CHAPTER III

MATERIALS AND METHODS

3.1 Animals

3.1.1 Greenback mullet (*Liza subviridis*)

Juvenile, male and female greenback mullet, *Liza subviridis* collected from canal in Samutprakarn Province were used in all experiments of this study. The mullets were acclimated in the laboratory tanks for at least a week prior to experimentation. During the acclimation, mullets were fed twice daily with artemia.

3.1.2 Zebrafish (*Danio rerio*)

Adult zebrafish (*Danio rerio*) purchased from aquarium fish shop in Bangkok was used in isolated *tif*2 RID cDNA sequence. Zebrafish were fed twice with commercial fish feed.

3.2 Nucleic acid extraction

3.2.1 DNA extraction

Genomic DNA was extracted from muscle according to CTAB method described by Winnepenninckx (1993). Genomic DNA was extracted by placing muscle (9-10 g) in mortar containing liquid nitrogen and ground to the fine powder. The tissue powder was added into 10 ml of preheated (55°C) CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0 and 0.2% v/v β-mercaptoethanol and mixed by inversion. Then, proteinase K (150 µl of 10 mg/ml stock) was added into solution and mixed by inversion, incubated at 55°C for 1-3 h, and mixed by inversion every 30 min. Then, proteinase K (150 µl of 10 mg/ml stock) was added into solution, incubated at 55°C overnight. The mixture was centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant was removed to new tube and extracted by adding equal volume of chloroform: isoamyl alcohol (24:1) and mixing by inversion for 10 min. The mixture was centrifuged 5,000 rpm for 10 min at 4°C (2 times) and the colorless aqueous phase was removed to a new tube. DNA was precipitated by adding 2/3 volume of isopropanol and left at room temperature for a

few min before centrifuging at 5,000 rpm for 10 min at 4°C. The DNA was spooled by a hooked Pasteur pipette into a new tube, washed with 70% and 100% ethanol, respectively, and centrifuged at 10,000 rpm for 10 min at room temperature. After supernatant was discarded, the pellet containing DNA was air-dried and redissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA) for overnight. RNA was then digested by the addition of RNase A (10 µg/µl) to a final concentration of 100 ng/µl and incubated at 37°C for 1 h. The DNA solution was further extracted (2 times) by adding equal volume of phenol, mixed by inversion for 15 min, and centrifuged at 10,000 rpm for 10 min at room temperature before transferring upper aqueous phase into a new tube and extracted by adding equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), mixed by inversion for 15 min, and centrifuged at 10,000 rpm for 10 min at room temperature. The upper aqueous phase was transferred into a new tube and extracted (2 times) by adding equal volume of chloroform: isoamyl alcohol (24:1) and mixing by inversion for 10 min. After centrifuging at 10,000 rpm for 10 min at room temperature, the upper aqueous phase was transferred into a new tube and DNA was precipitated by adding 1/10 volume of 3 M sodium acetate, pH 5.2 and 2 volume of chilled absolute ethanol. The mixture was incubated at -80°C for 30 min and centrifuged at 10,000 rpm for 10 min at room temperature. The DNA pellet was washed 2 times by adding 1 ml of 70% ethanol and centrifuging at 10,000 rpm for 10 min at room temperature. After the supernatant was discarded, the pellet containing DNA was air-dried, dissolved in 50 µl of TE buffer, and stored at 4°C for further use.

3.2.2 RNA Extraction

Total RNA was extracted by placing liver in mortar containing liquid nitrogen and ground to fine powder. The tissue powder was then transferred to microcentrifuge tube containing 1 ml of TRI REAGENT (1 ml/ 50-100 mg tissue), vortexes vigorously until homogeneous, maintained for 5 min at room temperature and centrifuged at 12000xg for 15 minutes at 4°C. After centrifugation, the mixture was separated into cell debris and red aqueous phase. The aqueous phase was transferred to a new 1.5 ml microcentrifuge tube and 0.2 ml of chloroform was added. The mixture was vortexes for 1 minute, left at room temperature for 2-5 minutes (3 times), and centrifuged at 12000xg for 15 min at 4°C. The mixture was separated

into 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 was left at room temperature for 10 min and centrifuged at 12000xg for 8 min 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.

3.3 First Strand cDNA Synthesis

Total RNA extracted from liver of estrogen treated and control mullet was 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 of oligo $(dT)_{15}$ primer and nuclease free water to make a final volume of 5 µl. The reaction was incubated at 70°C for 5 minutes and immediately placed on ice for 5 min. Then 5x reaction buffers, 25 mM $MgCl_2$, dNTP mix, RNasin was added to final concentrations of 1x, 3.25 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 25 °C for 5 min, 42 °C for 1 h and 30 min and 70°C for 15 minutes to terminate reverse transcriptase activity. Concentration and rough quality of first strand cDNA was examined by spectrophotometry (OD_{260}/OD_{280}) and was analyzed by electrophoresis (1% agarose gel). The first strand cDNA was kept at -20°C until required.

3.4 Determination of DNA, RNA and cDNA concentration using spectrophotometry and electrophoresis

3.4.1 Spectrophotometry

RNA, DNA, 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 the ratio of OD_{260}/OD_{280}. The acceptable ratios of approximately purified DNA and RNA were 1.8 and 3.0 respectively. The ratio lower than 1.8 indicated the concentrations of residual proteins or organic solvents whereas the ratio greater than this value indicated the contamination of RNA in the DNA solution.

3.4.2 Electrophoresis

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

3.5 Agarose gel electrophoresis

A 0.48 g of agarose gel (1.2% w/v) was weighted out and mixed with 40 ml of 1x TBE buffer. The gel slurry was boiled in a microwave oven to complete solubilization, and allowed to approximately 50°C before pouring into the gel mold. A comb was inserted and the gel was left to polymerize. 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 1 cm. Appropriate volume of sample was mixed with 10x loading dye (0.25% bromophenol blue and 25% ficoll in water) at the ratio of 5:1 and loaded into each well. A λ*Hin*dIII and 100 bp DNA ladder were used as the standard DNA markers. Electrophoresis was carried out at 5-6 volts/cm until bromophenol blue moved to approximately one-half of the gel. The electrophoreses gel was stained with an ethidium bromide solution (0.5 μg/ml) for 5-15 min and destained in 1x TBE buffer to remove unbound ethidium bromide 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 18-20 s.

3.6 Primer design

3.6.1 Primers for amplifying partial coding sequence.

Actin_MR primers for *Macrobrachium rosenbergii* actin cDNA sequence designed by Preechaphol (personal communication) were initially used to cross-amplify partial coding sequence of actin in the mullet. After the partial sequence of mullet actin was obtained, a new pair of primers specific to mullet actin was designed and used as reference housekeeping gene. Degenerate PCR primers for amplifying partial coding sequences of *tif2*, *ER*, *chg*-L, *chg*-H and *vtg*-1 were designed from the conserved amino acid sequences of these proteins obtained from the protein database of the Genbank. The details of these primers were shown in Table 3.1.

Table 3.1 Detail of primers used for amplifying partial coding sequences of target genes.

Primers	Sequence	Tm (°C)	Expected size (bp)
ER F	5'-GCN WSN GGN TAY CAY TAY GG-3'	56-68	116
ER R	5'-TCD ATN GTR CAY TGR TT-3'	42-52	
ER R-α	5'-TC RAA DAT YTC NGC CAT NCC-3'	54-64	731
chg-L F	5'-ATH GTN GAR TGY CAY TAY CC-3'	52-64	921
chg-L R	5'-GTN ACR TCN CCY TCC CAY TC-3'	58-68	
chg-H F	5'-GTN CAR TGY ACN AAR GAY GG-3'	54-66	750
chg-H R	5'-CNA CRA ANG TRA ACA TYT T-3'	46-56	
vtg-1 F	5'-TAY GTN TAY RAN TAY GAR GC-3'	48-62	407
vtg-1 R	5'-GTY TTR CAN ACN CCY TG-3'	46-56	
Actin_MRF	5'-CGA GAC CTT CAA CAC CCC AG-3'	60.1	332
Actin_MRR	5'-ACT GCC GCC TCC TCC TCT T-3'	61	
tif2 F1	5'-CAR RCN ATG GGN AAR MC-3'	46-58	1,325
tif2 R1	5'-TGN ARR TCR TCN ARD AT-3'	40-54	
tif2 F2	5'-AAR GAR AAR CAY AAR AT-3'	38-48	182
tif2 R2	5'-GCR TKN TCY TTY TTY TT-3'	40-52	
tif2 F	5'-AAR CCN GAY AAR TGY GC-3'	46-56	185
tif2 R	5'-AAR AAN CCR TCN ARN GCY TC-3'	52-66	
Danio rerio tif2 RID F	5'-CTG GGA TCG CCT GAT AGA AA-3'	57.7	695
Danio rerio tif2 RID R	5'-TTC TCG GTC TTA ATC CCA GT-3'	53.9	

3.6.2 RACE PCR primers

RACE PCR primers for amplifying *ER*, *chg* and *vtg* of *L. subviridis* were designed from the partial coding sequences of each gene. Primers were designed with the following criteria: the length of the primers was between 26-28 bases, the melting temperature was between 65-72°C Details of these primers were shown in Table 3.3.

Table 3.2 Detail of RACE PCR primers used for amplifying 33.1 and 53.1 sequences of target genes.

Primers	Sequence	Tm (°C)	Expected size (bp)
ER F RACE	5'-CTT CAA GAG GAG CAT CCA GGG TCA CAA-3'	70.7	1,764
ER R RACE	5'-CAT TGT GAC CCT GGA TGC TCC TCT TGA-3'	71.3	1,014
ER α R RACE	5'-GAC GCT TTG TGC TCT GGA TCT TTG GC-3'	71.2	1,240
ER β F RACE	5'-GGG GAA GAA ATC GGA GGA CGG AGT G-3'	71.9	2,000
ER β F1 RACE	5'-GCG GCC ACG TCT CGC TTC AGA GA-3'	73.5	1,200
chg-L F RACE	5'-GGC TGG GGA GGT GGA GCT CAT CCT AAT A-3'	73.2	128
chg-H R RACE	5'-CCG CCT TCC CCT TGT CCC AAT AGT GTG A-3'	76.1	1,200
chg-H F1 RACE	5'-CTA CCC TGT GGC CAA AGT ATT GAG G-3'	65.4	972
chg-H R1 RACE	5'-CCT TCA CCT TGT CCC AAG AGT GTG A-3'	66.5	704
vtg-1 F RACE	5'-AAT GGT GTG GTA GGG AAA CTG CTC GTC-3'	69.7	4,900
vtg-1 F1 RACE	5'-TGG TGC TGA ACA TCC ACA GAG GC-3'	66.8	4,854
vtg-1 R RACE	5'-GCT CTC GCC AAA CCT TCC TCT GAC-3'	67.6	600
vtg-3 R1 RACE	5'-CGG AGG CAT CAT AAC CCT CGG GC-3'	73.4	3,790
vtg-3 R2 RACE	5'-GTG GGG AAG ATG TTG TTC GCA CTT-3'	66.4	2,479

3.6.3 Genome walk primers

Genome Walk primer for *chg*-L was designed from the nucleotide sequence of *L. subviridis*. Primers were designed with the following criteria: the length of the primers was between 26-28 bases, the melting temperature was between 65-72°C Details of these primers were shown in Table 3.3.

Table 3.3 Detail of GenomeWalk primers used for amplifying genomic sequences of target genes.

Primers	Sequence	Tm (°C)	Expected size (bp)
chg-L GW R1	5'-TTC CTC CGC CAC CTT AAC TG-3'	60.2	-
chg-L GW R2	5'-GAG GAA GGC TGC TCA CAT TG-3'	58.0	-
chg-L D12 R1	5'-ACA GTT CCC CAG GGT GAG GTC ATT CG-3'	73.2	-
chg-L D12 R2	5'-CCC AAA CAT ATC CCT CCT GAC TTC CAC A-3'	71.2	-

3.6.4 Gene specific primers used in semi-quantitative analysis and other purposes.

Specific primers for *ER*, *chg*, *vtg*, and actin were designed from the nucleotide sequence of *L. subviridis* with the following criteria: the lengths of the primers were 18-24 bases, the melting temperature were 50-72°C. Details of these primers were shown in Table 3.4.

Table 3.4 Detail of gene specific primers used in semi-quantitative analysis and other purposes.

Primers	Sequence	Tm (°C)	Expected size (bp)
chg-L semi F	5'-ATG ACC TCA CCC TGG GGA AC-3'	60.6	559
chg-L semi R	5'-GCT GTG CGA GGC AAG AAC C-3'	61.3	
chg-H semi F	5'-CAC TAT TGG GAC AAG GGG AA-3'	57.3	337
chg-H semi R	5'-GTG CAT TCA CCA TTG ACC AA-3'	57.0	
vtg-3 semi F	5'-GGG ACT CAA CAC CTG AAG CT-3'	56.3	352
vtg-3 semi R	5'-ATT CTT GTG CCC ATG TCC TC-3'	57.0	
actin semi F	5'-CTG TCC CTG TAC GCC TCT G-3'	55.9	267
actin semi R	5'-TCC CAT CTC CTG CTC AAA GT-3'	56.9	
ERα F	5'-AAT GAA GGC GAC TGC GTT GAG GGC-3'	73.3	543
ERα R	5'-CGT ACT GCA GGA CGC CCG GAC CA-3'	74.7	
chg-L D12-D20 R	5'-TAC GAT TCT TTA GGA CCA GC-3'	53.3	1,030
3'*vtg*-1 internal F1	5'-GCC CGC TTT GAA GAC CTA-3'	56.0	-
3'*vtg*-1 internal F2	5'-AGC CAT CCA TGT GGA CGT-3'	55.8	-
3'*vtg*-1 internal R1	5'-AGG CTC GTA CCC ATT TGC-3'	55.6	-
3'*vtg*-1 internal R2	5'-TGA TGT GGT TCT GCT TGA T-3'	50.9	-

3.6.5 Specific primers for full length gene amplification

Specific primers for amplified *ERα* and *chg*-L full length (open reading frame) were designed from the nucleotide sequence of *L. subviridis* with the following criteria: the lengths of the primers were 18-24 bases, the melting temperature were 50-72°C. Details of these primers were shown in Table 3.5

Table 3.5 Detail of gene specific primers used in amplified full length cDNA

Primers	Sequence	Tm (°C)	Expected size (bp)
ERα full length F	53.1-GGG GGA TGA TTC ATG TAT AAG AG-33.1	57.4	1,885
ERα full length R	53.1-CCT TCC TGC CTC ATA AGA TGT-33.1	56.1	
chg-L full length F	53.1-GCT TGT CAC TGT GGA GCC ATG GT-33.1	66.3	1,293
chg-L full length R	53.1-TCA TGG ACA ACC GTG TTA AGC GAG-33.1	66.5	

3.7 Amplification of partial cDNA sequences of *ER*, *tif2*, *chg*, *vtg* and actin gene.

Partial cDNA sequences of *ER*, *chg*-L, *chg*-H, *vtg*-1, *vtg*-3, *tif2* and actin were amplified by PCR. Final concentration of the reaction for each gene was shown in table 3.6.

Table 3.6 Primers and PCR composition to amplify partial fragments of target genes

Gene	Template (ng)	Primers (μM)		*Taq* (U)	Buffer	$MgCl_2$ (mM)	dNTP (mM)	PCR condition
		Forward	Reverse					
ER	500*	*ER* F (0.5)	*ER* R (0.5)	1	1X	2	0.1	A
chg-L	521.4*	*chg*-L F (0.5)	*chg*-L R (0.5)	1	1X	1	0.1	B
chg-H	521.4*	*chg*-H F (0.5)	*chg*-H R (0.5)	1	1X	2	0.1	C
vtg-1	500*	*vtg*-1 F (0.5)	*vtg*-1 R (0.5)	1	1X	2	0.1	D
vtg-3	1,200*	*ER* F (1)	*ER* R-α (1)	1	1X	2	0.1	E
vtg-3	521.4*	*ER* F (0.5)	oligo dT *Sal*I (0.3)	1	1X	2	0.1	F
actin	500*	Actin_MRF (0.5)	Actin_MRR (0.5)	1	1X	2	0.1	B
tif2	1,000**	*Danio rerio tif2* RID F (0.5)	*Danio rerio tif2* RID R (0.5)	1	1X	2	0.1	C

* = liver 1st strand cDNA of *L. subviridis*
** = head and visceral organ 1st strand cDNA of *D. rerio*

PCR conditions were as follow.

Condition A

Initial denaturation step:	94 °C	3	min	for	1	cycle
Denaturation step:	94 °C	1	min			
Annealing step:	50 °C	1	min	for	35	cycles
Elongation step:	72 °C	2	min			
Extension step:	72 °C	7	min	for	1	cycle

Condition B

Initial denaturation step:	94 °C	3	min	for	1	cycle
Denaturation step:	94 °C	1	min			
Annealing step:	60 °C	1	min	for	35	cycles
Elongation step:	72 °C	2	min			
Extension step:	72 °C	7	min	for	1	cycle

Condition C

Initial denaturation step:	94 °C	3	min	for	1	cycle
Denaturation step:	94 °C	1	min			
Annealing step:	45 °C	1	min	for	35	cycles
Elongation step:	72 °C	2	min			
Extension step:	72 °C	7	min	for	1	cycle

Condition D

Initial denaturation step:	94 °C	3	min	for	1	cycle
Denaturation step:	94 °C	1	min			
Annealing step:	55 °C	1	min	for	35	cycles
Elongation step:	72 °C	2	min			
Extension step:	72 °C	7	min	for	1	cycle

Condition E

Initial denaturation step:	94 °C	3	min	for	1	cycle
Denaturation step:	94 °C	1	min			
Annealing step:	45 °C	1	min	for	5	cycles
Elongation step:	72 °C	2	min			
Denaturation step:	94 °C	1	min			
Annealing step:	50 °C	1	min	for	30	cycles
Elongation step:	72 °C	2	min			
Extension step:	72 °C	7	min	for	1	cycle

Condition F

Initial denaturation step:	94 °C	3	min	for	1	cycle
Denaturation step:	94 °C	1	min			
Annealing step:	45 °C	2	min	for	5	cycles
Elongation step:	72 °C	2	min			
Denaturation step:	94 °C	1	min			
Annealing step:	50 °C	2	min	for	30	cycles
Elongation step:	72 °C	2	min			
Extension step:	72 °C	7	min	for	1	cycle

3.8 Cloning and sequencing of target genes

3.8.1 Preparation of Competent Cells

Competent *E. coli* strain JM109 cells were prepared by $CaCl_2$ method described by Ausubel et al, (1989) with some modifications. 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 100 ml of LB broth and incubated at 37 °C until OD_{600} was approximately 0.4-0.6. The culture was then placed on ice for 30 min and centrifuged at 3,000 rpm for 15 min at 4 °C. The cell pellet was resuspended in 30 ml of chilled $MgCl_2$-$CaCl_2$ solution kept on ice for 45 min. After centrifugation at 3,000 rpm for 15 min at 4 °C, the pellet was resuspended in 2 ml of chilled 0.1 M $CaCl_2$ solution (contained 15% glycerol). The cell suspension was aliquot (100 µl each) into microcentrifuge tube and stored at -80 °C for subsequently used.

3.8.2 Elution of DNA fragment from agarose gel

The required DNA fragment was fractionated through agarose gel in duplication. DNA fragment was run side-by-side with a 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 min. 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 sterile razor blade. DNA was eluted from the agarose gel using a HiYield Gel Extraction kit (Real Biotech Corp.) according to the protocol recommended by the manufacture with some modifications. The excised gel was transferred into a microcentrifuge tube and 500 µl of the DF Buffer was added and mixed by inversion. The mixture was incubated at 55 °C for 15 min or until the gel slice was completely dissolved. The tube was inverted every 2-3 min during incubation. The mixture was transferred into a DF column inserted in a collection tube and centrifuged at 8,600 rpm for 1 min. The flow-through solution was discarded. After this step, 500 µl of Wash Buffer (EtOH added) was added to the DF column and centrifuged as above. The flow-through solution was discarded. The column was re-centrifuged at 14,000 rpm for 2 min to remove the trace amount of the Wash Buffer. The DF column was then placed into a sterile 1.5 ml microcentrifuge tube. DNA was eluted out by an addition of 10 µl of elution buffer to the center of filter membrane and left for 2 min until buffer elution buffer absorbed before centrifugation at 14,000 rpm for 2 min. The eluted sample was stored at -20°C until further required.

3.8.3 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 the reaction, followed by 0.5 µl (25 ng) of pGEM®-T Easy Vector, then PCR product (amount (ng) calculated from below equation), 1 µl of T_4 DNA ligase (3U/µl), and dH_2O were added to make 10 µl final volume. The reaction mixture was incubated overnight at 4 °C and stored at -20°C until required.

(25 ng vector x kb of insert x 4)/3.0 kb vector = ng of insert

3.8.4 Transformation of Ligation Product

Competent cell were thawed on ice for 5 min before 5 µl of the ligation mixture was added, gently mixed by pipette, and left on ice for 30 min. The transformation reaction was heat-shocked in a 42 °C water bath for exactly 1 min. The reaction tube was immediately placed on ice for 5 min. The mixtures were removed from the tube and added to a new tube containing 1 ml of SOC medium. The cell

suspension was incubated with shaking at 37 °C for 90 min. The mixture was centrifuged at 13,000 rpm for 30 sec at room temperature, gently resuspended in 100 µl of SOC medium, spreaded onto a selective LB agar plate containing 50 µg/ml of IPTG and 20 µg/ml of X-gal, and further incubated overnight at 37 °C. The recombinant clones containing inserted DNA were white whereas those without inserted DNA were blue.

3.8.5 Detection of Recombinant Clone by Colony PCR

An interesting colony was picked by a toothpick 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 dNTP mix, 2 mM $MgCl_2$, 0.1 µM each of pUC1 primer (53.1-CCGGCTCGTATGTTGTGTGGA-33.1) and pUC2 primer (53.1-GTGGTGCAAGGCGATTAAGTTGG-33.1), and 0.75 U of *Taq* DNA polymerase. PCR was carried out in a thermocycler consisting of predenaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 1 min and extension at 72 °C (extension time depending on the length of interested insert) and 1 cycle of last extension step at 72 °C for 7 min. The colony PCR products were electrophoreses through 1.2% agarose gel and visualized under a UV transilluminator after ethidium bromide staining.

3.8.6 Isolation of Recombinant Plasmid

Recombinant plasmid DNA was isolated by alkaline lysis method with modification. Transformed cell containing recombinant plasmid was inoculated into LB broth (3 ml) supplemented with ampicillin (150 µg). After incubation at 37 °C overnight with vigorous shaking, 1.5 ml of the culture was then poured into a microcentrifuge tube and centrifuged at 13,000 rpm for 1 min at room temperature. After centrifugation was complete and the medium was discarded, another 1.5 ml of the culture was added to the pellet and centrifuged again. Bacterial pellet was resuspended in 200 µl of ice-cold GTE buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0) and incubated at room temperature for 5 min. Freshly prepared alkaline lysis solution (0.2 N NaOH, 1% (w/v) SDS) (400 µl) was added to bacterial suspension. The tube was closed tightly and mixed gently by inverting the tube until mixture was clear before placing on ice for 5 min. Ice-cold 3 M potassium

acetate, pH 4.8 (300 µl) was added to the tube and mixed gently by inverting the tube several times. The tube was kept on ice 5 min prior to the centrifugation at 12000 rpm for 5 min at room temperature. The supernatant was transferred to a fresh tube and mixed with as equal volume of Tris-HCl (pH 8.0) equilibrated phenol for 15 min (2 times). The mixture was centrifuged at 12,000 rpm for 10 min at room temperature. The upper aqueous phase was transferred to a sterile microcentrifuge tube and further extracted once with phenol: chloroform: isoamyl alcohol (25:24:1) and 2 times with chloroform: isoamyl alcohol (24:1). Plasmid in an aqueous phase was precipitated by the addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volume of ice-cold absolute ethanol. After incubation at -80 ^{O}C for 30 min, the precipitated plasmid was recovered by centrifugation at 10,000 rpm for 10 min at room temperature and briefly washed twice with 70% ethanol. The pellet was air-dried and dissolved in 50 µl of TE buffer. RNase A (10 mg/ml) 1 µl was added to digest contaminating RNA. Plasmid DNA was incubated at 37 ^{O}C for 1 hour and store at -20 ^{O}C until further required.

Alternatively, plasmid DNA was extracted using HiYield^{TM} Plasmid MiniKit (Real Biotech). Briefly, 1.5 ml of the inoculated culture was transferred to a microcentrifuge tube and centrifuged for 1 min at 14,000 rpm. The supernatant was then discarded. The pellet of bacterial cells was resuspended in 200 µl of PD1 buffer (RNase A added) with vigorous vortex. Cell was lyses by adding 200 µl of PD2 buffer, mixed gently by inverting the tube 10 times, and left at room temperature for 2 min until the lysate was clear. The mixture was neutralized by adding 300 µl of PD3 buffer, mixed immediately by inverting the tube 10 times, and centrifuged at 15,000 rpm, for 15 min at 10°C. The supernatant was carefully collected, applied to the PD column, and centrifuged at 8,600 rpm for 1 min at room temperature. The flow-through solution was discarded. The PD column was washed by adding 400 µl of W1 buffer and centrifuged at 8,600 rpm for 1 min at room temperature. The flow-through solution was discarded. The PD column was washed by adding 600 µl of Wash buffer (ethanol added) and centrifuged at 8,600 rpm for 1 min at room temperature. The flow-through solution was discarded. The PD column was centrifuged at 14,000 rpm for 2 min at room temperature to remove the trace amount of the washing solution. To elute DNA, the PD column was placed in a clean microcentrifuge tube and 50 µl of elution buffer was added to the center of column. The column was left to stand for 2

min before centrifugation at 14,000 rpm for 2 min at room temperature. The flow-through solution containing plasmid DNA was stored at -20^{O}C until further required.

3.8.7 Restriction enzyme digestion

Plasmid DNA (1-5 µl) was digested with *Eco*RI and separated by agarose gel electrophoresis as procedure described above in 3.5. The condition of digestion reaction was as follow; 1.33X buffer H, 0.13 mg/ml BSA, 5.33 mM spermidine and 3 U of *Eco*RI (Promega) in total volume of 15 µl. The reaction was incubated at 37 °C for 4 h.

3.8.8 DNA Sequencing

The recombinant plasmid was unidirectional sequenced with the M13 reverse and/or M13 forward primers or specific primers with Sanger dideoxy method by Macrogen Company

3.8.9 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). Significantly probabilities of matched nucleotide/ proteins were considered when the probability (E) value was 10^{-4}. The deduced amino acid sequences were characterized by Prosite and SMART programmed.

3.9 Conduction of estrogenic treatment in juvenile *L. subviridis*

3.9.1 Preliminary study

Acclimated mullets were separated into 2 groups; mullets (4 Individuals) intra-peritoneally injected with 17β-estradiol at the dose of 15 mg/kg body weight and normal mullets (5 Individuals). One week after the treatment, liver were dissected from the collected mullets and subjected to RNA extraction.

3.9.2 Estrogenic response study

Acclimated mullets were separated into 7 groups and each group contained 20 mullets. Mullets from each group were intra-peritoneally injected with 17β-estradiol dissolved in soybean oil at the concentrations of 0 (control), 0.05, 0.1, 0.25, 0.5, 1, and 5 mg/kg body weight respectively. From each treatment, 3 mullets were collected at 3 and 6 days of post treatment. Liver were dissected from the collected mullets and subjected to RNA extraction.

3.10 Expression of 17β-estradiol-responsive genes detected by semi-quantitative RT-PCR

3.10.1 Trial RT-PCR condition for Semi-quantitative analysis

Semi-quantitative RT-PCR condition of *chg*-L, *chg*-H, *vtg*-3 and actin were determined as follow; liver 1st-cDNA of all treatment group was pool and used for determined the condition by vary cDNA conc. at 50, 100, 250, 500 and 750 ng in 50 µl of PCR reaction and collect PCR product at 15, 20, 25, 30 and 35 cycles in following gradient; 1X PCR buffer, $MgCl_2$; 2 mM (actin, *chg*-H), 1.5 mM (*chg*-L, *vtg*-3), 0.1 mM dNTP, forward and reverse specific primer; 0.5 µM (actin, *chg*-H and *chg*-L), 0.25 µM (*vtg*-3) and 1U of *Taq* (Finnzyme) in 50 µl reaction mixture.

3.10.2 Semi-quantitative analysis of target genes

After appropriate condition of each gene for Semi-quantitative analysis was obtained, RT-PCR reaction for each gene was conducted as shown in table 3.7.

Table 3.7 Primers and PCR composition for semi-quantitative analysis of target genes

Gene	Template (ng)	Primers (µM)		*Taq* (U)	Buffer	$MgCl_2$ (mM)	dNTP (mM)	PCR condition
		Forward	Reverse					
chg-L	250	*chg*-L semi F(0.5)	*chg*-L semi R(0.5)	1	1X	1.5	0.1	H
chg-H	250	*chg*-H semi F(0.5)	*chg*-H semi R(0.5)	1	1X	2	0.1	G
vtg-3	250	*vtg*-3 semi F(0.25)	*vtg*-3 semi R(0.25)	1	1X	1.5	0.1	H
actin	100	actin semi F(0.5)	actin semi F(0.5)	1	1X	2	0.1	G

RT-PCR condition was conducted as follow:

Condition G

Initial denaturation step:	94 °C	3	min	for	1	cycle
Denaturation step:	94 °C	1	min			
Annealing step:	50 °C	1	min	for	25	cycles
Elongation step:	72 °C	2	min			
Extension step:	72 °C	7	min	for	1	cycle

Condition H

Initial denaturation step:	94 °C	3	min	for	1	cycle
Denaturation step:	94 °C	1	min			
Annealing step:	55 °C	1	min	for	25	cycles
Elongation step:	72 °C	2	min			
Extension step:	72 °C	7	min	for	1	cycle

3.11 Statistic Analysis

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

3.12 Determination of full-length cDNA sequences by Rapid Amplification of cDNA Ends polymerase chain reaction (RACE-PCR)

3.12.1 mRNA purification

Liver mRNA was purified from liver total RNA by QuickPrep *micro* mRNA Purification Kit (Amersham Biosciences). RNA pellet was dissolved in 25 µl DEPC-H_2O, 400 µl of extraction buffer was added to RNA solution, and the solution was mixed until homogenous. Elution buffer (800 µl) was added to the solution and mixed until homogenous. RNA sample and oligo(dT) cellulose were centrifuged at 14,000 rpm for 1 min 30 sec at room temperature, resulting in the removal of the

buffer from oligo (dT) cellulose. Total RNA was then placed on the top of pellet of oligo (dT) cellulose, mixed gently by inverting the tube for 3 min, and centrifuged at 14,000 rpm for 1 min 30 sec. The supernatant was removed before the pellet of oligo (dT) cellulose was washed with 1 ml of high salt buffer 5 times and 1 ml of low salt buffer 2 times. The washed pellet was resuspended in 300 µl of low salt buffer, transferred to MicroSpin column inserted in collection tube, and centrifuged at 14,000 rpm for 1 min 30 sec. The effluent was discarded and the pellet of oligo (dT) cellulose in the column was washed with 500 µl of low salt buffer for 3 times. After the effluent was discarded, the column was transferred to a new appendorf tube. mRNA was eluted twice by adding 200 µl each of prewarmed elution buffer (65 °C) to the column and centrifuged at 14,000 rpm for 1 min 30 sec. The eluate containing mRNA from both elutions was mixed and placed on ice. Total RNA was determined by diluting 10 times and 10 µl of eluate was qualified by running on 1% agarose gel. mRNA was precipitated by adding 10 µl of glycogen solution, 40 µl of potassium acetate solution (1/10 volume of mRNA sample) and 1 ml of 95% ethanol, incubated at -30 °C for 30 min, centrifuged at 12,000 g for 5 min at 4 °C, and stored at -80 °C until required.

3.12.2 Synthesis of 5' and 3'-RACE first strand cDNA

5' and 3'-RACE first strand cDNA were synthesized with BD SMARTTM RACE cDNA Amplification Kit (BD Biosciences) with some modification. The ingredient for 5'-RACE cDNA synthesis was as follow. One µg of liver mRNA was combined with 1.2 µM 5'-CDS primer and 1.2 µM BD SMART II A oligo in 5 µl final volume. The mixture was incubated at 70°C for 2 min and cooled on ice for 2 min followed by the addition of the following ingredient: 1X first strand buffer, 2 mM DTT, 1 mM dNTP and 1 µl of BD PowerScript Reverse Transcriptase and amplified at 42°C for 1 hour and 30 min. 5'-RACE cDNA was diluted with Tricine-EDTA buffer, incubated at 72 °C for 7 min, and stored at -20 °C until required. The protocol for 3'-RACE cDNA synthesis was similar to that for 5'-RACE cDNA synthesis except 1.2 µM 5'-CDS primer and 1.2 µM BD SMART II A oligo was replaced with 1.2 µM 3'-CDS primer A and BD PowerScript Reverse Transcriptase was replaced with ImpromII-Reverse Transcriptase. The reaction was incubated at 42 °C for 2 hours.

3.12.3 RACE-PCR

5' and 3' cDNA end sequences of *ER*, *chg*-L, *chg*-H, *vtg*-1, *vtg*-3 were amplified by RACE-PCR. Final concentration of the reaction for each gene was shown in table 3.8.

Table 3.8 Primers and PCR composition to amplify 5' and 3' cDNA end sequences of target genes.

Step/ side of gene	Template (µl)	Total volume (µl)	Primers (µM)		*Taq* (U)	Buffer	$MgCl_2$ (mM)	dNTP (mM)	PCR condition
			Forward	Reverse					
1st3'ER*	3'RACE cDNA (1)	25	ER F-RACE (0.2)	UPM (1X)	1X	1X	included in buffer	0.8	I
2nd3'ER#	1st PCR pdt (1)	25	ER F-RACE (0.2)	NUP (0.2)	0.5	1X	1.5	0.2	J
1st5'ERα*	5'RACE cDNA (5)	25	UPM (1X)	ERα R (0.2)	1	1X	included in buffer	0.8	K
2nd5'ERα *	1st PCR pdt (1)	25	NUP (0.2)	ERα R (0.2)	1X	1X	included in buffer	0.8	L
1st5'ER#	5'RACE cDNA (1)	25	UPM (1X)	ER R-RACE (0.2)	0.5	1X	1.5	0.2	I
2nd5'ER#	1st PCR pdt (1)	25	NUP (0.2)	ER R-RACE (0.2)	1	1X	1.5	0.2	J
1st5'ER*	5'RACE cDNA (1)	25	UPM (1X)	ER R-RACE (0.2)	1X	1X	included in buffer	0.8	I
2nd5'ER*	1st PCR pdt (1)	25	NUP (0.2)	ER R-RACE (0.2)	1X	1X	included in buffer	0.8	J
1st3'ERβ*	3'RACE cDNA (5)	25	ERβ F-RACE (0.2)	UPM (1X)	1X	1X	included in buffer	0.8	L
2nd3'ERβ *	1st PCR pdt (1)	25	ERβ F-RACE (0.2)	NUP (0.2)	1X	1X	included in buffer	0.8	L
1st3'ERβ (1)*	3'RACE cDNA (1)	25	ERβ F1-RACE (0.2)	UPM (1X)	1X	1X	included in buffer	0.8	M
2nd3'ERβ (1)*	1st PCR pdt (1:50) (1)	50	ERβ F1-RACE (0.2)	NUP (0.2)	1X	1X	included in buffer	0.8	J
1st5' *chg*-L#	3'RACE cDNA (1)	25	UPM (1X)	*chg*-L D12 R1 (0.2)	0.5	1X	1.5	0.2	I
2nd5' *chg*-L#	1st PCR pdt (1)	25	NUP (0.2)	*chg*-L D12 R2 (0.2)	0.5	1X	1.5	0.2	J

Table 3.8 Primers and PCR composition to amplify 5' and 3' cDNA end sequences of target genes (cont.).

Step/ side of gene	Template (µl)	Total volume (µl)	Primers (µM)		*Taq* (U)	Buffer	$MgCl_2$ (mM)	dNTP (mM)	PCR condition
			Forward	Reverse					
1st3' *chg*-L*	3'RACE cDNA (1)	25	*chg*-L F-RACE (0.2)	UPM (1X)	1X	1X	included in buffer	0.8	I
2nd3' *chg*-L*	1st PCR pdt (1:50) (1)	25	*chg*-L F-RACE (0.2)	NUP (0.2)	1X	1X	included in buffer	0.8	J
1st 5'*chg*-H (*chg*-L)*	5'RACE cDNA (1)	25	NUP (0.2)	*chg*-H R-RACE (0.2)	1X	1X	included in buffer	0.8	I
2nd 5'*chg*-H (*chg*-L)*	1st PCR pdt (1)	25	NUP (0.2)	*chg*-H R-RACE (0.2)	1X	1X	included in buffer	0.8	J
1st 5'*chg*-H*	5'RACE cDNA (1)	25	UPM (1X)	*chg*-H R1-RACE (0.2)	1X	1X	included in buffer	0.8	N
2nd 5'*chg*-H*	1st PCR pdt (1:50) (1)	50	NUP (0.2)	*chg*-H R1-RACE (0.2)	1X	1X	included in buffer	0.8	N
1st 3'*chg*-H*	3'RACE cDNA (1)	25	UPM (1X)	*chg*-H F1-RACE (0.2)	1X	1X	included in buffer	0.8	N
1st 5'*vtg*-1*	5'RACE cDNA (1)	25	UPM (1X)	*vtg*-1 R RACE (0.2)	1X	1X	included in buffer	0.8	N
2nd 5'*vtg*-1*	1st PCR pdt (1)	25	NUP (0.2)	*vtg*-1 R RACE (0.2)	1X	1X	included in buffer	0.8	N
1st 3'*vtg*-1*	3'RACE cDNA (1)	25	UPM (1X)	*vtg*-1 F1 RACE (0.2)	1X	1X	included in buffer	0.8	O
1st 5'*vtg*-3 (1)*	5'RACE cDNA (1)	25	UPM (1X)	*vtg*-3 R1 RACE (0.2)	1X	1X	included in buffer	0.8	P
1st 5'*vtg*-3 (2)*	5'RACE cDNA (1)	25	UPM (1X)	*vtg*-3 R2 RACE (0.2)	1X	1X	included in buffer	0.8	N

Table 3.8 Primers and PCR composition to amplify 5' and 3' cDNA end sequences of target genes (cont.).

Step/ side of gene	Template (µl)	Total volume (µl)	Primers (µM)		*Taq* (U)	Buffer	$MgCl_2$ (mM)	dNTP (mM)	PCR condition
			Forward	Reverse					
2nd 5'*vtg*-3 (2)*	1st PCR pdt (1:50) (1)	25	NUP (0.2)	*vtg*-3 R2 RACE (0.2)	1X	1X	included in buffer	0.8	N

* mean amplified with BD Advantage 2 Polymerase mix

mean amplified with EXT *Taq*

RACE-PCR condition was conducted as follow:

Condition I

Denaturation step:	94 °C	30	sec	for 5 cycles
Elongation step	72 °C	3	min	
Denaturation step:	94 °C	30	sec	
Annealing step:	70 °C	30	sec	for 5 cycles
Elongation step:	72 °C	3	min	
Denaturation step:	94°C	30	sec	
Annealing step:	68 °C	30	sec	for 20 cycles
Elongation step:	72 °C	3	min	

Condition J

Denaturation step:	94 °C	30	sec	
Annealing step:	68 °C	30	sec	for 20 cycles
Elongation step:	72 °C	3	min	

Condition K

Denaturation step:	94 °C	1	min	for 25 cycles
Elongation step:	72 °C	4	min	

Condition L

Denaturation step:	94 °C	30	sec	for 5 cycles
Annealing step:	71 °C	30	sec	
Elongation step:	72 °C	3	min	
Denaturation step:	94 °C	30	sec	for 5 cycles
Annealing step:	70 °C	30	sec	
Elongation step:	72 °C	3	min	
Denaturation step:	94 °C	30	sec	for 20 cycles
Annealing step:	68 °C	30	sec	
Elongation step:	72 °C	3	min	

Condition M

Denaturation step:	94 °C	30	sec	for 5 cycles
Annealing step:	70 °C	30	sec	
Elongation step:	72 °C	3	min	
Denaturation step:	94°C	30	sec	for 20 cycles
Annealing step:	68 °C	30	sec	
Elongation step:	72 °C	3	min	

Condition N

Denaturation step:	94 °C	30	sec	for 20 cycles
Annealing step:	60 °C	30	sec	
Elongation step:	72 °C	3	min	

Condition O

Denaturation step:	94 °C	30	sec	for 20 cycles
Annealing step:	60 °C	30	sec	
Elongation step:	72 °C	5	min	

Condition P

Denaturation step:	94 °C	30	sec	for 5 cycles
Elongation step	72 °C	4	min	
Denaturation step:	94 °C	30	sec	
Annealing step:	70 °C	30	sec	for 5 cycles
Elongation step:	72 °C	4	min	
Denaturation step:	94°C	30	sec	
Annealing step:	68 °C	30	sec	for 20 cycles
Elongation step:	72 °C	4	min	

3.12.4 Full-length cDNA amplification

Full-length cDNA of each target gene was amplified using primers designed to covered start and stop codon. Long distance RT-PCR was conducted using EXT *Taq* DNA polymerase (Finnzyme). Final concentration of the reaction for each gene was shown in table 3.9.

Table 3.9 Primers and PCR composition to amplify full-length cDNA of target genes.

Gene	Template (ng)	Primers (0.5 µM)		*Taq* (U)	Buffer	$MgCl_2$ (mM)	dNTP (mM)
		Forward	Reverse				
ERα	1,000	*ERα* full length F	*ERα* full length R	1	1X	1.5	0.2
chg-L	1,000	*chg*-L full length F	*chg*-L full length R	1	1X	1.5	0.2

Long distance RT-PCR condition was conducted as follow:

ERα

Initial denaturation step:	94 °C	3 min		for 1 cycle
Denaturation step:	94 °C	1 min	}	for 5 cycles
Annealing step:	55 °C	1 min		
Elongation step:	72 °C	2 min		
Denaturation step:	94°C	1 min	}	for 30 cycles
Annealing step:	50 °C	1 min		
Elongation step:	72 °C	2 min		
Extension step:	72 °C	7 min		for 1 cycle

chg-L

Initial denaturation step:	94 °C	3 min		for 1 cycle
Denaturation step:	94 °C	1 min	}	for 35 cycles
Annealing step:	57 °C	1 min		
Elongation step:	72 °C	2 min		
Extension step:	72 °C	7 min		for 1 cycle

3.13 Determination of genomic DNA sequences by GenomeWalk

3.13.1 Construction of Genome Walk libraries

Genomic DNA (3.5 μg each) was separately digested by 3 restriction endonucleases with the condition shown in table 3.10.

Table 3.10 Ingredients of genomic DNA digestion

Enzyme	Buffer and supplements	Incubation temperature (°C)	Incubation time (h)	Total volume (µl)
*Dra*I (fermentas)	Y^+/Tango™ (1X)	37	6	100
*Ssp*I (fermentas)	G^+(+BSA) (1X)	37	4	100
*Hae*III (Promega)	C (1X)	37	2	100

After digestion, DNA was extracted once with equal volume of phenol, once with equal volume of chloroform: isoamyl alcohol (24:1), precipitated with 3.5 volume of ice-cold absolute ethanol and 1/10 volume of 3 M sodium acetate pH 5.2, and incubated at -80°C for 30 min. The DNA pellet was recovered by centrifugation at 13,000 rpm for 10 min at room temperature, washed with 70% ethanol, air-dried, and dissolved in 10 µl of TE buffer. Purified DNA (500 ng) was ligated with 9 µM GenomeWalk Adaptors in 1X ligation buffer with 3U of T4 DNA ligase in 10 µl final volume and incubated at 16°C for 16 hours. Ligation reaction was stopped by heat at 70°C for 5 min in water bath and addition 30 µl of TE buffer to ligate and stored at -20 °C until required.

3.13.2 GenomeWalk PCR

5' and 3' genomic sequences of *chg*-L and *vtg*-1 respectively were amplified by GenomeWalk PCR. Final concentration of the reaction for each gene was shown in table 3.11

Table 3.11 Primers and PCR composition to amplify genomic sequences of target genes.

Gene	Template (µl) in total volume (µl)	Primers (0.2 µM)		*Taq* (U)	Buffer	$MgCl_2$ (mM)	dNTP (mM)	PCR condition
		Forward	Reverse					
1st 5'*chg*-L-*Dra*I, *Ssp*I, *Hae*III (1)*	Ligation pdt 0.5/50	AP1	*chg*-L R1	1	1X	1.1	0.2	Q
2nd 5'*chg*-L-*Dra*I, *Hae*III (1)*	1st PCR pdt (1:50) 1/50	AP2	*chg*-L R2	0.4	1X	1.1	0.2	R
2nd 5'*chg*-L *Ssp*I (1)*	1st PCR pdt (1:50) 1/50	AP2	*chg*-L R2	1	1X	1.1	0.2	S
1st 5'*chg*-L-*Dra*I, *Ssp*I, *Hae*III (2)*	Ligation pdt 0.5/50	AP1	*chg*-L D12 R1	1	1X	1.1	0.2	Q
2nd 5'*chg*-L-*Dra*I, *Ssp*I, *Hae*III (2)*	1st PCR pdt (1:50) 1/50	AP2	*chg*-L D12 R2	1	1X	1.1	0.2	R
1st 5'*chg*-L-*Dra*I, *Ssp*I, *Hae*III (2)#	Ligation pdt 0.25/50	AP1	*chg*-L D12 R1	1	1X	1.1	0.2	Q
2nd 5'*chg*-L-*Dra*I, *Hae*III (2)#	1st PCR pdt (1:50) 0.5/25	AP2	*chg*-L D12 R2	1	1X	1.1	0.2	R
1st 3'*vtg*-1 *Dra*I, *Ssp*I, *Hae*III #	Ligation pdt 2/25	AP1	*vtg*-1 F-RACE	1	1X	1.5	0.2	T
2nd 3'*vtg*-1 *Dra*I, *Ssp*I, *Hae*III #	1st PCR pdt (1:50) 1/25	AP2	*vtg*-1 F1-RACE	1	1X	1.5	0.2	U

* = amplified by *Taq* (Finnzyme), # = amplified by *Taq* EXT (Finnzyme),

GenomeWalk PCR condition was conducted as follow:

Condition Q

Denaturation step:	94 °C	25	sec	for	7	cycles
Elongation step	72 °C	3	min			
Denaturation step:	94°C	25	sec	for	32	cycles
Elongation step:	67 °C	3	min			
Extension step:	67 °C	7	min	for	1	cycle

Condition R

Denaturation step:	94 °C	25	sec	for	5	cycles
Elongation step	72 °C	3	min			
Denaturation step:	94°C	25	sec	for	20	cycles
Elongation step	67 °C	3	min			
Extension step:	67 °C	7	min	for	1	cycle

Condition S

Denaturation step:	94 °C	25	sec	for	5	cycles
Elongation step	72 °C	3	min			
Denaturation step:	94°C	25	sec	for	30	cycles
Elongation step	67 °C	3	min			
Extension step:	67 °C	7	min	for	1	cycle

Condition T

Denaturation step:	94 °C	25	sec	for	7	cycles
Elongation step	68 °C	3	min			
Denaturation step:	94°C	25	sec	for	32	cycles
Elongation step	65 °C	3	min			
Extension step:	67 °C	7	min	for	1	cycle

Condition U

Denaturation step:	94 °C	25	sec	for	5	cycles
Elongation step	66 °C	3	min			
Denaturation step:	94°C	25	sec	for	20	cycles
Elongation step	65 °C	3	min			
Extension step:	66 °C	7	min	for	1	cycle