CHAPTER V

DISCUSSION

5.1 Cloning and characterization of ER gene

Results of study in *L. subviridis* indicate the existence of at least 2 isoforms of ER gene (ERα and ERβ gene). Numbers of *ER* isoforms are different in fish species. For examples, *Fundulus heterocltus* (Urushitani et al., 2003), *Zoarces viviparus* (Andreassen et al., 2003) and *Cyprinodon variegatus* (Karels and Brouwer, 2003) have 1 isoform (*ER*α) but *Carassius auratus auratus* (Choi and Habibi, 2003), *Danio rerio* (Menuet et al., 2002), *Dicentrarchus labrax* (Halm et al., 2004) and *Micropterus salmoides* (Attwood, Kroll and Denslow, 2004) have 3 isoforms (*ER*α, *ER*β, and *ER*γ).

ERα

Full length cDNA sequence of *ER*α in *L. subviridis* is the first report of full length of this gene in family mugilidae. *ER*α cDNA sequence was isolated from liver of juvenile mullet treated with E_2 and mature females which vitellogenic ovary. It is shown that *ER*α gene in this species expresses in the liver tissues under regulated of E_2 as reported in other fish species such as *ER*α cDNA sequence isolated from livers of gravid females of sheepshead minnow *Cyprinodon variegatus* (Karels and Brouwer, 2003), *ER* DEF domains was recloned from liver of female rainbow trout *Oncorhynchus mykiss* (Matthews and Zacharewski, 2000), *ER*α cDNA sequence was isolated from liver of male eelpouts *Zoarces viviparus* which treated with 1 mg/kg of E_2 for 14 days (Andreassen et. al., 2003), *ER*α cDNA sequence was isolated from estradiol treated adults (males and females) zebrafish *Danio rerio* liver (Menuet et al., 2002), *ER*α cDNA was isolated from adult female European sea bass *Dicentrarchus labrax* liver (Halm et al., 2004), in largemouth bass *Micropterus salmoides*, *ER*α expression predominated in liver, highly up-regulated in liver of E_2 injected males and female liver when plasma E_2 levels was elevated in the spring (Attwood, Kroll and Denslow, 2004), *ER*α mRNA expression in liver of mummichog *Fundulus heteroclitus* was induced by E_2-stimulation (Urashitani et al., 2003), *ER*α expression in liver of male and female at early stages of gonadal recrudescence of goldfish *Carassius auratus auratus* (Choi and Habibi, 2003).

A single transcript of 5' cDNA end was obtained while 4 variants of 3' cDNA ends of *ERα* were obtained. All of them contained stop codon, polyadenylation site, and polyA tails. Two of them were almost identical with the same length and these 2 variants were combined perfectly with the 5' end creating a single full length sequence of *ERα*. The other 2 were clearly different from the previous variants. Part of E and F domain were absent from their sequences.

Multiple transcripts with different sizes of *ERα* mRNA in other fish species have been reported. Northern blot analysis showed that 2 *ERα* mRNAs with 5.5 and 4 kb were expressed in liver of mummichog *Fundulus heteroclitus* (Urashitani et al., 2003). Three forms of *ERα* mRNAs with 4.8, 3.6 and 2.7 kb were expressed in ovary of largemouth bass *Micropterus salmoides* (Attwood, Kroll and Denslow, 2004). But in other fish species or, in some cases, the same species but in different tissues have reported the existence of only 1 transcript such as *ERα* mRNAs with 3.6 kb was found in liver of largemouth bass *Micropterus salmoides* (Attwood, Kroll and Denslow, 2004), *ERα* mRNA with 3.6 kb was expressed in liver, pituitary, testis, and ovary of eelpout *Zoarces viviparus* (Andreassen et. al., 2003). So the existence of 1 or multiple transcripts which different in size is species and tissues specific. In *L. subviridis* different in size of *ERα* mRNAs arose from variations in the lengths of 3' untranslated regions. In this study, incomplete *ERα* mRNA transcript was found.

ERα deduced sequence of *L. subviridis* determined in this study included 5 domains: A/B, C, D, E, and F. A/B domain possesses AF-1 function. C is a DNA binding domain which contains 2 zinc finger regions and 8 conserved cysteine residues (4 cysteine residues for each zinc finger). D is hinge region which is important for tertiary structure. E is a ligand binding domain which binds to estrogen and F domain is also involved with the estrogen binding. As reported in other fish species, A/B, D and F domain is poorly conserved but DNA binding domain and ligand binding domain is very highly and conserved, respectively. ERα of *L. subviridis* is also in agreement with those reported in other fish species such as *Danio rerio* (Menuet et al., 2002), *Carassius auratus auratus* (Choi and Habibi, 2003), *Fundulus heteroclitus* (Urashitani et al., 2003), *Dicentrarchus labrax* (Halm et al., 2004), *Micropterus salmoides* (Attwood, Kroll and Denslow, 2004). But in some species such as *Zoarces viviparus*, its ERα lack A domain which involved in the

repression of the activation function AF-1 that is normally constitutively active in the isoforms lacking the A-domain (Andreassen et. al., 2003). Additionally, in DNA binding domain contained P box (EGCKA) which interact with regulatory region of DNA were reported in *Micropterus salmoides* (Attwood, Kroll and Denslow, 2004), *Dicentrarchus labrax* (Halm et al., 2004), *Danio rerio* (Menuet et al., 2002) and D box (PATNQ) which involved receptor dimerization was reported in *Micropterus salmoides* (Attwood, Kroll and Denslow, 2004). It contained helices H3, H6, H8, H11, and H12 surrounding hydrophobic cavity as *Danio rerio* (Menuet et al., 2002).

ERβ

ERβ cDNA sequences were isolated from liver of greenback mullet *L. subviridis.* A full length ERβ cDNA sequence of *L. subviridis* is first report of this gene in family mugilidae. The existence of *ERβ* genes in this species is similar to the reports in other fish species. *Dicentrarchus labrax ERβ* cDNAs was isolated from vitellogenic ovary (Halm et al., 2004), *Danio rerio ERβ* cDNAs was isolated from estradiol-treated adults (males and females) liver (Menuet et al., 2002). *ERβ* expressed in liver, ovary, brain, and pituitary of *Micropterus salmoides* (Attwood, Kroll and Denslow, 2004). *ERβ* expressed predominantly in testis and ovary of sexually immature *Carassius auratus auratus* at early stage of gonadal recrudescence (Choi and Habibi, 2003). This *ERβ* cDNA sequence in this study is the first report in *L. subviridis.*

Two different sizes of 5' cDNA end were found. The difference in length was located in the UTR region. Two different sizes of 3' cDNA end were also found and the difference was found in both coding and 3'UTR regions. The existence of 2-4 different sizes of *ERβ* transcript is in agreement with the reports on multiple transcripts with different size of *ERβ* mRNA in some fish species. These include *ERβ* in *Micropterus salmoides* with the sizes of 3.5 and 5.6 kb expressed in liver and 2.7 and 3.5 kb *ERβ* transcript were expressed in ovary (Attwood, Kroll and Denslow, 2004). Short form full length *ERβ* cDNA sequence was determined encoded ERβ which contained C domain, P box, and D box as reported in other fish species including *Micropterus salmoides*(Attwood, Kroll and Denslow, 2004), *Dicentrarchus labrax* (Halm et al., 2004), *Danio rerio* (Menuet et al., 2002), *Carassius auratus auratus* (Choi and Habibi, 2003). *L. subviridis* ERβ lack some region of E domain and

overall region of F domain. It is presumably indicated that the short form of *L. subviridis* ERβ can not bind to estrogen. *ER* transcript which lacks some domain was reported in some species such as *ER* transcript at size 1.5 kb which lack DNA binding domain found in mouse ovaries (Hillier et al., 1989), short ER alpha does not have DNA binding domain and missing 50 % of ligand binding domain that it can not longer bind estrogen found in *Ictalurus punctatus*. However, if it still able to associate with other functional ER proteins, it could modify their activity (Patino et al., 2000). For example TERP-1 is an N-terminal ER alpha variant found in rat pituitary (Friend et al., 1997) that is similar size to *Ictalurus punctatus* short ER alpha, this mammalian ER variant does not bind estrogen but can modify ER-dependent promoter activity (Schreihofer et al., 1999).

5.2 Cloning and characterization of choriogenin (*chg*) gene

At least 2 forms of *chg* (*chg* L and *chg* H) were found in *L. subviridis*. *chg*-L and *chg*-H of *L. subviridis* is first report of this gene in family mugilidae. The number of *chg* isoforms is different in fish species. Three isoforms (*chg* L, *chg* H and *chg* H minor) were reported in Arctic Char (*Salvelinus alpinus*) (Westerlund et al., 2001), medaka (*Oryzias latipes*) (Kanamori et al., 2003), *O. mykiss* (Hyllner et al., 2001), while 2 isoforms (*chg* L and *chg* H) were found in *C. variegatus* (Knoebl, Hemmer, and Denslow, 2004) and *Oryzias javanicus* (Yu et al., 2006), and 1 isoform (ZP2) was found in *Danio rerio* (Islinger et al., 2003) and *Oncorhynchus mykiss* (Celius et al., 2000). The *chg* cDNA sequence of *L. subviridis* in this study was also the first report.

chg L

chg L was isolated from liver of juvenile greenback mullet *L. subviridis* exposed to 17β-estradiol (E_2) and mature females which have vitellogenic ovary. It is shown that this *chg* L gene expresses in the liver tissues under regulated of E_2 which is similar to the reports from other fish species such as *O. mykiss* (Hyllner et al., 2001), *Oryzias javanicus* (Yu et al., 2006), *C. variegatus* (Knoebl, Hemmer, and Denslow, 2004), *Salvelinus alpinus* (Westerlund et al., 2001), *Oryzias latipes* (Lee et al., 2002). *L. subviridis* Chg L contains ZP domain with 261 amino acid residues as earlier described about this domain by Bork and Sander (1992). ZP domain is a conserved module for polymerization of mouse zona pellucida into filaments of

similar supramolecular structure (double helical structure) and important for mouse ZP2 and ZP3 assembly on oocyte (Jovine et al., 2002). *L. subviridis* ZP domain contained 8 conserved cysteines residues and conserved N-glycosylation site as reported in *Oryzias javanicus* (Yu et al., 2006). Additionally, this Chg L contains proline rich region which was not found in *Oryzias javanicus* (Yu et al., 2006). *L. subviridis chg*-L contained 3 half-sites ERE which correspond with *Oryzias javanicus chg*-L have 5 half-sites ERE (Yu et al., 2006).

***chg* H**

chg H was isolated from the liver of greenback mullet *L. subviridis* under the regulation of E_2 similar to chg L and also as reported in other fish species such as *O. latipes* (Murata et al., 1997; Lee et al., 2002 and 2002), *Salvelinus alpinus* (Westerlund et al., 2001), *C. variegatus* (Knoebl, Hemmer, and Denslow, 2004) and *O. javanicus* (Yu et al., 2006). *L. subviridis* Chg H contain ZP domain have 275 amino acid residues as described about this domain (Bork and Sander, 1992) as reported in *O. javanicus* (Yu et al., 2006).

5.3 Cloning and characterization of *vtg*

At least 2 isoforms of *vtg* (*vtg* 1 and *vtg* 3) were identified in *L. subviridis*. *L. subviridis vtg*-1 is first report of full length of this gene in family mugilidae. *L. subviridis vtg*-3 is first report of this gene in family mugilidae. Various numbers of *vtg* isoforms are reported in many fish species such as 2 isoforms (*vtg* 1 and *vtg* 2) in *C. variegatus* (Knoebl, Hemmer, and Denslow, 2004) and *O. latipes* (Lee et al., 2002), 2 isoforms (*vtg* 1 and *vtg* 3) in Japanese common goby (*Acanthogobius flavimanus*) (Ohkubo et al., 2004), 1 isoform in *O. javanicus* (Yu et al., 2006), *O. mykiss* (Celius et al., 2000, Arukwe, Kullman, and Hinton, 2001, Arukwe et al., 2002), fathead minnows (*Pimephales promelas*) (Gordon et al., 2005), *Micropterus salmoides* (Bowman and Denslow, 1999), masu salmon (*Oncorhynchus masou*) (Fujita et al., 2005), *Rivulus marmoratus* (Kim et al., 2004), *Carassius auratus auratus* (Ishibashi et al., 2001), Japanese conger (*Conger myriaster*) (Mikawa et al., 2006), 3 isoforms (*vtg* 1, *vtg* 2, and *vtg* 3) in mosquitoefish (*Gambusia affinis*), 7 isoforms in *D. rerio* (Wang et al., 2005). *L. subviridis vtg* 1 cDNA sequence was first

report about full length cDNA sequence of *vtg* 1 in family mugilidae. *L. subviridis* *vtg* 3 cDNA sequence was first report about *vtg* 3 in family mugilidae.

vtg 1 and *vtg* 3 cDNA sequence were isolated from liver of greenback mullet *L. subviridis* (17β-estradiol (E_2) treated juveniles and mature females which vitellogenic ovary that shown this *vtg* genes in this species expressed in the liver tissues under regulated of E_2 as reported in other fish species such as Japanese common goby (*Acanthogobius flavimanus*) (Ohkubo et al., 2004), *D. rerio* (Wang et al., 2005), *Micropterus salmoides* (Bowman and Denslow, 1999). Vtg 3 of *L. subviridis* lack phosvitin domain as *D. rerio* (Wang et al., 2005), Japanese common goby (*Acanthogobius flavimanus*) (Ohkubo et al., 2004).

L. subviridis Vtg-1 contained lipoprotein N-terminal domain (LPD_N), beta shell regions and von Willebrand factor type D domain (VWD). *L. subviridis* Vtg-3 contained lipoprotein N-terminal domain (LPD_N) and beta shell regions. VWD required for multimerisation correspond with some evidence such as dimeric form of Vtg-1 found in *A. flavimanus* (Ohkubo et al., 2004), Vtg-1 purified from *Puntius conchonius* possesses both antibacterial and hemagglutinating activities *in vitro* (Shi, Zhang, and Pang, 2006)

5.4 Comparative sensitivity of *chg*-L, *chg*-H, and *vtg*-3 genes in liver of *L. subviridis* to estrogen.

Estrogen response at mRNA expression level of *chg*-L, *chg*-H, and *vtg*-3 after 6 days exposed was lower than 3 days exposed may involved degradation of estrogen which injected into mullet which catalyzed by enzyme caused amount of active estrogen decreased. Sulfotransferases (SULT) converts active estrogen to inactive estrogen sulfates found in fish (Kirk et al., 2003). In human, catechol-O-methyl-transferase (COMT) is involved in the degradation of estrogens (Mattias et al., 2004). Estrogen degradation at high rates in mullet may cause high activity of sulfotransferases and catechol-O-methyl-transferase enzyme. Estrogen response of *chg*-L, *chg*-H, and *vtg*-3 in *L. subviridis* were high variation may cause from variation of activity of sulfotransferases and catechol-O-methyl-transferase enzyme in individual of fish. This hypothesis was supported by evidences about genetic polymorphism of sulfotransferases (*SULT*) and catechol-O-methyl-transferase

(*COMT*) genes in human. In human functional polymorphism in the COMT gene (val158met) resulting in 60-75% difference in enzyme activity between the valine (high activity) and methionine (low activity) variants (Mattias et al., 2004). Studied in human found G to A transition at codon 213 (CGC/Arg to CAC/His) of *SULT1A1* gene, and individuals homozygous for the *His* allele have a substantially lower activity of sulfotransferase (SULT) 1A1 enzyme than those with other genotypes. The result from study suggest that homozygosity for the *SULT1Aa* His^{213} allele may be risk factor for breast cancer, and its effect may be modified by the exposure level of endogenous estrogens (Zheng et al., 2001). So functional polymorphism of activity of COMT and SULT in mullet caused susceptibility, sensitivity, and fold of induction of studied genes against estrogen exposure at highly different between individual of fish.

From the results of semi-quantitative RT-PCR analysis, at 3 days of estrogen exposure, the expression levels of *chg*-L, *chg*-H, and *vtg*-3 genes were increased dramatically at the dose of 5 mg/kg of estrogen. However, the sensitivity of these genes against estrogen appeared to be in the same level. At 6 days of estrogen exposure, the expression levels of *chg*-L and *chg*-H were increased dramatically at the dose of 5 mg/kg of estrogen but *vtg*-3 is not sensitive to estrogen in all dose of the study. In *L. subviridis* estrogenic sensitivity of *chg* higher than *vtg* correspond with *O. javanicus* (Yu et al., 2006), *O. latipes* (Lee et al., 2002), *O. mykiss* (Celius et al., 2000) and *S. alpinus alpinus* (Westerlund et al., 2001). From our results indicate that mRNA expression level of *chg*-L, *chg*-H, and *vtg*-3 in liver of juvenile and/or male *L. subviridis* can used for application in biomarker of xenoestrogen contamination in seawater. The efficiency is *chg*-L > *chg*-H > *vtg*-3, respectively.