Cloning and Expression of Two *CYP19* cDNAs in Protogynous Wrasse, *Halichoeres tenuispinis*

by

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Cloning and Expression of Two *CYP19* cDNAs in Protogynous Wrasse, *Halichoeres tenuispinis*

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(Supervised by Professor **Se Jae Kim)**

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ABSTRACT

P450 aromatase (P450arom, CYP19), a *CYP19* gene product, is a member of the cytochrome P450 superfamily, which catalyzes the formation of aromatic C18 estrogen from C19 androgen. It has been reported that there are two types of P450arom gene in teleost fish, termed *CYP19a*, *CYP19b*, respectively.

In this study, two *CYP19* genes were isolated from the ovary and brain of protogynous wrasse (*Halichoeres tenuispinis*). *CYP19a* cDNA, isolated from the ovary cDNA library, is about 2 kb in length, which encodes 519 amino acids. The amino acid sequence of *CYP19a* has 71-77% homology with ovarian aromatase of other teleost fish. *CYP19b* cDNA was also isolated from brain cDNA using 5' and 3' rapid amplification of cDNA ends (RACE) analysis. The size of *CYP19b* cDNA is about 2.6 kb which encodes 496 amino acids. The deduced amino acid sequence has 61-81% homology with brain aromatase of other teleost fish. From Northern blot analysis, a single 2.6Kb transcript of *CYP19b* and a 2.2 kb transcript of *CYP19a* were observed in the brain and ovary, respectively. The tissue-specific expression of *CYP19* genes was analysed by RT-PCR assay. *CYP19a* was expressed highly in the ovary, and less in other tissues, but not in the intestine. However, *CYP19b* expression was higher in the brain and ovary, but lesser in the other several tissues. The expression levels of *CYP19* genes were also determined in the ovary and brain during reproductive period. From this result, the difference and change of expression levels of the *CYP19* genes might be correlated with the physiological and reproductive functions of estrogen in reproductive period. In addition, expression of *CYP19* in non-steroidogenic

tissues of wrasse might suggest its novel function to be further investigated.

Key word: wrasse, *CYP19a, CYP19b*, cDNA library, RACE, RT-PCR

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INTRODUCTION

P450 aromatase (P450arom, CYP19), a *CYP19* gene product, is a member of the cytochrome P450 superfamily, which catalyzes the formation of aromatic C18 estrogen from C19 androgen. The conversion of androgens to estrogens is modulated by the aromatase complex which consist of a steroid cytochrome P450 aromatase and a ubiquitous flavoprotein, NADPH-dependant cytochrome P450 reductase (Conley *et al*., 2001; Gelinas *et al*., 1998; Valle *et al*., 2002). *CYP19* gene is highly conserved all vertebrates especially among mammals (Callard and Tchoudakova, 1997, Conley and Hinshelwood, 2001). Besides, the *CYP19* gene is expressed in a wide variety of tissues including ovary, testis, placenta, brain, adipose tissue and skin in human (Simpson *et al*., 1997). The aromatase activity has been identified in amphibians (Yu *et al*., 1993), reptiles (Jeqasurea and Place, 1998), and birds (Elbrecht and Smith, 1992; Wartenberg *et al*., 1992). Also, it has been cloned from ovary and brain of teleosts, including freshwater teleosts, seawater teleosts and a cartilaginous fish (Valle *et al*., 2002). In teleost fish, at least two types of *CYP19* genes, termed *CYP19a* and *CYP19b,* have known to be expressed in the ovary and brain (Tchoudakova and Callard, 1998; Kishida and Callard, 2001; Kwon *et al*., 2001).

The steroid hormone has many significant roles in vertebrates. Especially, estrogen is a key steroid hormone to control sexual differentiation and reproduction in all vertebrates (Valle *et al*., 2002). In

teleost fish, estrogen is essential for the biosynthesis of yolk protein in the liver and oocyte development in the ovary. In the brain, the synthesis of estrogen by aromatization of androgen seems to be related with the neuroendocrine functions, sexual behavior and differentiation during the development of the central nervous system (Kihsida and Callard, 2001; Kwon *et al*., 2001).

The wrasse, *Halichoeres tenuispinis*, is a marine protogynous hermaphrodite fish. The female period has female function for several years of life, thereafter, some females begin to change sex. Therefore, there are three sex types in wrasse, composed of initial-phase (IP) males (primary males), IP-females (primary females), and terminal-phase males (TP, secondary males).

In the process of sex reversal, the role of steroid hormone is very important. In the previous studies, the level of estrogen was closely correlated with gonadal regression and recrudescence in hermaphrodite fish (Chang and Lin, 1998; Kitano *et al*., 1999; Lee *et al*., 2001). Because P450 aormatase synthesizes estrogen, the quantity of P450arom gene product is considered to be directly correlated with the degree of estrogen synthesis.

Most of the studies, related sex reversal of teleosts, have aimed to produce monosex populations in the aquaculture industry (Pandian and Sheela, 1995). Studies in gonadal sex differentiation in fish have been carried out based on the morphological changes, for example, the histological and ultrastructural changes during sex differentiation in the

presence of steroid hormones (Nakamura *et al*., 1998). In recent years, there have been several studies investigating the molecular mechanism to understand the gonadal differentiation and various sex characteristics of teleosts.

The present study aimed to investigate the molecular mechanism of P450 aromatase of wrasse. Two types of *CYP19* genes were isolated and compared with other CYP19 species. In addition, the size, number and abundance of *CYP19* transcripts in the ovary and brain were determined using Northern blot analysis. And the tissue-specific expression of ovarian and brain aromatase was analyzed in various tissues by RT-PCR assay.

MATERIALS AND METHODS

1. Animal and Tissue preparation

The protogynous wrasse were collected from the Jeju Sea from May to August. After anesthetizing the fish on ice, their body weight and height were measured. The tissues were dissected, rinsed in DEPC-H_2O and measured the weight of gonad. The tissues were stored in liquid nitrogen until RNA extraction.

2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from frozen tissues (7-12 cm adult female) using Tri reagent (MRC, Cincinnati, OH, USA) according to manufacturer's protocol. Briefly, 100 mg of each tissue was homogenized in 1 mL of Tri reagent and then added 0.2 mL chloroform to the homogenate, mixed vigorously, and centrifuged at 12,000× g for 15 min at 4℃. The aqueous phase was transferred to a fresh tube and total RNA was precipitated from the aqueous phase by mixing with 0.5 mL of isopropanol. Samples were stored at room temperature for 10 min and centrifuged at 12,000× g for 8 min at 4℃. The supernatant was discarded and the RNA pellet was washed with 80% DEPC-EtOH, and centrifuged at 7,500× g for 5 min at 4℃. After the ethanol was removed, the RNA pellet was briefly air-dried for 5 min at room temperature and dissolved in DEPC-H_2O. To calculate

the amount and degree of purity of the RNA, the absorbance was measured at 260 nm and 280 nm. The RNA samples with an A_{260}/A_{280} ratio from 1.6 to 2.0 were selected for synthesis of cDNA.

The first strand of cDNA were synthesized with 1 μg of total RNA using cDNA synthesis kit (Clontech, Palo Alto, Ca, USA) according to manufacturer's manual. After cDNA synthesis, reaction mixture was diluted to 100 μL final volume by adding 80 μL of DEPC water.

3. Oligonucleotide primers and Polymerase Chain Reaction (PCR)

The primer sets (Table 1) for amplification of ovarian *CYP19* fragment were designed from conserved regions after multi-alignment of *CYP19a* cDNAs of several teleosts (GenBank accession No.); tilapia (AF009336), goldfish (AB009336), zebrafish (AF004521), fathead minnow (AJ277866), gilthead sea bream (AF399866), Japanese flounder (AB017182), red sea bream (AB0511290), medaka (D82968). For the amplification of brain *CYP19* cDNA, the brain-type primer sets (Table 2) of *CYP19* were designed within conserved region by multi-alignment; Nile tilapia (AF306786), Mozampique tilapia (AF135850), goldfish (AB009335), zebrafish (AF226619), channel catfish (AF17239), Japanese medaka (AY319970). The amplification of cDNA was carried out in reaction buffer [10 mM Tris-HCl of pH 9.0 at 20℃, 50 mM KCl, Triton-X-100, 1.2 mM $MgCl_2$, 0.2 mM dNTP mix (Promega, Madison, WI, USA), containing 100 pmol of each primer sets, 2 units of Taq DNA polymerase (Promega, Med

Table 1. Primers used in cloning and RT-PCR analysis of wrasse ovarian *CYP19*

Primer	Sequence	Remark
aroA-1F	5'-CATGAATGAACGAGGCATCA-3'	forward primer for *CYP19a* cDNA fragment
aroA-1R	5' CCGGTGTTCAGAATGATGTT 3'	reverse primer for *CYP19a* cDNA fragment
aroA-2F	5' TGGATCAACGGAGAGGAAAC 3'	forward primer for RT-PCR
aroA-2R	5' TGGATCAACGGAGAGGAAAC 3'	reverse primer for RT-PCR
aroA-3F	5' TGGATCAACGGAGAGGAAAC 3'	forward primer for RT-PCR and probe for Northern blot
aroA-3R	5' CCAACACATACTGCCTCACG 3'	reverse primer for RT-PCR and probe for Northern blot
aroA-4F	5' CTCTGTGGAAGAAGATACG 3'	forward primer for primer walking sequencing of *CYP19a* cDNA
aroA-4R	5' CCTCGGTAAGAACCTCATGC 3'	reverse primer for primer walking sequencing of *CYP19a* cDNA
β-actin F	5' ACTACCTCATGAGAGTCCTG 3'	forward primer for internal standardization of RT-PCR
β-actin R	5' TTGCTGATCGACATCTGCTG 3'	reverse primer for internal standardization of RT-PCR

Table 2. Primers used in RT-PCR, cloning and 5' and 3'-RACE of wrasse brain *CYP19*

Primer	Sequence	Remark
aroB-1F	5' TGTGGATAAGCGGAGAGGAG AC 3'	forward primer for RT-PCR, cloning and probe for Northern blot
aroB-1R	5' AGGCTGATGGACAGAGTGTC 3'	reverse primer for RT-PCR, cloning and probe for Northern blot
aroB-2F	5' CTGACCAGAGAACCAGACAG 3'	forward primer for sequencing of *CYP19b* cDNA
aroB-2R	5' GACTTACAGGCTGCAGTTTC 3'	reverse primer for sequencing of *CYP19b* cDNA
B-GSP-1R	5' TCAACCACAGGATGAAACCG CAGAG 3'	gene-specific primer for RACE
B-GSP-1F	5' CTGTGTGCTGGCAGTATGGTG ATGG 3'	gene-specific primer for RACE
B-NGSPR	5' CGATCACCATACTGCCAGCAC ACAG 3'	nested primer for 5'- RACE
B-NGSPF	5' TCAACCACAGGATGAAACCGC AGAG 3'	nested primer for 3'- RACE
β-actin F	5' ACTACCTCATGAGAGTCCTG 3'	forward primer for internal standardization of RT-PCR
β-actin R	5' TTGCTGATCGACATCTGCTG 3'	reverse primer for internal standardization of RT-PCR

ison, WI, USA)]. The amplification was performed with 30 cycles of denaturation (45 sec at 94℃), annealing (45 sec, 50℃), extension (90 sec, 72℃). For cloning, the PCR product was inserted into the pGEM T easy vector (Promega, Medison, WI, USA) and sequenced. After conforming of type specificity, the amplified fragments were used for ovarian and brain *CYP19* screening.

4. Construction and Screening of cDNA library for *CYP19a*

cDNA library was constructed in λ TriplEx2 vector (Clontech, Palo Alto, CA, USA) with ovary cDNA by using the SMART cDNA library construction kit (Clontech, Palo Alto, CA, USA) according to manufacturer's protocols. Briefly, first strand cDNA were synthesized with 1 ㎍ total RNA and primer sets of SMART IV oligonucleotide and CCS Ⅲ/3' PCR primer. cDNA amplification was carreid out using the advantage 2 PCR kit (Clontech, Palo Alto, CA, USA) by method of Long-distance (LD) PCR. PCR was performed to the 20 cycles of 5 sec at 95℃ and 6 min at 68℃ after denaturation at 95℃ for 20 sec. The amplified cDNA was subjected to proteinase K treatment and *Sfi* Ⅰ digestion for cloning. After digestion, cDNA was purified by CHROMA SPIN-400 column and ligated into λ TriplEx2 vector. The DNA mixture was packaged into packaging extract (Stratagene, La Jolla, Ca, USA) by standard packaging reaction according to supplier's mannual. After the titering of unamplified library, the library was amplified to the phages with 5×10^4 pfu/mL

efficiencies. Amplified library on the positively-charge nylon membranes (Osmonics, Westborouhg, MA, USA) was hybridized with the [^{32}P]-α-dCTP-labeled *CYP19a* cDNA probe radiolabeled with random-primed labelling kit (Fermentas, Hanover, MD, USA). Hybridization was performed at 42℃ for overnight in a solution containing 6× SSPE, 5× Denhart's solution, 0.5% SDS, 0.1 mg/mL fish sperm DNA and 5 mg/mL radiolabeled cDNA probe. The hybridized membranes were washed under low stringency condition with 2× SSC, 0.1% SDS at RT and high stringency condition with 0.1× SSC, 0.1% SDS at 65℃, respectively. The membranes were exposed to X-ray film at -70℃ for 120 hours. Positive plaques, detected by screening, were conversed to pTriEx2 plasmid by *in vivo* excision and circularization in *E. coli* BM 25.8.

5. Rapid amplification of cDNA ends (RACE) for *CYP19b*

The RACE for *CYP19b* cloning was carried out with SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's manual. First-strand cDNA were synthesized with 1 ㎍ of total brain RNA and primers provided in the kit. The cDNA for 5'-RACE was synthesized using modified lock-docking oligo (dT)primer, termed 5'-RACE CDS primer (5'-CDS) and SMARTⅡ A oligo. The 3'-RACE cDNA was synthesized with a special oligo (dT) primer, termed 3'-RACE CDS primer (3'-CDS). *CYP19b* specific primers (Table 1, 2) for RACE were designed to adjust of condition (GC content, 50-70%; Tm≥70℃).

The first PCR was performed with UPM and gene-specific primer (GSP) (5'- RACE, B-GSP-1R; 3'- RACE, B-GSP-1F) using the 50 fold diluted cDNA as template by 3-step program of touchdown PCR. The PCR was carried out as follows: 1) 5 cycles of 94℃ for 5 sec, 72℃ for 3 min; 2) 5 cycles of 94℃ for 5 sec, 70℃ for 10 sec, and 72℃ for 3 min; 3) 25 cycles of 94℃for 5 sec, 68℃ for 10 sec, and 72℃ for 3 min. The nested PCR was carried out with the NUP and internal nested gene-specific primer (NGSP) (5'- RACE, B-NGSPR; 3'- RACE, B-NGSPF) using the 25 fold diluted first PCR product as a template. The nested PCR was performed for 20 cycles under the condition of 94℃ for 5 sec, 68℃ for 10 sec and 72℃ for 3 min. After the cDNA fragment was amplified by RACE, the product was separated on a 1.2% agarose gel, and then the band at the expected size was excised and purified with Nucleo Trap gel extraction kit (Clontech, Palo Alto, CA, USA). The purified DNA fragment was cloned in pGEM-T easy vector and sequenced.

6. RT-PCR analysis

Total RNAs, isolated from the wrasse tissues, were used for synthesis of cDNA as described above. PCR reactions were carried out with 5 μL of the synthesized cDNA. The amplification of cDNA was performed in reaction buffer [10mM Tris-HCl, pH 9.0, 50 mM KCl, Triton-X-100, 1.2 mM $MgCl_2$, 0.2 mM dNTP mix (Promega, Madison, WI, USA), containing 100 pmol of each primer set, 2 units of Taq DNA polymerase (Promega,

Madison, WI, USA)]. The type specific primers (Table 1 and 2) were used for amplification of cDNA fragments. RT-PCR for *CYP19a* was performed with 30 cycles of 94℃ for 45 sec, 50℃ for 45 sec, and 72℃ for 90 sec, and 30 cycles of 94℃ for 45 sec, 55℃ for 45 sec and 72℃ for 90 sec for *CYP19b*. The sizes of amplified fragments of *CYP19a* and *CYP19b* were 400 bp and 380 bp, respectively. Actin primers were used as an internal control and the size of amplified fragment was 510 bp. The PCR products were analyzed on a 1.5% agarose gel.

7. Northern blot analysis

Poly(A)$^+$ mRNAs were extracted from the total RNA of ovary and brain tissues according to the manufacture's manual (Promega, Madison, WI, USA) by using a biotinylated oligo(dT) primer. The poly(A)$^+$ mRNAs were electrophoresed on a 1.2% formaldehyde agarose gel with the RNA Markers (Promega, Madison, WI, USA), blotted onto a positive-charge nylon transfer membrane (Schleicher & Schuell BioScience, USA) by vacuum transfer. The membrane was fixed with UV-crosslinker at 120 mJ/㎠. The cDNA fragments, amplified by RT-PCR from ovarian and brain RNA using a specific primer sets, were radiolabeled with [^{32}P]-α-dCTP (Amersham Pharmacia Biotech, NY, USA) using random priming (Fermentas, Hanover, MD, USA). The membrane was pre-hybridized at 68℃ for 1 hour in ExpressHyb solution (Clontech, Palo Alto, CA, USA), subsequently hybridized at 68℃ for 3.5 hours with the 10 ng/mL of

denatured probe. After washing of the membrane in low stringency (5 times in 2× SSC, 0.05% SDS at RT for 45 min) and high stringency (3 times in 0.1× SSC, 0.1% SDS at 50℃ for 45 min), then the membrane was expressed to X-ray film (Biomax-MS, Kodak, NY, USA) for 50 hours at -70℃.

8. Sequence analysis

The nucleotide and amino acid sequences were analyzed by using BLASTN and BLASTX (National Center for Biotechnology Information, NIH, USA) database. The translation of amino acid sequences of cDNA and multiple alignments were confirmed by using EditSeq and MegAlign software of DNASTAR version 4.05.

RESULTS

1. Isolation of two *CYP19* cDNAs

1) Ovarian *CYP19*

The *CYP19a* cDNA was isolated from a wrasse ovarian cDNA library. A fragment (about 660 bp) was amplified with the primer, designed from conserved region of other teleost fishes *CYP19a*. After confirmation that the fragment was ovarian type, it was used as a probe to screen the ovarian *CYP19*. After screening of the 5×10^4 clones, several positive plaques were detected, isolated and determined their sequences. The sequence of one clone among these positive clones was 2020 bp in length, contained a 38 bp 5'- untranslated region (UTR), a 1557 bp open reading frame (ORF) and a 425 bp 3'- UTR including poly(A)$^+$ tail. The deduced amino acid sequence was 519 in length (Fig. 1).

2) Brain *CYP19*

A 2666 bp cDNA encoding brain *CYP19* was isolated by 5' and 3' rapid amplification cDNA ends(RACE). Initially, a fragment of 380 bp was amplified by RT-PCR with the primer designed from conserved regions of other teleost fishes brain *CYP19*s. After confirmation that the fragment is a type of brain *CYP19*, the primer sets were designed from this fragment for 3' and 5' RACE: The 5' and 3' RACE products (1.2 kb, 1.4 kb) were isolated, and subsequently subjected to the nested PCR. These products

were cloned into pGEM T-easy vector and sequenced. The combined sequence of brain *CYP19* cDNA contained a 282 bp 5'- UTR, a 1488 bp ORF and a 896 bp 3'- UTR including poly(A)$^{+}$ tail. The deduced protein was 496 amino acids in length (Fig. 2).

2. Sequence analysis

The deduced amino acid sequence of wrasse ovarian CYP19 was compared with ovarian CYP19 of other vertebrates. The sequence homologies are followed: black porgy (83%), sea bass (83%), red sea bream (82%), Nile tilapia (77%), Mozambique tilapia (77%), rainbow trout (74%), zebrafish (63%), goldfish (62%), frog (52%), chicken (49%), respectively (Fig. 3). The deduced amino acid sequence of wrasse CYP19b with other brain CYP19s showed different homologies with: Nile tilapia (81%), Mozambique tilapia (79%), catfish (63%), goldfish (61%), zebrafish (61%) (Fig. 3). However, wrasse CYP19a and CYP19b showed 59% homology between their amino acid sequences. Based on this result, the ovarian and brain type CYP19 were separated in the phylogenetic tree. And the CYP19 of high vertebrates and teleost fishes branched off in distinct two groups (Fig. 4).

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                                    ACGCGGGGGATCTCTCTCTGCAGACTCGGTGAATCCAC    38
ATG GAT CTG ATC TCT GCT TGT GAA CGG ACC ATG AGT CCT GTA GGT                    83
 M   D   L   I   S   A   C   E   R   T   M   S   P   V   G                     15
TTA GAC GCT GAG GTG GGT GAC CTG GGA TAT ATG TCC CAG AAT GCA                   128
 L   D   A   E   V   G   D   L   G   Y   M   S   Q   N   A                     30
ACT GTG GTA GTA TTG CAG GGA GTG TCG ACA GCA ACC AGG ACG CTG                   173
 T   V   V   V   L   Q   G   V   S   T   A   T   R   T   L                     45
GTT TTG CTC TTT TGT GTG CTG CTC GCC GCC TGG AGC CAC ACA GAG                   218
 V   L   L   F   C   V   L   L   A   A   W   S   H   T   E                     60
AAG AAA TCT GTA CCA GGC CCA TCC TTC TGT CTG GGT TTG GGG CCA                   263
 K   K   S   V   P   G   P   S   F   C   L   G   L   G   P                     75
CTT CTG ACA TAT TTA AGA TTC ATC TGG ACT GGT ATC GGC ACA GCA                   308
 L   L   T   Y   L   R   F   I   W   T   G   I   G   T   A                     90
TCT AAC TTC TAC AGC ACA AAG TAC GGA GAC ATT GTC AGA GTT TGG                   353
 S   N   F   Y   S   T   K   Y   G   D   I   V   R   V   W                    105
ATC TAC GGA GAG GAG ACG CTC ATT CTC AGC AGG GCA TCA GCT GTG                   398
 I   Y   G   E   E   T   L   I   L   S   R   A   S   A   V                    120
CAC CAC GTC CTG AAG AGT AGC AGC TAT ACC TCA CGT TTT GGG AGC                   443
 H   H   V   L   K   S   S   S   Y   T   S   R   F   G   S                    135
AAG CAG GGC CTC AGC TGC GTC GGC ATG AAT GGA AGA GGG ATC ATA                   488
 K   Q   G   L   S   C   V   G   M   N   G   R   G   I   I                    150
TTT AAC AAC AAT GTG CCT CTG TGG AAG AAG ATA CGC ACA TAT TTC                   533
 F   N   N   N   V   P   L   W   K   K   I   R   T   Y   F                    165
ACC AAA GCG CTG ACA GGT CCA GGT CTG CAG CAG ACG GTG GAG GTC                   578
 T   K   A   L   T   G   P   G   L   Q   Q   T   V   E   V                    180
TGC GTC TCA TCC ACT CAG AGT CAC CTG GAT GAT CTG GAC AGT TTG                   623
 C   V   S   S   T   Q   S   H   L   D   D   L   D   S   L                    195
GAT CAG GTG GAC GTG ATC AGT CTG CTG CGC TGC ACC GCG GTC GAC                   668
 D   Q   V   D   V   I   S   L   L   R   C   T   A   V   D                    210
ATC TCC AAC AGA CTC TTC CTG GAT GTT CCT GTG AAT GAG AAA GAG                   713
 I   S   N   R   L   F   L   D   V   P   V   N   E   K   E                    225
CTG ATG GTG AAG ATT CAT AAG TAC TTT GAC ACG TGG CAG ACT GTG                   758
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 L   M   V   K   I   H   K   Y   F   D   T   W   Q   T   V    240
CTC ATC AAA CCG GAC ATT TAC TTC AAG TTG GAC TGG ATT CAG CAG    803
 L   I   K   P   D   I   Y   F   K   L   D   W   I   Q   Q    255
AAG CAC AAG ATG GCA GCC CAG GAG CTG CAA GGT GCT ATC GAG AGC    848
 K   H   K   M   A   A   Q   E   L   Q   G   A   I   E   S    270
CTC GTG GAG CAG AAG AGG AGA GAG ATG GAG CAG GCG GAG AAA CTA    893
 L   V   E   Q   K   R   R   E   M   E   Q   A   E   K   L    285
GAC GAC ATC AAC TTC ACA GCA GAG CTC ATC TTT GCT CAG AGC CAC    938
 D   D   I   N   F   T   A   E   L   I   F   A   Q   S   H    300
GGT GAG CTG TCT GCA GAG AAC GTG AGG CAG TAT GTG TTG GAG ATG    983
 G   E   L   S   A   E   N   V   R   Q   Y   V   L   E   M    315
GTG ATC GCA GCA CCT_GAC ACT CTG TCA ATC AGC CTC TTT TTC ATG   1028
 V   I   A   A   P   D   T   L   S   I   S   L   F   F   M    330
CTG CTG CTC CTC AAA CAG CAT CCT GAT GTG GAG CTG CAG CTG CTG   1073
 L   L   L   L   K   Q   H   P   D   V   E   L   Q   L   L    345
CAG GAG ATA GAC ACT GTT GTT GGT GAC AGA CAG CTC CAG AAC GGA   1118
 Q   E   I   D   T   V   V   G   D   R   Q   L   Q   N   G    360
GAC CTC CAC AAG TTG CCG TTG CTG GAA AGC TTC GTC AAC GAA TGC   1163
 D   L   H   K   L   P   L   L   E   S   F   V   N   E   C    375
TTA CGC TTC CAC CCT GTG GTG GAC TTC ACC ATG CGC CGC GCC TTG   1208
 L   R   F   H   P   V   V   D   F   T   M   R   R   A   L    390
TCT GAT GAC ATC ATA GAA GGC TAC AGG GTT CCC AAA GGG ACA AAC   1253
 S   D   D   I   I   E   G   Y   R   V   P   K   G   T   N    405
ATC ATT CTG AAC ACA GGC CAC ATG CAC AGG ACT GAG TCT TTC CAC   1298
 I   I   L   N   T   G   H   M   H   R   T   E   S   F   H    420
AAA CCA AAT GAC TTC AAT CTG AAA AAC TTT GAA AAA AAT GCT CCT   1343
 K   P   N   D   F   N   L   K   N   F   E   K   N   A   P    435
CGT CGT TAC TTC CAG CCG TTT GGC TCG GGG CCT CGC TCG TGC GTG   1388
 R   R   Y   F   Q   P   F   G   S   G   P   R   S   C   V    450
GGA AAA CAC ATC GCC ATG CTG ATG ATG AAA TCC ATC CTG GTG ACT   1433
 G   K   H   I   A   M   L   M   M   K   S   I   L   V   T    465
CTG CTG TCC CAG TAC TCA GTT TGT CCT CAT AAG GGT CTG ACC CTG   1478
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 L   L   S   Q   Y   S   V   C   P   H   K   G   L   T   L    480
GAC GGC CTC ACG CAG ACC AAT GAC CTG TCC CAG CAG CCG GTG GAG  1523
 D   G   L   T   Q   T   N   D   L   S   Q   Q   P   V   E    495
CAT CAT CAG AAG AAC GAG CCG CTC GGC ATG AGG TTC TTA CCG AGG  1568
 H   H   Q   K   N   E   P   L   G   M   R   F   L   P   R    510
CAG AGA GGA TCC TGG GAA AAT CTC TGA                          1595
 Q   R   G   S   W   E   N   L   *                           519
GAGCCCCTGTTGTGTTCAGCTCTTTATTTCTACCTGTACATATAAAGCTAATATTGTTA  1654
CTTTATGCTTTTTATGACTGTATAAAGATTTTTTAAGATTTCTATTTGAGTGACTGAGT  1713
ACTTTATGAAGTTAATGTTCCAAATACTGACAGTAAATTTCAGTGCAGTTAGTTTGTGT  1772
TGTTATCTTTTTGTGGTGGATTTATTGAATATGTTTACTTATTTTTCTCACTTTGTTTT  1831
CATGTTGCAGTAATTGCTTAAATTATGTGAGGTCCTTTCAATAATCTTGGTACTGTTTG  1890
ACTTTTTTCAAAAACATCTCTTTTTTTTGTTGCATGAACTGGATTTTATGCAACTCGAT  1949
GTGTGTACAGAGTAAAAATAAATAAAATCTGTAATAATTCCAAAAAAAAAAAAAAAAAA  2008
AAAAAAAAAAAA                                                 2020
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Figure 1. Nucleotide sequences and deduced amino acid sequences of wrasse *CYP19a* cDNA. The start and stop codons are in bold italics. This nucleotide and deduced amino acid sequence data was registered in the GenBank with accession No. AY489061.

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 T   Y   S   R   F   I   W   T   G   I   G   T   A   C   N     60
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 V   D   I   S   N   K   L   F   L   R   V   P   I   N   E    195
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Y N K H K K A A Q E L Q D V M 240

GAA AGC TTG CTG GTG ACA AAG AGG AAG ATG ATA AAG GAG TCT GAG 1047

E S L L V T K R K M I K E S E 255

AAG TTG GAT GAT GAT CTT GAC TTC GCA ACA GAG CTG ATC TTT GCT 1092

K L D D D L D f A T E L I F A 270

GAG AGT CAT GGA GAG CTC TCA GCT GAT AAC GTC AGA CAG TGT GTG 1137

E S H G E L S A D N V R Q C V 285

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L E V W R S Q L Q Y T L S I S 300

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L F F M L M L L K Q N P D V E 315

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D G N L D Y Q S L N V L E S F 345

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I N E S L R F H P V V D F T M 360

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k G T N I I L N t G L M H K T 390

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E F F P H P T E F S L T N F D 405

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K T V P S R F F Q P F G C G P 420

CGC TCC TGC GTG GGC AAA CAC ATC GCC ATG GTG ATG ATG AAG GCC 1587

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CAA CCT GTG GAG GAC GAG CAC AGC CTG GCC ATG CGG TTT ATC CCT  1722
 Q   P   V   E   D   E   H   S   L   A   M   R   F   I   P    480
CGA GCA ACA CAA CCT CCA CTC AGT CAC ATA TTT AGT CAG GAA ATG  1767
 R   A   T   Q   P   P   L   S   H   I   F   S   Q   E   M    495
TAG                                                          1770
 *
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AAAAAAAAAA                                                    2666
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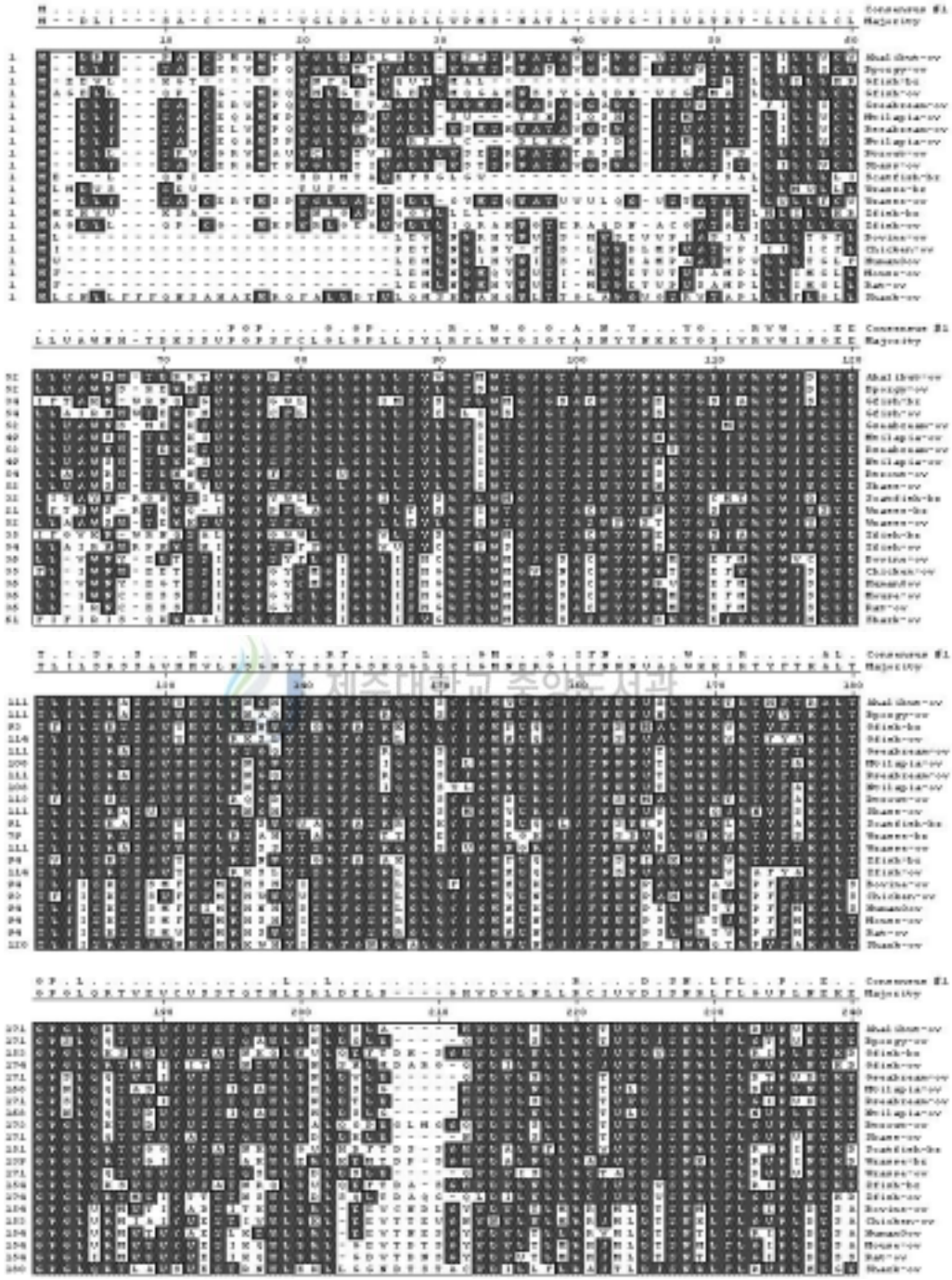
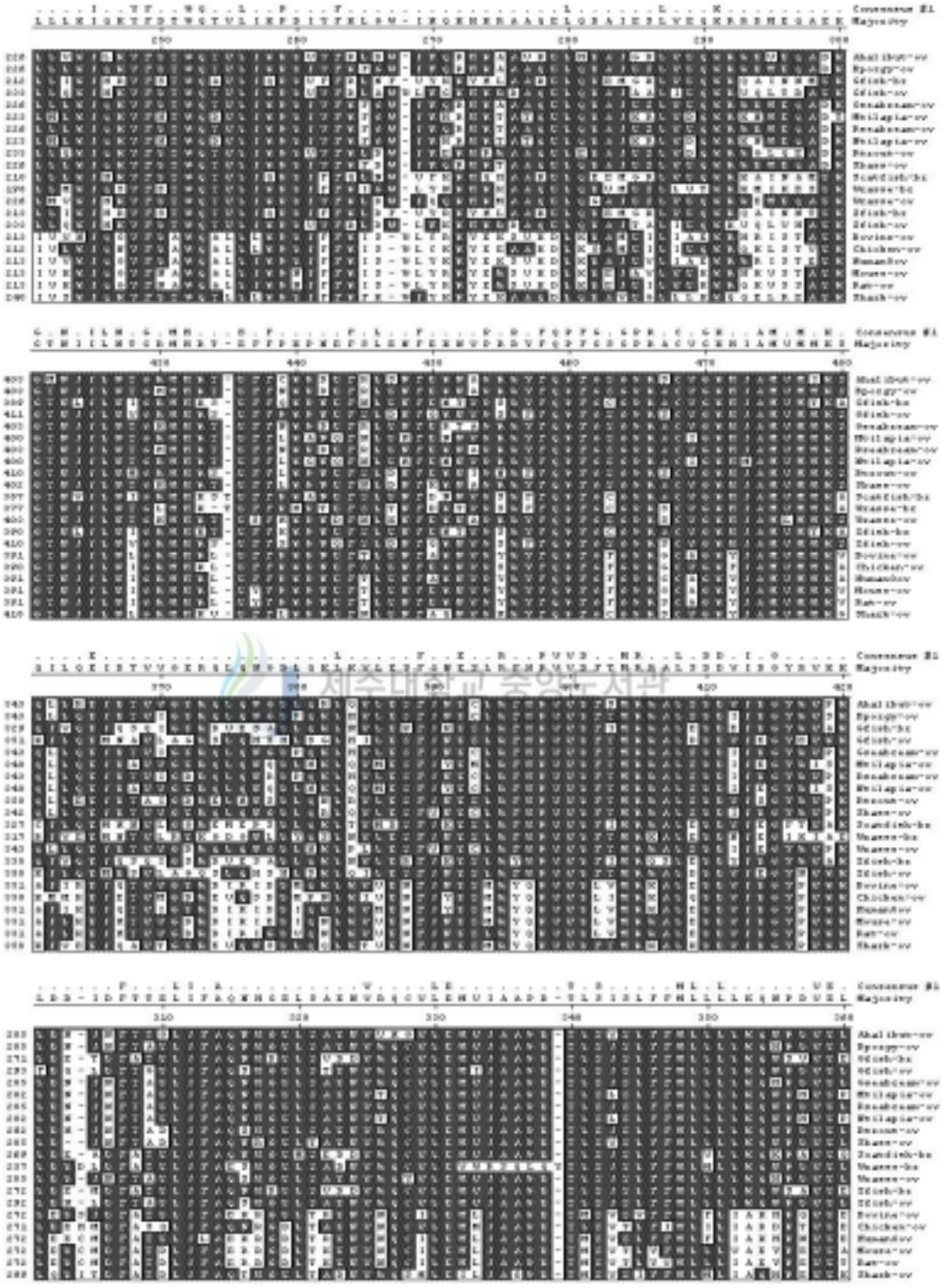
Figure 2. Nucleotide sequences and deduced amino acid sequences of wrasse *CYP19b* cDNA. The start and stop codons are in bold italics. This nucleotide and deduced amino acid sequence data was registered in the GenBank with accession No. AY489060.





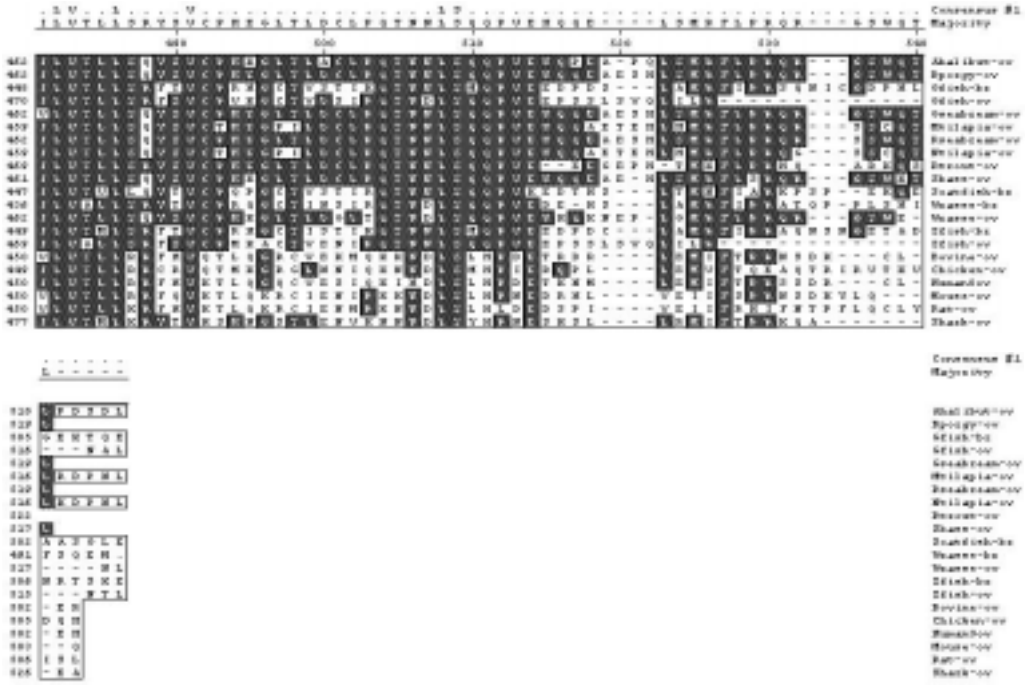


Figure 3. Multiple alignment of wrasse CYP19 amino acids with other CYP19s. The conserved amino acids are black-boxed. Ahalibut; atlantic halibut, Bporgy; black porgy; Gfish, goldfish; Gseabream, gilthead sea bream; Rseabream, red sea bream; Rtrout, rainbow trout; Sbass, sea bass; Scatfish, Southern catfish; Zfish; zebrafish; ov, ovarian type CYP19; br, brain type CYP19.

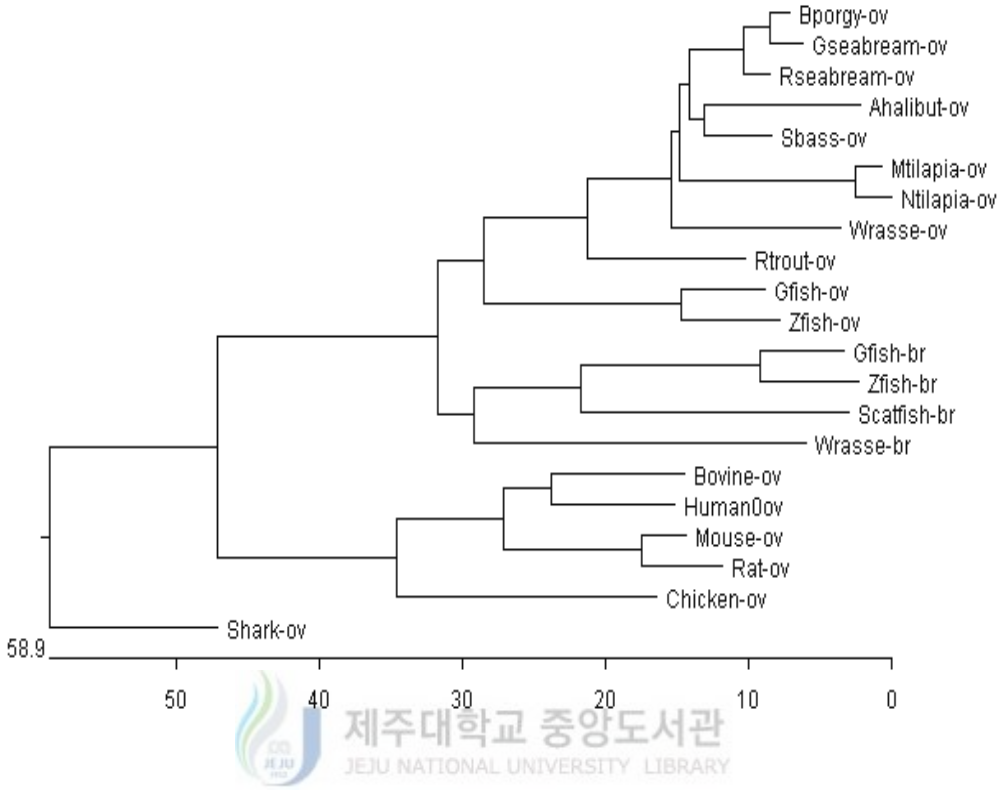


Figure 4. Phylogenetic tree of wrasse and vertebrate CYP19s. Amino acid sequences of CYP19 were analysed by DNASTAR. Bporgy, black porgy; Gseabream, gilthead sea bream; Rseabream, red sea bream; Ahalibut, atlantic halibut; Sbass, sea bass; Gfish, goldfish; Zfish, zebrafish; Scatfish, Southern catfish; ov, ovarian type CYP19; br, brain type CYP19

3. mRNA expression of *CYP19* in various tissues

Total RNAs were prepared from the various tissues including ovary, brain, liver, gill, kidney, spleen, muscle, intestine and heart from females and testis from males. The expressions of *CYP19a* and *CYP19b* in the ovary and brain were investigated during the maturing and spawning period (May to August). The *CYP19a* was expressed highly in the ovary from maturing to spawning period, reaching the peak level in July (Fig 5A). In the brain, the ovarian *CYP19* was expressed very weakly and its expression level was gradually increased following the spawning periods (Fig 5B). However the expression level of *CYP19b* in brain was gradually increased from May to August, which was similar to that in ovary (Fig. 5B). Also, we measured the expression level of *CYP19* genes in the liver, gill, testis, kidney, spleen, muscle, intestine and heart of wrasse. The ovarian *CYP19* was expressed highly in the ovary and kidney than other tissues, and not detected in the intestine. In contrast, the brain *CYP19* was expressed comparably in all tissues (Fig. 6).

4. Northern blot analysis of *CYP19*

The size and number of *CYP19* transcripts were examined with Northern blot analysis (Fig. 7). One transcript (about 2.2 kb) was detected in ovary by using ovarian-specific cDNA probe, and the other transcript (about 2.6 kb) was detected in brain with brain-specific cDNA probe. However, the

abundance of mRNAs from ovary or brain might be not enough to verify transcripts of *CYP19b* or *CYP19a,* respectively. Because only little amount of cDNA could be detected in RT-PCR analysis, the expression of *CYP19a* and *CYP19b* in the ovary and brain was verified in RT-PCR analysis, but not in Northern blot analysis (Fig. 6).

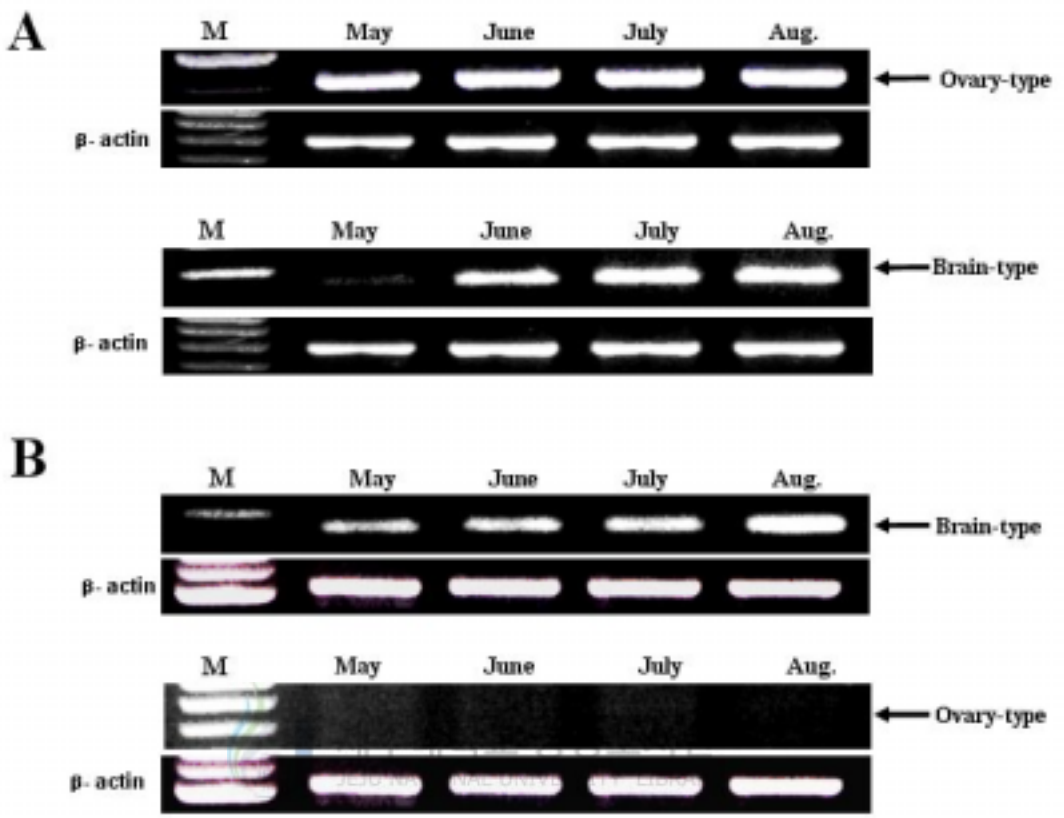
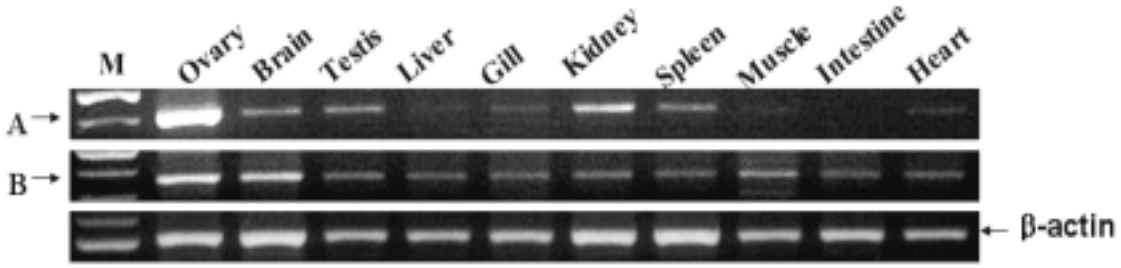


Figure 5. The expression of *CYP19a* and *CYP19b* in the ovary(A) and brain(B) during reproductive seasons. M; 100 bp DNA ladder marker.

1



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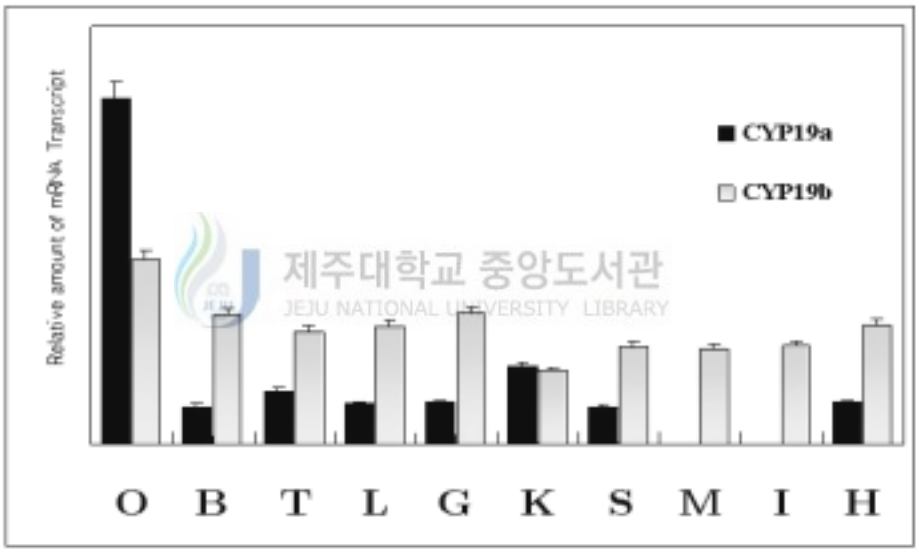

Figure 6. Tissue-specific expression of *CYP19a* and *CYPb* of adult wrasse by RT-PCR analysis. (1) A; *CYP19a*, B; *CYP19b*, (2) the relative expression level of *CYP19a* and *b,* which were normalized with that of β-actin: O, ovary; B, brain; T, testis; L, liver; G, gill; K, kidney; S, spleen; M, muscle; I, intestine; H, heart; M, 100 bp DNA ladder marker.

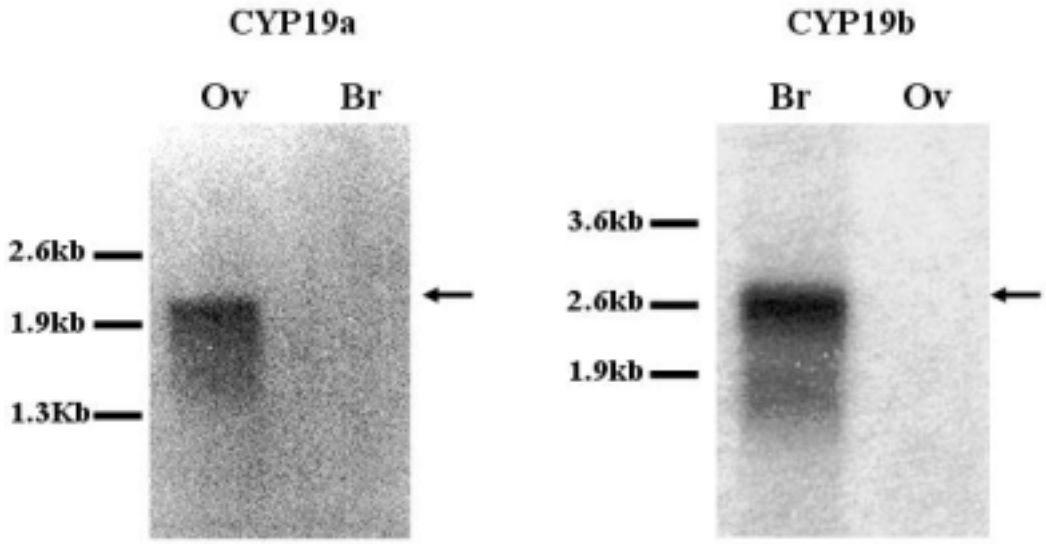

Figure 7. Northern blot analysis of the wrasse ovarian and brain transcript of *CYP19*. The ovarian and brain transcript were hybridized with a [^{32}P]-labeled ovarian and brain cDNA fragment. Transcript size of *CYP19a* and *CYP19b* was approximately 2.2 kb, 2.6 kb, respectively. Ov, ovary; Br, brain.

DISCUSSION

(1) Isolation of two *CYP19* cDNAs

In the present study, the two types of *CYP19* of wrasse were cloned and characterized. The comparison of CYP19 amino acids between wrasse and other species reveals that the proteins are belongs to the same family. The ovarian and brain CYP19 showed a lower homology (59%), and it was confirmed that the ovarian CYP19 is different from the brain CYP19 of wrasse from the phylogenetic tree analysis.

The size of the ovarian *CYP19* transcript of wrasse was about 2.2 kb from Northern blot analysis. The transcript size of wrasse *CYP19a* is similar to those of zebrafish (2.1 kb; Kishida and Callard, 2001) and European sea bass (2.2 kb; Vally *et al*., 2002) but shorter than those of rainbow trout (2.6 kb; Tanaka *et al*., 1992), medaka (2.6 kb; Tanaka *et al.,* 1995), catfish (3.4 kb; Trant, 1994) ovarian aromatase. The transcript size of brain *CYP19* was estimated as about 2.6 kb, which is similar to that of Nile tilapia (2.6 kb; Kwon *et al.,* 2003) but shorter than those of rainbow trout (3.8 kb; Valle *et al.,* 2002), zebrafish (4.4 kb; Kishida and Callard, 2001) and goldfish (3 kb; Gelinas *et al.,* 1998). Although it was reported about the variants of *CYP19* transcript in the same tissue of rainbow trout (Valle *et al*., 2002), the present study showed only one transcript of *CYP19* in the ovary and brain, respectively.

(2) Tissue-specific expression of *CYP19* genes

It has been reported that *CYP19* is expressed in various tissues. Because aromatase enzyme catalyzes the conversion of androgen to estrogen, the expression of *CYP19* is directly correlated with the estrogen activity. From previous studies, the degree of C*YP19* mRNA expression has been connected with the aromatase enzyme activity (Chang *et al.,* 1997; Gelinas *et al.,* 1998; Gen *et al.,* 2001; Gonzalez and Piferer, 2003). However, the brain and ovarian aromatase enzymes did not show any significant difference to catalyze androgen to estrogen in eukaryotic cells (Tchoudakova and Callard, 1988).

In fact, sex hormones have been regarded as products of steroidogenic tissues, such as ovary or testis. However, recent studies suggested that non-steroidogenic tissues also have the estrogen activity, which might be related with the expression of the *CYP19* gene. In the present study, the expression of *CYP19* genes was detected in the non-steroidogenic tissues, such as brain, liver, gill, kidney, spleen, muscle, intestine and heart of adult wrasse.

Although the liver is generally considered as a non-steroidogenic tissue, there are several reports showing the expression of *CYP19* in the human and rat liver (Gonzalez and Piferer, 2003). But in fish, there has been no report of the expression of *CYP19* genes in the liver. The result of study is, so far, the first report showing the expression of *CYP19* genes in the liver. More recently, Gonzales and Piferrer (2003) reported aromatase

activity in the liver of European sea bass. Assisi *et al.* (2000) also suggested that the aromatase activity might be correlated with the estrogen activity of converting the circulating testosterone (T) into estradiol-17β (E_2), which might be a possible complement to ovarian-secreted E_2 to induce hepatic vitellogenin synthesis.

However, there are many evidence about the steroidogenic property of the kidney and spleen. The fish kidney can synthesize, androgen and estrogen (Watts *et al.*, 1995) and also has the steroidogenic activity (Morohashi *et al*., 999; Kobayashi *et al*., 1996). The steroidogenic activity is based on the existence of adrenal gland on the kidney of teleost fish (Milano *et al.*, 1997) contrary to tetrapod (Milano *et al*., 1997). The expression of two types of *CYP19* in wrasse kidney may be the first report in teleost fish. The expression of ovarian *CYP19* in the spleen has been reported in the human fetus (Price *et al*., 1992) and Japanese flounder (Kitano *et al*., 1999). We also detected the expression of two types of *CYP19* in the wrasse spleen. From the previous studies, the steroidogenic property of spleen could be explained by the presence of transcription factors or precursors, such as steroidogenic factor-1 (SF1) or cholesterol side-chain cleavage cytochrome P450 (P450scc) (Morohashi *et al*., 1999).

Although estrogen is a female hormone, it is also essential for the male. The fact that the existence of estrogen in the testicular cell has given much of interest to investigators. Although the physiological function of estrogen has been suggested in the male gonad, the precise role of

estrogen in testis is still unknown (Carreau, 2001; O'Donnell *et al*., 2001). The evidence of the function of estrogen has based on the existence of estrogen-receptors (Brinkmann *et al*., 1972; Kato *et al.,* 1974; Abney, *et al.,* 1976) or the expression level and localization of *CYP19* in the process of testis development (Lavallet and Carreau, 1997; Carreau, 1999).

Although the study about testicular aromatase has been investigated with various parameters (immunolocalization, aromatase activity, and aromatase mRNA expression) (Carreau, 1999), such studies have been restricted mainly in mammals. Therefore, the further study is also required in low vertebrates, such as fish. In this study, the expression of *CYP19* genes was investigated in the wrasse testis. The degree of testicular expression of *CYP19* was very low, but the pattern was similar to that of ovarian *CYP19* in the ovary during reproductive seasons (data not shown). Taken together, the expression of *CYP19* in wrasse testis might suggest the possible involvement of the estrogen in the process of spermatogenesis in fish.

In the other tissues, very weak and inconsistent expression of ovarian *CYP19* was observed in the gill, muscle and heart, whereas similar expression pattern of brain *CYP19* was observed in the gill, muscle and heart. However, in the intestine, only *CYP19b* was detected, which is inconsistent with the previous studies (Valle *et al*., 2002). Although the expression of *CYP19* genes in several tissues of fish has been reported, the function is sill unknown. It could be simply considered that the expression might be correlated with the steroid activity in various tissues.

We also examined the change of *CYP19* expression level in the ovary and brain of wrasse during the reproductive seasons (May to August). In the ovary, the expression of ovarian *CYP19* was gradually increased in May to July but slightly decreased in August. This result is consistent with the change in plasma concentration of estradiol-17β and the GSI value of the female wrasse (data not shown). Therefore, the expression of *CYP19* may function for the production of estrogen and the development of ovary, which is agreement with the report from red sea bream (Gen *et al*., 2001). In the brain, the expression level of brain *CYP19* gradually increased along the reproductive seasons. Especially, the peak time (August) of brain *CYP19* expression suggests the correlation of sexual behavior in reproductive period. From the previous studies (Tchoudakova and Callard, 1998; Trant *et al*., 1997; Kitano *et al*., 1999), the expression of ovarian *CYP19* in the brain has been generally accepted. But contrary to the earlier studies, the expression of *CYP19b* in the ovary was very strong and the expression level of *CYP19b* in the ovary was increased during reproductive seasons, which was similar to that of *CYP19b* in the brain.

Wrasse is a protogynous hermaphrodite fish. Although the wrasse, used in this study, were adult females in the time of sex reversal, the evidence about sex reversal related with the expression level of *CYP19* gene is not enough. Thus, the comparative studies on the expression of *CYP19* should be done during natural and inducing change of sex.

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국 문 초 록

놀래기에서의 에스트로겐 합성효소 유전자의 분리 및 발현에 관한 연구

최 진 영

CYP19 유전자의 발현에 의해 생성되는 P450 aromatase (CYP19, P450 arom)는 웅성호르몬인 안드로겐을 자성호르몬인 에스트로겐으로 변환시키는 효소이다. 모든 척추동물에서 에스트로겐은 다양한 측면에서 중요한 기능을 하며, 특히 어류에서는 생식소의 분화 및 발달, 뇌에서의 성적 특성 및 행동에 관계된 신경내분비학적 면에서 필수적인 기능을 한다고 알려져 있다. 어류에서는 두 가지 타입의 aromatase가 있다고 알려져 있는데, 난소 특이적인 *CYP19a*와 뇌 특이적인 *CYP19b*가 그것이다. 본 연구에서는 암컷의 일부가 일생 중에 성전환 하는 제주산 놀래기에서 두 가지 타입의 *CYP19* 유전자를 분리하였고, 성숙 및 산란시기에 걸친 발현양상을 비교하였다.

CYP19a cDNA는 놀래기의 난소 cDNA library screening에 의해 분리되었으며, 총 염기서열의 길이는 2020 bp로, 1557 bp의 open reading frame (ORF)을 포함하며, 425개의 아미노산을 구성하는 것으로 나타났다. 뇌 특이적인 *CYP19b* cDNA는 5‘ and 3′ rapid amplification cDNA ends (RACE) 방법으로 분리되었으며, 1488 bp의 ORF를 포함하는 2666 bp의 염기서열로 구성, 496개의 아미노산을 구성하는 것으로 추정된다.

놀래기의 난소와 암컷의 뇌에서 성숙 및 산란기 동안의 두 유전자의 발현양상을 RT-PCR 방법으로 분석하였으며, 전사체의 크기 및 수를 알아보기 위하여 Northern blot analysis를 실시하였다. 난소 특이적인 *CYP19a*는 난소에서는 전 시기에 걸쳐 강하게 발현되었으며, 특히 산란기에 속하는 7월경에 가장 강하게 발현된 것으로 나타났다. 뇌 특이적인 *CYP19b*는 뇌에서 산란 및 퇴화기인 8월로 갈수록 점차 발현양이

증가하는 것으로 나타났다. 이는 놀래기의 생식주기와 관련된 생식소의 발달과 성적 행동과 관련된 뇌에서의 에스트로겐의 합성과 직접적인 관련이 있다고 여겨진다. 그 외 정소, 간, 아가미, 신장, 비장, 근육, 장, 심장 등의 조직에서도 *CYP19a*와 *CYP19b*의 발현 여부를 살펴보았으며, 이를 통해 다양한 조직에서의 aromatase의 기능에 관해 추정해 볼 수 있었다. 또한 난소와 뇌에서 분리된 mRNA로 Northern blot analysis를 한 결과, 각각 2.2 kb, 2.6 kb정도의 전사체를 확인할 수 있었다. 놀래기의 *CYP19a*는 black porgy, sea bass의 *CYP19a*와 83%, *CYP19b*는 Nile tilapia의 *CYP19b*와 81% 의 상동성을 나타냈으며, 계통유전학적 분석을 통해 분리된 두 유전자가 서로 다른 타입임을 확인할 수 있었다.

주요어: 놀래기, *CYP19a*, *CYP19b*, cDNA library, RACE, RT-PCR

감사의 글

지난 2년의 시간은 그 무엇보다 사람의 소중함을 일깨워주는 시간들이었습니다. 개인적으로 무척 힘들었던 시기에 대학원에 들어왔고 그간 많은 일들이 있었지만, 주위의 좋으신 분들의 관심과 사랑으로 지금의 이 논문을 쓸 수 있었다고 자신 있게 말할 수 있습니다. 또 다른 시작을 앞두고 있는 시점에서 두려운 마음이 앞서지만 그동안 다져진 몸과 마음으로 전진해 나갈 것을 다짐해 봅니다.

먼저 지난 5년 이라는 긴 시간 동안 한결같으신 모습으로 학문에 대한 자세와 가르침을 주신 김세재 교수님께 특별히 감사의 말씀을 드리고 싶습니다. 늘 건강하시길 빕니다.

또한 바쁘신 와중에도 본 논문에 대한 관심을 가져주신 김원택 교수님과 박덕배 교수님께도 감사의 말씀을 드립니다. 그리고 6년 이라는 배움의 과정동안 생물학에 대한 흥미와 열정을 키워주신 오문유 교수님, 김문홍 교수님, 이용필 교수님, 오덕철 교수님, 고석찬 교수님과 이화자 교수님께도 감사의 마음을 전해드립니다.

그리고 멀리서나마 항상 관심을 가져 주시고 진심어린 충고와 조언을 아끼지 않으셨던 해양과학대학의 이제희 교수님과 제주 TIC에 계신 문상욱 박사님께도 감사의 말씀을 전하고 싶습니다.

5년 동안의 연구실 생활 중에 가장 많은 가르침을 주셨던 박지원 선생님과 정형복 선배님께 특별히 고마움의 마음을 전합니다. 여러 면에서 많이 모자랐던 이 후배를 포기하지 않으시고 한결같은 자세로 배움의 길로 이끌어 주신 점, 잊지 않겠습니다. 그 외에도 많은 도움을 주셨던 김기옥 박사님, 강신해 선생님, 박수영 선생님, 윤지현 선배님과 이동헌 선생님, 김영미 선배님, 임희경 선배님께도 감사의 마음을 전해드립니다. 그리고 짧은 시간이었

지만 존재만으로 나에게 든든함을 주었던 대주오빠, 명천오빠, 경만, 재영에게도 고맙다는 말을 하고 싶습니다.

또한, 생물학이라는 학문에 대한 진정한 애정과 연구자로서의 자세를 보여주신 송관필 선생님과 정용환 선생님, 그리고 한상현 선배님께 감사의 말씀을 드립니다.

대학원 동기로서 그간 많은 정이 들었던 영준오빠와 은아에게도 고마움의 마음을 전하며 앞으로도 좋은 친구로서의 연이 이어지기를 기대해 봅니다.

학부 때부터 지금까지 한결같은 모습으로 곁에서 지켜봐주며, 어려울 때마다 다시 일어설 수 있는 힘을 주었던 친구, 미선과 준호, 명옥에게 특별히 감사의 말을 전합니다. 그리고 힘든 시절에 만나서 지금까지 늘 내게 웃음을 주고, 같은 자리에 머물지 않도록 나를 일깨워 주었던, 진정한 사랑이란 어떤 것인가를 보여준 후배, 은영에게 고마움의 마음을 전하고 싶습니다.

마지막으로, 이 길을 택함에 있어서 누구보다 큰 힘과 용기를 주시고, 아낌없는 사랑의 표현으로 언제나 정신적인 버팀목이 되어주신 아버지와 어머니, 그리고 나의 든든한 후원자 동생 승재에게 이 자리를 빌어 사랑을 고백하고 싶습니다.

그리고 항상 곁에 머무시어 홀로 걸어가는 이 길을 외롭지 않게 해 주신, 이토록 많은 사람들을 보내주신 하느님께도 사랑을 고백하며, 이 논문을 바칩니다.

감사합니다.