Mediterranean Aquaculture Journal 2025 12 (1): 1-12

Mediterranean Aquaculture Journal

https://maj.journals.ekb.eg

Print ISSN: 1687-7616 Online ISSN: 1687-7187

Original Article

Genetic improvement of cold tolerance and growth of Nile tilapia (*Oreochromis niloticus*)

Dalia M. Thabet[1], Ramadan M. Abou Zied[1], Gamal M. Hassan[2] and Samy Y. El-Zaeem[3]

[1,] Department, Faculty of Agriculture, Fayoum University, Egypt
[2,] Genetic Department, Faculty of Agriculture, Fayoum University, Egypt
[3,] Animal and Fish Production Department, Faculty of Agriculture, (Saba-Bacha), Alexandria University, Alexandria, Egypt

ABSTRACT

This research aimed to develop a cold-tolerant Nile tilapia, *Oreochromis niloticus*, via genetically modified breeding by incorporating fragmented pure DNA extracted from Atlantic salmon into the gonads. The results indicated a significant enhancement ($P \leq 0.05$) in the majority of growth performance and feed utilization metrics of genetically modified *Oreochromis niloticus* treated with the gsr gene and reared at various low temperature levels up to 8ºC, in comparison to the control fish maintained at the same temperatures . Genetically modified *O. niloticus* displayed better traits results ($P \leq 0.05$) compared to the other fish within the same conditions. The results of the Inter Simple Sequence Repeat (ISSR) fingerprinting showed a highly genetic polymorphic percentage (35.95%) among fish receiving foreign DNA and their control using different primers. The results of this study indicate that genetically modified *O. niloticus* exhibiting enhanced growth rates and cold temperature resistance can be developed by an efficient and rapid process.

Key Words: Cold temperature tolerance, *Oreochromis niloticus*, productive performance, genetically modified, DNA, gsr gene, ISSR; PCR

1. INTRODUCTION

All biological populations and ecosystems have been affected by the severe climate and environmental alterations resulting from global warming, which encompass atypical snowfall and recurrent winter cold fronts (Agha *et al.*, 2018).

The sustainable growth of agricultural production stability and safety presents a significant problem for all nations today (Cattiaux *et al.*, 2012). Nile tilapia is a tropical and subtropical eurythermal freshwater fish, which can tolerate a wide range of environmental temperatures, ranging from a lower lethal of 7–10°C to an upper lethal of 42°C (Blasco *et al.*, 2022).

This fish is susceptible to low water temperatures. Prolonged exposure of Nile tilapia to low temperatures (7–10°C) impacts physiology, development, reproduction, and metabolism, particularly in temperate and subtropical locations marked by seasonal water temperature variations (Panase *et al.*, 2018). At the molecular level, reduced temperatures diminish enzymatic reaction rates and the diffusion and transport of biomolecules, potentially decelerating protein denaturation and disaggregation (Zhang *et al.*, 2012).

Correspondence: Ramadan M. Abou Zied Mail: rma01@fayoum.edu.eg
Department of Animal production, Faculty of Agriculture, Fayoum University, Egypt
Received: Jan. 30, 2025 Revised: Mars. 9, 2025 Accepted: Mars. 12, 2025

Furthermore, low temperatures can impede transcription and translation, damage cellular cytoskeletal components, modify membrane permeability, and disrupt energy production in cells (Sonna *et al.*, 2002).

Alongside several genetic research studies investigating low-temperature tolerance in various tilapia species, several countries have also created cultured tilapia strains demonstrating varying levels of cold tolerance through selective breeding and hybridization (Cnaani *et al.*, 2000).

Gene transfer refers to the introduction of exogenous DNA fragments into the nucleus or cytoplasm of gametes, zygotes, embryos, or somatic cells using physical or chemical methods, enabling the replication and expression of foreign genes within the host cells. These alien DNA pieces may originate from the host genome, similar species, or whole distinct species. Subsequent to such a transfer, a gene fragment would enable the protein to function actively within the host cell. When subjected to this transfer procedure, the fish exhibit characteristics of nature and manifest the genetic features encoded by the foreign genes, thereby being classified as genetically modified or transgenic fish (Tsai, 2003).

A prevalent technique for introducing foreign DNA involves microinjection into the nucleus or cytoplasm of fertilized ova. This method, however, necessitates a certain level of expertise, presents challenges, and is time-intensive (Inoue *et al.*, 1990; Sin *et al.*, 1993).

To circumvent the challenges associated with microinjection, more efficient procedures are necessary, particularly for their application in aquaculture to expedite the breeding of commercially significant species. The predominant potential mass methods include electroporation of fertilized eggs (Xie *et al.*, 1993), electroporated sperm (Symonds et al., 1994), utilizing sperm cells as vectors for foreign DNA introduction into fish eggs (Khoo *et al.*, 1992), and direct injection of foreign DNA into fish gonads (Lu, 2002).

The objective of this study was to genetically improve the cold tolerance, harvest body weight, and growth rate of Nile tilapia (*O. niloticus*) at low temperature.

2. MATERIALS AND METHODS

The experimental work was undertaken in two areas: Fish farm and the laboratory of breeding and production of fish, animal and fish production department, Faculty of Agriculture (Saba-Bacha), Alexandria University, and Al-Delta for scientific services, Alexandria, Egypt. Some experiments were undertaken at the laboratory of the Animal Production Department, Faculty of Agriculture, Fayoum University.

2.1 .Fish origin

The Nile tilapia used in this study descended from a randomly mating population at the fish farm in Tolombat Bersek, Kafr El-Dawar, El-Behera Governorate, Egypt.

2.2 .Genomic DNA isolation from Atlantic salmon

The DNA was extracted using Qiagen DNeasy spin columns (Promega, Madison, Wisconsin) according to the manufacturer's standard protocol. Pieces of fin, operculum, and scale tissue were cut and weighed, and all weights were standardized between 5 and 25 mg according to the manufacturer's specifications. A final volume of 300 µL was eluted from each spin column.

2.3 .PCR amplification of glutathione reductase gene (gsr)

For detection of the glutathione reductase gene (gsr), two specific primers were used (supplied by Amersham Fermentas Biotech. NJ. USA).

The sequence of the forward primer was 5´-GATTGCTTTCACCCTCCCACA-3; the reverse primer was 5´ GCCCATAGCAGACAGTCCAC-3´ (Table1).

The primers were designed according to the sequence of the glutathione reductase gene (gsr) sequence of Atlantic salmon, is available GenBank, to design a pair of primers (product size: 883 bp). Specific PCR primers were used for the isolation of the glutathione reductase gene (gsr). PCR using this pair of primers was optimized to amplify DNA isolated from Atlantic salmon. PCR was performed in an Eppendorf thermal cycler (Master Cycler Pro S) gradient using a PCR amplification, DreamTaq

Green PCR Master Mix (2X) (K1081, Thermo Fisher, USA), was used for specific gene amplification according to the manufacturer protocol through the Creacon (Holland, Inc.) Polymerase Chain Reaction (PCR) system cycler (Table2). gsr glutathione reductase primer sequences were designed via the primer Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The reaction was conducted in a Techne thermo cycler (TC3000, UK), programmed for an initial denaturation at 94°C for 5 minutes, followed by 45 cycles consisting of 1.5 minutes at 94°C, 2 minutes at 60.54°C, and 3 minutes at 72°C, culminating in a final extension of 7 minutes at 72°C for 35 cycles.

Table (1). Primer of gsr glutathione reductase gene under study.

Primer 9	**Product length**	**Tm**
GATTGCTTTCACCCTCCCACA	**883**	**60.55 °C**
GCCCATAGCAGACAGTCCAC		**60.46 °C**

Table (2): Master Mix component for PCR reactions

Master Mix component	**Amount**	**Final concentration**
Sterile nuclease free water	23 µl	
Green *taq* (Dream*Taq*) master mix	25 µl	1.0 x
ISSR Primer(5pmol /µl)	1.0 µl	5.0 pmol
DNA extracted sample (50ng /µl)	1.0 µl	25.0 ng
Total	50.0 µl	

2.4 .Agarose gel electrophoresis and detection of the amplification products

A 1.0% agarose solution was made by dissolving 0.75 g of agarose in 50 ml of 1x TBE electrophoresis buffer within a 50 ml flask. The agarose was subsequently dissolved by heating in a microwave oven.

The agarose was cooled to 50°C. A comb was placed in the electrophoresis apparatus, and agarose was subsequently poured into it. Meticulous attention must be exercised while pouring the agarose to prevent the creation of bubbles. The gel solidified within 15 minutes and became opaque. The electrophoresis apparatus was filled with buffer, and the comb was removed, resulting in the formation of 6 or 10 wells for sample application, alongside a DNA ladder (peqGOLD 1 kb DNA-Ladder, Peqlab, VWR) as per the manufacturer's protocol. The electrodes were linked to the power supply, which was subsequently activated. It was calibrated to 80 volts for 100 minutes. The gel was extracted from its support and placed in the staining tray for a 30-minute staining with ethidium bromide, followed by a 20-minute destaining in distilled water.

2.5 .Injection of gsr DNA into Nile tilapia gonads

Adult Nile tilapia, *O. niloticus*, with an average live weight of 187.00 ± 4.56 g for males and 165.00 ± 6.24 g for females, were selected. The readiness of females to spawn was determined by assessing the extent of swelling of the urogenital papilla (Hussain et al., 1991). Males were assessed using sperm stripping (Wester and Foote, 1972).

Six selected females and three men were directly injected with foreign DNA (gsr gene) into their gonads and muscles using a hypodermic needle, in addition to a control group consisting of three males and three females. The adult fish were inoculated by inserting the needle into the oviduct and sperm duct openings (El-Zaeem, 2001; Lu *et al.,* 2002). Following the administration of DNA treatments, three replicates (N = 15) from each treated fish and control were individually stocked (15 fry per hapa) in fiberglass tanks (total volume 350 liters) filled with dechlorinated water and sufficient aeration.

Brood fish were fed to satiation twice a day with pellets containing 25% protein, six days a week.

2.6 .Base generation (F0) Culture conditions

Offspring from genetically engineered Nile tilapia and their control, designated as base generation (F0), were collected, enumerated, and weighed. Part of the fry were transferred separately to 6 hapas (2 × 3 × 0.7 m) at a density of 15 fish/m, three hapas treated with foreign DNA and three hapas for control. Fries are fed a diet containing 30% CP to help them with the best growth. At the middle of December 2023, fries reached approximately 50 g and were then transferred into the six fiberglass aquaria in the fish lab. and exposed to cold temperatures gradually, with recorded fish dying.

2.7 .Quantitative traits measurements

The relevant metrics were established: initial and final body weight (g), daily gain (g/day), specific growth rate (SGR %/day), total body length (cm), feed intake, feed conversion ratio (FCR), protein efficiency ratio (PER), and protein and energy retention percentages (PR% and ER%). Gross energy contents of feed were calculated according to NRC (2011). The gross energy of fish was determined by their chemical composition using factors of 5.7 for protein and 9.5 for fat, as per Viola *et al.* (1981). Body composition studies for moisture, crude protein, and fat contents were conducted initially and finally, following the standard AOAC (1984) techniques.

2.8 .Fish acclimation to low water temperature

Nile tilapia juveniles (50-55 g/fish) were transferred from hapas suspended in a commercial fish farm, Tolombat Bersek, Kafr El-Dawar, El-Behera Governorate, Egypt. Two treatment groups of fish were examined, each consisting of three replicates of 15 fish. 1) The experimental group (n= *45*) consisted of fish housed in 3 fiberglass tanks (100 L each), acclimatized to laboratory settings for two weeks, with a consistent water temperature of 25°C throughout the trial duration, and fed a commercial meal containing 30% crude protein. After the termination of the conditioning period, fish in each tank were counted, and their physical appearance was checked to assure that they were healthy and did not have any injuries or deformities. At the end of the experiment, the tissue (muscle) samples were taken from 3 fish (one fish per tank). 2) Control group: the same conditions as the treated group. The temperature was reduced by one degree every 12 hours. Feeding was discontinued when the fish ceased to eat at 16°C. The water temperature was reduced by one degree every two days. Upon the water temperature reaching 6–8°C, the fish began exhibiting signs of mortality. At this step, tissue (muscle) samples were collected from the fish groups. Each death was documented, noting the time and temperature; thereafter, the fish were extracted from the cooling tanks, and tissue samples were collected from the deceased fish and frozen for genetic analysis.

2*.9 .Characterization of genetically modified Nile tilapia and their wiled type by Inter-simple sequence repeat (ISSR) analysis*

2.9.1 .Genomic DNA Extraction

Approximately 100 mg of the test fish's muscle was excised and placed into a 1.5 mL microcentrifuge tube containing 800 µL of 70% ethanol solution. The samples were preserved at −20°C and frozen for the extraction of genomic DNA. The MasterPure™ DNA Purification Kit (Epicenter, Madison, WI, USA) was utilized to recover genomic DNA from Nile tilapia. A muscle sample was transferred to a new 1.5 mL microcentrifuge tube, followed by the addition of 800 µL of tissue and cell lysis solution and 2 µL of proteinase K. The samples were thoroughly mixed and incubated in a 55°C oven for 6-12 hours. Upon complete dissolution of the tissue, 400 µL was transferred into a new centrifuge tube, followed by the addition of 250 µL of protein precipitation reagent, which was mixed thoroughly. The solution was centrifuged at 10,000× g and 4°C for 10 minutes. Subsequently, 500 µL of supernatant was transferred to a new 1.5 mL microcentrifuge tube, followed by the addition of 500 µL of isopropanol, which was combined many times to yield dehydrated DNA pellets. The pellets were centrifuged to the bottom of the tube utilizing a microcentrifuge (Spin mini), and the supernatant

was decanted. Subsequently, 100 µL of 70% alcohol was introduced into the 1.5 mL microcentrifuge tube for two washes. Following centrifugation to eliminate residual alcohol, the tube was positioned in an oven at 55°C for 10 minutes to ensure complete evaporation of the alcohol. Subsequently, 200 µL of dd H_2O was added, and the tube was incubated in a dry bath at 37°C for 20 minutes to facilitate DNA dissolution.

2.9.2 .PCR reactions for ISSR analysis

The methodology outlined by Bornet and Branchard (2001) was employed to conduct the PCR reactions for ISSR analysis. Five primers were evaluated for amplification at varying annealing temperatures of Nile tilapia genomic DNA. The primers listed in Table (3) containing anchored ISSR demonstrated adequate amplification and band resolution. The PCR amplification utilized 25 ng of genomic DNA, 2.5 mM $MgCl_2$, 1 U of Taq DNA polymerase, 1X PCR buffer devoid of $MgCl_2$, 1 µM ISSR primer, and 0.2 mM dNTP mix. The volume was adjusted to 50 µl. PCR reactions were conducted in a Perkin Elmer GeneAmp 9600 thermocycler under the following conditions: denaturation at 94ºC for 7 minutes; 35 cycles consisting of denaturation at 94ºC for 30 seconds, primer annealing at a temperature specific to each primer for 45 seconds, and primer extension at 72ºC for 2 minutes; followed by a final extension step at 72ºC for 7 minutes.

Table (3): Master Mix component for PCR reactions

Master Mix component	Amount	Final concentration
Sterile nuclease free water	23 µl	
Green taq (DreamTaq) master mix	25 µl	1.0 x
ISSR primer(5pmol /µl)	1.0 µl	5.0 pmol
DNA extracted sample (50ng /µl)	1.0 µl	25.0 ng
Total	50.0µl	

The PCR products of ISSR reactions were resolved on a 1.4% agarose gel in 1X TBE buffer pre-stained with ethidium bromide (1 µg/ml), and electrophoresis was carried out at 90 volts for 1.5 h followed by 70 volts for 2 h. The 100 bp DNA Ladder RTU (GeneDireX, Germany) was used as a standard DNA was visualized under UV light on a UV- Transillu-minator. The gel was photographed using a Gel documentation system .

Table (4) Nucleotide sequence of primers used for ISSR analysis of genetically modified Nile tilapia and their wiled type

Primer set	Primer code	Primers sequence {5'-3'}	Annealing temperature
1	814	5'(CT)8 TG 3'	44
2	HB8	5' (GA) 6 GG 3'	48
3	HB9	5' (GT) 6 GG 3'	48
4	HB10	5' (GA) 6 CC 3'	48
5	HB15	5' (GTG)3GC 3'	52

2.9.3 .Data analysis

The ISSR amplification products were recorded as "1" for presence and "0" for absence. The genetic relationships among isolates were assessed by computing Jaccard's similarity coefficient for pairwise comparisons, based on the proportion of common bands generated by the primers. The similarity matrix underwent cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA), resulting in the generation of a dendrogram.

The calculations were executed utilizing the software NTSYS–PC, Version 2.02h (Rohlf, 1997). The Jaccard similarity matrix underwent principal component analysis. This coordinating method employs a multidimensional analysis of observable relationships. PCA simplifies intricate interactions into a function of fewer, more straightforward components. This technique involves deriving the data matrix from the distances or similarities among the operational taxonomic units.

2.9.4. Computational analysis (Blast) and construction of phylogenetic tree

The nucleotide sequences of the ISSR obtained from the six fish (three treatments and three controls) were compared, and the alignments of the sequences were performed with the help of the program CLUSTALW 2.1 multiple sequence alignment (Larkin *et al.,* 2007).

3 .RESULTS AND DISCUSSION

3*.1 .Effect of injection Nile tilapia broodstock with foreign DNA, on growth performance of progeny*

The initial body weight (IBW) was achieved by genetically modified *O. niloticus* treated with Salmo salar DNA insignificantly ($P \leq 0.05$) from those of the control fish (Table 5). The genetically modified *O. niloticus* fed with *Salmo salar*-DNA exhibited the highest mean values of final body weight (FBW), daily gain (DG), and specific growth rate (SGR), which were considerably greater ($P \leq 0.05$) than those of the control group. Despite the adverse effect of low temperature on growth, the genetically modified *O. niloticus* showed higher growth performance than the control.

This may be attributed to the effect of growth hormone. Rahman *et al.* (1998) and Meri and Devlin (1999) indicated that the growth hormone gene in transgenic fish increases plasma growth hormone levels by 10 to 13 times, exceeding 40 times that of non-transgenic fish.

Furthermore, Martinez *et al.* (1996, 1999, and 2000), Pitkanen *et al.* (1999), Rahman and Maclean (1999), El-Zaeem (2001), and Mori *et al.* (2007) documented that transgenic fish exhibit greater weight compared to non-transgenic fish. No notable variations were detected in the condition factor of genetically modified *O. niloticus* subjected to *Salmo salar* DNA treatment. The control group had the lowest survival rate (93.61%), which was substantially different ($P < 0.05$) from the treated group.

The superior outcomes of the current study can be ascribed to the effective transfer and expression of exogenous DNA (Wen *et al.*, 1993; Ali, 2001; El-Zaeem, 2001, 2004a, 2004b; Hemeida *et al.,* 2004; El-Zaeem and Assem, 2006; Assem and El-Zaeem, 2005). Nile tilapia is a tropical species; however, several studies have evaluated different factors that may influence the tilapia to show a good performance when subjected to temperatures below its ideal (Abdel-Ghany *et al.,* 2019; Agouz et al., 2016; Corrêa et al., 2018; He *et al.,* 2017). One of these known factors is genetics.

Behrends *et al.* (1996) showed that the selection of tilapia more tolerant to cold did not result in a tilapia strain with a lower lethal temperature; however, these animals had progenies with higher growth at suboptimal temperatures (21°C).

Table (5). Effect of injected Nile tilapia brood stock foreign DNA on growth performance of progeny

Item	Foreign DNA	Control
IBW g	0.420 ± 0.10	0.419 ± 0.10
FBW g	55.87^{a} ± 13.39	49.14^{b} ± 16.08
FL, cm	14.21 ± 1.61	13.70 ± 1.08
WG, g	55.45^{a} ± 13.39	48.72^{b} ± 16.08
DG g/day	0.358 ± 0.08	0.314 ± 0.10
SGR%/ day	3.13^{a} ± 0.148	3.03^{b} ± 0.222
Condition factor (K)	1.91 ± 0.14	1.85 ± 0.18
Survival rate, %	100^{a} ± 0.00	93.61^{b} ± 2.610

Means within each comparison in the same rows with the different superscripts differ significantly ($P \leq 0.05$).

3.2 .Effect of injected Nile tilapia brood stock foreign DNA, on body chemical composition and feed utilization of progeny

At the end of the experiment, no notable variations in moisture content were observed among the treatments. However, crude protein levels were insignificantly lower ($P \leq 0.05$) in non-genetically engineered *O. niloticus* treated with *Salmo salar* DNA compared to the control group. Furthermore, the control fish exhibited the highest mean lipid content, which did not differ substantially ($P <$

0.05) from that of genetically modified *O. niloticus* treated with *Salmo salar* DNA (Table 6).
The average feed intake values were the same across all fish groups. The optimal food conversion ratio (FCR) and the superior protein efficiency ratio (PER) were attained by the treated fish, exhibiting a significant difference ($P < 0.05$) compared to the control group.

The enhanced FCR and PER in fish injected with *Salmo salar* DNA compared to the control may be ascribed to the increased levels of growth hormone in the plasma of the treated fish. Rahman et al. (1998) reported that growth hormone binds to specific cell receptors, which induces the synthesis and secretion of insulin-like growth factors (IGF-1 and IGF-II), resulting in the promotion of somatic growth through improved appetite, feeding efficiency, and growth rate (De la Fuente and Castro, 1998). Oakes et al. (2007) reported that the improved performance of transgenic Coho salmon resulted from increased nutritional intake. Numerous authors (Cook *et al.*, 2000; Wu *et al.*, 2003; Hallerman *et al.*, 2007) observed that growth hormone transgenic fish exhibited superior feed efficiency compared to their non-transgenic counterparts.

Table (6). Effect of injected Nile tilapia brood stock foreign DNA on body chemical composition and feed utilization of progeny

Item	At start	Foreign DNA	Control
DM	27.16	29.03± 1.22	28.41± 1.32
CP	48.91	58.26 ± 3.35	60.57± 5.82
EE	9.37	18.88 ± 1.51	19.14 ± 6.26
Feed intake		164.70 ± 0.00	164.70 ± 0.00
FCR		3.12 ± 0.70^{b}	$3.78^{a} \pm 1.36$
PER		1.21 ± 0.29^{a}	$1.06^{b} \pm 0.35$

Means within each comparison in the same rows with the different superscripts differ significantly *($P \leq 0.05$)*.

3.3 .PCR amplification of gsr gene of salmo salar

Amplification of the gsr gene of *Salmo salar* using the primers yielded a single product estimated by gel electrophoresis of approximately 883 bp obtained from all the PCR amplifications for Salmo salar fish (Figure 1).

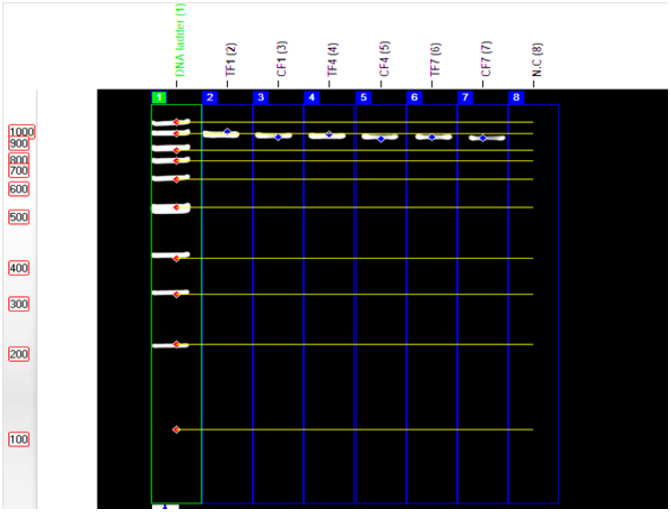

Figure (1): molecular weight detection for amplicons of the gsr glutathione reductase gene for samples

3.4 .Genetic variability assay for genetically modified Nile tilapia and their wild type by Inter-simple sequence repeat (ISSR) analysis

Five primers selected to study the genetic diversity among Nile tilapia and their wild type generated unambiguously amplified bands, furnishing a varied number of amplicons depending on the primer and specimen combinations. The number of bands observed for all the genotypes examined with each primer is presented in Table 7. Major and minor ISSR fragments ranging from 200 to 2500 bp were attained. All of the primers revealed a varying degree of polymorphism among the genotypes, in the range of 41.17–82.14%. The profiles obtained with ISSR primers are shown in Figure 2.

Table (7): Data parameters of the Inter-Simple Sequence Repeat (ISSR) fingerprinting technique of five primers for two treatments with three replicates

Primer 1

DNa ladder (1)		TF1 (2)		CF1 (3)		TF4 (4)		CF4 (5)		TF7 (6)		CF7 (7)	
Band No	MW	Band No	MW	Band No	MW	Band No	MW	Band No	MW	Band No	MW	Band No	MW
1	**300.000**	1	173.899	1	172.261	1	170.648	1	172.261	1	177.252	1	175.562
2	**200.000**	2	144.527	2	117.887	2	143.179	2	144.527	2	148.620	2	147.248
3	**150.000**	3	116.708	3	89.537	3	117.887	3	114.373	3	114.373	3	115.536
4	**100.000**	4	93.762	4	80.296	4	76.378	4	94.801	4	73.583	4	93.762
5	**75.000**	5	72.132	5	34.152	5	35.000	5	77.719	5	36.710	5	77.719
6	**50.000**	6	35.852	6	23.882	6	24.596	6	35.852	6	24.596	6	37.578
7	**35.000**	7	24.596	7	*8.889*	7	*8.148*	7	21.211	7	*8.889*	7	23.882
8	**20.000**	8	*9.259*					8	*8.889*			8	*9.259*
9	**10.000**												

Primer 2

TF1 (20)		CF1 (21)		TF4 (22)		CF4 (23)		TF7 (24)		CF7 (25)	
Band No	MW	Band No	MW	Band No	MW	Band No	MW	Band No	MW	Band No	MW
1	243.629	1	169.058	1	169.058	1	165.942	1	162.907	1	162.907
2	169.058	2	133.962	2	128.870	2	132.677	2	127.618	2	133.962
3	131.400	3	97.911	3	100.000	3	98.952	3	101.056	3	97.911
4	96.873	4	70.652	4	44.216	4	40.275	4	72.132	4	42.185
5	70.652	5	41.216	5	*7.778*	5	16.020	5	42.185	5	15.606
6	41.216	6	15.606			6	10.530	6	15.208	6	*7.778*
7	15.208	7	10.530					7	*7.407*		
8	*9.630*										

Primer 3

TF1 (26)		CF1 (27)		TF4 (28)		CF4 (29)		TF7 (30)		CF7 (31)	
Band No	MW	Band No	MW	Band No	MW	Band No	MW	Band No	MW	Band No	MW
1	169.058	1	164.415	1	165.942	1	223.006	1	172.261	1	170.648
2	139.181	2	139.181	2	137.864	2	165.942	2	141.838	2	143.179
3	114.373	3	114.373	3	112.071	3	140.505	3	113.218	3	114.373
4	73.583	4	92.718	4	75.000	4	113.218	4	93.762	4	94.801
5	36.710	5	75.000	5	14.100	5	75.000	5	72.132	5	73.583
6	21.211	6	35.852	6	*8.889*	6	35.852	6	36.710	6	36.710
7	10.000	7	22.507			7	21.211	7	26.072	7	26.072
		8	*9.259*			8	*8.519*	8	14.825	8	*9.259*
								9	*8.889*		

Primer 4

TF1 (8)		CF1 (9)		TF4 (10)		CF4 (11)		TF7 (12)		CF7 (13)	
Band No	MW	Band No	MW	Band No	MW	Band No	MW	Band No	MW	Band No	MW
1	220.552	1	178.969	1	180.716	1	182.492	1	182.492	1	182.492
2	165.942	2	157.041	2	154.192	2	151.388	2	154.192	2	152.785
3	139.181	3	122.688	3	122.688	3	122.688	3	121.475	3	122.688
4	112.071	4	97.911	4	83.934	4	82.749	4	82.749	4	102.120
5	90.608	5	82.749	5	41.216	5	42.185	5	42.185	5	82.749
6	67.628	6	41.216	6	28.392	6	28.392	6	29.189	6	43.183
7	33.309	7	26.833	7	16.020	7	10.797	7	10.530	7	29.189
8	22.507	8	10.797	8	10.797					8	10.797
9	*7.407*										

Primer 5

TF1 (14)		CF1 (15)		TF4 (16)		CF4 (17)		TF7 (18)		CF7 (19)	
Band No	MW	Band No	MW	Band No	MW	Band No	MW	Band No	MW	Band No	MW
1	172.261	1	169.058	1	175.562	1	175.562	1	178.969	1	173.899
2	140.505	2	113.218	2	144.527	2	144.527	2	117.887	2	147.248
3	113.218	3	70.652	3	114.373	3	114.373	3	75.000	3	114.373
4	75.000	4	32.470	4	94.801	4	95.837	4	34.152	4	92.718
5	43.183	5	20.000	5	72.132	5	70.652	5	20.000	5	72.132
6	18.881	6	*5.926*	6	20.594	6	20.000	6	*7.407*	6	22.507
7	*5.556*			7	*6.296*	7	*7.407*			7	*5.926*

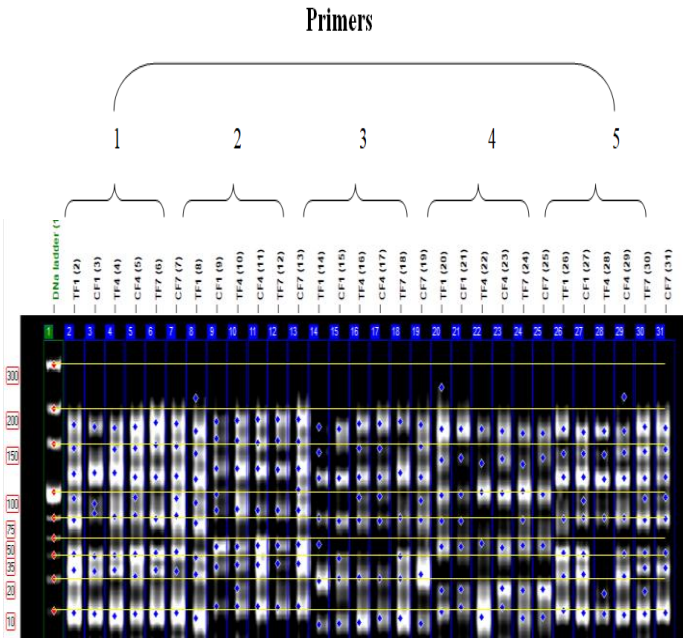

Figure (2): Computerized detection of Inter-Simple Sequence Repeat (ISSR) fingerprinting technique of five primers for two treatments with three replicates

3.5. Computational analysis (BLAST) and construction of phylogenetic tree.

The results of the phylogenetic tree derived from ISSR analysis are presented in Figure 3. The results indicated a genetic variance between the control fish and the genetically engineered fish. In this aspect, the phylogenetic analysis for DNA sequences was performed using ClustalW (1.82) MEGA-6 V.4 software. The phylogeny was divided into two main clusters; the control sample CF4 and treated sample TF4 formed one cluster, and the second sub-cluster was divided into two sub-sub-cluster. The first sub-sub-cluster contained CF1 and CF7, and the second sub-sub-cluster contained treated samples TF1 and TF7.

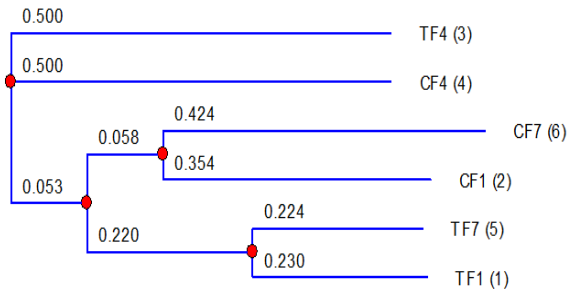

Figure (3): Phylogenetic tree for all five primers of samples according to Inter-Simple Sequence Repeat (ISSR) fingerprinting technique results .

CONCLUSION

The present study demonstrated that incorporating the gsr gene into Nile tilapia (*Oreochromis niloticus*) via direct gonadal injection is a promising method for enhancing cold tolerance and growth performance in this economically important species. Genetically modified fish exhibited significantly higher final body weights, daily growth gains, specific growth rates, and survival rates at low temperatures of up to 8°C compared to the control group. These findings suggest that this gene transfer approach could offer a rapid and efficient biotechnological tool to enhance cold tolerance and productivity traits in Nile tilapia, which is particularly valuable for aquaculture in regions prone to low winter temperatures. Considering the growing importance of Nile tilapia in Egypt, genetic improvement of the species must be given more attention with a focus on streamlining research interventions towards developing a well-planned Nile tilapia selective breeding program.

REFERENCES

Abdel-Ghany, H.M., El-Sayed, A.F.M., Ezzat, A.A., Essa, M.A. & Helal, A. M. 2019. Dietary lipid sources affect cold tolerance of Nile tilapia (*Oreochromis niloticus*). *Journal of Thermal Biology*, 79, 50–55. https:// doi.org/10.1016/j.jtherbio.2018.11.009.

Agha, R., Gross, A., Gerphagnon, M., Rohrlack, T. & Wolinska, J. 2018. Fitness and eco-physiological response of a chytrid fungal parasite infecting planktonic cyanobacteria to thermal and host genotype variation. *Parasitology*,145(10), 1279-1286 .

Agouz, H.M., Soltan, M.A., Esaid Abd-Elkarem, E. & El-Qabatti Abd Elbary, A. 2016. Effect of feed supplementation with L-carnitine on growth and cold tolerance of Nile tilapia, Oreochromis niloticus. *Egyptian Journal of Aquatic Biology & Fisheries*, 20 (3), 67–75. https:// doi.org/10.21608/EJABF.2016.10608.

Ali, A.M. 2001. The role of genomic DNA and introns in gene transfer. *Int. J. Biotechnol*ogy. 3: 411-418.

AOAC, 1984. *Official Methods of Analysis of the AOAC*, 13th ed. Assoc. Official Analytical Chemists, Washington, D.C. 1285 pp.

Assem, S.S. & El-Zaeem, S.Y. 2005. Application of biotechnology in fish breeding. II: Production of highly immune genetically modified redbelly tilapia, *Tilapia zillii*. *Afr. J. Biotechnol*ogy. 5: 449- 459.

Behrends, L.L., Kingsley, J.B. & Bulls, M.J. 1996. Cold tolerance in maternal mouthbrooding tilapias: Heritability estimates and correlated growth responses at suboptimal temperatures. *The Third International Symposium on Tilapia in Aquaculture, 41, 257.*

Blasco, F., Taylor, E.W., Leite, C.A., Monteiro, D.A., Rantin, F.T.& McKenzie, D.J. 2022. Tolerance of an acute warming challenge declines with body mass in Nile tilapia: evidence of a link to capacity for oxygen uptake. *Journal of Experimental Biology*. 225(16): jeb244287.

Bornet, B. & Branchard, M. 2001. 'Nonanchored inter simple sequence repeat (ISSR) markers: Reproducible and specific tools for genome fingerprinting', *Plant Molecular Biology Reporter*, vol. 19, pp. 209–215.

Cattiaux, J., Quesada, B.. Arakelian, A., Codron, F., Vautard, R., You, P. 2012. North-Atlantic dynamics and European temperature extremes in the IPSL model: sensitivity to atmospheric resolution. Clim Dyn. DOI 10.1007/s00382-01436-7.

Cnaani, A., Gall, G.& Gideon, H. 2000. Cold tolerance of tilapia species and hybrids. *Aquaculture International*, 8: 289-298 .

Cook, J.T., McNiven, M.A. & Sutterlin, A.M. 2000. Growth rate, body composition and feed digestibility/conversion of growth-enhanced transgenic Atlantic salmon (*Salmo salar*). *Aquaculture*, 188: 15-32.

Corrêa, C.F., Nobrega, R.O., Block, J.M. & Fracalossi, D.M. 2018. Mixes of plant oils as fish oil substitutes for Nile tilapia at optimal and cold suboptimal temperature. *Aquaculture*, 497, 82–90.

De La Fuente, J. & Castro, F.O. 1998. Gene transfer in an aquatic organism. Springer-Verlag Berlin Heidelberg and Landes Bioscience Georgetown, Texas, USA.
doi.org/10.1016/j.aquaculture.2018.07.034.

El-Zaeem, S.Y. 2001. Breeding Studies in Tilapia. Egypt. Ph.D. Thesis. Fac of Agric. (Saba-Bacha), Alex. Univ., Egypt.

El-Zaeem, S.Y. 2004a. Alteration of the productive performance characterestics of Oreochromis niloticus and Tilapia zillii under the effect of foreign DNA injection. Egypt. *J. Aquat. Biol. Fish*. 8: 261-278.

El-Zaeem, S.Y. 2004b. Evaluation of the first and second generations delivered from fast growing genetically-modified *Tilapia zillii*: A productive approach. Egypt. *J. Aquat. Biol. Fish.* 8: 53- 66.

El-Zaeem, S.Y. & Assem, S.S. 2006. Evaluation of the first and second generations delivered from fast growing genetically-modified *Tilapia zillii*: Histological and genetic approach. *J. Appl. Sci. Res*. 7: 364-374.

Hallerman, E.M., Mclean, E. &Fleming, I.A. 2007. Effects of growth hormone transgenes on the behavior and welfare of aquaculture fishes: A review identifying research needs. *Appl. Anim. Behavior Sci.* 104: 265-294.

He, Y.F., Wang, L.M., Zhu, W.B., Dong, Z.J. & Liu, N. 2017. Effects of salinity on cold tolerance of Malaysian red tilapia. *Aquaculture International*, 25(2), 777–792.
https://doi.org/10.1007/s1049 9-016-0077-y.

Hemeida, A.A., Riad, S.A. & El-Zaeem, S.Y. 2004. Genetic alteration following the production of genetically modified Nile Tilapia (*Oreochromis niloticus*). *Egypt. J. Genet. Cytol.* 33: 369-387.

Hussain, M.G., Chtterji, A., McAndrew, B.J. & Johnstone, R. 1991. Triploidy induction in Nile tilapia, *Oreochromis niloticus* L. using pressure, heat and cold shock. *Theor. Appl. Genet*. 81: 6- 12.

Inoue, K., Yamashita, S., Hata, J.S., Kabino, S., Asada, E., Nagahisa & Fujita, T. 1990. Electroporation as a new technique for producing transgenic fish. *Cell Differ. Dev*. 29: 123-128.

Khoo, H.W., Ang, L.H., Lim, H.B., Wong, K.Y. 1992. Sperm cells as vectors for introducing foreign DNA into zebrafish. *Aquaculture*, 107: 1-19.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H.

.2007. Clustal W and Clustal X version 2.0. *Bioinformatics*., 23: 2947–2948.

Lu, J., Hua, B.F., Jen-Leh, Wu. & Chen, T.T. 2002. Production of transgenic silver sea Bream (*Sparus sarba*) by different gene transfer methods. *Marine Biotechnol*. 4: 328-337.

Martinez, R., Amilcar, A., Mario, P., Fidel, H., Vivian, H., Jose, V., Teresita, S. & De la Fuent, J. 1999. Mendelian transmission transgene dosage and growth phenotype in transgenic tilapia (*O hornorum*) showing ectopic expression of homologous growth hormone. *Aquaculture*, 173: 271-283.

Martinez, R., Estrada, M.P., Berlanga, J., Guillen, I., Herradez, O., Cabrena, E., Pimentel, R., Morales, R., Herrea, F., Moralesm, A., Pina, J.C., Abad, J., Sanchez, V., Melamed, P., Lieonat. R. & De la Fuente, J. 1996. Growth enhancement in transgenic tilapia by ectopic expression of tilapia growth hormone. *Mol. Mar. Biol. Biotechnol.* 5(1): 62-70.

Martinez, R., Juncal, J., Zaldivar, C., Arenal, A., Guillen, I., Morera, V., Carrillo, O., Estrada, M.; Morales, A. & Estrada, M.P. 2000. Growth efficiency in transgenic tilapia (*Oreochromis sp*.) carrying a single copy of an homologous cDNA growth hormone. *Biochem. Biophs. Res. Commun*. 267(1): 466-472.

Meri, T. &Devlin, R.H. 1999. Transgene and host growth hormone gene expression in pituitary and non tissue of normal and growth hormone gene transgenic salmon. *Mol. Cell Endocrinol*. 149: 129-139.

Mori, T., Hiraka, I., Kurata, Y.H., Kawachi, R.H., Devlin, H.N. & Araki, K. 2007. Changes in hepatic gene expression related to innate immunity, growth and iron metabolism in GH- transgenic amago salmon (*Oncorhynchus masou*) by cDNA subtraction and microarray analysis. General and Comparative Endocrinology 151: 42-54.

NRC (National Research Council) 2011. Nutrient requirements of fish. National Academy Press, Washington DC.

Oakes, J.D., Higgs, D.A., Eales, J.G. & Devlin, R.H. 2007. Influence of ration level on growth performance and body composition of non-transgenic and growth-hormone-transgenic Coho salmon. *Aquaculture*, 265: 309-324.

Panase, P., Saenphet, S. & Saenphet, K. 2018. Biochemical and physiological responses of Nile tilapia *Oreochromis niloticus* Lin subjected to cold shock of water temperature. *Aquaculture Reports*.11:17–23.

Pitkanen, T.I., Krasnov, A., Teerijoki, H. & Molsa, H. 1999. Transfer of growth hormone (GH) transgenes into Arctic charr (*Salvelinus alpinus* L.): I. Growth response to various GH constructs. Genet. *Anal. Biomol. Eng*. 15: 91-98.

Rahman, A., Mak, R., Ayad, H., Smith, A., & Maclean, N. 1998. Expression of a novel piscine growth enhancement in transgenic tilapia (*Oreochromis niloticus*). *Transgenic Res.* 7: 357-369.

Rahman, M.A. & Maclean, N. 1999. Growth performance of transgenic tilapia containing an exogenous piscine growth hormone gene. *Aquaculture*, 173: 333-346.

Rohlf, F.J. 1997. NTSYS-PC. Numerical taxonomy and multivariate analysis system ver. 2.02h. *Applied Biostatistics*, Setuaket, NewYork, USA.

Sin, F.Y.T., Bartley, A.L., Walker, S.P., Sin, I.L., Symonds, J.E., Hawke, L., Hopkins, C.L. 1993. Gene transfer in chinook salmon (*Oncorhynchus tshawytscha*) by electroporating sperm in the presence of pRSV-lacZ DNA. *Aquaculture*, 117: 57-69.

Sonna, L., Fujita, J., Gaffin, L. & Lilly, M. 2002. Effects of heat and cold stress on mammalian gene expression. *J. Applied Physiology: Respiratory, Environmental and Exercise Physiology*, 92(4):1725-42.

Symonds, J.E., Walker, S.P. & Sin, F.Y.T. 1994. Electroporation of Salmon sperm with plasmid DNA: evidence of enhanced sperm/DNA association. *Aquaculture*, 119: 313-327.

Tsai, H. 2003. Transgenic Fish: Researches and Application. *J. Fish Soc.* Taiwan 30(4): 263-277.

Viola, S., Mokadi, S., Rappaport, U. & Arieli, Y. 1981. Partial and complete replacement of fish meal by soybean meal in feeds for intensive culture of carp. *Aquaculture*, 26: 223-236.

Wen, G.L., Wang, T. & Chen, H. 1993. Alteration of fish melanic Characteristics following transfer of total DNA. [Abstract]. *Aquaculture*, 111: p. 309.

Wester, R.C. & Foote, R.H. 1972. Quotation effect on bovine spermatozoa motility and testosterone binding. *Proc. Soc. Exp. Biol. Med*. 141: 26- 30.

Wu, G., Sun, Y. & Zhu, Z. 2003. Growth hormone gene transfer in common carp. *Aquat. Living Resour*. 16: 416-420.

Xie, Y., Liu, D., Zou, J., Li, G. & Zhu, Z. 1993. Gene transfer via electroporation in fish. *Aquaculture*, 111: 207-213.

Zhang, W., Luo, Q., Miao, L., et al. 2012. Self-assembly of glutathione S-transferase into nanowires. *Nanoscale* 4:5847–51.