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Expression profile of sex steroid hormone estrogen receptors (ERs) in the development of juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus polyphekadion* ♂)

Eric Amenyogbe[1,2*], Gang Chen[1,2], Zhongliang Wang[1,2], JianSheng Huang[1,2], Xiaoying Lu[1,2], Mingde Lin[1,2] and Zhi Xiong Guo[1,2]

[1]College of Fisheries, Guangdong Ocean University, Zhanjiang 524025, China.
[2]Guangdong Provincial Key Laboratory of Aquaculture in the South China Sea for Aquatic Economic Animal of Guangdong Higher Education Institutes, Laboratory of Fish Aquaculture. Zhanjiang 524025, China.

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Recognized for its traditional roles, estrogen is ever-present in all vertebrates, regulates reproduction by binding and activating estrogen receptors (ERs), and also controls several functions of vertebrates, including reproductive immune, and central nervous systems. In order to access any other possible functions of the estrogen receptors in the development of the juvenile Hybrid grouper (*Epinephelus fuscoguttatus* ♀× *Epinephelu polyphekadion* ♂), full-length of ERs cDNA sequences were isolated and analyses were found to be 2391 bp for hgERα, 2626 bp for gERb1 and 2339 bp for hgERb2, respectively. The results of amino acid and phylogenetic analysis revealed that each hgER was grouped in consistent taxonomic groups of perciformes and demonstrated great evolutional conservation in functional domains. Real-time PCR examination discovered that the receptors expressed in all tissues examined, though, at a different level, the ERα mRNA level expressed higher than ERβ1, and ERβ2 in tissues examined. The ERα mRNA level of expression was found to be highest in the tissue of the heart, followed by muscle, and liver. The ERβ1 mRNA level was greatest in heart tissue, trailed by liver and muscle and ERβ2 was highest in the heart trailed by stomach and liver. The minimal expression was recorded in the kidney, the gill, and the brain for ERα, ERβ1, and ERβ2 respectively. These results put forward that steroid hormone estrogen receptors might be playing a significant part in the controlling of social behavior complexity, plasticity behavior, and the assessment of a gratifying inducement in Hybrid grouper.

Key words: Estrogen receptors, Real-time POR, tissue expression, hybrid grouper.

INTRODUCTION

Well known for its critical roles, Estrogen is known for regulation of reproduction through binding and activation

*Corresponding author. E-mail: gdoucg@126.com.

of estrogen receptors (ERs) (Chen et al., 2011), some functions in vertebrates are also controlled by estrogen, for example; reproduction, the immune system, and the central nervous system (Bakker and Brock, 2010; McCarthy, 2010; Vasudevan and Pfaff, 2008). Several authors have researched the biological importance of estrogen in vertebrates including fish, mostly in regeneration (Hewitt and Korach, 2002; Wang, 2005). Two forms of estrogen receptors are reported in several vertebrates, ERα and ERβ, except, in teleost fish, where three models are detailed: ERα, ERβ1, and ERβ2. It seems that the ERαγ form in fish is genetically related to ERβ which might be due to gene duplication within the teleosts fish. Due to this result, ERβ and ERγ are named ERβ1 and ERβ2 (Katsu et al., 2011; Hawkins et al., 2000). According to Thornton (2001), the "ancestral condition for jawed vertebrates is considered to contain two forms of ER, corresponding to ERα and ERβ" (Katsu et al., 2010c). In mammals, two types of estrogen receptors are reported; examples are fishes, birds, reptiles, and amphibians. As well established, the estrogen is the key steroid hormone (Chen et al., 2011) that regulates, differentiate and plays essential roles in "growth of oocyte maturation for female reproduction" (Diotel et al., 2018; Ni et al., 2013; Lassiter et al., 2002; Pepe et al., 2002; Pelletier et al., 2000), and also play a precarious role in controlling the survival of spermatogonia and development of mature "spermatogenesis for male reproduction" in vertebrates (Ni et al., 2013; Makinen et al., 2001; Ebling et al., 2000).

The physiological functions of estrogens are reportedly mediated through the "specific cell surface receptors - the estrogen receptors" (Fu et al., 2014; Mermelstein and Micevych, 2008; Beyer et al., 2003). It is therefore necessary to critically look at the physiological role played by the estrogen receptors. Available literature has proven that the superfamily of nuclear hormone receptors (Perrotti, 2017) of which estrogen receptors belongs to (Blumberg and Evans, 1998) have many characteristics in common, in which proteins of this superfamily can be grouped into six distinct domains. "A/B domain which has a transactivation function, the C domain which consists of two zinc finger motifs" and consists of eight cysteine residues are essential for DNA binding. They also have a "D area which is the hinge region and enables the protein to change its conformation" (Fu et al., 2014); there is also the E domain which has the possibility of being the domain of ligand-binding and that of the F domain whose function is not clearly understood (Fu et al., 2014; Todo et al., 1996). Other authors have reported the third ER subtype of estrogen receptors (ER-b2) in many species of teleost such as the Atlantic croaker (Wang et al., 2005; Hawkins et al., 2000), goldfish (Ma et al., 2000), zebrafish (Bardet et al., 2002; Menuet et al., 2002) and largemouth bass (Sabo-Attwood et al., 2004), this has proven that at "least three subtypes" of estrogen receptors exist in teleosts (Guo et al., 2017; Wang et al., 2005).

To advance understanding of molecular endocrinology of phylogenetically hybrid grouper fish and also provide further data on the "evolution of teleosts estrogen receptors", estrogen receptors encoding cDNA sequences were sequestered and clones (Katsu et al., 2006) from juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀× *Epinephelus polyphekadion* ♂). Currently, little or no material information exist concerning estrogen receptors cloning and qPCR expression profile of hybrid grouper, hence, the objective of this study was to clone (Yu et al., 2008) the full length of juvenile hybrid grouper estrogen receptors and also studies their expression in the various tissues samples and analyzed the possible other roles played by the estrogen receptors in the development of the hybrid grouper fish. The data of this work could be helpful in researches intended at improving the production of hybrid grouper. Assessment of the resultant sequence data was done to determine their other possible role played by these receptors other than their traditional role of reproduction in vertebrates using COFACTOR server (Zhang et al., 2017) to analyze the Gene Ontology.

MATERIALS AND METHODS

Experimental fish

Groupers (*Epinephelus* spp*)* are teleosts, typical of them being "monandric protogynous hermaphrodites, meaning they mature as females" and can change sex after sexual maturity (Erisman et al., 2009; DeMartini et al., 2011). Hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus polyphekadion*) is an essential aquaculture fish recently developed through cross-breeding to provide a new aquaculture strain (James et al., 1999). Interestingly, "the hybrid grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus polyphekadion*♂) mature as a male meaning it grows from female to male and can change sex after sexual maturity between the ages of 3-5 years" (Amenyogbe et al., 2019). It must be noted that the fish developed gonads at its maturity which makes the study in gonad impossible at this stage of the hybrid grouper. A cumulative of 3 "female hybrid juvenile grouper (3 - 4 month) were used for the experiment with an average body weighing 82.3 ± 4.32 g, together with the length of 13.73 ± 0.13 cm obtained from Guangdong, Hengxing Group Co.LTD., Guangdong Province" (Amenyogbe et al., 2019). "Live fish procedures followed the guidelines by Institutional Animal Care and the Fisheries and Aquaculture College, Laboratory of Fish Breeding, Guangdong Ocean University, China" (Amenyogbe et al., 2019). Tissue samples of "brain, heart, intestine, muscle, head, kidney, liver, stomach, gill, and spleen were immediately dissected, iced up, and kept at -80°C with liquid nitrogen until use".

RNA isolation

The "total RNA from the brain, gill, liver, muscle, intestine, spleen, stomach, head, kidney and heart tissues of the hybrid grouper was extracted by the use of MiniBEST Universal RNA extraction kit (TransGen Biotech, China) and using Trizol reagent (Invitrogen) following the manufacturer´s instructions". The "quality of total RNA was analyzed by the use of 1% agarose gel electrophoresis and UV spectrophotometry (Nandrop 2000, Thermo, USA)".

Table 1. The polymerase chain reaction primers used in this study of ERS.

Primers Sequence	
M13 : CGCCAGGGTTTTCCCAGTCACGAC RV : GAGCGGATAACAATTTCACACAGGA	Vector (Pmd-18)
UPM-long: CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT UPM-short: CTAATACGACTCACTATAGGGC	Universal race primers
Al-F3: TACTCTGCTCCTCTGGAYGCMCACG Al-R3: GCATCTCCAGCAGCAGGTCGTAYAG B1- S1: CACTWCTGTGCTGTGTGYCACGACT B1-R3: CTCACCCTGGAYGTGGCRGCTATCA B2-S2: CGTCTACAAYGAACCCAGCCCACA B2-A2: AGAGTCTGCTGCTGGGTCGWCA	Partial
3B1-S1: TAACAGGACCAGCGTTGGGTTCATT	3utr
3B1-S2: GCCAAGAAGATTCCAGGATTCATAG	3utr
3A-S2: TCCTCCGATTCCTGTCAATAGTGC	3utr
3A-S3: ACTGGACCTGTAGACGGGTGTTGA	3utr
3B2-S1: CACAATGGACTACATCTGCCCCGC	3utr
3B2-S4: CTCCAGACTTCAAACTCAGCAGGG	3utr
5B1-A2: ACTTCACAGCATTTGCGGAGGCGAC	5utr
5B1-A3: CTTACGGCGGTTCTTGTCTATGGTG	5ut
5B2-A1: TTGATTAGTGGCGGGGCAGATGTA	5utr
5B2-A2: TTAGTGGCGGGGCAGATGTAGTCC	5utr
5A-A3: ATGATGAAAGGAGGTGTGCGTAAG	5utr
5A-A4: CTCATCTTTGCCCAGGACCTCATA	5ut
GP-βactin(F) –TACGAGCTGCCTGACGGACA GP-βactin(R)- GGCTGTGATCTCCTTTTGCA EXPA-F2: ATGCCCACTTCGTAACAC EXPA-R2: CACCTCAACACCCGTCTA EXPβ1-F3: CTGGTCGTGTGAGGGGTGTA EXPβ1-R3: GCCTTTGGTCTGTTGGTTCG EXPβ2-F3: TCATTGCCTTCAGACAGAC EXPβ2-R3: GCTCATCGACATCACCACC	RT-qPCR

Where “M13, RV, UPM-Long and short are all universal primers, GP- βactin is Grouper βactin”, A1-F3, A1-R3, B1-S1, B1-R3, B2-S2, B2-A2 represent partials, and 3B1-S1, 3B1-S2, 3A-S2, 3A-S3, 3B2-S1, 3B2-S4 indicates 3UTR, while 5B1-A2, 5B1-A3, 5B2-A1, 5B2-A2, 5A-A3, 5A-A4 indicates 5UTR and EXPA-F2, EXPA-R2, EXPβ1-F3, EXPβ1-R3, EXPβ2-F3, and EXPβ2-R3 all indicates the primers used for gene expression of “estrogen receptor genes, and 3&5UTR stands for untranslated regions”.

Cloning of estrogen receptors (ERs)

To clone “a partial cDNA fragment of ERα, ERβ1, and ERβ2, primers, as shown in Table 1, were designed”. Primers were designed for amplification of hybrid grouper ERα, ERβ1, and ERβ2 gene coding sequences using the existing full-length sequences of *Epinephelus coioides-estrogen receptor alpha -(GU721076.1); Acanthopagrus schlegelii-estrogen receptor alpha-(AY074780.1); Sparus aurata-estrogen receptor alpha-(AF136979.2) and Acipenser schrenckii-estrogen receptor alpha1-(AB435631.1)* ; *Epinephelus coioides estrogen receptor beta 1-(GU721077.1); Sparus aurata-receptor type beta1-(AF136980.1); Acanthopagrus schlegelii –estrogen receptor beta1-(AY074779.1), and Acipenser schrenckii-estrogen receptor beta1-(AB435633.1)*; *Epinephelus coioides estrogen receptor beta2- (GU721078.1); Acanthopagrus schlegelii estrogen receptor beta 2-(EU346949.1) and Micropterus salmoides estrogen receptor beta2-(AY211021.1).*

Primer 5 software was used to design primers (Table 1). cDNA synthesis was performed using the TRANSgen First-strand cDNA synthesis kit with a total volume of 20 µl of reaction mixture following the manufacturer’s guidelines. The partial cDNA fragments of ERs were “amplified from the first-strand cDNA from” a mix of the liver, heart, and brain tissues. The PCR reaction was performed in 20 µl volumes of the reaction mixture. The amplification was performed following reaction conditions, “94°C for 5 min, followed by 35 cycles for 30 s at 94°C, for 30 s at 58°C, for

35 s at 72°C and 10 min at 72°C". The separation of PCR products was done by using electrophoresis, and the "DNA Bands were recycled and purified by the use of the SMART RACE cDNA purification Kit (Clontech, Palo Alto, CA)". The "purified DNA portions were then subcloned into the pMD18-T vector (Takara, Japan) and converted into competent *Escherichia coli* DH5a cells". A maximum of "three different positive clones each were selected and send to Sangon Biological engineering (Guangzhou) LTD". for sequencing (Amenyogbe et al., 2019). To "clone *3*′ and *5*′ untranslated region (UTR) end of ERα, ERβ1 and ERβ2, two sense primers were designed depending on the partial cDNA sequence of ERα, ERβ1 and ERβ2 as shown in Table 1 using the SMART RACE cDNA Synthesis Kit (Clontech, Palo Alto, CA) according to the manufacturer's guidelines". A "clear fragment was purified, cloned, and sequenced using the same procedure as described earlier".

3′ RACE was done in "both first and second amplification" using Amenyogbe et al. (2019) method. Additionally, "5′ RACE was carried out using the same procedures and methodologies that made use of reverse primers (Table 1) in place of sense primers". "PCR amplification was done in a 15-µl volume of the reaction mixture" following Amenyogbe et al. (2019) method.

Sequence analysis

The "sequences of partial, 3′ and 5′ UTR were assembled to form the full-length cDNA of the target gene by the use of LaserGene software" (*https://*dnaman.software*.informer.com)*. The "Gene translation, predictions of the amino acid sequence" was done using EXPASY web tools (http://expasy.org/tools). The conserved domains search was done using Marchler-Bauer et al. (2011) to ascertain conserved domain sequences among designated ER superfamily genes. To establish genetics relations, phylogenetic analysis was carried out, and a consensus tree was built using MEGA 6.0 software 6 (Tamura et al., 2013). We examined the physical and chemical properties of the protein sequences using the PROTEAN program (version 5.07; DNASTAR Inc.: Madison, WI, USA, 2003.) to ascertain their chemical compositions. COFACTOR server (Zhang et al., 2017) method was used for gene ontology GO terms predictions.

Tissue mRNA expression of ERs by qRT-PCR

The "mRNA levels of ERα, ERβ1, and ERβ2 in tissues were resolute by Real-time qRT-PCR" (Amenyogbe et al., 2019) in the liver, muscle, brain, stomach, spleen, intestine, gill, head kidney, kidney and heart using Transtar "Tip Green qPCR Supermix (TransGen Biotech, China)" the manufacturer´s instructions were followed, a Bio-Rad Connect ("Roche Light Cycler®96 SW1.1" Real-time Detection) in 10 ul reaction. We used *β-actin* as a control gene. The primers for ERα, ERβ1, and ERβ2 and *β-actin* were designed using primer5 designers (Table 1). The total mixture for the reaction for quantitative Real Time-PCR consisting of 5 ul Transtar "Tip Green qPCR Supermix (TransGen Biotech, China), and 0.4 ul of each sense and antisense primer, 3.6 ul of H_2O and 0.6 ul of cDNA". A melting curve was performed to detect the specificity (Fu et al., 2014). The "reaction conditions of for the qRT-PCR were as follows: 30 s at 95°C; and was amplified for 40 cycles for 15 s at 95°C, annealing for 15 s at 59°C and extension for 15 s at 72°C". Each sample "verified in triplicate". The $2^{-\Delta\Delta Ct}$ methods (Livak, and Schmittgen, 2001) was used to calculate the results.

Statistical analysis

The data in this study were articulated as a means±SEM. The significant differences in the data between ERs are presented using one-way ANOVA followed by Duncan's posthoc test and a probability level less than 0.05 ($P < 0.05$) was used to indicate significance. Also, the Independent samples T-test was also used, and the significance level was set at $P < 0.05$. All statistical analysis were performed using SPSS 16.0 (SPSS, Chicago, IL, USA) (Cui et al., 2017).

RESULTS

Cloning and characterization of hybrid grouper ESRs

By the use of standard PCR procedures, the partial DNA fragments were augmented from Hybrid grouper (*Epinephelus fuscoguttatus* ♀× *Epinephelus polyphekadion* ♂) liver, heart, and brain RNA. The DNA fragment acquired, and sequence examination exhibited that the fragments had a resemblance to ERα, ERβ1, and ERβ2. The RACE technique was used "to clone full-length" of hybrid grouper ERα, ERβ1 and ERβ2 cDNAs with the following (GenBank accession no. MK575468, MK544841, and MK570511 for ERα, ERβ1, and ERβ2 respectively. The sequence analysis of cDNA for ERα show 621 amino acids and considered molecular mass 61.04 kDa and ERβ1 comprises 546 amino acids and a determined molecular mass 60.47 kDa, and ERβ2 show 244 amino acids and a determined molecular mass 27.49 kDa. The hybrid grouper ERs "sequence can be grouped into five domains based on its sequence identity to other species' ERs" (Figure 2) as suggested by Katsu et al. (2010c). Amino acid sequences of hybrid grouper hgERα shared the identity of 42.3 and 40.3% with hgERβ1 and hgERβ2 respectively while hgERβ1 show the identity of 56.8% with hgERβ2. Hybrid grouper sequences of ERs equated with different species (*Homo sapiens, Mus musculus, Epinephelus coioides, Dicentrarchus labrax, Oreochromis aureus, Sebastes schlegelii*, *Acanthopagrus latus* and *Sparus aurata*), Hybrid grouper ERα shared 81.9-98.8, and 57.4-958% identities in the C, and E domains, respectively (Figure 1A). In contrast, Hybrid grouper ERβ1 shared 63.3-97.5, and 66.1-98.7% identities in the C, and E domains, respectively (Figure 1B) and hybrid grouper. The clone protein sequence of ERβ2 shared 22.9-75.2% identities in the E domains. Therefore, "C domain or DNA-binding domain and E domain or the ligand-binding domains" were conserved in all vertebrate ERs considered. The general identities of hybrid grouper ERα with ERα in (*Homo sapiens, Mus musculus, E. coioides, D. labrax, and S. schlegelii*) were 46.1, 46.6, 89.1, 88.3, and 90.2%, respectively, the overall identities of hybrid grouper ERβ1 with ERβ1 from the (*Homo sapiens, Mus musculus, E. coioides, O. aureus, and S. schlegelii*,) were 48.2, 48.3, 91.5, 79.0, and 85.7%, respectively. The overall identities of hybrid grouper ERβ2 with ERβ2 from the (*Homo sapiens, Mus musculus, E. coioides, A. latus,* and *S. aurata*) species were 44.9, 44.4, 74.5, 69.1 and 70.0% respectively. The phylogenetic analysis showed that each of these ERs

A

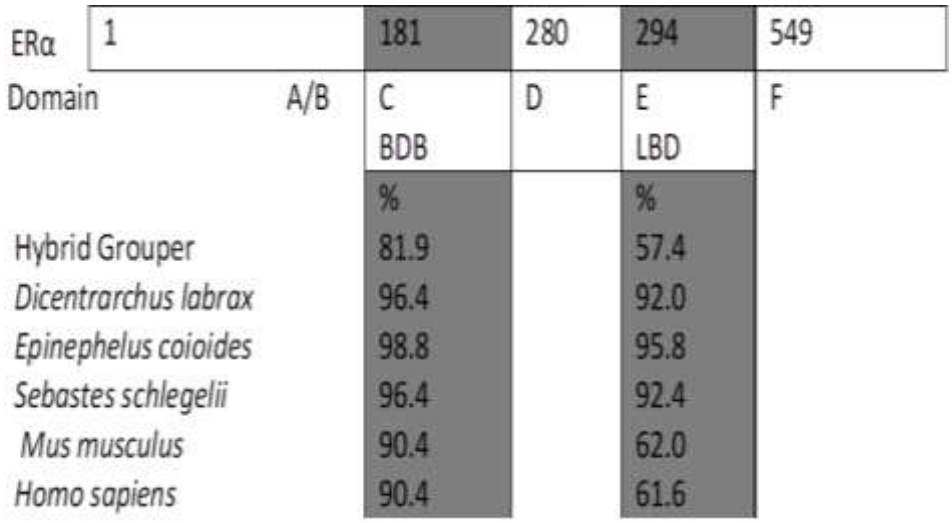


Figure 1a. The "DNA and ligand-binding domains" of Hybrid grouper ERα showing homology with some ERs. The figure presents the percentage "identities in the DNA and ligand-binding domains DBD and LBD" of the Hybrid grouper ERα in A with some sequences of the ER subfamily indicated. The Genbank accession numbers of ER sequences used are listed under the phylogenetic tree.

B

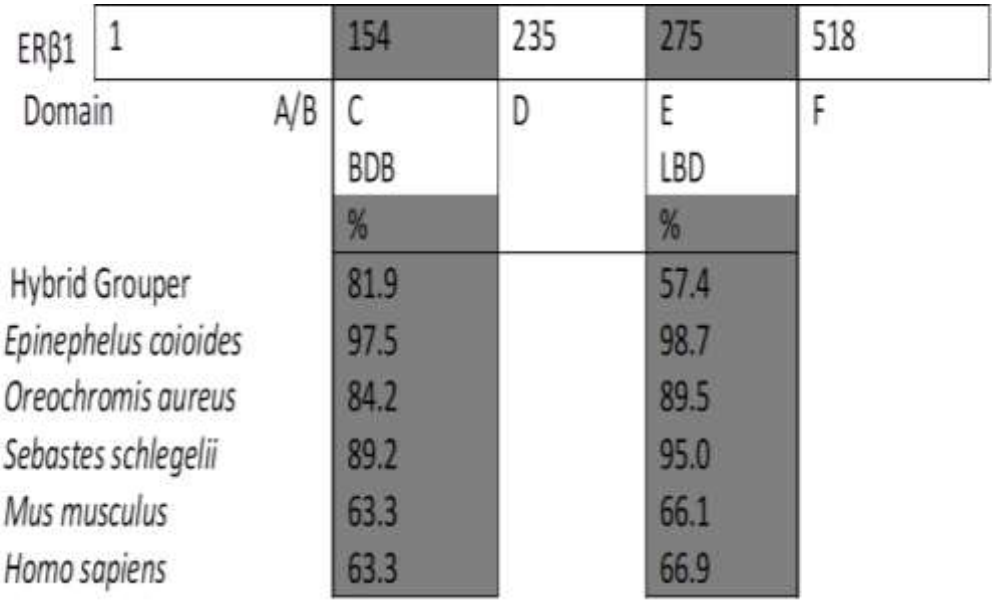


Figure 1b. The "DNA and ligand-binding domains" of hybrid grouper ERβ1 showing homology with some ERs. The figure presents the percentage identities in the "DNA and ligand-binding domains" of the hybrid grouper ERβ1 in B with some sequences from ER subfamily are indicated. The Genbank accession numbers of ER sequences used are listed under the phylogenetic tree.

belongs to ER superfamily (Figure 3).

The sequence alignments of ERs of different vertebrates including Hybrid grouper are demonstrated indicating the various domains. The Genbank accession numbers of ER sequences used are as same as ones used in the phylogenetic analysis. The MAPK, "P, and D-boxes from DBD", cAMP, PKC, AF-2 are indicated by Elbow Double-Arrow connector. The down arrow indicates the eight "cysteine residues of the zinc-finger motif". "A/B, B/C, C/D, D/E, and E/F" domains are marked with Double Arrows.

The phylogenetic examination was done using "Clustral W". The different ER sequences obtained from the Genebank database. Genebank accession numbers of

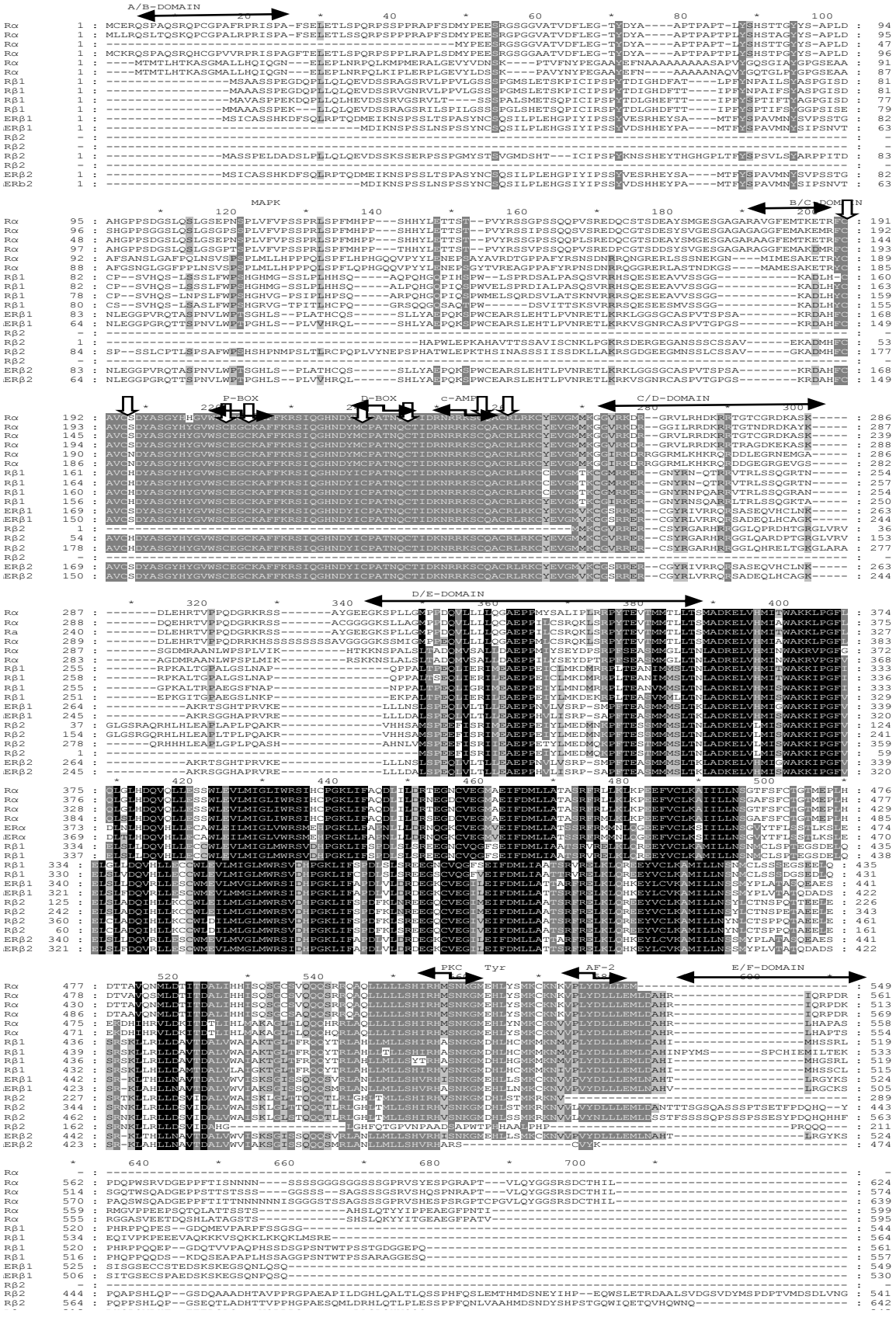


Figure 2. The essential amino acids in DNA and ligand-binding domains are highly conserved in hybrid grouper ERs.

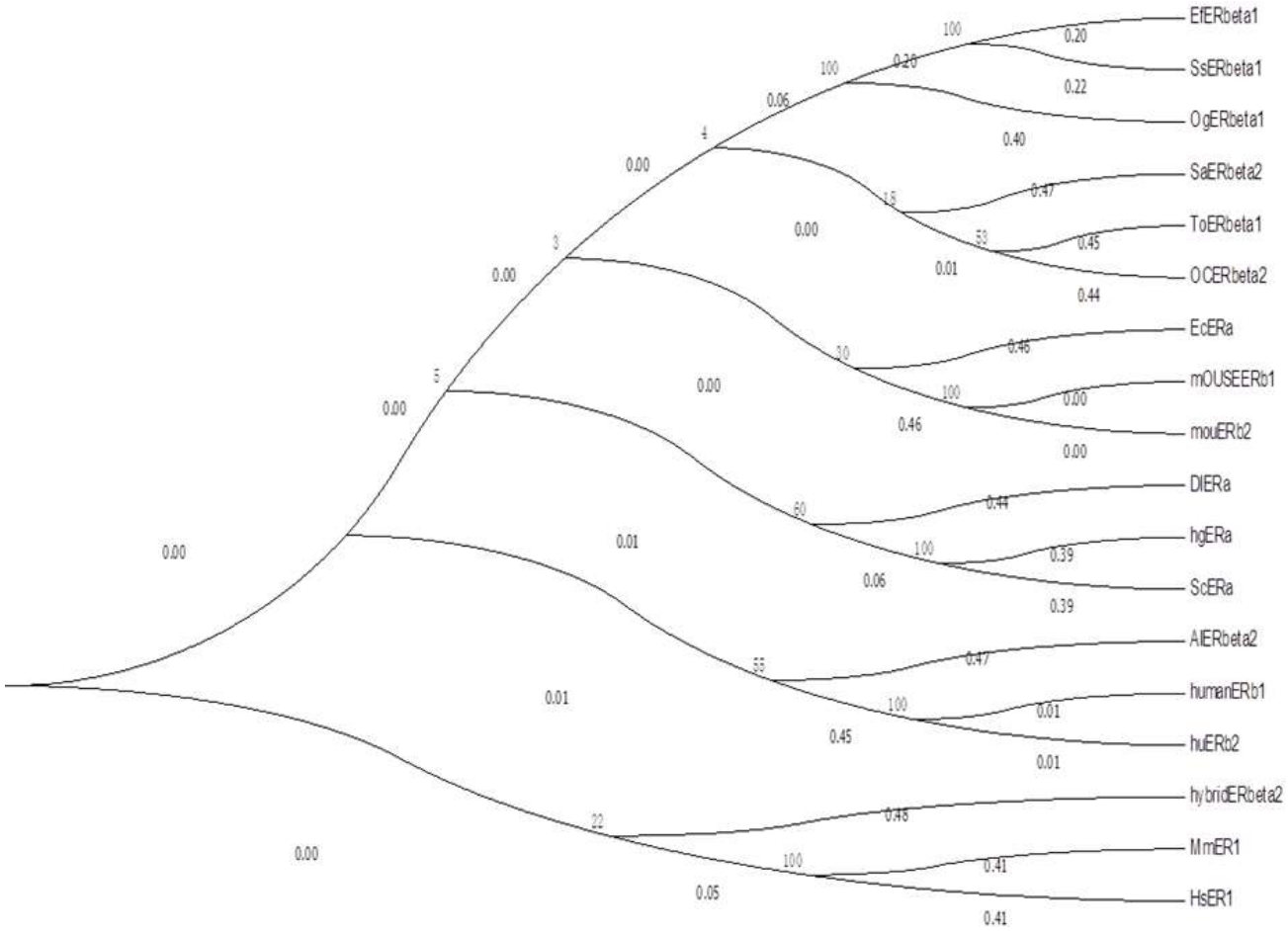


Figure 3. A phylogenetic tree is displaying the affiliations of the hgER with other ERs from other species.

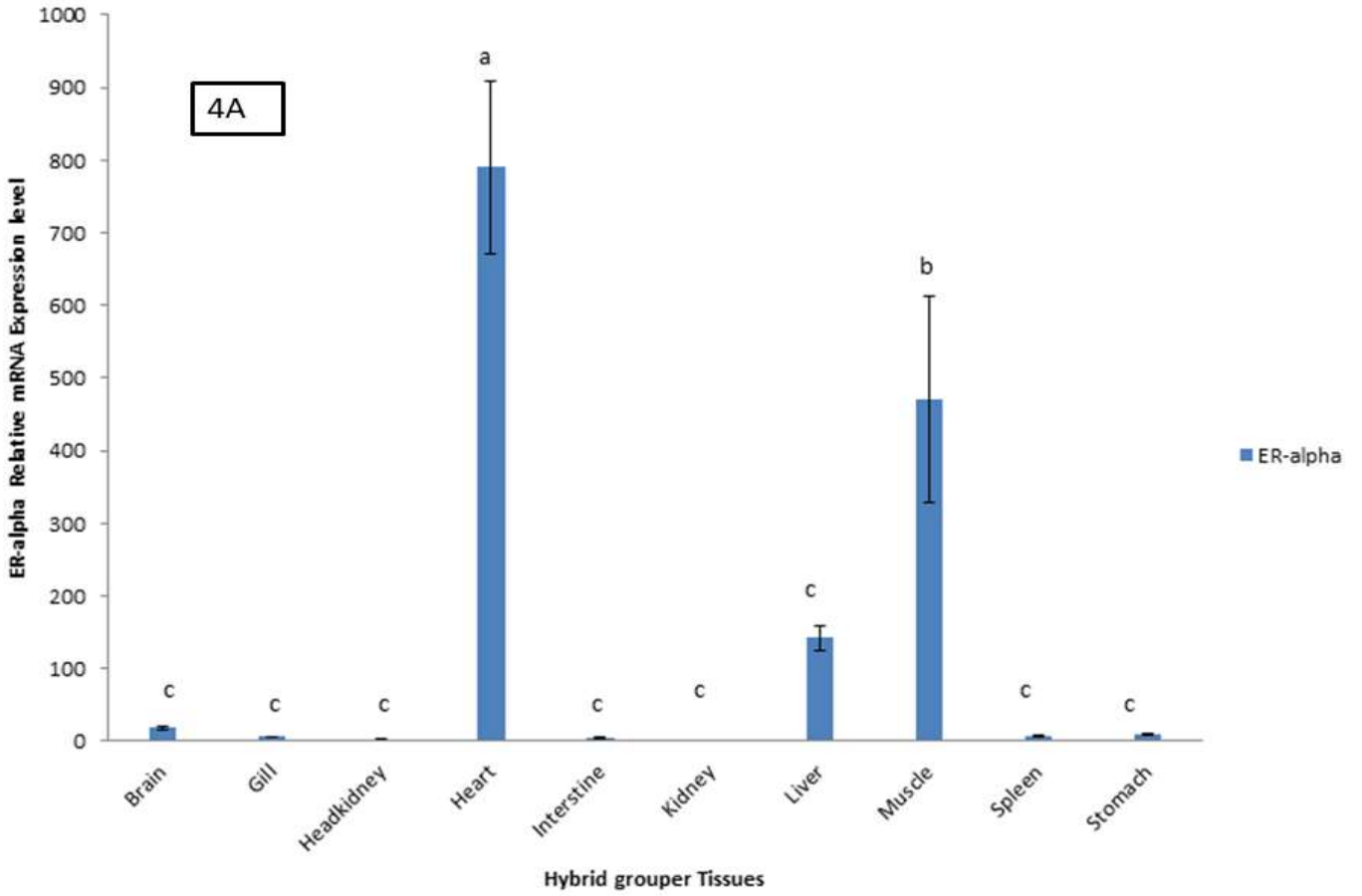
the sequences are: hybrid grouper ERα, MK575468; *E. coioides* ERα, ADK90033; *D. labrax* ERα, CAD43599; *S. schlegelii* ERα, ACN39246; *Mus musculus*-estrogen receptor isoform 1, NP_031982; *Homo sapiens*-estrogen receptor isoform 1, NP_000116; hybrid grouper ERβ1, MK544841; *E. coioides* ERβ1, ADK90034; *O. aureus* ERβ1, ACF75102; *S. schlegelii* ERβ1, ACN38898; *Mus musculus*-estrogen receptor beta isoform 2, NP_034287; *Homo sapiens*-estrogen receptor beta isoform 1,NP_001428 XP_495993; Hybrid grouper ERβ2, MK570511; *E. coioides* ERβ2, ADK90035; *A. latus* ERβ2, AEX68679; *S. aurata* ERβ2, CAE30471; *Mus musculus*, NP_034287 and *Homo sapiens,* NP_001258805. Where EFERbeta1- hybrid grouper ERβ1, SsERbeta1- *S. schlegelii* ERβ1, OgERbeta1- *E. coioides* ERβ1, mOUSEERb1- *Mus musculus*, ToERbeta1- *O. aureus*, SaERbeta2- *S. aurata* ERβ2, EcERa- *E. coioides* ERα, mouERb2- *Mus musculus*, DlERa- *D. labrax* ERα, HgERa-hybrid grouper ERα, SCERa- *S. schlegelii* ERα, AlERbeta2- *A. latus* ERβ2, humanERb1- *Homo sapiens*, HuERb2- *Homo sapiens*, HgERbeta2-hybrid grouper ERβ2, MmER1- *Mus musculus* and HsER1- *Homo sapiens.*

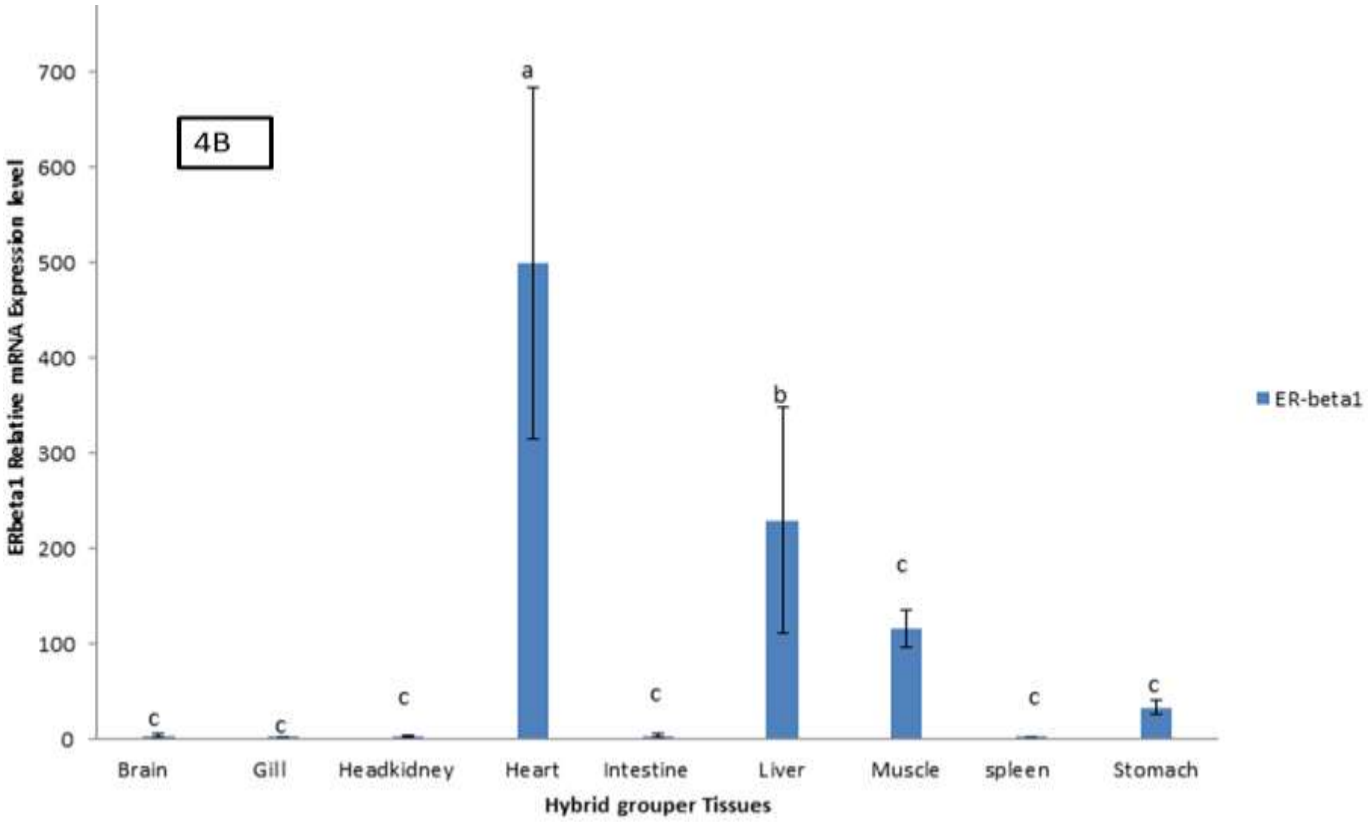
Gene expression

While at a different level, hybrid grouper estrogen receptors (hgERs) were expressed in all tissues samples that were studied. The *hgER*α expression level was found to be highest in the heart among the ten tissues examined, followed by the muscle. The *hgER*β1 was noticeably paramount also in the heart, followed by the liver. The *hgER*β2 expressed most profoundly in the liver, followed by the stomach. Generally, the highest mRNA expression levels of hgERα, *hgERβ1* and *hgER*β2 were all found in the heart. There was a substantial difference between the expression in the heart and liver and other samples as shown in Figure 4a, b, and c. All hybrid grouper ERs studied were expressed in all tissues samples examined, although at different levels. Interestingly, *hgER*α expressed relatively higher in all tissues as compared to the *hgER*β1 and *hgER*β2.

Physiochemical properties and sequence analysis

Scrutiny of the physical and chemical possessions of the


4A
ER-alpha Relative mRNA Expression level
1000
900
800
700
600
500
400
300
200
100
0
a
b
c
c
c
c
c
c
c
c
c
Brain
Gill
Headkidney
Heart
Interstine
Kidney
Liver
Muscle
Spleen
Stomach
Hybrid grouper Tissues
ER-alpha


4B
ERbeta1 Relative mRNA Expression level
700
600
500
400
300
200
100
0
a
b
c
c
c
c
c
c
c
Brain
Gill
Headkidney
Heart
Intestine
Liver
Muscle
spleen
Stomach
Hybrid grouper Tissues
ER-beta1

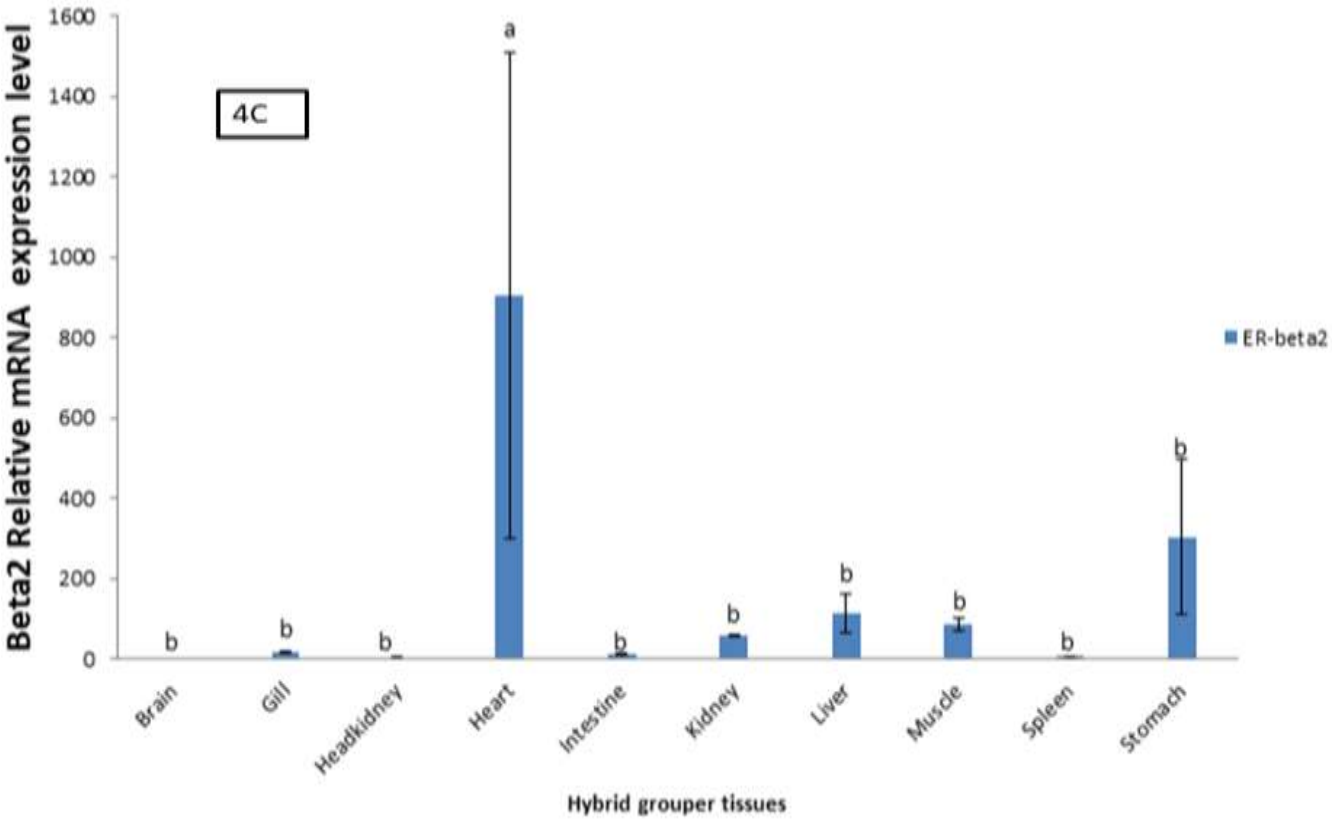


Figure 4. The mRNA expression profile of a) ERα, b) ERβ1, and c) ERβ2 respectively, in various tissues studies in Hybrid grouper. Statistics presented as the "mean ± SEM of triplicate experiments". Letters a, b, and c "indicate statistical differences at P < 0.05".

ERα sequence revealed the "molecular structural formula" of ERα to be $C_{2663}H_{4209}N_{759}O_{802}S_{42}$ "with a total atom number" of 8475. ERα has theoretically "predicted ion isoelectric value" of 8.19 and instability index of 65.10, classifying it as an unstable protein "with the molecular weight" of 68.40 kDa. While the scrutiny of the physical and chemical possessions of the ERβ1 sequence revealed the "molecular structural formula" of ERβ1 to be $C_{2632}H_{4203}N_{763}O_{783}S_{44}$ "with a total atom number" of 8425. ERβ1 has a "theoretical predicted ion isoelectric value" of 8.03 and instability index of 70.10, classifying it as an unstable protein with "molecular weight" of 60.47 kDa. On the other hand, the physical and chemical possessions of the ERβ2 sequence revealed the "molecular structural formula of ERβ1" to be $C_{1224}H_{1929}N_{329}O_{356}S_{17}$ with a "total atom number" of 3855. ERβ2 has "theoretically predicted ion isoelectric value" of 5.18 and instability index of 56.76, classifying it as an unstable protein "with the molecular weight" of 27.49 kDa.

Amino acid composition and protein secondary structure

The "molecular examinations of the amino acid sequence of ERα showed that the protein contained 162 hydrophobic residues" (25.049%), 61 "acidic residues" (10.92%), 67 basic residues (14.27%) and 193 "polar amino acids" (30.06%). Aliphatic catalogue and "a grand average of the hydropath city (GRAVY) of growth was 70.73 and −0.403, correspondingly". The total quantity of "a negatively charged residue (Asp and Glu) was 56, and the total number of positively charged residues (Arg and Lys) was 60". The examination of "amino acid sequence of ERβ1 indicated that the protein contained 163 hydrophobic residues (29.02%), 53 acidic residues (10.78%), 56 basic residues (13.44%) and 152 polar amino acids (26.63%). Aliphatic catalogue and grand average of hydropath city (GRAVY) of growth were 81.25 and −0.309, correspondingly". The total quantity of "a negatively charged residue (Asp and Glu) was 53, and the total number of positively charged residues (Arg and Lys) was 56". Molecular "analysis of the amino acid sequence" of ERβ2 contained 84 hydrophobic residues (33.61%), 34 acidic residues (15.41%), 21 basic residues (10.91%) and 50 polar amino acids (20.01%). Aliphatic catalogue and grand average of hydropath city (GRAVY) of growth were 91.93 and −0.223, respectively. The total quantity of "a negatively charged residue (Asp and Glu) was 34, and the total number of positively charged

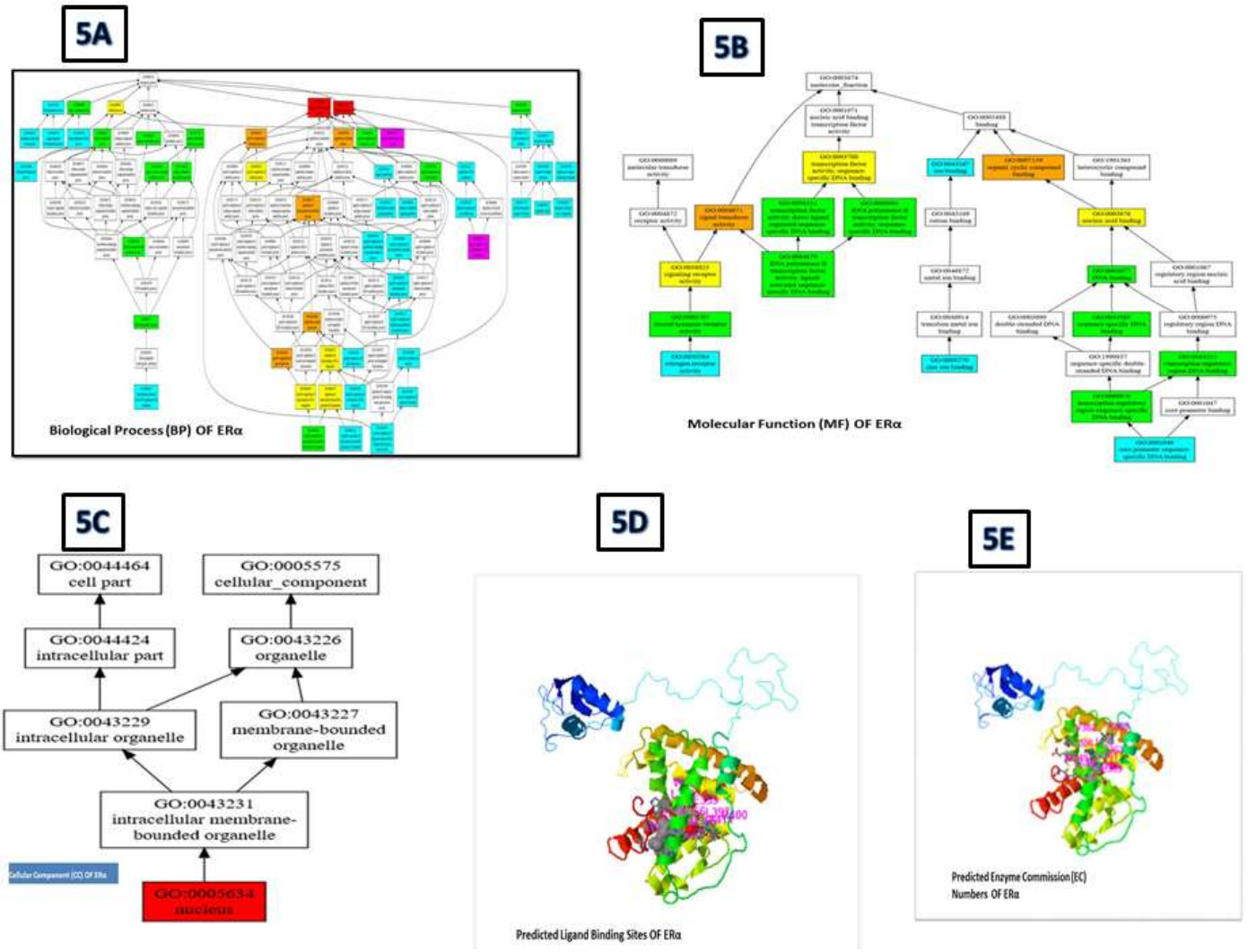


Figure 5a, b, c, d and e. The ERα's biological processes, molecular functions and cellular component.

residues (Arg and Lys) was 21".

Predicted Gene Ontology analysis of hybrid grouper estrogen receptors using COFACTOR software

Using COFACTOR server to analyze Gene Ontology, two main functions were identified; namely, biological process and molecular function with Estrogen receptor mRNAs regulation relationships in the development of hybrid grouper by the GO. It has been found that the hybrid grouper "regulated by estrogen receptor alpha" is involved in many biological processes, including "biological regulation, regulation of biological process, regulation of macromolecule metabolic process, regulation of gene expression, positive regulation of biological process, regulation of cellular process, positive regulation of gene expression, regulation of transcription", DNA-templated, positive regulation of cellular process, positive regulation of transcription, DNA-templated, cellular process, regulation of transcription from RNA polymerase II promoter, positive regulation of transcription from RNA polymerase II promoter, organic substance metabolic process, response to stimulus, primary metabolic process, single-organism process, cellular metabolic process, organic substance biosynthetic process, developmental process etc (Figure 5a). Additionally, it also has molecular functions which includes, organic cyclic compound binding, signal transducer activity, transcription factor activity, sequence-specific DNA binding, signaling receptor activity, nucleic acid binding, DNA binding, RNA polymerase II transcription factor activity, sequence-specific DNA binding, transcription factor activity, direct ligand regulated sequence-specific DNA binding, sequence-specific DNA binding, RNA polymerase II transcription factor activity,

ligand-activated sequence-specific DNA binding, steroid hormone receptor activity, transcription regulatory region DNA binding, transcription regulatory region sequence-specific DNA binding, ion binding, estrogen receptor activity, zinc ion binding and core promoter sequence-specific DNA binding (Figure 5b). Added to this are the presence of cellular function (Figure 5c), predicted ligand binding sites (Figure 5d) and predicted Enzyme commissions (Figure 5e).

Furthermore, it has revealed that the hybrid grouper regulated by estrogen receptor beta1 is also involved in many biological processes that includes, regulation of biological process, regulation of macromolecule metabolic process, regulation of gene expression, positive regulation of biological process, regulation of cellular process, positive regulation of gene expression, regulation of transcription, DNA-templated, positive regulation of cellular process, positive regulation of transcription, DNA-templated, cellular process, regulation of transcription from RNA polymerase II promoter, positive regulation of transcription from RNA polymerase II promoter, metabolic process, organic substance metabolic process, primary metabolic process, cellular metabolic process, organic substance biosynthetic process, organic cyclic compound metabolic process etc (Figure 6a). In addition, it has molecular functions which include, organic cyclic compound binding, signal transducer activity, transcription factor activity, sequence-specific DNA binding, receptor activity, signaling receptor activity, RNA polymerase II transcription factor activity, sequence-specific DNA binding, nucleic acid binding and DNA binding (Figure 6b). Other functions such as cellular function (Figure 6c), predicted enzyme commissions (Figure 6d) and predicted ligand binding sites (Figure 6e).

In a similar functions, the study revealed that the hybrid grouper regulated by estrogen receptor beta2 in many biological processes, including regulation of biological process, organic cyclic compound biosynthetic process, primary metabolic process, cellular process, regulation of gene expression, regulation of cellular process, positive regulation of biological process, positive regulation of gene expression, RNA biosynthetic process, regulation of cellular metabolic process, regulation of transcription, DNA-templated, positive regulation of transcription, DNA-templated, single-organism process, regulation of transcription from RNA polymerase II promoter, positive regulation of transcription from RNA polymerase II promoter, response to stimulus, transcription initiation from RNA polymerase II promoter, signal transduction, developmental process, anatomical structure development, single-organism cellular process etc (Figure 7a). Additionally, it has molecular functions which includes, organic cyclic compound binding, signal transducer activity, heterocyclic compound binding, ion binding, metal ion binding, transition metal ion binding, transcription factor activity, sequence-specific DNA binding, receptor activity, signaling receptor activity, nucleic acid binding, RNA polymerase II transcription factor activity, sequence-specific DNA binding, DNA binding, steroid hormone receptor activity, transcription factor activity, direct ligand regulated sequence-specific DNA binding, zinc ion binding, and RNA polymerase II transcription factor activity, ligand-activated sequence-specific DNA binding (Figure 7b). Other functions such as cellular function (Figure 7c), predicted enzyme commissions (Figure 7d) and predicted ligand binding sites (Figure 7e).

DISCUSSION

Estrogen receptors play very important traditional roles in the development of the reproductive system in vertebrates mostly in gonads and testis. Understanding and pinpointing the dissemination of ERs in hybrid grouper will help in understanding the possible other roles of estrogen in hybrid grouper development. Consequently, to our knowledge and understanding, there has been no study of the expression or the role of the ER in juvenile hybrid grouper a fish that has not developed gonads or testis yet. Estrogen is known traditionally to have a multiplicity of physiological functions and is tangled in regulating vertebrate metabolism, reproduction, cell proliferation, differentiation and inflammation through cellular machinery (estrogen receptors) required to warrant that estrogen executes these functions. Reproduction “activities in vertebrates, such as gonadal differentiation, maturation of the female reproductive tract, and procreative behaviors” are all associated with estrogen (Moore et al., 2005; Iguchi et al., 2001; McLachlan, 2001). In vertebrates, “estrogens seem to persuade both genomic and non-genomic cellular actions through the nuclear and perhaps G-coupled membrane receptors” (Moore et al., 2005; Iguchi et al., 2001; McLachlan, 2001). In 1990, the rainbow trout ER full-length sequence was reported in fish (Katsu et al., 2010c; Bjornstrom and Sjoberg, 2005). Ever since several other sequences have been recounted for teleost fishes, and three different types of ERs have been sequestered to date in a teleost (Katsu et al., 2010c; Hawkins et al., 2000).

In this study, full-length cDNA “sequences of distinctive ERα, ERβ1, and ERβ2” were cloned (Hu et al., 2018) from the juvenile hybrid grouper and characterized using PCR and the position of expressed ERs in the various tissues were examined. Hence, the prospective role of ERs in juvenile hybrid grouper development can be implicit. Upon aligning the amino acid sequence of the gene and sequences from different fish species, it has been found that the cDNA sequence of hybrid grouper estrogen receptors and their deduced amino acid sequence replicated a high degree of homology with the ERs homologs recognized from other animals and also the ERα and ERβ1 of juvenile hybrid grouper consist of

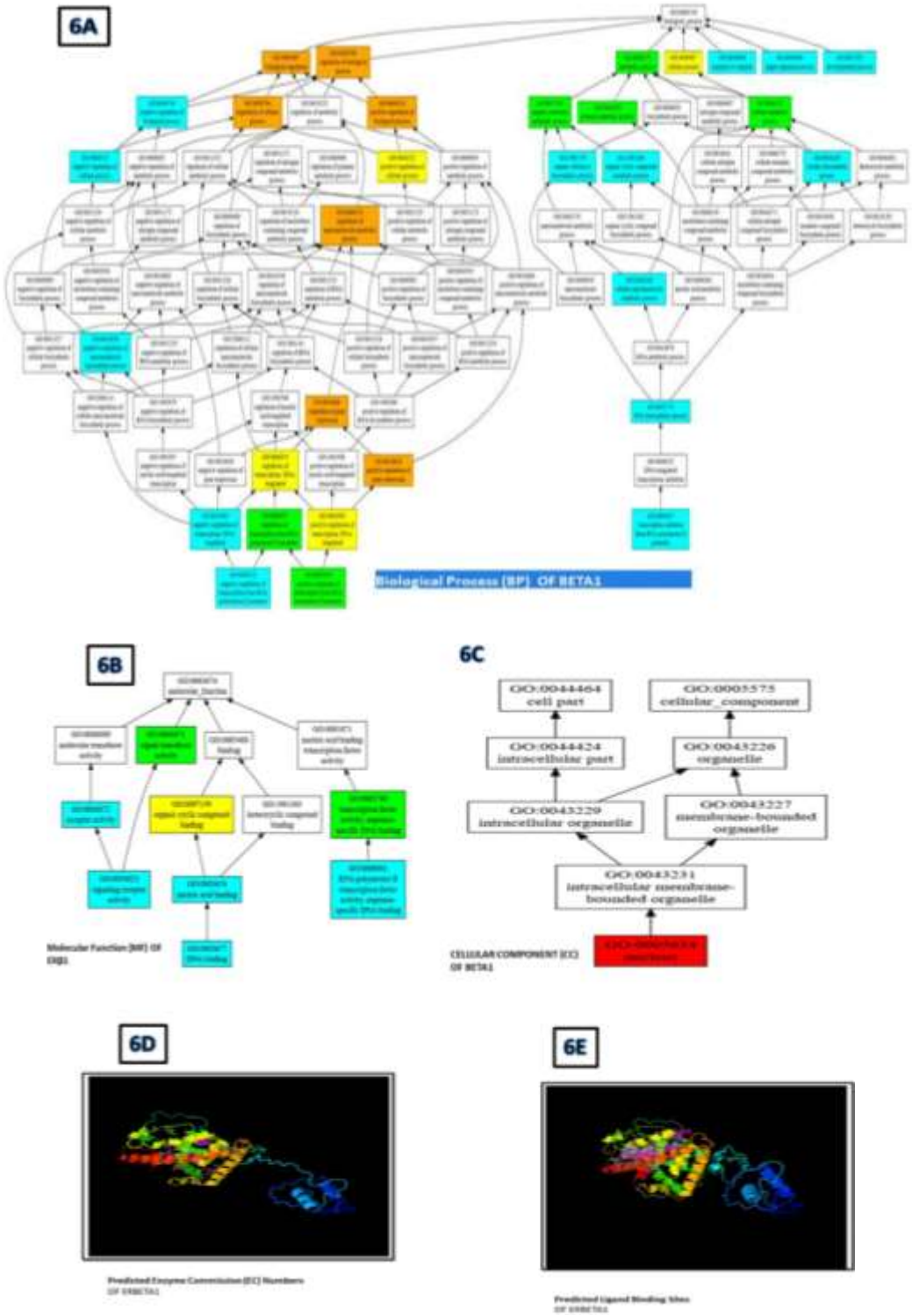


Figure 6a, b, c, d and e. The ERβ1's biological processes, molecular functions and cellular component.

well-known A/B, C, D, E and F molecular domains (Figure 2). This is an indication that this newly isolated cDNA encoded the hybrid grouper ERs protein.

Compared to other teleost fish, the A/B domain of the juvenile hybrid grouper ERα, and ERβ1, the C and E domains were less conserved (Ding et al., 2016). It was

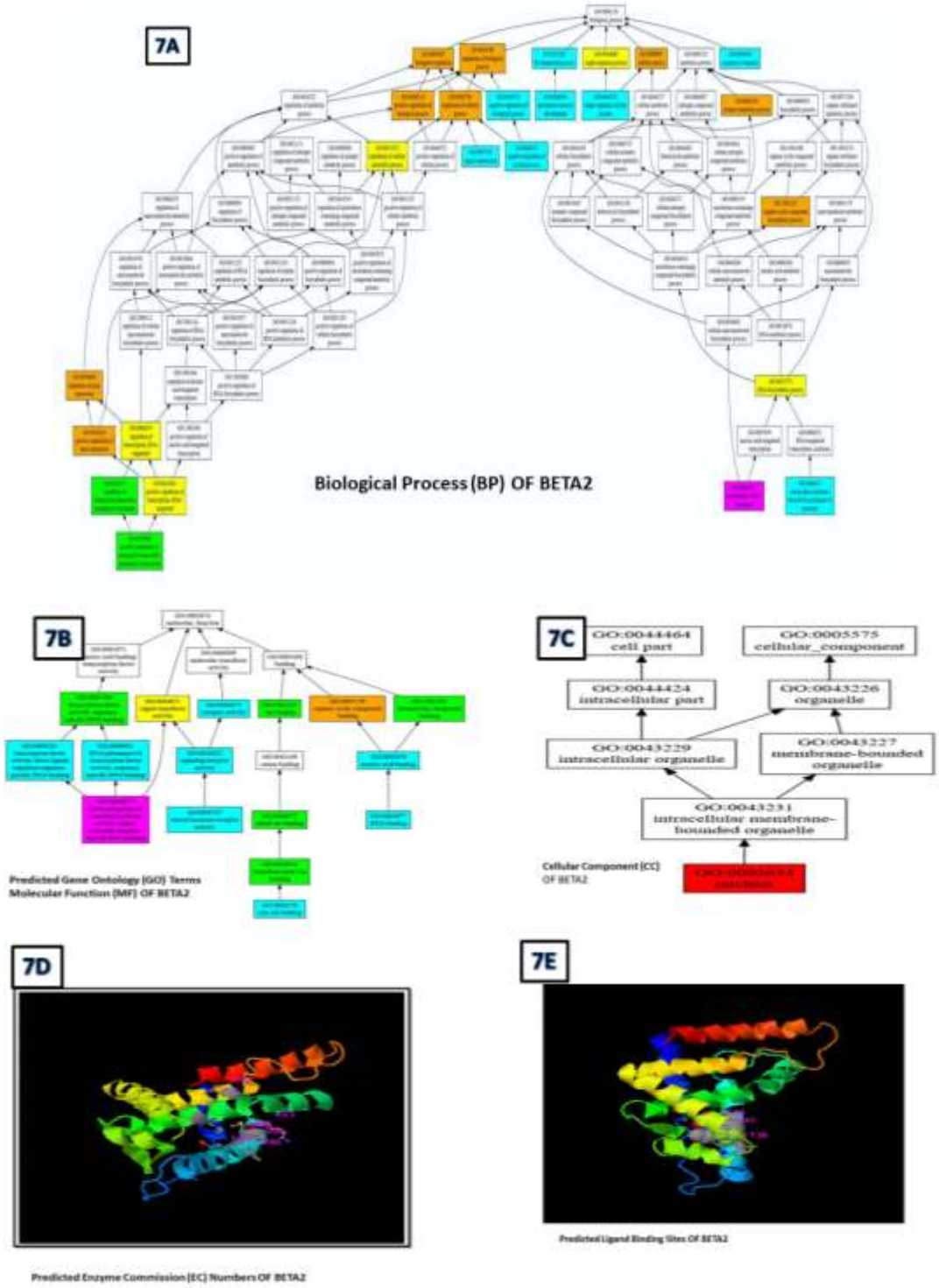


Figure 7a, b, c, d and e. The ERβ1's biological processes, molecular functions and cellular component.

also noted that the "phosphorylation sites known by mitogen-activated protein kinase (MAPK) were existent in the A/B domain of ERα and ERβ1", an indication that ERα and ERβ1 may influence the activation of MAPK pathway (Kato et al., 1995; Ding et al., 2016). It has also been noted that the AF-2 Activation domain (DLLLEML) occurred between amino acid residues 535 and 549 of the LBD domain, indicating that transcriptional activity was dependent on ligand binding (Ding et al., 2016; Kumar et al., 1987).

The hybrid grouper ERs genes shared high ranks of protein distinctiveness between the "DNA-binding domains" and contained the conserved motifs and elements believed to be essential for specific nuclear localization and command for target genes (Cui et al., 2017; Hall et al., 2002). There was also the reasonable uniqueness in the area called E/F domains or ligand-binding domains of the estrogen receptors which may be accountable "in part for the ligand-specificity and the different" answers to estrogen (Cui et al., 2017; Danielian et al., 1992; Kumar et al., 1987). The "ligand-binding domain", the AF-2 which is the "estrogen-dependent activation domains" were also observed to be conserved, indicating the similarity with other fishes, including *Scatophagus argus*, *E. coioides,* and *S. schlegelii* (Cui et al., 2017; Chen et al., 2011; Kim et al., 2003). Accepted functional sites of the protein for *hgER*s exhibited consistency with *ERs*. It is observed that the following domains "cysteine residues for two zinc fingers, P-box, D-box, and a cAMP site in the DBD domain, an AF-2 site, and a PKC phosphorylation site in the LBD domain" are conserved in *hgER*s (Figure 2). According to Mu et al. (2013), P- and D-boxes are shown to be crucial for DNA-binding. The importance of "PKC sites in all *ER*s" publicized by Härd and Gustafsson (1993) in which the initiation of PKC noticeably improves *"ER*-mediated transcriptional activation in a ligand-dependent manner" (Fu et al., 2008). The recognized A/B domain which contains the MAPK phosphorylation site was also observed to be conserved in the *ER*α subtype in sequence and position, as noted by others (Kato et al., 1995; Cho and Katzenellenbogen, 1993). Socorro et al. (2000) reported that the MAPK pathway could influence the "ligand-independent transcriptional activity of ER in both mammalian ERα and ERβ" (Fu et al., 2008).

While increasing literature exists on a phylogeny for numerous vertebrate steroid receptors (Howarth et al., 2008; Bury and Strum, 2007; Pakdel et al., 1990), scarce literature is available on hybrid species. Our study enhances essential material in this catalog. The analysis of sequences showed that two ERs protein sequences made up of the unique domain structures for NR superfamily while the hgERβ2 demonstrated the difference (Hu et al., 2006) slightly. A cautious examination of the phylogenetic tree discovered that this is in agreement with the case for ERs. At least "three sub-clusters of ERs were found in juvenile hybrid grouper even though maximally two ERs subtypes were isolated in many species" (Wang et al., 2005). The two ER-b subforms were reported in species of teleosts, such as the "Atlantic croaker, the Nile tilapia, and fugu, though not distributed in the same two sub-clusters as did the two ER-bs from zebrafish and goldfish" (Wang et al., 2005). An indication that "at least one of the two ER-b subtypes in the tilapia, Atlantic croaker, and fugu, tilapia-fugu ER-b2 clade has a diverse source from those of the zebrafish and goldfish, zebrafish ER-b1 clade" (Wang et al., 2005; Robinson-Rechavi et al., 2001b). It is possible that two consecutive lineage-specific replications might have transpired independently. Together they "took place after the divergence of teleosts". It is possible "the former took place only in zebrafish lineage, and the latter" transpired in the other teleosts deprived of the zebrafish lineage. The findings of the present study backed the proposition that most replicas of "fish genes arose more recently than the divergence of major fish groups" (Wang et al., 2005; Robinson-Rechavi et al., 2001b).

Examination of the tissue distribution of ERα, ERβ1, and ERβ2 offers comprehension into the prospective for targeting specific tissues. In the present research, we examined the expression configuration of ERs that is ERα, ERβ1 and ERβ2 mRNA in diverse tissues of juvenile hybrid grouper. The study revealed that ERs was expressed in all tissues of juvenile hybrid grouper examined. In goldfish, gilthead seabream and gilthead seabream the estrogen receptors were found to express mainly in gonads, but the overall expression in heart, liver, stomach, muscle intestine and head kidney showed largely corresponding expression patterns in hybrid grouper (Kato et al., 1995), the profiles of hgERs expression are similar to previously reported in tissue samples that were studied for all *ERs* in sea bass, with similar echelons among tissues (Lannigan, 2003) indicating possible similar function in hybrid grouper. The analysis of tissue expression in various tissue samples discovered all the three estrogen receptors expressed widely in hybrid grouper tissues which are in agreement with results from other studies (Cui et al., 2017; Cheng et al., 2015; Chen et al., 2011; Filby and Tyler, 2005; Halm et al., 2004; Kim et al., 2003) with the highest expression level in the heart contrary to study in goldfish which reported that ERα mRNA expression level was highest in the pituitary gland (Choi and Habibi, 2003). The expression of three estrogen receptors at differential expression configurations in tissues indicates that they might have diverse physiological roles. The hybrid grouper ERs gene was found to be highest in the heart but significantly lower in the kidney. Studies on *S. aurata* reported that ERα was expressed mainly in the liver and pituitary gland (Ding et al., 2016; Pinto et al., 2006), in partial agreement with the present study, whilst *Oreochromis mykiss* ERα and ERβ1 was found to expressed highest in the lever (Nagler et al., 2007). Altogether, the three estrogen receptors expressed highly in the heart, muscle, and liver, which suggests all of them, may be involved in growth and reproduction regulation in hybrid grouper (*Epinephelus fuscoguttatus* ♀× *Epinephelus polyphekadion* ♂). The ERs expression levels were comparatively high in the heart of ERα, ERβ1, and ERβ2. Even though ERs expressed in the liver, and the manifestation levels were low, this is a contradiction to other studies. This finding was contrary to the expression patterns of ERs in goldfish, *S. aurata*, and *O. mykiss*. The reason for these occurrences is not readily

known and further study is necessary to elucidate the implications.

Conclusions

This study established the actuality of three estrogen receptors in juvenile hybrid grouper and demonstrated that ER-alpha, ER-beta1 and ER-beta2 are expressed throughout all tissues which implies that estrogen through these receptors may be responsible for the regulations of physiological and pathological functions in Hybrid grouper. The copious expression of hybrid grouper ERs advocates a broad expression pattern as in mammalian ERs. These results put forward that steroid hormone estrogen receptors might be playing a significant part in the controlling of social behavior complexity, plasticity behavior, and the assessment of a gratifying inducement in Hybrid grouper. Based on this study, further study is necessary to elucidate the effect of ERs in developmental stages.

There is also need for research of the spatial configurations of ER-transcript expression in adult hybrid grouper tissues and also further dichotomization of the part estrogen receptors might be playing in regulating the incredible malleability of social behavior within hybrid grouper.

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CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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