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Identification of the First Transgenic Aquatic Animal in Iran by PCR-Based Method and Protein Analysis

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Abstract

In the recent years, there is evidence of training a red type of zebrafish which differs from wild-type in body color. There is not any document how it reaches to the ornamental fish farms of Iran but at first, it was a doubt it belongs to a morphotype or genetic modification (GM). First of all, a set primer was designed to validate zebrafish species. Mitochondrial 16S rDNA was selected and amplified. The validation of 16S rDNA gene sequences data detected three haplotypes deposited in the GenBank. By seeking scientific articles about transgenic zebrafish, the first attention went to Glowfish (color transgenic zebrafish). We decided to build a foundation of PCR based detection for finding a gene correspondence red color of zebrafish. This sequence is specific for producing a red color in the sea anemone and it was not in genome database of wild zebrafish. A set primer was designed according to the red color of sea anemone (dsRed) to make a fragment about 680 bp in red zebrafish and cream zebrafish specimens (they had red parents). Fragment sequencing (ds/Red) revealed that it belongs to the sea anemone and none of the samples nucleotides differ from each order and positive sequence from a plasmid containing dsRed. Also, protein samples from cream and red zebrafish were extracted and evaluated with fluorescence microscope by TxRed control color. A dark and a red spread was detected for protein smear of the cream and redfish respectively. A dark spread was detected for cream fish protein smear but a red smear was marked for redfish protein. This research simply demonstrates the existence of transgenic zebrafish in Iranian ornamental fish industry.

Key words: Zebrafish; *16S rDNA* gene; *ds/Red* gene; Genetic modification

Introduction

The zebrafish (*Danio rerio*) is a tropical freshwater fish belonging to the minnow family (*Cyprinidae*) of the order *Cypriniformes*. Native to the Himalayan region, it is a popular aquarium fish, frequently sold under the trade name zebra danio. The zebrafish is also an important and widely used vertebrate model organism in scientific research and was among the first vertebrates to be cloned. It is particularly notable for its regenerative abilities and has been modified by researchers to produce many transgenic strains. This fish is a member of the Iranian ornamental fish industries and in recent years applied for human diseases model. Transgenic fish technology is useful for basic science such as organ developmental research in vertebrates, as a model in finding gene function, especially in human disease, and is helpful for producing ornamental fish. Furthermore, the most important application related to develop high-quality white meat fish for human consumption . Two important transgenic fish approved by FDA are *GlowFish* and *AquAdvantage* Salmon. *GlowFish* is a genetically modified zebrafish (*Danio rerio*) for ornamental and bioreactor application introduced by Gong *et al*., 2003 (Gong *et al.*, 2003) in Singapore and *AquAdvantage is* a genetically modified

salmon approved by FDA for food consumption and commercialization in November 2015 (Waltz, 2016). Transgenic salmon produced by "all-fish" genes; has DNA construct containing the promoter and terminator regions of the antifreeze protein (AFP) gene from the ocean pout (*Macrozoarces americanus*) linked to the GH coding region (cDNA) from the chinook salmon (*Oncorhynchus tshawytscha*) (Du *et al*., 1993; Butler and Fletcher, 2009). *GlowFish* is a zebrafish modified by insertion of Fluorescent protein genes (GFP, YFP, BFP or RFP/ dsRED) and chromoproteins. These proteins are from jellyfish (*Aequorea victoria*), site direct mutagenesis of GFP, *Discosoma sp*, and carpet sea anemone (*Stichodactyla haddoni*), respectiveley for GFP, YFP, and BFP, RFP/dsRed, (Chiang *et al.*, 2014) and chromoprotein following a strong muscle specific promoter such as the *mylz-2* (Gong *et al.*, 2003; Zeng *et al.*, 2005) and registered for commercialization in ornamental fish trade (Gong *et al.*, 2006). Freshwater ornamental fish breeding and distribution is growing and has achieved a combined annual turnover of more than USD 15 million. At least 85 different species are currently bred in Iran. The intention of the government is to expand this activity by importing annually up to five new species to be bred and distributed. Production is centered in Kashan, Tehran, Arak and Gilan. The number of species handled by the industry will increase to 115 species during the period of the plan. Zebrafish is one of these species easily accepted as pet fish for many years. However, in recent years, there is some evidence for increased reproducing of red color zebrafish than wild type in Iran's land commercial ornamental fish farms as a result of higher market demand for color. In spite of advantages of the transgenic animal in producing high quality white meat and have commercial benefit for ornamental color fish producing, but any escape of transgenic fish maybe have damage for our important endemic and economic aquatic species. Therefore this research could be show and identified scientific data for more attention to protecting aquatic animal resources in Iran. Main goal of this study is about pay attention to field of transgenic animal, benefit, disadvantages and molecular detection of transgenic fish. There is not any document how this type of zebrafish introduced into the Iran, but we decided to determine if this type is a kind of transgenic color zebrafish by molecular technique and protein analysis.

Materials and Methods

Sample collection, DNA extraction and species validation of zebrafish by PCR-based assay and sequencing of *16S rDNA* gene

A total number of 10 red zebrafish breeder and cream zebrafish (Fig. 1) offsprings specimens left from the red male and female zebrafish were collected from a farm breeding ornamental fish in Gilan province, respectively. DNA was extracted from all fish with 10 mg fresh muscle tissue with tissue extraction kit (Qiagen) according to the manufacture's protocol. A wild type zebrafish DNA sample was applied for control in transgenic zebrafish PCR assay. PdsRed-1 plasmid was used for PCR amplification of dsRed fragment as positive control. 16S rDNA mitochondrial gene was selected to validate *Danio rerio* (zebrafish) species. Primers for 16S rDNA gene designed according to multiAlign all haplotypes of 16S rDNA gene for zebrafish. Primer concentrations in the PCR assay were 1 mM of primer F 16S rDNA and 1 mM for primer R 16S rDNA. DNA concentration was 1 ng/µl in each tube. Annealing temperature: 60ºC, $MgCl_2$ concentration: 2 mM. PCR cycling conditions: initial activation step: 5 min at 94ºC, 35 cycles of 30 sec. at 94ºC, 30 sec. at the annealing temperature 58ºC, 30 sec. at 72ºC, followed by a final extension step of 10 min at 72ºC. 16S rDNA gene amplified a fragment approximately 240 bp (Fig. 2A). Sequencing was done by Macrogen Company (South Korea). All three zebrafish haplotypes mentioned in NCBI (A, B, C) were observed in our samples and total of 20 samples were *Danio rerio* species according to accession number of 16S rDNA gene for zebrafish were deposited in GenBank for zebrafish (Fig. 3A).

Fig. 1. Red and cream zebrafish

Primer design for dsRed fragment in muscle tissue and positive control of dsRed in plasmid containing dsRed gene, Sequencing of amplified fragments and analyzing

Primer for the dsRed gene (dsRed positive) was designed according to the sequence of this gene in pdsRed-1 Vector from the first nucleotide to the end. Primer concentrations in the PCR assay were 0.5 mM of primer F dsRed and 0.5 mM for primer R dsRed. DNA concentration was 2 ng/µl in each tube. Annealing temperature: 55ºC, MgCl2 concentration: 1.5 mM. PCR cycling conditions: initial activation step: 10 min at 94ºC, 35 cycles of 30 sec. at 94ºC, 30 sec. at the annealing temperature 55ºC, 45 sec. at 72ºC, followed by a final extension step of 10 min at 72ºC. All of the extracted DNA samples and vector contain dsRed gene amplified a fragment approximately 680 bp (Fig. 2B). A wild type zebrafish DNA did not amplify any dsRed fragment. The sequences of both the red zebrafish and the cream ones who were the red one's offsprings amplified the dsRed and pdsRed-1 plasmid (positive control of dsRed) fragments. It confirmed that these fragments are identical in the sequence, indicating any variation was found even in one nucleotide (Fig. 3B).

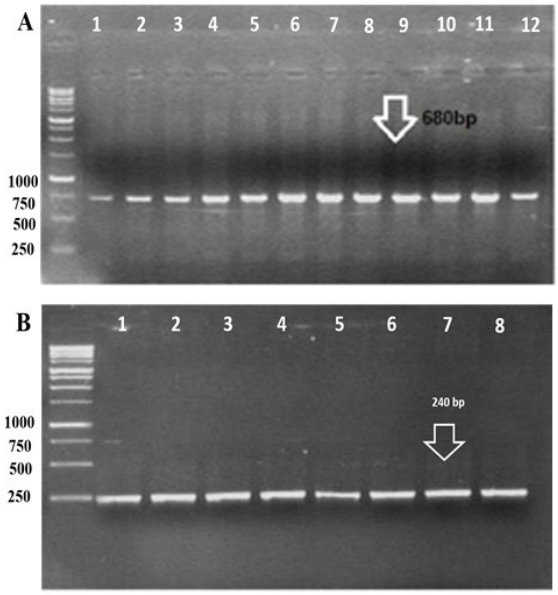

Fig. 2. Agarose gel electrophoresis results: **(A)** Agarose gel electrophoresis for *dsRed* gene fragment; Lane 1:1kb size marker, lanes 2-8 *dsRed* fragments amplified in cream zebrafish with red parents, 9-12 dsRed fragments amplified in red zebrafish.13 dsRed fragments amplified by vector containing dsRed gene. Size of fragment is approximately 680bp. **(B)** Agarose gel electrophoresis for 16*S rDNA* gene fragment for zebra Danio detection; Lane 1:1kb size marker, lanes 2-9 *16S rDNA* amplified in red and cream zebrafish. Size of fragment is approximately 240 bp

DsRED fluorescence Protein extraction from muscle tissue and protein fluorescence detection

Total protein was extracted by homogenizing one volume of fish body mass (red and cream zebrafish) with nine volume of 20mM Tris-HCl pH 8.0 and centrifuged on 14000g for 20 min. after centrifuge one drop of supernatant smeared on a glass slide. Red and cream fish slides investigated by fluorescence microscope (Olympus BX43) under TxRed control color and photo Camera ASI applied spectral and 40X objectives were used to take slide protein photo (Fig.4). A dark and a red color spread were detected for the cream and red fish protein drop, respectively.

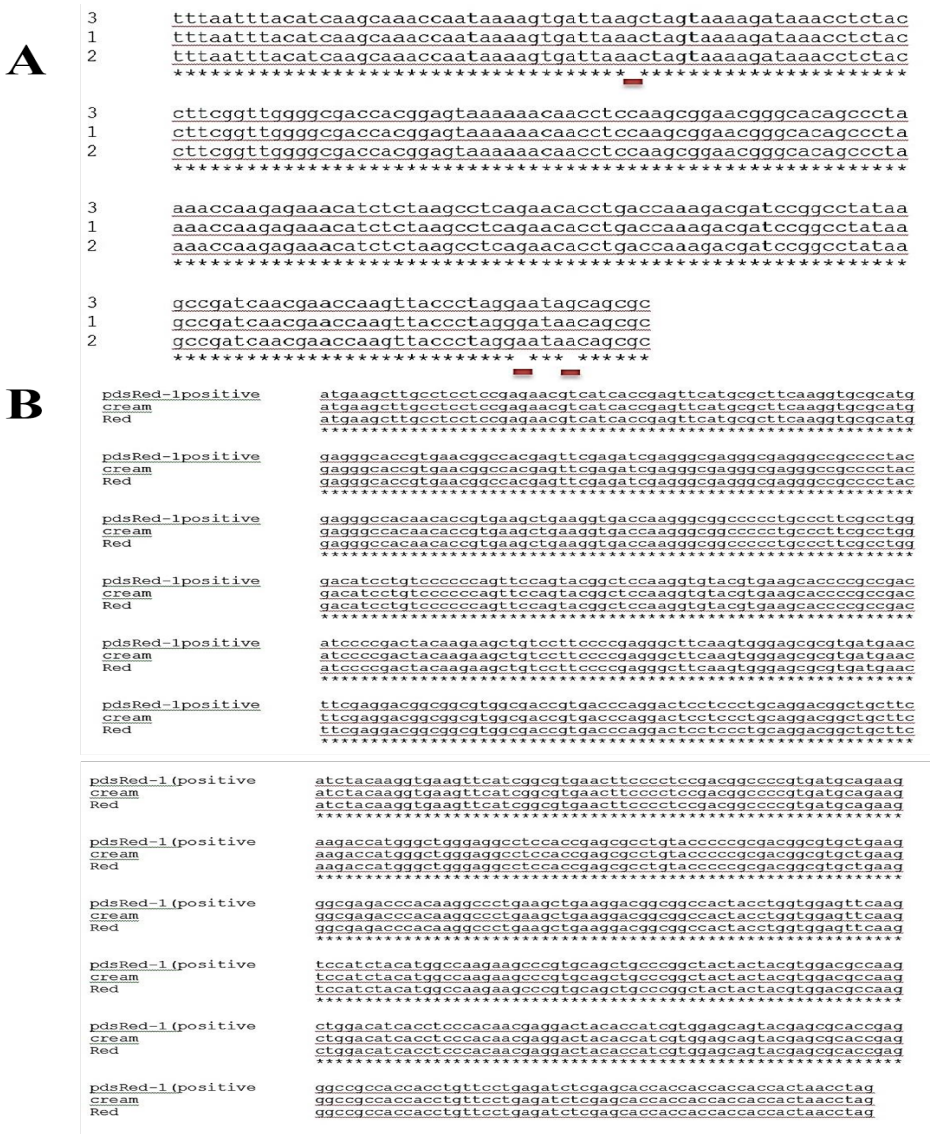

Fig. 3. Multi-alignment of sequences: **(A)** Multialign of three sequence amplified for 16S rDNA mitochondrial gene for zebrafish species detection; this sequences showed all of fish belong to zebra danio. Three different genotypes for zebra fish were amplified for *16S rDNA* in our research and marked by red lines in this figure. **(B)** Multialign sequences of three different dsRed amplified by polymerase chain reaction in pdsRed-1(positive control for dsRed) DNA, Red zebrafish specimens DNA and cream zebrafish specimens DNA (offspring of male and female red color).

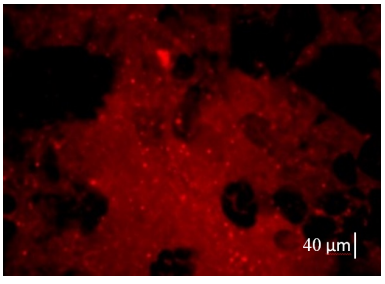

Fig. 4. Color spread of red zebrafish body muscle protein under fluorescence microscope (TxRed control color).

Discussion

This research confirmed for the first time GM fish in Iran. There is not any document to detect how this GM fish entered in Iran. Fertile red zebrafish breeders have been imported into Iran used and used to produce population of red zebrafish in ornamental fish industry as a result of high demand for this zebrafish color rather than cream or wild-type. Amplified sequences of 16S rDNA showed all of specimens belong to *Danio rerio* but amplified dsRed fragment showed this gene was not

belong to zebrafish genome according to BLAST tool in ensemble and NCBI database for zebrafish genome data. dsRed gene belongs to *Discosoma sp*. and applied for Glowfish production, respectively (Wan *et al*., 2002). In Germany and Netherlands, RFP and YFP positive zebrafish has been reported and shown by a PCR-assay and SSCP method, respectively. It was noticed that the red color gene (dsRed) involved in red zebra fish shows differences in some nucleotides, indicating the diversities found in this gene may associate with the point mutation (Rehbein and Bogerd, 2007). Furthermore, the transgenic red zebrafish and the cross-hybrids between transgenic and non-transgenic zebrafish have been reported by the PCR-based method in Italy, achieving hybrid GM fish should also be included in the GMO organisms list regulated by legislative Decree224/2003 (Ofelio *et al*., 2012). In our research the entire sample from cream and red zebrafish received from an ornamental fish farm and the most cause of finding a one type red gene related to this reason. It is suggested to get the samples from numerous ornamental fish farms throughout the country to carry out a better and more comprehensive survey of colored zebrafish. There are severe restricted laws with sanction up to 51700 € in Europe (Plan *et al*., 2010) for transgenic organism maintain and trading. Some states of USA and some Asian country banned to hold and trade genetically modified organisms (GMO) but there is not any accepted internal legal control on GMO especially animals import in Iran. Only, our country approved the Cartagena Protocol in 2001 and officially has been obligated to accomplish it from the beginning of 2004. It is necessary to pay attention to the important point that Iran has many endemic and commercial fishes. Since there is a risk that GM fish breeders escape to nature, the population of our important endemic and economic aquatic species most probably threatens with extinction.

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