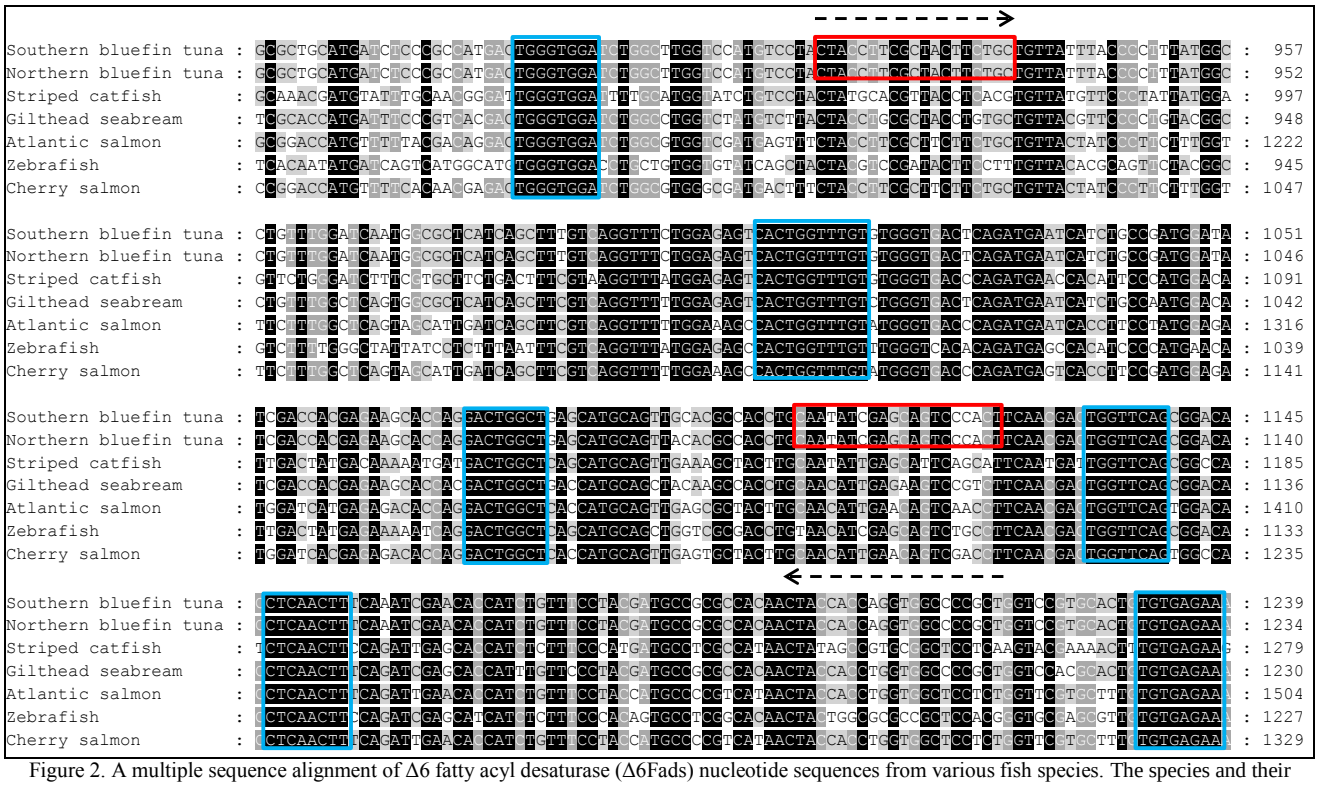

Figure 2. A multiple sequence alignment of Δ6 fatty acyl desaturase (Δ6Fads) nucleotide sequences from various fish species. The species and their accession numbers were Southern bluefin tuna *(Thunnus maccoyii*, HM032095.1), Northern bluefin tuna (*Thunnus thynnus*, HQ214238.1), Striped catfish (Pangasianodon hypophthalmus, JX035811.1), Gilthead seabream (Sparus aurata, GQ162822.1), Atlantic salmon (Salmo salar, NM_001172281.1), Zebrafish (Danio rerio, AF309556.1), and Cherry salmon (Oncorhynchus masou, AB074149.1). All accession numbers are from the GenBank database.

DISCUSSION

A southern bluefin tuna cell line designated SBT-E1 grows healthy under a normal atmosphere condition (25^{o}C) in a complete medium as described in the procedure. It is likely that cells with the optimal condition and medium have established as effective. The temperature of cell incubation was determined created on the normal body temperature of bluefin tuna, ranged 25^{o}C-30^{o}C (Carey & Teal, 1969). The optimal medium for cells seeded in Leibovitz's L-15 supplemented 10% of FBS. In a case of cell line culture, some studies reported that fish cell line are commonly used to detect and characterize pathogenic infections in fish species (Crane et al. 2000; Chen et al. 2004; Gong et al. 2011; Hasoon et al. 2011). In addition, fish cell lines have also been used as in vitro models of lipid metabolism (Tocher & Dick 1990; Gregory et al. 2011; Minghetti et al. 2011). During the establishment of the SBT-E1, there has been no evidence of infection or contamination.

Recently, the expression of *desaturase* and *elongase* genes has been investigated in fish species (Morais 2011; Gregory et al. 2010). Other study that explained that besides melting temperature, proper primer design was the primary factor that affects the function of the oligonucleotides (Dieffenbach et al. 1993).

In case of multiple alignment visualization, when the Δ6FadscDNAs from various fish species were annotated and retrieved in the GeneBank database to compare with the SBT Δ6FadscDNAs, the SBT Δ6Fads nucleotide sequence was very similar to the other fish Δ6Fads nucleotide sequences. In some regions, the sequences from the different fish species were 100% identical. The regions that are identical were likely to code for active site of the enzyme. This alignment feature is characteristic of an amino acid sequence of the SBT Δ6Fads comparing to other fish species sequences. Clearly, the Δ6Fads sequence has an N-terminal extension in SBT and NBT but is not likely similar to the regions of the other fish.

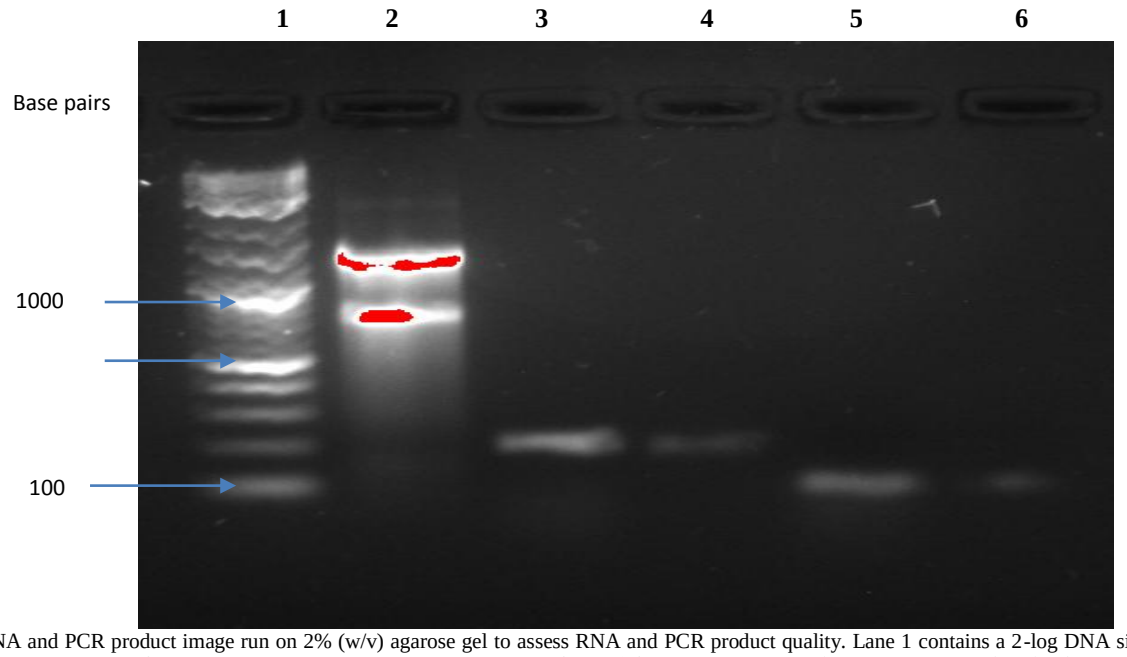

Figure 3. RNA and PCR product image run on 2% (w/v) agarose gel to assess RNA and PCR product quality. Lane 1 contains a 2-log DNA size marker. Lane 2 contains 3.5 µg RNA from the SBT cell line cultured in the presence of 10% (v/v) FBS. Lane 3 contains 2.3 µg PCR product for the Δ6Fads cDNA; lane 4 contains 0.2 µg cleaned-up PCR product for the Δ6Fads cDNA; lane 5 contains 2.5 µg PCR product for β-actin cDNA; lane 6 contains 0.2 µg cleaned-up PCR product for β-actin cDNA.

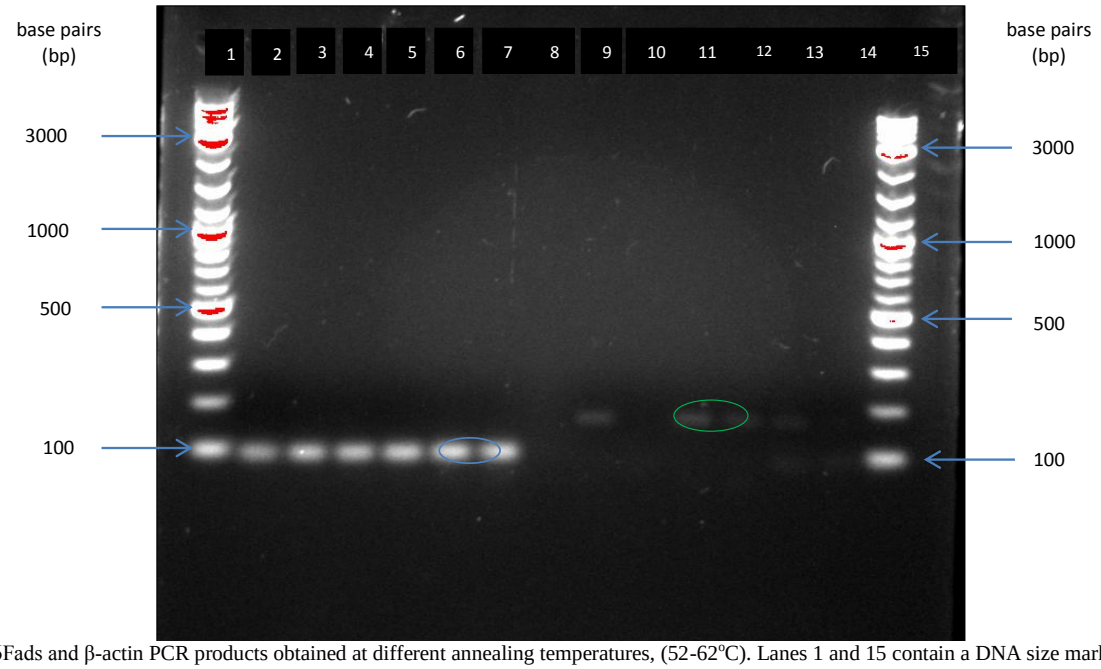

Figure. 4. Δ6Fads and β-actin PCR products obtained at different annealing temperatures, (52-62^{o}C). Lanes 1 and 15 contain a DNA size marker (100-3000 bp). Lanes 2-7 contain 5 µl of β-actin PCR reaction mixture obtained at annealing temperatures of 61.4^{o}C, 59.4^{o}C, 57.8^{o}C, 54.9^{o}C, 54.4^{o}C, and 53^{o}C, respectively. Lanes 8-14 contain 5 µl of Δ6Fads PCR reaction mixture obtained at annealing temperatures of 61.4^{o}C, 59.4^{o}C, 57.8^{o}C, 54.9^{o}C, 54.4^{o}C, and 53^{o}C, respectively. Green and blue circles indicate that the optimal annealing temperatures for the Δ6Fads and β-actin PCR primers were 54.9^{o}C and 53^{o}C, respectively.

This study found that the primer pairs were designed can amplify the *SBT Δ6Fads* gene. Thus, this gene or enzyme has also been proposed to have a role in PUFA *de novo*. The presence of the observed bands indicated that the primers were successfully designed and amplified the target genes.

Additionally, the optimal annealing temperature (57^{o}C) for the standard PCR with Δ6Fads and β-actin primers was determined based on the bright visible bands. Thus, *Δ6Fads* gene performance can be amplified well by 5'-ACTACCTTCGCTACTTCTGC-3' for forward direction and 5′-AGTGGGACTGCTCGATATTG-3′ for reverse direction.

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