CHAPTER II

MATERIAL AND METHOD

2.1 Animals

Black tiger shrimp, *Penaeus monodon* at the size of approximately 15-20 g purchased from the local shrimp farm in Pathumtani Province were used in all experiments of this study. The shrimps were acclimated in the laboratory tanks (0.75x0.75x0.75 m^3) with air-lift circulating seawater (salinity at 15 ppt, ambient temperature at 27-28 ^{o}C) for at least a week prior to experimentation. During the acclimation shrimps were fed twice daily.

2.2 Preparation of *Vibrio harveyi*

The method used in this experiment was modified from the method described by Doungpanta (2004). Culture stock of *V.harveyi* stain 1526 was streaked on marine agar plate to obtain colonies. A single colony was then inoculated in marine broth and incubated at room temperature for 16 h. The titer of culture was monitured by plate count method in marine agar plate (modified from Austin, 1988). The culture was diluted to the experiment condition at the concentration of 10^6 CFU/ml for challenge test.

2.3 Nucleic acid Extraction

2.3.1 RNA Extraction

Total RNA was extracted by placing target tissues (gills and haemocytes) in mortar containing liquid nitrogen and ground to the fine powder. The tissue powder were then transferred to microcentrifuge tube containing 500 µl of TRI REAGENT® (1 ml/ 50 mg tissue) and mixed. The mixture was left at room temperature for 5 minutes before adding 0.2 ml of chloroform. The mixture was vortexed for 15 seconds, left at room temperature for 2-5 minutes, and centrifuged at 12000xg for 15 minutes at 4°C. The mixture was separated into the lower red, phenol-chloroform phase, the interphase, and the colorless upper aqueous phase. The aqueous phase inclusively containing RNA was transferred to a new 1.5 ml microcentrifuge tube. RNA was precipitated by an addition of 0.5 ml of isopropanol and mixed thoroughly. The mixture were left at room temperature for 10-15 minutes and centrifuged at 1200xg for 10 minutes at 4°C. The supernatant was removed and RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 7500xg for 5 minutes at 4°C. After ethanol was removed, RNA pellet was air-dried for 5-10 minutes. RNA was dissolved in DEPC-treated H_2O for immediately used. Alternatively, the RNA pellet was kept under absolute ethanol in a -80°C freezer for long storage.

2.4 First Strand cDNA Synthesis for Semi-quantitative RT-PCR

Total RNA extracted from gills and hemocyte of oxidative stressed, osmotic stressed and handling stressed *P. monodon* were subjected to first strand cDNA synthesis using an ImProm-II™ Reverse transcription System Kit (Promega). Total RNA (1.5 µg) was combined with 0.5 µg oligodT_{15} and nuclease free water to make the final volume of 5 µl. The reaction was incubated at 70°C for 5 minutes and immediately placed on ice for 5 minutes. Then 5x reaction buffer, $MgCl_2$, dNTP Mix, RNasin was added to final concentrations of 1x, 2.25 mM, 0.5 mM and 20 units, respectively. Finally, 1 µl of ImProm-II™ Reverse transcriptase was added and gently mixed. The reaction mixture

was incubated at 70°C for 15 minutes to terminate reverse transcriptase activity. Concentration and rough quality of first stranded cDNA was examined by spectrophotometry (OD_{260}/OD_{280}) and was analyzed by electrophoresis (1.2% agarose gel). The first stranded cDNA was kept at -20°C until required.

2.5 Determination of RNA and cDNA Concentration using Spectrophotometry and Electrophoresis

2.5.1 Spectrophotometry

DNA, RNA and cDNA was quantified by measuring the optical density of the solution at 260 nanometer (OD_{260}). An OD_{260} of 1.0 corresponds to 40 μg/ml of single strand RNA and 33 μg/ml for single strand cDNA (Sambrook et al., 2001). Therefore, the concentrations of DNA, RNA and cDNA samples were estimated using the following equation:

Concentration of DNA (RNA or cDNA) = OD_{260}x dilution factor x 50 (40 for RNA or 33 for cDNA)

The purity of DNA and RNA samples can be evaluated from a ratio of OD_{260}/OD_{280}. The acceptable ratios of approximately purified DNA and RNA were 1.8 and 2.0, respectively. The ratio lower than 1.8 indicated the concentration of residual proteins or organic solvents whereas the ratio greater than this value indicated the contamination of RNA in the DNA solution.

2.5.2 Electrophoresis

The quality of DNA, RNA and cDNA were observed by analyzing the sample in agarose gel electrophoresis. The size and the amount of DNA were evaluated by comparing with lambda DNA.

2.6 Agarose Gel Electrophoresis

A 0.28 g of agarose gel (0.8% w/v) was weighted out and mixed with 35 ml of 1x TBE buffer. The gel slurry was boiled in a microwave oven to complete solubilization, and allowed to approximately 60°C before pouring into the gel mold. A comb was inserted and the gel was left to solidify. When needed, the comb was carefully removed and the gel was submerged in a chamber containing enough amount of 1x TBE buffer to cover the gel for approximately 0.5 cm. Appropriate volume from each sample was mixed with one-fifth volume of the 10x loading dye (0.25% bromophenol blue and 25% ficoll in water) and loaded into each well. A 100 bp DNA ladder was used as the standard DNA marker. Electrophoresis was carried out at 5-6 volts/cm until bromophenol blue moved to approximately one-half of the gel. The electrophoresed gel was stained with an ethidium bromide solution (0.5μg/ml) for 5-15 minutes and destained in running tap water to remove unbound ethidium bromine from the gel. The fragments were visualized under UV transilluminator and photographed through a red filter using Formapan Classic 100 film. The exposure time was 10-20 seconds.

2.7 Primer Design

PCR primers to amplify genes manganese superoxide dismutase (Mn-SOD), copper/zinc superoxide dismutase (Cu/Zn-SOD), arginine kinase (AK), defender against cell death (DADI), thioredoxin peroxidase, heat shock protein 70 (HSP70), heat shock protein 90 (HSP90), and Actin were designed with the sequences from the DNA database of the GenBank and cDNA library of *P. monodon*, using Primer Premier 5.0 program with the following criteria: the length of the primers were between 18-15 bases, the melting temperatures were between 55-70°C (Table 2.1).

Table 2.1 Details of primers designed with the sequences from the DNA database of the GenBank and cDNA library of *P. monodon*

Primers	Sequence	Tm (°C)	Expected size (bp)
Actin 1	5' GGTATCCTCACCCTCAAGTA 3'	51.8	327
Actin2	5' AAGAGCGAAACCTTCATAGA 3'	47.7	
Mn-SOD-F	5' ATGGCTGAGGCAAAGGAAG 3'	51.1	731
Mn-SOD-R	5' TCAAGACCGAGCAATGGAA 3'	48.9	
AKF	5' ATGGCTGACGCTGCTGTTA 3'	51.1	538
AKR	5' TCTGCTGGACTTCCTTGCTC 3'	53.8	
HSP70F	5' CCTCTATCACTCGTGCTCGC 3'	55.9	719
HSP70R	5' GTCCCTCTGCTTCTCATCGT 3'	53.8	
HSP90F	5' TCCACGAGGATTCCACCAACC 3'	56.3	612
HSP90R	5' TCGGCATCCGCCTTTGTCTCA 3'	56.3	
TPxF	5' CGAAGTGGTTGCTTGCTCTA 3'	51.8	233
TPxR	5' CTGGCAGGTCATTGATTGTT 3'	49.7	
DAD1F	5' CGATGCCTACCTCTTCTACG 3'	53.8	214
DADIR	5' GATGAAATCAGCAAAGCCTC 3'	49.7	
Cu/Zn-SOD-F	5' GGHAADCAYGGHTTCCA 3'	46.5	272
Cu/Zn-SOD-R	5' AARTCRTCNACNCCNGCRTG 3'	49.7	

2.8 Amplification of Mn SOD, Cu/ZnSOD, and AK genes

PCR reaction of each gene was performed in a 25 µl reaction mixture containing 1X PCR buffer (free Mg^{2+}), 2 mM $MgCl_2$, 100 µM of each dNTP, 10 µM Forward and Reverse primer, 1 unit Taq DNA polymerase and 100 ng cDNA template from hemocyte of *P. monodon*. PCR was carried out in thermocycler consisting of predenaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute and extension at 70 °C for 1 minute. The final extension was carried out at 72 °C for 7 minutes.

2.9 Cloning and sequencing of target genes

2.9.1 Preparation of Competent Cells

Competent *E.coli* stain JM109 cells were prepared by calcium chloride method described by Ausubel *et al*, (1989) with some modification. A single colony of *E.coli* was inoculated into 5 ml of LB broth and incubated at 37°C overnight with shaking. The culture was sub-inoculated by adding 1 ml of the culture into 50 ml of LB broth and incubated at 37°C until the OD_{600} was approximatelu 0.4-0.6. The culture was then placed on ice for 30 min and centrifuged at 3,000 rpm for 10 min. The cell pellet was resuspended in 50 ml of chilled 50 mM $CaCl_2$ solution and kept on ice for 45 min. After centrifugation at 3,000 rpm for 10 min, the pellet was resuspended in 2 ml of chilled 0.1 M $CaCl_2$ solution. Glycerol was added to make the final concentration of 15%. The cell suspension was aliquot (200 µl each) into a microcentrifuge tube and stored at -80°C for subsequently used.

2.9.2 Ligation of PCR Product

Purified DNA from PCR product of each target genes was ligated into pGEM®-T Easy Vector (Promega, U.S.A.). The ligation reaction was conducted as described by company provided protocol. Briefly, 5 µl of 2x Rapid Ligation Buffer were added to reaction. Then, 0.5 µl (25 ng) of pGEM®-T Easy vector was added, then 1 µl of PCR product, 1 µl T_4 DNA ligase and dH_2O were added to make 10 µl final volume. The reaction mixture was incubated overnight at 4°C.

2.9.3 Transformation of Ligation Product

Competent cell were thawed on ice for 5 minutes before 2-4 µl of the ligation mixture was added, gently mixed by pipetting, and left on ice for 30 minutes. The transformation reaction was heat-shocked in a 42°C water bath for exactly 1 minute. The reaction tube was immediately placed on ice for 2-3 minutes. The mixture were removed from the tubes and added to a new tube containing 1 ml of prewarmed SOC medium. The cell suspension was incubated with shaking at 37°C for 90 minutes. The mixture was centrifuged for 20 seconds at room temperature, gently resuspended in 100 µl of SOC medium, and spreaded onto a selective LB agar plates containing 50 µg/ml of IPTG and 20 µg/ml of X-gal and further incubated at 37°C for 16 hours. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue.

2.9.4 Detection of Recombinant Clone by Colony PCR

An interesting colony was picked by a pipette tip and served as the template in PCR reaction. The reaction was performed in a 25 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 100 µM of each dNTP mix, 2 mM $MgCl_2$, 0.1 µM each of pUC1 primer (5'-CCGGCTCGTATGTTGTGTGGA-3') and pUC2 primer (5'-GTGGTGCAAGGCGATTAAGTTGG-3'), 0.5 unit of DNA polymerase. PCR was carried out in a thermocycler consisting of predenaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94oC for 30 seconds, annealing at 50°C for 1 minute and extension at 72°C for 1.5 minutes. The colony PCR products were electrophoresed though 1.2% agarose gel and visualized under a UV transilluminator after ethidium bromide staining.

2.9.5 Isolation of Recombinant Plasmid

Recombinant plasmid DNA was isolated by alkaline lysis method with modification. Transformed cell containing recombinant plasmid was inoculated in LB broth (3 ml) supplemented with amplicilin (150 µg) and incubated at 37°C for 16 hours with vigorous shaking. The culture was then poured into a microcentrifuge tube and centrifuged at 12000 rpm for 30 seconds at 4°C. After centrifugation was complete, the medium was removed by aspiration, leaving the bacterial pellet as dry as possible. Bacterial pellet was resuspended in 200 µl of ice-cold GTE buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10mM EDTA, pH 8.0) by vigorous vortexing. Freshly prepared alkaline lysis solution (0.2 N NaOH, 1% (w/v) SDS) (400 µl) was added to the bacterial suspension. The tube was closed tightly and inverting the tube rapidly 5 times before placing on ice for 3-5 minutes mixed the content. Ice-cold 3 M potassium acetate, pH 4.8 (300 µl) was added to the tube and alkaline lysis solution was dispersed through the viscous bacterial lysate by inverting the tube several times. The tube was kept on ice for 3-5 minutes prior to the centrifugation of the bacterial lysate at 12000 rpm for 5 minutes at 4°C. The supernatant was transferred to a fresh tube. Plasmid was extracted by mixing

with as equal volume of Tris-HCl (pH 8.0) equilibrated phenol for 15 minutes. The mixture was centrifuged at 12000 rpm for 10 minutes at room temperature. The upper aqueous phase was transferred to a new sterile microcentrifuge tube and further extracted once with phenol and once with chloroform: isoamyl alcohol (24:1). One-tenth volume of 3 M sodium acetate (pH 5.2) was added. Plasmid was precipitated by an addition of two volume of ice-cold absolute ethanol and incubated at -80°C for 30 minutes. The precipitated Plasmid was recovered by centrifugation at 12000 rpm for 15 minutes at room temperature and briefly washed twice with 70% ethanol. The pellet was air-dried and resuspended in 50 µl of TE buffer. RNase A was added to a final concentration of 100 µg/ml to digest contaminating RNA. Plasmid DNA was incubated at 37°C for 1 hour and store at -20°C until further required.

Alternatively, plasmid DNA was extracted using QIAprep® Miniprep Kit (QAIGEN GmbH, D-40724 Hilden). Briefly, about 1.5 ml of the inoculated culture was transferred to a microcentrifuge tube and spin for 1 minute at 13,000 rpm. The supernatant was then discarded. It was possible to increase the amount of the cultured cells to the same tube by adding more cell culture and the process was repeated. The pellet of bacterial cells was resuspended in 250 µl of buffer P2 (Lysis buffer contains sodium hydroxide), the tube was inverted gently 4-6 times. After adding 350 µl of buffer N3 (Neutralization buffer contains guanidine hydrochloride), the tube was inverted gently 4-6 times and centrifuged for 10 minutes at maximum speed in tabletop microcentrifuge (13,000rpm). The supernatant (about 850 µl) was carefully collected and applied to the QIAprep column and centrifuged at 13,000 rpm for 1 minute. The flow-though solution was discarded. The QIAprep column was washed by adding 750 µl of PE buffer (wash buffer contains ethanol). The QIAquick column was centrifuged at 13,000 rpm for 1 minute to remove the trace amount of the washing solution. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl EB buffer (10 mM Tris-HCl, pH 8.5) or water was added to the center of the column and let to stand for 2 minutes then centrifuged for 1 minute. Plasmid DNA was store at -20°C until further required.

2.9.6 Restriction enzyme digestion

Two microlitres of plasmid DNA were digested with appropriate restriction enzymes (*Eco*RI). The digested plasmid DNA was separated by agarose gel electrophoresis as procedure described above in 2.6.

2.9.7 Elution of DNA Fragment from Agarose Gel

The required DNA fragment was fractionated through agarose gels in duplication. DNA fragment was run side-by-side with a 100 bp DNA marker and the other was loaded into the distal well of the gel. After electrophoresis, lanes representing the DNA standard and its proximal DNA sample were cut and stained with ethidium bromide (0.5 μg/ml) for 5 minutes. Position of the DNA marker and the ethidium bromide stained fragment were used to align the position of the non-stained target DNA fragment.

The desired DNA fragment was excised from the agarose gel with a sterile razor blade. DNA was eluting from the agarose gels using a QIAquick gel Extraction kit (QIAGEN) according to the protocol recommended by the manufacture. The excised gel was transferred into a microcentrifuge tube and weighed. Three volumes of the QG buffer was added and mixed by inversion of the tube. The mixture was incubated at 50-55°C for 10 minutes or until the gel slice was completely dissolved. The mixture was transferred into a QIAquick column inserted in a 2 ml collection tube and centrifuged at 12000 rpm for 1 minute. The flow-though solution was discarded. Another 0.5 ml of the QG buffer was added to the QIAquick column and recentrifuged for 1 minute. After this step, 0.75 ml of the PE buffer was added to the QIAquick column and centrifuged as above. The flow-though solution was discarded. The column was recentrifuged to remove the trace amount of the washing solution. The QIAquick column was then placed into a sterile 1.5 ml microcentrifuge tube. DNA was eluted out by an addition of 15 0.5μl of EB buffer (10 mM Tris-HCl, pH 8.5) or H_2O to the center of QIAquick membrane and left for 1 minute. The eluted sample was stored at 20°C until further required.

2.9.8 DNA Sequencing

The recombinant plasmid was undirectional sequenced using a Thermo Sequence Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham Biosciences, Sweden) with the M13 reverse or M13 forward primers on an automated DNA sequencer (MegaBace1000, Amersham BioScience).

2.9.9 Data analysis

Nucleotide sequences of PCR product were compared with those previously deposited in the GenBank using BLAST*N* (nucleotide similarity) and BLAST*X* (translated protein similarity) (Altschul *et al.*, 1990; available at http://www.ncbi.nlm.nih.gov). Significant probabilities of matched nucleotides/ proteins were considered when the probability (E) value was 10^{-4}.

2.10 Single Stranded Conformational Polymorphism (SSCP) Analysis

2.10.1 Non-Denaturing Polyacrylamide Gel Electrophoresis

Non-denaturating polyacrylamide gels were used for size-fractionation of both single- and double-stranded DNA. As a general rule, double-stranded DNAs migrate through these gels at rates that are inversely proportional to the $\log_{10}$ of their size. However, their base compositions and sequences also affect electrophoretic mobility, so that duplex DNAs of exactly the same size can differ in mobility by up to 10%.

2.10.2 Preparation of Glass Plate

The long glass plate was thoroughly wiped with 2 ml of 95% commercial grade ethanol in one direction with a tissue. This process was then repeated twice. Afterwards, the long glass plate was coated with 1 ml of freshly prepared Bind silane (4 µl of Bind silane, 995 µl of 95% ethanol and 5 µl of 5% glacial acetic acid) and left for approximately 10-15 minutes. Excess binding solution was removed with a piece of tissue. The long glass plate was further cleaned with 95% ethanol for 3 times. The short glass plate was treated as described above with the exception that the binding solution was replaced by the Repel silane (2% dimethyldichlorosilane in octamethylcyclotetrasitoxone). The cleaned glass plates were assembled with a pair of 0.4 mm spacer. The bottom and both sides of assembled glass plates were sealed with the plastic tape.

2.10.3 Preparation of SSCP Gel

The glass plates (PROTEIN II xi Cell) were cleaned and prepared as described previously. Different concentration of low crosslink non-denaturing polyacrylamide gel (37.5: 1 or 75:1 of acrylamide and bis-acrylamide) was prepared by dilution of a 40% stock solution to required gel concentration. The acrylamide gel solution (30-40 ml) were mixed with glycerol (5% concentration), if desired and 240µl of 10% APS and 24 µl of TEMED. The analytical comb was inserted into the prepared gel and allow for polymerization for 4 hours or overnight.

2.10.4 Electrophoresis

The gel sandwich was placed in the vertical sequencing apparatus with the short glass plate inward. The gel sandwich was securely clamped with the integral gel clamps along the sides of the sequencing apparatus. The upper and lower buffer chambers were filled with approximately 300 ml of 1xTBE. Six microlitres of the acrylamide gel loading dye (98% formamide, 200 µl EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol)

was loaded into each well. The gel was pre-run at 30-40 W for 20 minutes. Six microlitres of the amplified products were mixed with 24 µl of the SSCP loading dye (98% formamide, 200 µl EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured in boiling bath for 5 minutes, and immediately cooled on ice for 3 minutes. The denatured PCR products were electrophoretically analyzed in native polyacrylamide gels (different gel concentration of 37.5:1 or 75:1 crosslink with and/or without glycerol) at 250-300 volts for 16-24 hours at 4°C. The electrophoresed bands were visualized by silver staining described previously with the extraction that the gel was rinsed for 3 times for 3 minutes each after the fix/ stop step.

2.10.5 Silver Staining

The gel plates were carefully separated using a plastic wedge. The long glass plate with the gel was placed in a plastic tray containing 1.5 litres of the fix/ stops solution and agitates well for 25-30 minutes. The gel was soaked with shaking 3 times for 2 minutes with deionized water. The gel was lifted out from the tray between each wash and allowed the washed water draining out of gel for 4 seconds. The gel was transferred to 0.1% silver nitrate (1.5 litres) and incubated with agitation at room temperature for 30 minutes. The gel was soaked in 1.5 litres of deionized water with shaking (10 forward and 10 backward agitations) and immediately placed in the tray containing 1.5 litres of the chilled developing solution. This step was crucial and the time taken to soak the gel in the water and transfer it to chilled developing solution should be no longer than 5-10 seconds. The gel was well agitated until the first band was visible (usually 1.5-2 minutes). The gel was then transferred to another tray containing 1.5 litres of chilled developer and shook until bands from every lane observed (usually 2-3 minutes). One litre of the fix/stop solution was directly added to the developing solution and continued shaking for 3 minutes. The strained gel was soaked in deionized water twice for 3 minutes each. The gel was left at room temperature.

2.11 Conduction of stress treatment in *P.monodon*

2.11.1 Oxidative stress

Acclimated shrimps were separated into 2 groups: shrimps challenged by *V.Harveyi* to induce oxidative stress and control shrimps. Each group (with 2 replications) contained 40 shrimps. In the stressed tank, *V.Harveyi* was applied to the tank to make up the final concentration of 10^6 CFU/ml. After challenged, 3 shrimps from each treatment were collected at 0, 6, 12, 24, 48, and 72 h of post induction. Gills and haemocytes were separated from the collected shrimps and subjected to RNA extraction.

2.11.2 Handling stress

Similar experimental condition to the oxidative stress induction was conducted for handling stress. Shrimps were separated into 2 groups: stressed and non-stressed (control) tanks. In the stressed tank, water was disturbed by circulation with external force for 5 minutes. The water disturbance was carried out for 4 times every 30 minutes and the shrimps were kept out of the water for 1 minute before putting back to the tanks. After challenged, 3 shrimps from each treatment were collected at 0, 6, 12, 24, 48, and 72 h of post induction. Gills and haemocytes were separated from the collected shrimps and subjected to RNA extraction.

2.11.3 Osmotic stress

Shrimps acclimated in seawater at the salinity of 15 ppt. for more than a week were separated into 5 laboratory tanks (30 shrimps each) containing seawater at the salinities of 0, 15, 30, 45 and 60 ppt. Mortality of the experiment shrimps were recorded. From each treatment, 3 shrimps were collected at 0, 6, 12, 24, 48, and 72 h of experiment. Gills and haemocytes were separated from the collected shrimps and subjected to RNA extraction.

2.12 Expression of Stress Response Genes Detecting by Semi-quantitative RT-PCR

Reverse transcription was conducted to make first strand cDNA using total RNA extracted from gills and hemocyts of stressed animals. The reaction was performed in the final volume of 20 µl, at 42 °C, for 90 minutes using Improm-II™ reverse transcription kit condition (1 U of Improm-II™ reverse transcription, 2µl of 1x Improm-II™ reactive buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTP mix, 0.5 µg Oligo dT, and 2.0 U of Ribonuclease inhibitor). Quantitative RT-PCR was conducted using exact concentration of first-stranded cDNA as template with the concentration of 5, 100, 100, 25, 100, 100, and 100 ng for Actin, Mn-SOD, AK, HSP70, HSP90, DADI, and Thioredoxin peroxidase, respectively. Primers were designed from the DNA database of the GenBank and cDNA library of *P. monodon* (Table 2.1). For PCR condition, samples were supplemented with the addition of 1x buffer (10 mM Tris-HCl, 8.8, 50 mM KCl, 0.1% Triton x-100), 2 mM $MgCl_2$ were used for all genes, 0.5 µM each of forward and reverse primer were used for all genes except DAD-I used 1 µM, respectively, 100 µM of each dNTP, and 1U Taq DNA polymerase. The PCR reaction for Actin, AK, Mn-SOD, HSP70, HSP90, DADI, and thioredoxin peroxidase genes was performed as follow.

Actin

Initial denaturation step :	94°C	for 1 cycle
Denaturing step:	94°C	
Annealing step:	55°C	for 25cycles
Elongation step:	72°C	
Extension step:	72°C	for 1 cycle

Mn-SOD gene

Initial denaturation step :	94°C	for 1 cycle
Denaturing step:	94°C	
Annealing step:	60°C	for 25cycles
Elongation step:	72°C	
Extension step:	72°C	for 1 cycle

AK gene

Initial denaturation step :	94°C	for 1 cycle
Denaturing step:	94°C	for 25 cycles
Annealing step:	60°C	
Elongation step:	72°C	
Extension step:	72°C	for 1 cycle

HSP70 kDa gene

Initial denaturation step :	94°C	for 1 cycle
Denaturing step:	94°C	for 25 cycles
Annealing step:	65°C	
Elongation step:	72°C	
Extension step:	72°C	for 1 cycle

HSP90 kDa gene

Initial denaturation step :	94°C	for 1 cycle
Denaturing step:	94°C	for 25cycles
Annealing step:	55°C	
Elongation step:	72°C	
Extension step:	72°C	for 1 cycle

DADI gene

Initial denaturation step :	94°C	for 1 cycle
Denaturing step:	94°C	for 30 cycles
Annealing step:	55°C	
Elongation step:	72°C	
Extension step:	72°C	for 1 cycle

Thioredoxin peroxidase

Initial denaturation step :	94°C	for 1 cycle
Denaturing step:	94°C	for 25 cycles
Annealing step:	55°C	
Elongation step:	72°C	
Extension step:	72°C	for 1 cycle

2.13 Statistic Analysis

All the measurements were made in tree replicates. The results were analyzed using the ANOVA and Duncan new multiple range test ($p < 0.05$) at 95% confidence level by SPSS.