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Thesis for the Degree of Master of Fisheries Science

Differentially expressed genes associated with a growth of Pacific abalone, *Haliotis discus hannai*

by

Doyeon Rho

Department of Fisheries Biology

The Graduate School

Pukyong National University

February 2016

Differentially expressed genes associated with a growth of Pacific abalone, *Haliotis discus hannai*

참전복 (*Haliotis discus hannai*) 의 성장과 연관된 유전자의 발현 차이

Advisor: Prof. Jong-Myoung Kim

by

Doyeon Rho

A thesis submitted in partial fulfillment of the requirements
for the degree of

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In the Department of Fisheries Biology, The Graduate School,
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Differentially expressed genes associated with a growth of Pacific abalone, *Haliotis discus hannai*

A dissertation

By

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February, 2016

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Differentially expressed genes associated with a growth of Pacific abalone, *Haliotis discus hannai*

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Abstract

Pacific abalone (*Haliotis discus hannai*) is one of the commercially important marine gastropods in Korea aquaculture industry. In order to identify the gene associated with a faster growth of abalones, RNA was isolated from soft tissues of abalone's spats for 200 days post-fertilization (dpf). Abalones were divided into three groups (small, medium and large) by size. Up to 20-fold variations in weights were noticed among small, medium, and large size groups ranging from 0.26±0.09 g, 1.43±0.405 g, and 24±1.09 g, respectively. RNA-Seq analysis was carried out to examine the transcriptome and differentially expressed genes associated with the faster growth. Transcript levels were quantified by RPKM values with 0.3 threshold value for differential expression. Based on transcriptome analysis and functional annotation, several transcripts were selected as candidates associated with faster growth in abalones. A transcript encoding vitelline envelope zona pellucida (ZP) domain 4 showed up-regulation in a larger size abalones. In order to examine its development stage-dependent

expression, RT-PCR analysis are carried out with abalones collected from egg, 50, 100, 150 and 200 dpf. RT-PCR using specific primers to ZP domain 4 gene indicated its female-specific expression in large group abalones of 200 and 300 dpf. During the study, RNA isolation from hepatopancreas and gonad, in particular from the large size abalones, seemed to be challenging. The difficulty in the following RT-PCR might be due to the presence of inhibitory materials co-precipitated with RNA. An efficient and simple method was developed for extracting high quality RNA from pacific abalones without inhibitory materials.

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Differentially expressed genes associated with a growth of Pacific abalone, *Haliotis discus hannai*

Ⅰ. Introduction

Abalones are commercially important marine gastropods in eastern Asia. Six speices of abalone have been identified in Korea coast: *Haliotis discus hannai, H. discus discus*, *H. madaka*, *H. gigantea*, *H. diversicolor*, *and H. diversicolor supertexta* (Kim et al., 1988). Pacific abalone, *Haliotis discus hannai,* is one of the most valued seafood and is the major species for abalone aquaculture industry in Korea. Production of abalones have been rapidly increased since 2000s as shown by its production of 20 tons in 2000 to 6,601 tons in 2011 (Korea National Statistical Office, KOSTA). However, the rate of production has been decreased in recent years possibly due to deteriorated environmental and genetic condition such as inbreeding depression (Park & Kim et al., 2013).

Various approaches have been employed to enhance the productivity of abalone industry. Nutritional approach was tried to increase the growth rate of juveniles, *Haliotis asinina*, by providing an artificial feed (Hahn et al., 1989; Emmanuel et al., 1996). Growth characteristics of many cultured molluscs including abalones and other gastropods were also shown to be improved through the selection of the aquaculture stocks using the selective genetics (York et al., 2011). Selection of fast-growing abalone species is important for the profit of the industry by reducing time to reach the market size (Beaumont & Hoare et al., 2003; Hahn et al., 1989). Since the growth rate of abalones as known to be associated with genders, sex differentiation and formation on gonads are also major determinants for commercial abalone production (Arcos et al., 2009). Therefore, it is important to understand of the sexual development-associated biology and gonad development to select the abalone with a faster growth. Doublesex/male abnormal-3-related transcription factor-1 (DMRT-1), vitelline envelope zona pellucida domain (VEZP) and vitellogenin 1(VTG1) were characterized as gender-specific genes in *Haliotis asinina* (Klinbunga et al., 2009; Amparyup et al., 2010). Some sex-related DNA markers have also been characterized in *Penaeus monodon* (Khamnamtong et at., 2006) and *Haliotis asinina* by using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) (Tang & Menasveta et al., 2004).

The objective of this study was to analyze the transcritome of *H. discus hannai* in association with its growth. For this, soft tissues of Pacific abalone were subjected to analysis for identifying growth-related gene and genes involved in sex differentiation. Several genes were identified to be associated with fast growth from transcriptomes analysis of *H. discus hannai* with different growth rates. In particular, one of the candidates was shown to be associated with sex differentiation. The gender-specific trasncript encodes vitelline envelope zona pellucida (ZP) domain 4. Expression levels of the trasncript was analyzed upon different stages of development to examine its association with a growth.

Extraction of RNA from biomaterials is a prerequisite for analyzing the transcriptome, levels of gene expression using reverse transcription polymerase chain reaction (RT-PCR). Isolation of intact RNA without degradation or inhibitory materials for cDNA-synthesis or PCR amplification is critical for the transcriptome and tissue-specific expression analysis. In this study, RNA isolation was carried out by using Trizol family of reagent. RNA isolation from hepatopancreas and gonads of abalones with sexually mature phenotype seemed to be challenging as the difficulty in the following RT-PCR was noticed. This is might be due to the presence of inhibitory materials co-precipitated with RNA.

In this study, three RNA isolation methods were compared for their

efficient removal of the inhibitors during lysis of the cells, separation of RNA containing aqueous phase and precipitation. As a result, an efficient and simple method was developed for extracting high quality RNA from hepatopancreas and gonads of adult Pacific abalones.

Ⅱ. Materials and Methods

1. Materials

Various sizes of Pacific abalones (*Haliotis discus hannai*) at 200 dpf were collected from the Genetics & Breeding Research Center of the National Fisheries Research Development Institute (NFRDI, Geoje, Korea). Abalone eggs and abalones at 50, 100, 150, 200 and 300 dpf were obtained from Abalone Research Institute of JeollaNamdo Marine-Fisheries & Development Institute (Wando, Korea). Fully mature abalones were collected from Namcheon Fish Market & Abalone Research Institute of JeollaNamdo Marine-Fisheries & Development Institute. Total RNA was isolated using FavorPrep™ Tri-RNA reagent obtaining from FAVORGEN Biotech Corporation (Ping-Tung, Taiwan) and Hybrid^{R} columns obtaining from GeneAll (Seoul, Korea). RNase-free DNaseI was obtained from Roche (Indianapolis, ILUSA). RNASeq and transcriptome analysis are carried out from Insilicogen Inc (Suwon, Korea). M-MLV cDNA synthesis kits were purchased from Enzynomics (DaeJeon, Korea). HIQ-Mix and oligonucleotide primers were obtained from Genotech (DaeJeon, Korea).

2. RNA isolation

Various sizes of *H. discus hannai* (n=80) grown for 200 dpf were obtained from NFRDI, and divided into three groups (small, medium and large) by size. Weights and shell lengths, heights, and widths were measured for each abalone. Immediately after the collection, soft tissues were dissected from abalones, frozen on lipid nitrogen, and then stored at −80℃. RNA were extracted from each tissue using Tri−RNA reagent (Favorgen) as follows. Soft tissue (~0.1g) was added with 1ml Tri−RNA reagent and grounded in a glass homogenizer. Upon addition of 200 ㎕ chloroform to the homogenate followed by centrifugation at 15,000 x g, 4℃, for 5 min, and 500㎕ supernatent (aqueous phase) were mixed with an equal volumn of isopropanol. RNA was precipitated upon centrifugation at 15,000 x g, 4℃, for 5 min and RNA pellet was washed with 70% ethanol followed by centrifugation at 15,000 x g, 4℃, for 5 min. RNA pellet suspended in RNase−free water was treated with RNase−free DNase I at 37℃ for 15 min. RNA was further purified by using Hybrid^{R} column following the manufacturers instructions. RNA integrity was indentified by 2 % agarose gel electrophoresis followed by staining with ethidium bromide (Etbr). Amount of RNA was measured by Micro−volumn Nucleic Acid spectrophotometer (ASP−2680, V4.1, ACTGene).

3. Construction of transcriptome library and transcriptome analysis

RNA was further isolated by using Truseq RNA sample prep Kit (Illumina) including polyA^{+} RNA isolation was used for construct mRNA library. cDNA library construction was produced using reverse transcriptase, random primer and Truseq adaptor connected to phosphorylated cDNA-terminal. Sequencing was carried out by Illumina HiSeq2000 (Illumina, Inc., USA). Low quality base and adaptor were removed using CLC Assembly Cell package (ver 4.2.0, CLCBio, Arhaus, Denmark), and subjected to *de novo* transcript assembly using CLC_assembler (CLC Assembly Cell). CLC_mapper was used to select the best transcriptomes by short sequence reads using transcriptome assembly. Functional analysis of transcriptome was mapped by Blastx program using Metazoa database of UniProt. Arrangement of the best-matched UniProt entry was carried out with $1E^{-5}$ of threshold. Gene Ontology (GO) and KEGG analysis were carried out by annotation information from UniProt entry. Expression level of the transcripts was compared by the RPKM (Reads Per kb per Million reads) methods (Mortazavi et al., 2008) and transcripts with higher than 0.3 threshold RPKM value were regarded to be expressed (Ramskold D et al., 2009).

Fold change and p-value (Audic and Clavaie's method) were calculated through the comparison of RPKM values of three groups. The false discovery rate (FDR) was obtained to multiple testing correction using Benjamini-Hochberg method (Benjamini-Hochberg et al., 1995). Differentially expressed genes were selected by 2 (FDR<=0.05), 4 (FDR<=0.01), and 10 (FDR<=0.001) fold change values.

4. RT-PCR

To confirm the developmental stage-dependent expression of the transcript, total RNAs were extracted from eggs and large abalones collected at 50, 100, 150, and 200 dpf. And differential expression of the selected transcripts was confirmed by RT-PCR. For this, total RNAs were extracted from individual Pacific abalones, *H. discus hannai*, ($n \geq 8$) grown for 200 and 300 dpf divided into large and small group. cDNA synthesis was performed in total 40$\mu\ell$ reaction using M-MLV cDNA synthesis kit (Enzynomics, Korea). This includes 80 μM oligo dT primer annealed and 2μg of total RNA in a total volume of 20 $\mu\ell$ incubated at 70℃ for 5 min and cooling down to 4℃. Reverse transcription was performed by an addition of 20 $\mu\ell$ mixture including 10X M-MLV RT buffer, 2 μM dNTP, 40 U RNase inhibitor and 200 U

M-MLV reverse transcriptase. Total of 40 ㎕ mixture was incubated at 42 ℃ for 60 min followed by inactivation heat at 72℃ for 10 min.

Primers were designed for sequences corresponding to the differentially expressed transcript candidates associated with a faster growth of abalones (Table 1). RT-PCR was performed in a 20 ㎕ reaction composed of 2 ㎕ cDNA prepared above, 1 ㎛M target-specific primers in 1X HiQ-PCR Mix (Genotech, Korea). Standard PCR was composed of pre-denaturation at 95℃ for 3 min, followed by cycles of denaturation at 95℃ for 30 sec, annealing at 55℃ for 30 sec, and extension at 72℃ for 30 sec (Biorad thermal cycler T1000). Final extension was carried out at 72℃ for 5 min. Transcript specific to ribosomal protein L3 was included as a positive control for normalization of cDNA template.

To examine tissue- and female-specific expression of the selected transcript, tissues including tentacle, gill, intestine, hepatopancreas, gonad, mantle and muscle were dissected from fully mature *H. discus hannai*. RNAs isolated from individual tissues were used for cDNA synthesis and RT-PCR as described above.

5. Comparison of RNA extraction methods from hepatopancreas and gonads of abalones

To compare the efficiencies of RNA isolation, hepatopancreas, gonad and muscle were dissected from fully mature female and male *H. discus hannai*.

5-1. RNA extraction methods

Three different methods were tested for comparing efficiency of RNA extraction. The common protocol (Method Ⅰ) used Trizol reagent to lyse the cells and seprate into an phase upon addition of chloroform. Isopropanol was used to precipitate the RNA. Two other methods (Method Ⅱ and Ⅲ) include additional steps in the protocol. Method Ⅱ includes on the physical separation step using centrifugation before chloroform treatment in Method Ⅰ. Method Ⅲ includes a lithium chloride (LiCl) precipitation additional to the RNA prepared from Method Ⅱ.

Method Ⅰ. Common method based on trizol reagent

Tissue (~0.1g) was homogenized in 1ml of trizol (FAVORGEN) in a glass homogenizer. Homogenate was thoroughly mixed with 200㎕

chloroform followed by centrifugation at 15,000 x g, RT, 5 min. The supernatent was mixed with an equal volumn of isopropanol and incubated at room temperature for 10 min. Upon centrifugation at 15,000 x g, RT, 5 min, RNA pellet was washed with 70% Ethanol. RNA pellet was suspended in RNase-free water.

Method Ⅱ. RNA isolation method involving centrifugation to remove debris

Tissue (~0.1g) homogenized in 1ml of trizol as described in Method Ⅰ were centrifugated twice at 15,000 x g, for 5 min at RT. The supernatant (800㎕) transferred to a fresh tube was mixed with 160㎕ chloroform and then centrifugated at 15,000 x g for 5 min. The following steps including isopropanol precipitation and ethanol washing were the same as described in Method Ⅰ.

Method Ⅲ. Additional lithium chloride precipitation

Tissue (~0.1 g) were grounded in Trizol, centrifuged twice, treated with chlroform and isopropanol as the same in Method Ⅱ. RNA pellet was dissolved in 600㎕ of DEPC-water and then added with 400㎕ 5M LiCl. The mixture was incubated at -20℃ for 3 hours followed by centrifugation at 15,000 x g at RT for 5 minutes. Upon washing with 70% Ethanol, RNA pellet was used for cDNA synthesis.

5-2. RT-PCR

RNAs prepared from three isolation methods were treated with RNase-free DNaseI at 37℃ for 15min and then subjected to Hybrid^{R} column purification according to the manufactruer's instructions. Total 2 ㎍ RNA was used to synthesize the first-strand cDNA as discribed before in section 4. RT-PCR was performed in a 20㎕ reaction including 2 ㎕ cDNA and 1 ㎛M primers specific to ribosomal protein L3 in 1X HiQ-PCR Mix (Genotech, Korea). Amplification reaction cycles were composed of predenaturation at 95℃ for 3 min, deneturation at 95℃ for 30 sec, annealing at 55℃ for 30 sec, and extension at 72℃ for 30 sec (BioRad thermal cycler T1000). The final extension was carried out at 72℃ for 5 min. PCR product was indentified by agarose gel electrophoresis.

5-3. Test for PCR- and cDNA synthesis inhibitor

PCR inhibitor. To test whether the materials copurified with RNA inhibit the PCR amplification step, PCR was carried out with different ratios of cDNA prepared from different methods. Total RNAs extracted from hepatopancreas of fully mature abalones using three isolation methods are subjected to cDNA synthesis as described above. cDNA was synthesized with 2 ㎍ RNA isolated from hepatopancreas by using

Method Ⅰ and Ⅲ, respectively. PCR amplification was carried out with different ratios of cDNA templates (10:0, 7.5:2.5, 5:5, 2.5:7.5, and 0:10). cDNA synthesized with RNA isolated from hepatopancreas using Method Ⅲ and 3' DW were included a positive control.

cDNA synthesis inhibitor. To test whether the materials inhibit cDNA synthesis step, cDNA was synthesized with RNAs mixed with different ratios tissues isolated using different extraction methods. Total RNA of muscles and hepatopancreas extracted by Method Ⅰ and Ⅲ the concentrations were adjusted to 200ng/㎕. RNAs isolated from hepatopancreas using Method Ⅰ and muscles using Ⅲ were mixed together in different ratios (10:0, 7.5:2.5, 5:5, 2.5:7.5, and 0:10). Mixture of hepatopancreas and muscles extracted using Method Ⅲ was a positive control. Mixtures of RNAs were subjected to cDNA synthesis as described above.

PCR was performed in a 20㎕ reaction including 10 ㎕ of cDNA mixture, 1 ㎛M Ribosomal protein L3 primers and HiQ-PCR Mix (Genotech, Korea). Amplification reaction was same discribed condition above, but cycles was normalized depending on template amount.

6. Data analysis

PCR products were identified on 2% agarose gel electrphoresis followed by staning with dthidium bromide and quantified by Gel Doc System/Station (BIORAD, USA) with background subtraction.

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Ⅲ. Results and Discussion

1. Differential growth in Pacific abalones

Various size of Pacific abalones, *Haliotis discus hannai*, grown for 200 dpf were collected for transcriptome analysis. Abalones were divided into three (small, medium, and large) groups by sizes as measured by weight, shell width, shell lengh and shell height (Table 2). They showed up to 20-fold differences in weights as shown by 0.26±0.09 g, 1.43± 0.405 g, and 5.24±1.09 g, respectively, of small, medium, and large groups (n=10 for each group). Up to 3-fold differences in shell length were detected between small and large groups (Table 2). Abalones with various size and developmental stages (Table 3) were also collected to confirm the results. Table 3 showed differences in weights of abalones collected at 100 to 300 dpf.

2. Transcriptome analysis of Pacific abalones

To examine the genes associated with differential growth in abalones, RNAs were isolated from soft tissues of abalones with variation in sizes.

To reduce the problems associated with deviation stemmed from a single organism, RNAs isolated from ten different abalones in the same group were mixed. According to Ramskold D et al. (2009), expression of transcripts are compared by RPKM values with higher than 0.3 threshold value. A total of 117,537 reference contigs were obtained (Table 4A) through *de novo* assembly and BLASTx, and transcripts of 28,981 (24.7%) best hit by BLASTx(Table 4B). Gene ontology(GO) assignment revealed three categories of transcripts including molecular functions (MF), biological process (BP), and cellular component (CC). Trascripts categorized in molecular functions (MF) was revealed 17,872 (61.9%) involved zinc ion binding, ATP binding at the top. A total of 12,647 (43.6%) transcripts were categorized in the biological process (BP) comprised predominantly those involved in DNA integration and proteolysis (Table 4 and Figure 1). Differentially expressed transcripts were determined from comparison of RPKM values of genes from different size groups (Table 5). In case of transcripts showing more than 10-fold differential expression between large and small group, 543 up-regulated transcripts and 428 down-regulated transcripts were identified from the large size abalone.

RT-PCR was carried out with at least two sets of templates prepared different batches of abalones to confirm differences observed in RNAseq analysis. PCR primers corresponding to the sequences obtained from

RNAseq analysis were designed and analyzed by OligoAnalyzer 3.1 (IDT, USA).

3. Differentially expressed genes in Pacific abalones

Differential expression observed in transcriptome analysis was confirmed by RT-PCR using first with cDNA templates prepared from RNA used for DEG analysis at 200 dpf. As a result, six transcripts out of 34 candidate transcripts associated with fast growth showed an expression profiling consistent with RNAseq analysis. Differential expression pattern was confirmed with two independent sets of templates by RT-PCR (Table 1 and Figure 2). Transcript encoding a ribosomal protein L3 (Tsc1) showing a constant expression in all three groups(0.8-1.2 FC) was included as an internal control to normalize the expression level of the transcripts. Five transcripts encoding sequences similar to putative incilarin A (Tsc 2), perlucin (Tsc 5), transforming growth factor-beta-induced protein ig-h3 (Tsc 8), vitelline envelope zona pellucida (ZP) domain 4 (Tsc 34) and defensin (Tsc 35) were up-regulated in a larger size abalones. One trancript encoding a sequence similar to tomoregulin-2 (Tsc 20) was confirmed to be down-regulated in a large size abalone by RT-PCR. Expression pattern of Tsc 34

related to sexual maturation and reproduction of oocytes in ovary were further tested.

3-1. Female-specific expression of Tsc 34

Fully mature abalones were tested for determining expression pattern of Tsc 34. Primers corresponding to a vitelline envelope ZP domain 4 of *H. discus hannai* were designed (Figrue 3 and Table 1). To test the tissue-specific expression profile of Tsc 34, RNAs of tissues including ganglion, tentacle, gill, heart, hepatopancreas, intestine, gonad, mantle, muscle were extracted for cDNA synthesis as described above. The result in Figure 4A indicated that the transcript was detected only in gonads but not in other tissues including ganglion, tentacle, gill, heart, hepatopancreas, intestine, gonad, mantle, muscle in female (Figure 4A).

In order to verify gender-specific expression of Tsc 34, RT-PCR was carried out with cDNA synthesized from RNA isolated from fully developed gonads. Sexually mature 13 female and 6 male abalones were subjected to RNA isolation. The result showed that Tsc 34 was expressed exclusively in gonads from females but not from males (Figure 4B).

Various sex-related genes genes including double-sex male abnormal-3-related transcription factor 1 (DMRT1), tektinA1 (TekA1), sperm lysin (SL) and vitelline envelope zona pellucida domain 2

(VEZPD2) in *H. asinina*, *H. laevigata* and *Penaeus monodon*were already reported (Amparyup et al., 2010; Omar et al., 2014; Klinbunga et al., 2009; Khamnamtong et al., 2006). Vitelline envelope ZP domain 4 (Tsc 34) is known to be expressed in growing oocytes in ovary (Lira et al., 1990; Akatsuka et al., 1998), and in an external part of the egg coat of marine gastropod abalone. It is also a prominent gene out of various vitelline envelope (VE) proteins used for identify abalone species (Aagaard et al., 2006). As a result, Tsc 34 exclusively expressed in a female abalone can be used as a marker for distinguishing the gender in *H. discus hannai*.

3-2. Developmental stage-dependent expression of Tsc 34

To examine the developmental stage-dependent expression of Tsc 34 together with gonad formation and sex differentiation in Pacific abalones, RNAs were isolated from at least two large-size individuals including eggs, and abalones collected at 50, 100, 150, and 200 dpf. Amplification of cDNA synthesized was carried out as described above using Tsc 34. Comparison of the levels of Tsc 34 and Tsc 1, a control, were shown in Figure 5. Expression of Tsc 34 was clearly detected in eggs. The result clearly revealed the detection of Tsc 34 in abalones of at least 200 dpf. The result indicated that sexual maturation for female in abalones was decided at least 200 dpf and identificated by RT-PCR using Tsc 34.

Molecular mechanisms of gonad development in abalone have been interest in aquaculture industres (Klinbunga et al., 2009; Amparyup et al., 2010). While most studies were carried out to understand development of adult gonads (Tomita et al., 1967, 1968), a little was known for information on gonad formation and sex differentiation processes of abalone during the juvenile stages (Awaji & Hamano et al., 2004). Maturation of gonads development were identified from abalones with the shell lengths of juveniles, *H. discus hannai*, ranging from 15 to 34 mm at about 8 months after settlement (Awaji et al., 2004). Abalones at 200 dpf used in this study were shell length 32.70 ± 3.41 (Table 3), as similar to result reported Awaji et al (2004). Threrfore, sex determination and gonad formation would be identified from juvenile at least 200 dpf of Pacific abalones. The results, together with the fact that gender of abalones at 200 dpf was not distinguishable by physical examination, indicated that Tsc 34 can be used as a marker.

3-3. Differential expression of a female-specific gene associated with fast growth in abalones

Expression of Tsc 34 was further analyzed to examine the relationship between growth rate and sexual development in abalones. The previous result showed that more than 10-fold differences in expression of Tsc

34 was detected between groups of two different size (small and large) in RNAseq analysis. RNA was isolated from soft tissues of small and large abalones. Differential expression of Tsc 34 was confirmed in abalones of 200 and 300 dpf by RT-PCR (Figure 6) although the detection frequencies of Tsc 34 varied depending on ages of abalones. In case of 200 dpf abalones, Tsc 34 was detected in three of four individuals belongs to the large group but not in a small size group (Figure 6A). For abalones of 300 dpf, Tsc 34 was detected in one out of four abalones in a small size group, and three out of four in a large size group (Figure 6B). The results indicating a higher frequency detection of Tsc 34 in a large size suggest a fatster growth rate of female than that of male. This, together with its female specific expression, indicated that Tsc 34 may be used as a marker for identification of fast growth and female-specific sex determination of *H.discus hannai*. The result also provide clues for differences in growth rate between females and males.

4. Comparison of RNA isolation methods

Most common RNA isolation methods are based on the Trizol reagents including acid guanidinium thiocyanate-phenol-chloroform extraction

solution (Chomczynski & Sacchi et al., 1987). RNAs were extracted by treating tissues with trizol reagent to lyse the cell followed by chloroform the addition to facilitate separating of aqueous and organic material. RNA was precipitated with isopropanol and collected by centrifugation. The most popular Trizol reagent method allowed simultaneous processing of many samples and the entire procedure was completed in short time.

During the study involving RNA extraction from the soft tissues of abalones with different developmental stages, this method seemed to be challenging from hepatopancreas and gonads of mature abalone as there was a difficulty in detecting the transcript by RT-PCR (Figure 7). Figure 7 showed the RT-PCR results using RNAs isolated from various tissues of sexually immature and mature abalones, respectively. Abalones were divided into mature and immature abalone by their visually identifiable gonad morphologies (Figure 7A). Mature abalone refers to the one with clearly distinguishable gonads development identified by visual observation. RNAs were isolated from each tissues by Method Ⅰ. While there were successful in amplification five tissues, no PCR amplified bands were detected in cDNA prepared from gonad and hepatopancreas of mature abalones (Figure 7B).

This result was further tested for hepatopancreas and gonads of several abalones collected from different developmental stages (Figure 8

and Table 6). Some hepatopancreas using Method Ⅰ showed a difficulty in amplifying the Tsc 1 (Figure 8A). This was also confirmed in RNA isolation from tissues mixed with hepatopancreas and gonads was not detected by RT-PCR (Figure 8B) while no difference was detected in RNAs isolated from the muscle of the same abalones. However, the A260/280 ratio and RNA yield was similar to three methods (Figure 9), RNA extracted from hepatopancreas and gonad depending on thair maturation states using Method Ⅰ was unsuitable for RT-PCR amplification.

In order to isolate RNA from hepatopancreas and gonads of abalone of at 2-5 years that may contain large amounts of inhibitory materials, Method Ⅱ and Ⅲ were developed to add several steps and tested using many abalones. Method Ⅱ and Ⅲ were modified from a protocol developed by Manickavelu used RNA isolation from wheat pistils (Manickavelu et al., 2006). Little differences in the A260/280 ratio and RNA yield were noticed among RNA isolated by three methods (Figure 9).

Method Ⅱ involved centrifugation before adding chloroform to remove impurity or all debris. The following steps including chloroform extraction and isopropanol precipitation were the same as in Method Ⅰ. However, amplification of cDNA was not detected in RNA isolated from hepatopnacreas using method Ⅰ consistently (Figure 10A). The result

also indicated a difficulty in RNA isolated from hepatopancreas and gonads by using Method Ⅰ and Ⅱ (Figure 10B) while no difference was detected in RNAs isolated from the muscle of the same abalones. These RNA isolated by two methods may not be sufficient for the demanding high-quality with hepatopancreas and gonads of *H. discus hannai* because of high levels of inhibitors.

Another Method Ⅲ included a lithium chloride treatment step in addition to method Ⅱ. Precipitation by LiCl was suggested to be a good quality RNA isolation method (Hong et al., 1995), in particular for removing inhibitors of translation or cDNA synthesis (Cathala et al., 1983). While all three methods did not show any difference in the A260/280 ratio and RNA yield (Figure 9), total RNA isolated from abalones by Method Ⅲ including an additional precipitation by LiCl seemed to be high quality without inhibitor problems in RT-PCR (Figure 10A, B). cDNA synthesized using total RNAs were isolated from muscle as a positive control was clearly detected (Figure 10A, B). The result indicated that the Method Ⅲ is the most reliable RNA isolation method from hepatopancreas and gonads of mature female and male as shown to be successful for the following RT-PCR (Figure 10).

Extraction of high quality RNA are essential for construction of cDNA libraries and identification by RT-PCR analysis (Liu et al., 2005). However some differential were noticed for isolated RNA without

inhibitory materials from hepatopancreas and gonads from mature abalones depending on RNA isolation methods. Most of the known inhibitors were believed to be organic compounds such as phenol, polysaccharides, sodium dodecyl sulphated (SDS) and different proteins such as collagen, haemoglobin, immunoglobin G (IgG) (Rossen et al., 1992). Oysters and bivalves were also known to contain inhibitors such as glycogen, polysaccharides (Atmar et al., 1993, 1995; Richards et al., 1999). Polysaccharides were seemed to be partition into the aqueous phase during phase separation and co-precipitate with RNA in the RNA precipitation step. It may interfere with downstream applications such as RNA purification, cDNA synthesis and RT-PCR (Dellacorte et al., 1994; Kansal et al., 2008). Method Ⅲ is thus a simple, rapid and high-throughput procedure for the isolation of RNA from hepatopancreas and gonads of *H. discus hannai* without inhibitors.

4-1. Identification of PCR- and cDNA synthesis inhibitor

Amplification of RNA isolated from hepatopancreas and gonads of some mature abalones using Method Ⅰ may not be suitable by RT-PCR analysis as an absence of the amplified products refering to the presence of copurified the materials that may inhibit (Schrader et al., 2012). This may result from either difficulties in intact RNA isolation from hepatopancreas and gonads or the presence of copurified materials that

may inhibitory to cDNA synthesis or PCR amplification. There were little differences in RNAs isolated by three methods in terms of the RNA yield determinated by A260/A280 rates, and intactness obseved by gel electrophoresis (Figure 9). RNAs extracted from hepatopancreas and gonads were not major point. The results indicated may that inhibition occur in RT-PCR or cDNA synthesis steps. In order to test whether the inhibitor affect PCR amplification or cDNA synthesis, the following expreiments were carried out.

To test inhibitory effect on PCR inhibitor, mixture of cDNAs prepared from different methods were tested by RT-PCR. For this, cDNA of hepatopancreas using Method Ⅰ was mixed in different ratios (0:10, 1:3, 1:1, 3:1, 10:0) together with hepatopnacreas cDNA using Method Ⅲ (Figure 11A). Each cDNA was included as as a positive control (Figure 11B). The result reflect the amount proportional to the template from Method Ⅲ. cDNA synthesized from Method Ⅰ did not inhibit reaction at any ratios. That is, inhibitors including hepatopancereas were not acted on PCR stage.

To test further whether inhibitors acted on cDNA synthesis, cDNAs were synthesized mixtures of RNAs isolated from hepatopancreas using Method Ⅰ mixed with muscle using Method Ⅲ. The ratio range from 0:10, 1:3, 1:1, 3:1, 10:0. The result indicated a clear inhibitory action of RNAs isolated from hepatopancreas using Method Ⅰ by RT-PCR

(Figure 12A). In contrast, cDNA synthesized with total RNA mixture isolated from muscle and hepatopancreas d using Method Ⅲ showed little differences in amplification (Figure 12B). That is, RNA of hepatopancreas isolated using Method Ⅰ may contain materials inhibitory to cDNA synthesis (Figure 12A). The result indicated that hepatopancreas have an inhibitor to disturb cDNA synthesis. Moreover, some RNAs isolated using Method Ⅰ were not suitable for removal inhibitor to synthesize cDNA.

There are various inhibitors to interfere RT-PCR amplification or cDNA synthesis. One of inhibitors, phenols, may cross-link RNA under oxidizing conditions and thus impeded RNA isolation (Su and Gibor et al., 1988). Presence of organic compouds such as polysaccharide was also suggested to interfere with cDNA synthesis because of the inhibitor was entrapped nucleic acids and co-precipitated with RNA (Wang et al., 2009). Reverse transcription may be also inhibited by direct interaction of the enzyme with melanin (Eckhart et al., 2000). The modified or degraded DNA may cause interference of PCR amplipication. For example, annealing of the primers to the DNA template may be disturbed by certain PCR inhibitors (Chandler et al., 1998). Lithium chloride precipitation was often used to isolate RNA containing materials such as polysaccharide or phenolic compounds (Wan & Wilkins et at., 1994; Yao & Wang et al., 2008). So, we predicted inhibitor including

hepatopancreas and gonads of Pacific abalones such as polysaccharide or phenolic compounds to inhibit cDNA synthesis.

In conclusion, RNA purified from hepatopancreas and gonads of mature Pacific abalone may contain copurified materials inhibitory cDNA synthesis. While these inhibitors were not removed by commonly RNA isolation method, addition of a preliminary centrifugation and a lithium chloride treatment (Method Ⅲ) may be able to remove inhibitors effectively. The proposed protocol (Method Ⅲ) is simple, efficient and effective particularly for removal of inhibitor.

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Gene ID	RPKM			E value	Description	bp	Primer 5' – 3'
	Large	Medium	Small				
Tsc1	1294.33	1227.59	1392.61	0	Ribosomal protein L3	1401	F:5' – TGTCACCAT CCTTGAGGCAC R:5' – CAGGAACAGGCTTCTCCAGG
Tsc2	10.03	4.08	0.88	5.00E-16	Incilarin A	709	F:5' – GGCGGCTACCTGGTTGAAAT R:5' – CCCACGATGTCTGGTCCATC
Tsc5	19.26	11.71	2.74	7.00E-52	Perlucin	375	F:5' –CCTCTTGGGTTTATGCAGCAC R:5' –CGG ACT GTC TCA TTT CCA GAC
Tsc8	33.68	23.4	7.37	3.00E-8	Transforming growth factor-β-induced protein ig-h3	399	F:5' – GTA CGC ATG GTC ACC TAC CC R:5' – TCGTAGCAGGGGTATGTTGTTA
Tsc20	45.46	25.82	374.98	1.00E-23	Tomoregulin-2	722	F:5' –ACTGTGACCATGAGCGTGAG R:5' –CAGCTACGTGGAGATCACGG
Tsc34	2.28	0.6	0	0	Vitelline envelope zona pellucida domain 4	990	F:5' –ATCAGCTGCTACTTCCAGCC R:5' –ACGGATCCCCATTCACGATG
Tsc35	155.23	89.83	33.46	1.00E-32	Defensin	614	F:5' –CTGCTTCTGCTGTGTTTGGT R:5' –CGACAGACACAGACGCCATT

Table 1. Transcripts associated with a fast growth of Pacific abalones. Levels of transcripts identified to be differentially expressed in large, medium and small size groups of abalones were indicated.

Group (200 dpf)	S (n=10)	M (n=10)	L (n=10)	Mean±SD (n=80)
Weight (g)	0.26 ± 0.09	1.43 ± 0.40	5.24 ± 1.09	2.10 ± 1.81
Shell length (mm)	13.53 ± 1.34	24.12 ± 2.49	36.15 ± 2.21	24.63 ± 8.17

Table 2. Sizes of Pacific abalones collected at 200 days post-fertilization for RNA-seq anaylsis.

Group (100 dpf)	S (n=31)	L (n=29)	Mean±SD (n=60)
Weight (g)	0.03 ± 001	0.07 ± 0.05	0.05 ± 0.04
Shell length (mm)	7.09 ± 0.72	9.27 ± 2.06	8.14 ± 1.87
Group (150 dpf)	**S (n=28)**	**L (n=30)**	**Mean±SD (n=58)**
Weight (g)	0.29 ± 0.12	0.65 ± 0.18	0.48 ± 0.24
Shell length (mm)	14.88 ± 2.55	19.91± 1.70	17.49± 3.31
Group (200 dpf)	**S (n=19)**	**L (n=10)**	**Mean±SD (n=29)**
Weight (g)	0.37 ±0.87	3.79 ±0.98	1.55 ±1.88
Shell length (mm)	8.25 ±2.02	22.82 ±1.74	13.28 ±7.30
Group (300 dpf)	**S (n=20)**	**L (n=20)**	**Mean±SD (n=40)**
Weight (g)	2.03 ± 0.50	7.22 ± 2.09	4.63 ± 3.30
Shell length (mm)	27.43 ± 2.58	40.72 ± 3.18	34.07 ± 7.31

Table 3. Various sizes of Pacific abalones used for identification of developmental stage-dependant expression of Tsc 34.

<A>

#. Contigs	Residues	Average	Minimum	Maximum
117,537	94,559,630	804.5	200	18,558

<B>

#. Best hit	GO Assignment		
28,981 (25%)	Cellular component	Molecular function	Biological process
	10,487 (36%)	17,872 (62%)	12,647 (44%)

e-value: 1e-05

Table 4. *De novo* assembly and BLASTx of differentially expressed transcripts associated with a fast growth of Pacific abalones.

Samples (Total: RPKM>=0.3)		FC >=4, FDR <=0.01			FC >=10, FDR <=0.001		
		Up	Down	Total	Up	Down	Total
Large vs Medium	22,680	1,301	908	2,209	190	141	331
Medium vs Small	22,338	4,216	1,907	6,123	812	514	1,326
Large vs Small	**24,414**	**2,859 (12%)**	**1,545 (6%)**	**4,404 (18%)**	**543 (2.22%)**	**428 (1.7%)**	**971 (3.9%)**

Table 5. Numbers of differentially expressed transcripts identified from comparison of RPKM values.

	Female (n=5)	Male (n=3)
Weight (g)	155.7+16.8	141.0+11.0
Shell length (mm)	110.8 +4.7	106.5+3.4

	Female (n=10)	Male (n=10)
Weight (g)	13.90±4.39	14.61±3.17
Shell length (mm)	52.14±3.75	53.52±4.04

Table 6. Sizes of Pacific abalones with sexually mature phenotype used for comparing RNA isolation methods. Abalones were collected from a regional Namcheon Fish Market and collected from Abalone Research Institute of JeollaNamdo Marine-Fisheries & Development Institute.

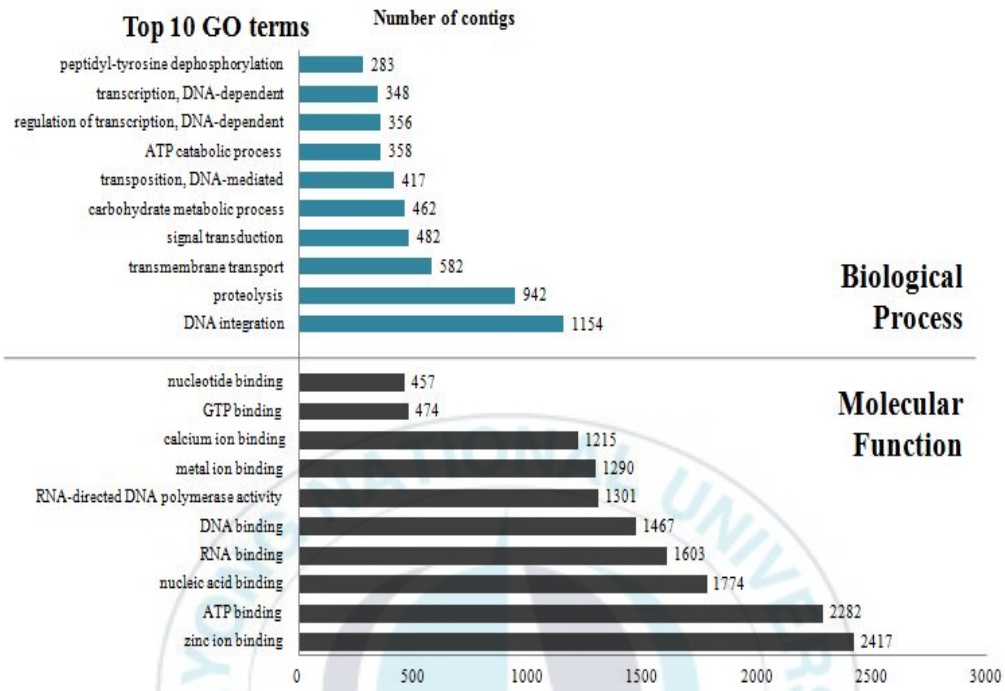


Figure 1. Gene ontology analysis of transcripts in *H. discus hannai*. Genes were categorized by involvement in biological processes and molecular function.

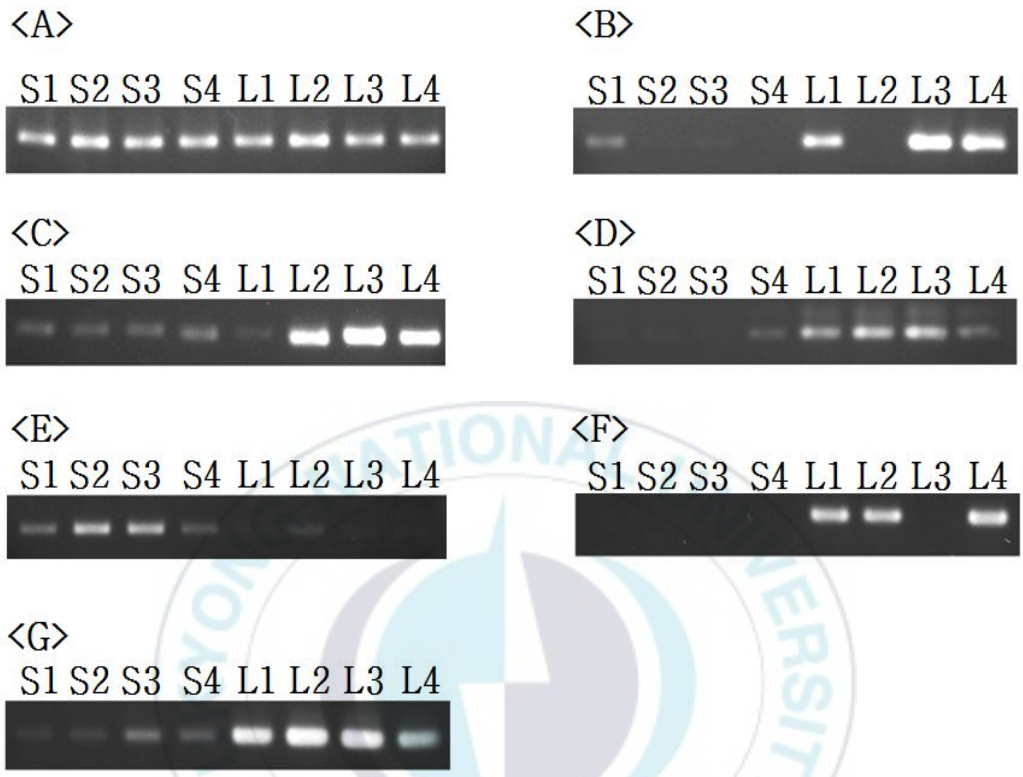


Figure 2. Levels of the transcripts in small (S1-S4) and large (L1-L4) Pacific abalones collected at 200 dfp. Primers encoding Ribosomal protein L3 (Tsc 1, A) included a positive control. Transcripts encoding Incilarin A (Tsc 2, B), Perlucin (Tsc 5, C), Transforming growth factor-beta-induced protein ig-h3 (Tsc 8, D), Tomoregulin-2 (Tsc 20, E), Vitelline envelope zona pellucida domain 4 (Tsc 34, F) and Defensin (Tsc 35, G) were identified to be associated fast growth using RT-PCR.

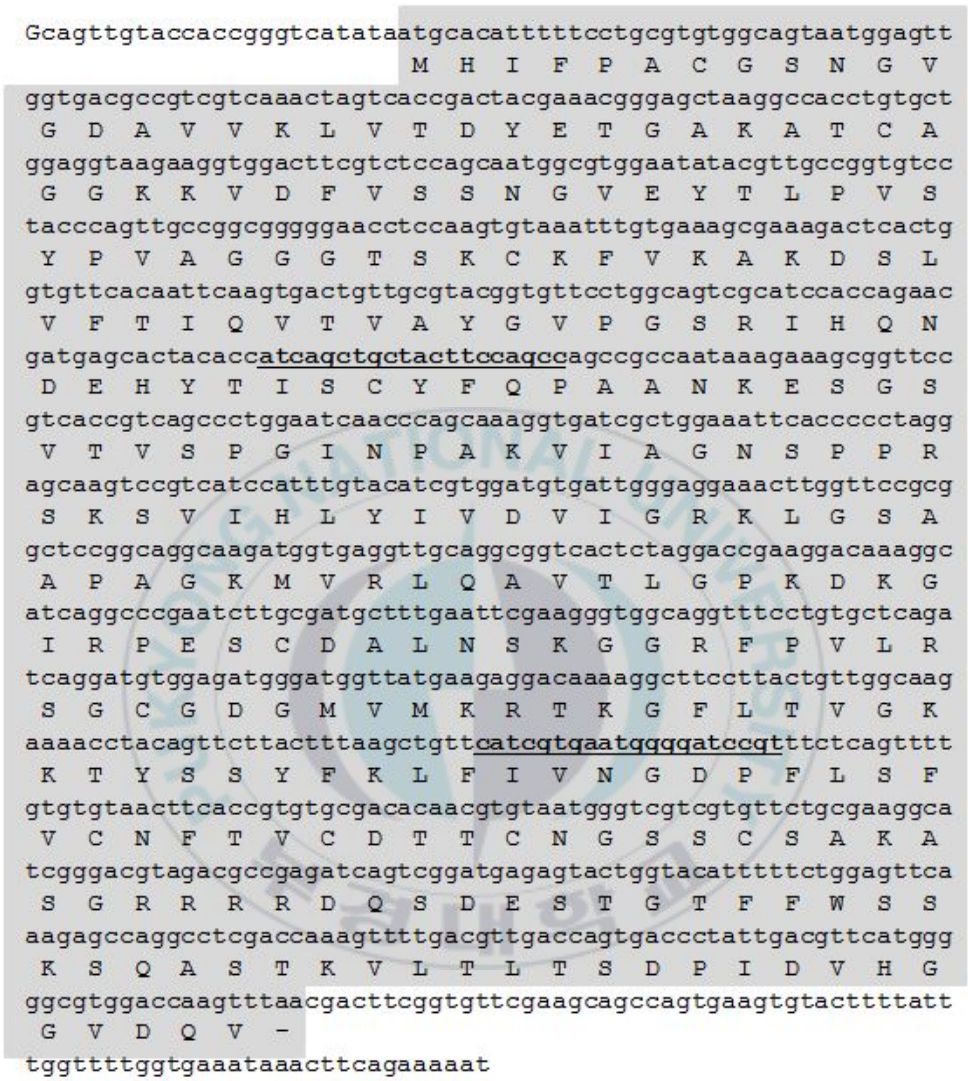


Figure 3. Sequence of vitelline envelope ZP domain 4 of *H. discus hannai.* The shaded region revealed sequence of the protein from the start codon to the stop codon. Forward and reverse primers designed in this study were underlined. Sizes of the amplified product is 391 bp.

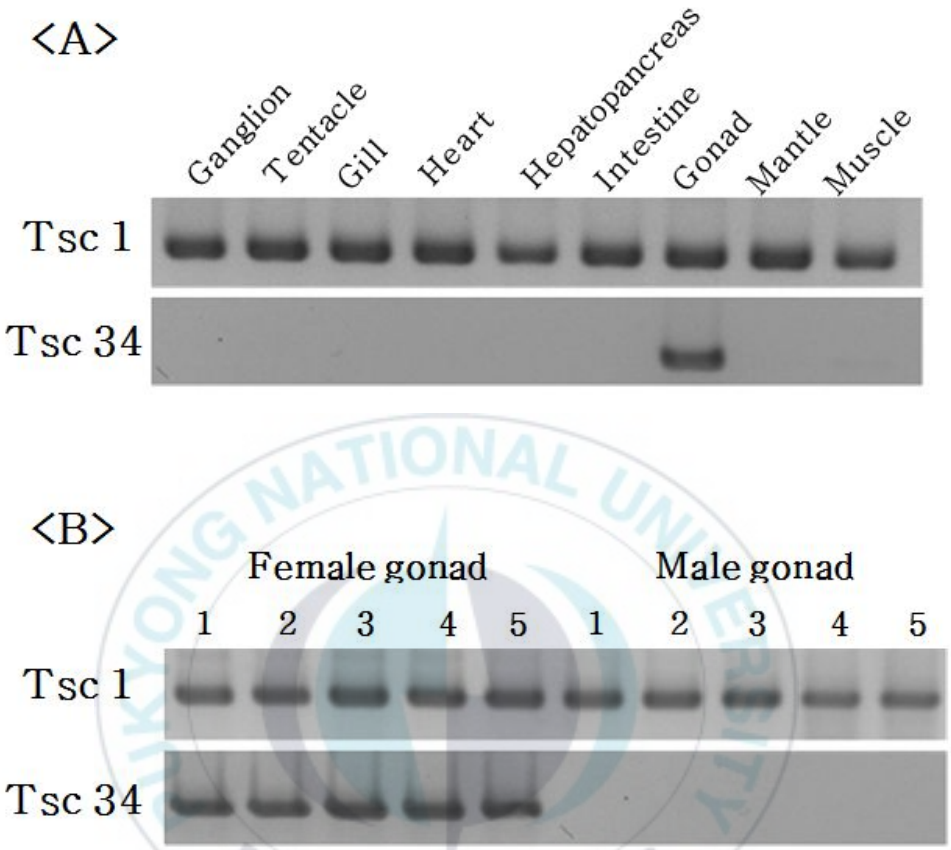


Figure 4. Verification of a female-specific gene Tsc 34 by RT-PCR. <A> Tissue distribution of Tsc 34. Tissues including ganglion, tentacle, gill, heart, hepatopancreas, intestine, gonad, mantle and muscle were tested. Trancripts encoding Tsc 1 was also included as a control. <B> Expression of Tsc 34 were analyzed with RNAs isolated from gonads of the sexually matured females (n=5) and males (n=5). Tsc 1 was included as a positive control.

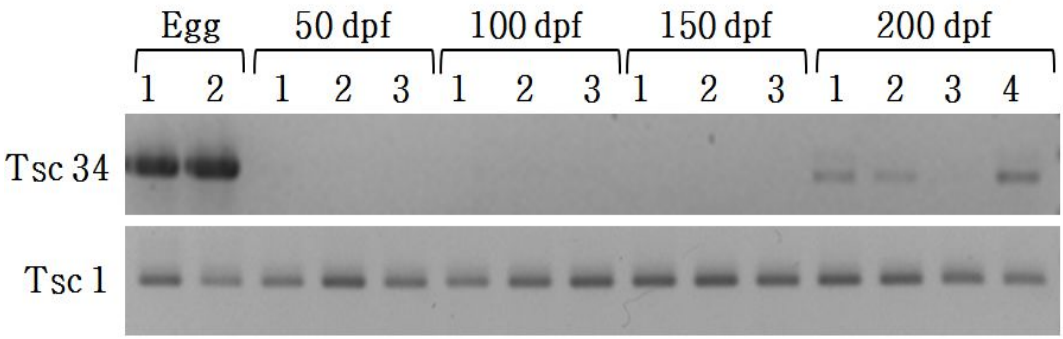


Figure 5. Developmental stage-dependent expression of Tsc 34. RNAs isolated eggs of abalones and ablaones at 50, 100, 150 and 200 dpf from at least two individual. Amplification of Tsc 1 was included as a control.

Figure 6. Expression of Tsc 34 detected from soft tissues of abalones collected at 200 dpf <A> and 300 dpf <B>. RNAs were isolated from four individuals in small (S1-S4) and large (L1-L4) abalones for 200 and 300 dpf were tested for examining the level of Tsc 34. Tsc 1 was included on a positive control.

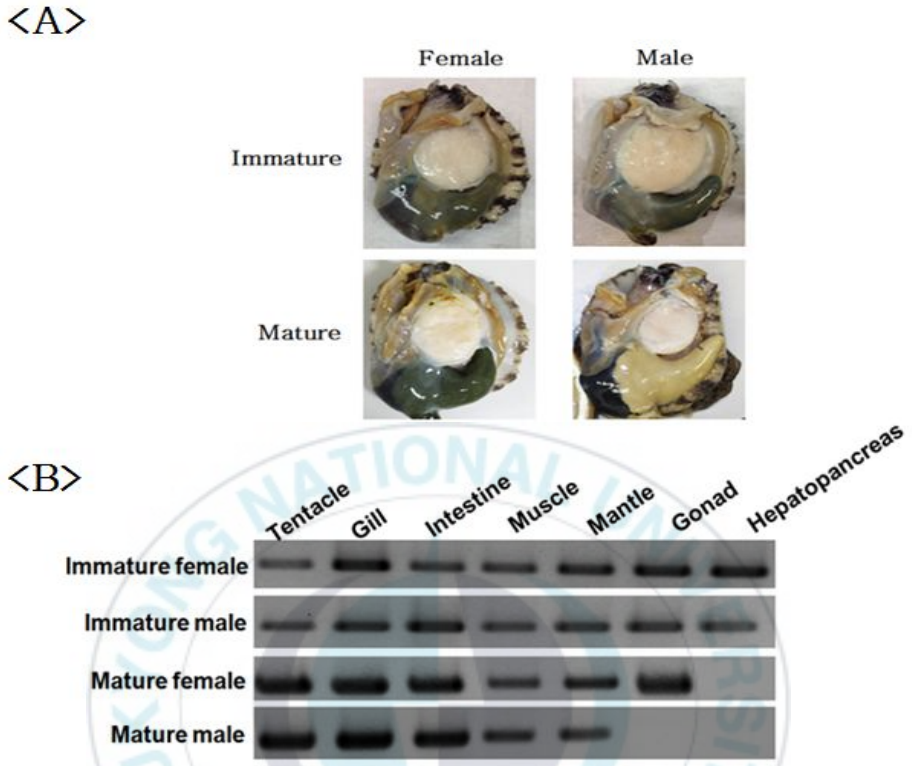


Figure 7. RT-PCR analysis of cDNA synthesized by RNA isolated using Method Ⅰ. <A> Photographs of sexually immature and mature abalones used for RNA isolation. Immature and mature abalones were distinguished by colors of gonads. Sexual determination in abalones was also confined by microscope. <B> Total RNA was isolated from tentacle, gill, intestine, muscle, mantle, gonad and hepatopancreas of female and male using Method Ⅰ. PCR amplification was carried out using Tsc 1.

Figure 8. RT-PCR analysis of RNA isolated from hepatopancreas and gonads of female (F1-F7) and male (M1-M6) abalones using Method Ⅰ. <A> Female (n=5) and male (n=3) abalones tested were collected from Namcheon Fish Market. RNAs were isolated from hepatopancreas. <B> Female (n=7) and male (n=6) abalones tested were collected from Abalone Research Institute of JeollaNamdo Marine-Fisheries & Development Institute. RNAs were isolated from mixed tissues of hepatopancreas and gonads. Amplification was carried out using Tsc 1. RNA isolated from muscle was used as a control for consistent detection.

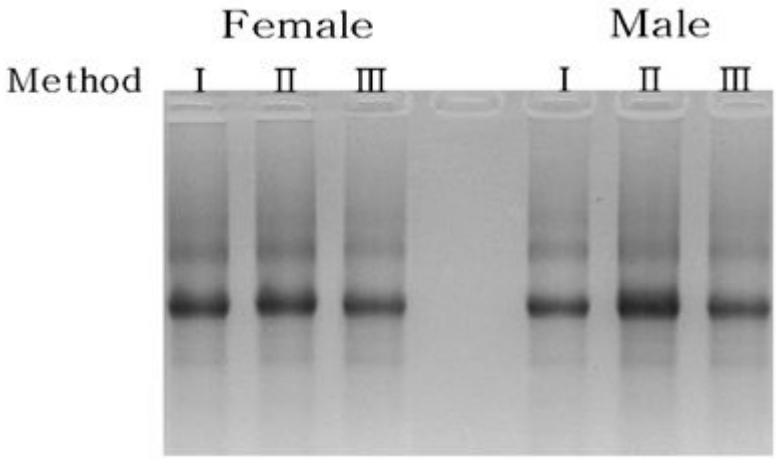


Figure 9. Electrophoretic analysis of RNA isolated from hepatopancreas using different extraction method Ⅰ, Ⅱ and Ⅲ. Tissues were dissected from fully mature female and male abalones. Total RNA was analyzed on 2% agarose gel electrophoresis followed by staining with ethidium bromide.

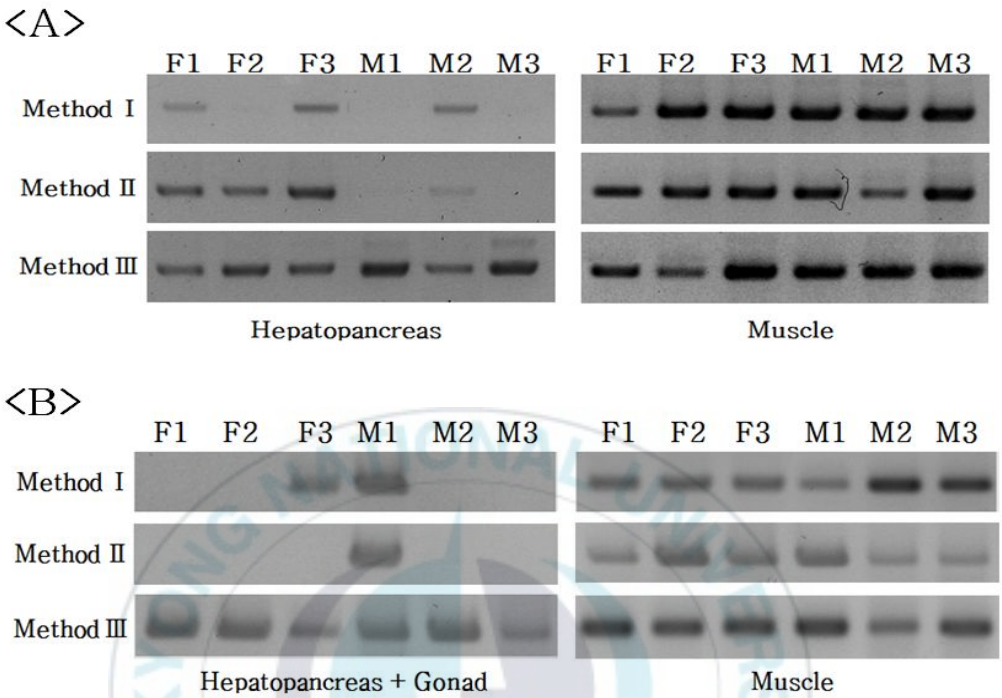
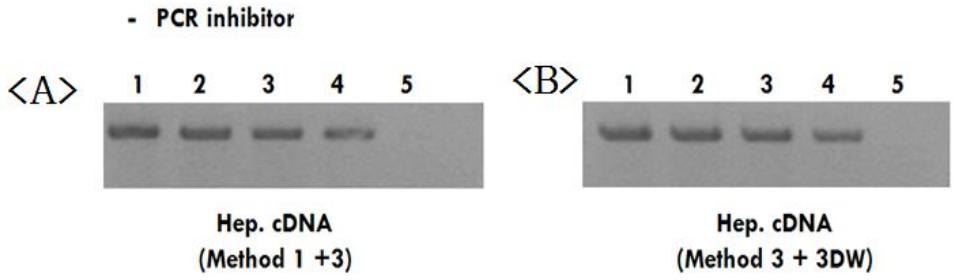


Figure 10. RT-PCR analysis of RNA isolated from hepatopancreas and muscles of female (F1-F3) and male (M1-M3) abalones using different methods. RNA isolated muscle as a control for its consistent detection by three methods. Abalones collected from Namcheon Fish Market <A>, and abalones collected from Abalone Research Institute of JeollaNamdo Marine-Fisheries & Development Institute <B> were used.



	Method 1 cDNA	Method 3 cDNA
1	0	10
2	2.5	7.5
3	5	5
4	7.5	2.5
5	10	0

(+)	3DW	Method 3 cDNA
1	0	10
2	2.5	7.5
3	5	5
4	7.5	2.5
5	10	0

Figure 11. RT-PCR analysis using different ratios cDNA templates prepared from RNA isolated by Method Ⅰand Ⅲ, respectively. <A> cDNA templates were prepared from RNA isolated from hepatopancreas (Hep.) using Method Ⅰand Ⅲ. Different ratios cDNA templates ranging from 0:10, 1:3, 1:1, 3:1 and 10:0 mixture of cDNA constructed from RNA isolated by Method Ⅰand Ⅲ, respectively were indicated. <B> The same amounts of cDNA templates prepared from hepatopancreas RNA were diluted with water for the control.

	Method 1 Hep. RNA	Method 3 Mus. RNA
1	0	10
2	2.5	7.5
3	5	5
4	7.5	2.5
5	10	0

(+)	Method 3 Hep. RNA	Method 3 Mus. RNA
1	0	10
2	2.5	7.5
3	5	5
4	7.5	2.5
5	10	0

Figure 12. RT-PCR analysis was carried out with cDNA templates synthesized from mixtures of RNAs isolated by Method Ⅰ and Ⅲ with different ratios, respectively. <A> RNA mixtures were prepared from hepatopnacreatic (Hep.) RNA isolated by Method Ⅰ and muscle RNA isolated by Method Ⅲ. Different ratios ranging from 0:10, 1:3, 1:1, 3:1 and 10:0 of RNAs isolated from hepatopancreas and muscle, respectively. <B> As a positive control, mixtures were prepared from RNA of hepatopancreas (Hep.) isolated by Method Ⅲ and RNA isolated from muscle by Method Ⅲ.

Ⅳ. Abstract (Korean)

참전복의 성장과 연관된 유전자들의 발현 차이

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참전복(*Haliotis discus hannai*) 은 양식 산업에서 중요한 해양 복족류 중 하나이다. 전복의 속성장과 연관된 유전자를 확인하기 위하여, 동일한 조건에서 200일 동안 자란 전복을 크기에 따라 small, medium 그리고 large group으로 나눴다. Small과 large group은 약 20 배 정도의 무게 차이를 보였으며 그룹별로 분류한 전복의 연조직으로부터 RNA를 추출하였다. RNA-Seq analysis을 시행하여 속성장과 연관된 전사체와 differentially expressed genes을 조사하였고, RPKM 값 0.3 이상의 전사체를 대상으로 목록을 구성하였다. 전사체 분석과 기능적인 annotation을 기반으로 하여, 몇몇의 전사체를 전복의 속성장과 연관된 후보로 선별하였다. 그 중 Vitelline envelope zona pellucida (ZP) domain 4을 암호화하는 전사체는 large 크기의 전복에서 up-regulation이 나타나는 것을 확인하였다. 속성장에 연관된 vitelline envelope ZP domain 4를 확인 하기 위하여 200일 그리고 300일의 두 그룹(small and large)과 발달 단계별 전복(egg, 50, 100, 150 그리고 200 일)으로부터 RNA 추출 후 RT-PCR 분석을 시행하였다. Vitelline envelope ZP domain 4 의 특이적인 primer를 사용하여 RT-PCR을 시행한 결과 200일과 300일의 large group의 전복 개체에서 주로 발현되는 것을 확인하였다. 조직별로는 암컷 생식소에 특이적으로 발현되며 적어도 200일은 자란 전복에서 나타난다는 것을 RT-PCR을 통해 확인하였다.

RNA 추출 실험 결과, 성숙한 전복의 간췌장과 생식소로부터의 RNA 추출시료에 따라 RNA 수율과 RT-PCR 확인에 차이가 있음을 발견하였으며, 이는 RNA와 co-precipitated 하는 물질이 cDNA 과정을 저해하는 물질임을 증명하였다. 따라서, 참전복의 간췌장과 생식소에서 inhibitory material가 없이 RNA를 추출하기 위한 간단하고 효과적이며 신뢰성있는 방법을 개발하였다.

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