Partial Sequence Characterization of Insulin-like Growth Factor-I (IGF-I) Gene in Mekong Giant Catfish (*Pangasianodon gigas*) and Striped Catfish (*Pangasianodon hypophthalmus*)

ลักษณะของลำดับนิวคลีโอไทด์บางส่วนของยีนอินซูลินไลค์โกรทแฟคเตอร์วัน (IGF-I) ในปลาบึก (*Pangasianodon gigas*) และ ปลาสวาย (*Pangasianodon hypophthalmus*)

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บทคัดย่อ: ยีนอินซูลินไลค์โกรทแฟคเตอร์วัน (IGF-I) มีบทบาทสำคัญในพัฒนาการและการเจริญเติบโตของสัตว์หลายชนิด รวมทั้งในปลา โดยยีน IGF-I อาจเป็นส่วนหนึ่งของลักษณะการเจริญเติบโตของปลาบึก (*Pangasianodon gigas*) ซึ่งเป็นปลาน้ำจืดที่ใกล้สูญพันธุ์ขนาดใหญ่ที่สุดและปลาสวาย (*Pangasianodon hypophthalmus*) ที่เป็นปลาที่มีความสำคัญทางเศรษฐกิจ แต่อย่างไรก็ตามยังไม่พบว่ามีข้อมูลของยีน IGF-I ในปลาทั้ง 2 ชนิดนี้ปรากฏอยู่ ดังนั้นการศึกษาในครั้งนี้จึงมีวัตถุประสงค์เพื่อหาลำดับนิวคลีโอไทด์บางส่วนของยีน IGF-I และเปรียบเทียบข้อมูลที่ได้กับปลาและสิ่งมีชีวิตชนิดต่าง ๆ โดยไพรเมอร์ถูกออกแบบ จากบริเวณอนุรักษ์ของยีน IGF-I ในปลาชนิดที่ใกล้เคียงกันคือปลากดอเมริกา (*Ictalurus punctatus*) ตัวอย่างดีเอ็นเอจากปลาบึกและปลาสวายถูกเพิ่มปริมาณ โคลน และหาลำดับนิวคลีโอไทด์ ผลพบว่ามีขนาดชิ้นส่วนดีเอ็นเอเท่ากับ 146 bp เมื่อเปรียบเทียบลำดับนิวคลีโอไทด์ของปลาทั้ง 2 ชนิด พบว่าลำดับนิวคลีโอไทด์ของยีน IGF-I มีความเหมือนกันเท่ากับ 99.3 เปอร์เซ็นต์ และยังพบจุดซิงเกิลนิวคลีโอไทด์โพลีมอร์ฟิซึม (SNPs) ที่ตำแหน่ง g.60C > T นอกจากนี้เมื่อวิเคราะห์การจัดกลุ่มโดยใช้วิธี Neighbour Joining method พบว่าสามารถจัดกลุ่มปลาหนัง (ปลาบึก ปลาสวายและปลากดอเมริกา) ออกจากปลาชนิดอื่นๆ ด้วยเปอร์เซ็นต์ความเหมือนเท่ากับ 24.4 เปอร์เซ็นต์ ดังนั้นการศึกษาในครั้งนี้เป็นครั้งแรกที่นำเสนอลำดับนิวคลีโอไทด์บางส่วนของยีน IGF-I ในปลาบึกและปลาสวาย ซึ่งผลการศึกษาดังกล่าวสามารถช่วยให้ข้อมูลเรื่องความสัมพันธ์ บทบาทหน้าที่ทางพันธุกรรมของยีน IGF-I ในปลาบึกและปลาสวายในต่อไปในอนาคตได้

คำสำคัญ: อินซูลินไลค์โกรทแฟคเตอร์วัน ลักษณะทางพันธุกรรม ปลาบึก ปลาสวาย

Abstract: Insulin-like growth factor-I (IGF-I) plays an important role in development and growth of multiple species including fishes. IGF-I may also contribute to high growth traits of an endangered species and the largest fresh-water fish, the Mekong giant catfish (*Pangasianodon gigas*) as well as an economically important species, striped catfish (*Pangasianodon hypophthalmus*). Nevertheless, nucleotide sequences of IGF-I in these two species has not been available. Thus, the goals of this research are to study sequence isolation and comparative sequencing of IGF-I of the other species. Degenerate primers were designed from a highly conserved region of a closely related species, channel catfish (*Ictalurus punctatus*). A fragment of 146 bp was amplified, cloned and sequenced from *P. gigas* and *P. hypophthalmus* DNA samples. A sequence comparison showed 99.3% of sequence similarity, and comparative sequencing identified the single nucleotide polymorphisms (SNPs) at g.60C > T position between *P. gigas* and *P. hypophthalmus*. The Neighbour Joining clustering analyses grouping the catfish *(P. gigas*, *P. hypophthalmus* and *I. punctatus*) with 24.4% of sequence similarity from consensus sequence of other species. This is the first report on nucleotide sequence of the *P. gigas* and *P. hypophthalmus* IGF-I gene. The results provide essential information for sequence relationship study and further understanding functional roles of IGF-I in Mekong giant catfish and striped catfish.

Keywords: Insulin-like growth factor-I, molecular characterization, *Pangasianodon gigas*, *P. hypophthalmus*

Introduction

Insulin-like growth factor-I (IGF-I) is a peptide hormone structurally similar to proinsulin and plays an important role in mammalian growth and development (Daughaday and Rotwein, 1989). In teleost IGF-I is primarily expressed in liver, but it is also produced in variety of tissues (Maures *et al*., 2002). Moreover, IGF-I also has a role in regulating postnatal growth in mammals and fish (Florini *et al*., 1996; Duan, 1997; Reinecke and Collet, 1998; Moriyama *et al*., 2000). The production of IGF-I depends on growth hormone and other endocrine factors, such as thyroid hormone (Schmid *et al*., 2003) and/or estrogen (Riley *et al*., 2004). In several fish species, blood and tissue IGF-I levels positively correlate with dietary ration, protein content, and body growth rate (Beckham *et al*., 2004; Duan, 1998; Pérez-Sánchez *et al*., 1995). The blood IGF-I also increase during the growing season (Mingarro *et al*., 2002), increase temperature (Beckham *et al*., 1998), day length (McCormick *et al*., 2000), metabolism (Castillo *et al*., 2004), development (Greene and Chen, 1999; Pozios *et al*., 2001), reproduction (Maestro *et al*., 1997), and osmoregulation in seawater (McCormick, 2001).

The highly conserved IGF system is generally composed of the ligands IGF-I, IGF-II (Reinecke and Collet, 1998) and IGF-III (Wang *et al*., 2008). Mature IGF-I peptides share high levels of amino acid sequence similarity between vertebrates (Duan, 1997). Not only sequence similarity of amino acids, but also the gene expression and function have been demonstrated to be conserved between fishes and mammals (Duan, 1997) possibly via evolutionarily conserved polypeptides (Duan, 1997; 1998). Although the nucleotide sequence of IGF-I gene are reported in Genbank databases for several fish such as channel catfish (*Ictalurus punctatus*) (Clay *et al*., 2005), *Oreochromis niloticus* (Wang *et al*., 2008), *Danio rerio* (See *et al*., 2014), the information is limited for Pangasius catfish species.

The Pangasius catfishes such as Mekong giant catfish (*P. gigas*) and striped catfish (*P. hypophthalmus*) are ecologically and economically important freshwater catfish in Thailand. The Mekong giant catfish is the largest freshwater fish which could reach a weight up to 300 kilograms (Roberts and Vidthayanon, 1991; Zanden *et al*., 2004). Despite their ecological and economic importance, genetic information is still limited. Only the complete mitochondrial DNA sequence (Jondeung *et al*., 2007) and growth hormone-encoding cDNA (Lemaire *et al*., 1994) of the Mekong giant catfish have been reported. IGF-I has gained our attentions since it may functionally contribute to the high growth potential and performance of the Mekong giant catfish based on large body size and weight. Therefore, in this study we aimed to identify DNA sequence and relationship of IGF-I in *P. gigas* and *P. hypophthalmus* using comparative sequencing approach and degenerate primers designed from the most conserved exon 3 and 4 of *I. punctatus* (Clay *et al*., 2005; Peterson *et al*., 2005).

Materials and Methods

Fish samples

A total of 40 individual catfish of *P. gigas* (n = 20) and *P. hypophthalmus* (n = 20) were obtained from Chiangrai and Chiangmai Inland Fisheries Research and Development Center, Department of Fisheries, Thailand.

DNA Isolation

Genomic DNA was extracted from fin samples using phenol-chloroform extraction procedure slightly modified from the protocol described by Harris *et al*. (1991). The samples were minced and digested at 37 °C overnight in 700 µL of TNES-urea buffer (1mM Tris-HCl pH 8.0, 6 M NaCl, 0.5M EDTA pH 8.0, 10% sodium dodecyl sulphate, and 4M urea) containing 4 µL of proteinase K solution (20 µg/mL) (Invitrogen, USA). An equal volume of phenol-chloroform (1:1 v/v) was then added and mixed until an emulsion formed. Separation of the DNA contained aqueous phase was achieved by centrifugation at 10,000 rpm for 10 min. The phenol-chloroform extraction step was repeated twice. DNA was precipitated with 1/10 of the volume of sodium acetate (3.0 M, pH 5.2) and an equal volume of isopropanol, washed twice with 70% ethanol and followed by air dry. Finally, the DNA was dissolved in a Tris-EDTA buffer and stored at -20 °C. The DNA concentration was determined using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific Inc., USA) and assessed for integrity on 1% agarose-gel electrophoresis.

Cloning and Sequencing

A sequence alignment of IGF-I across multiple fishes showed a highly conserved region corresponding to exon 3-4 of the channel catfish. Degenerate primers were designed from the channel catfish (*Ictalurus punctatus*) sequence (DQ 088971). The primer sequences are 5´-TTGCACAACCGTGGCATCG-3´ (forward primer) and 5´-TTTGGTGTTTTGGGCGTGTC-3´ (reverse primer). Each of eight DNA pools was generated from five individual of *P. gigas* or *P. hypophthalmus* (total n=40) for a comparative sequencing experiment. A PCR fragment was amplified using a touchdown PCR condition: 94 °C for 3 min, followed by 12x cycles of [94 °C for 30 s; 60 - 52 °C for 30 s (minus 0.5 °C for each cycle); 72 °C for 1 min] and 20x cycles of [94 °C for 30 s; 52 °C for 30 s; 72 °C for 1 min], and final extension of 72 °C for 5 min. The PCR product were purified and ligated into the

pGEM®-T vector (Promega, USA). The recombinant DNA was transformed into competent cells (DH5α strain) and the blue-white screening method was used to identify a positive clone harboring the insert DNA fragments according to the manufacturer's protocol. Plasmid DNA was extracted from bacteria culture using GenElute™ Plasmid Miniprep Kit (Qiagen) for sequence analysis. Nucleotide sequence was determined using CEQ 8000 Genetic Analysis System (Beckman Coulter) using Dye Terminator Cycle Sequencing (GenomeLab™ DTCS) Quick Start Kit (Beckman Coulter). The similarity search was performed using basic local alignment search tool namely BLAST at the NCBI webpage. The search analysis blast the query sequence against all sequences deposited across the database. The phylogenetic tree was generated using Neighbor-Joining method with 1000 bootstrap trials (Saitou and Nei, 1987) implemented in the MEGA 4.0.2. The sequence similarity was evaluated by CLUSTAL W software in BioEdit Sequence Alignment Editor.

Results

Partial sequence of *P. gigas* and *P. hypophthalmus* IGF-I

The degenerate primers designed from channel catfish IGF-I corresponding to exon 3 and 4 were able to amplify a PCR fragment in all *P. gigas* and *P. hypophthalmus* DNA samples (Figure 1). The fragment length was 146 bp which was the same size as in channel catfish. The sequence alignment of *P. gigas* and *P. hypophthalmus* IGF-I showed 99.3% similarity which 145 out of 146 bp were identified. Comparing between a consensus sequence of Mekong giant catfish, striped catfish and the reference of channel catfish IGF-I showed 98% similarity (144/146 bp) among these three species. A sequence comparison of IGF-I cloned from different DNA pools of *P. gigas* and *P. hypophthalmus* showed sequence difference at the position g.60C > T. Two clones derived from the DNA pool number 2 and 4 of *P. gigas* showed T allele, while the other two clones from DNA pool

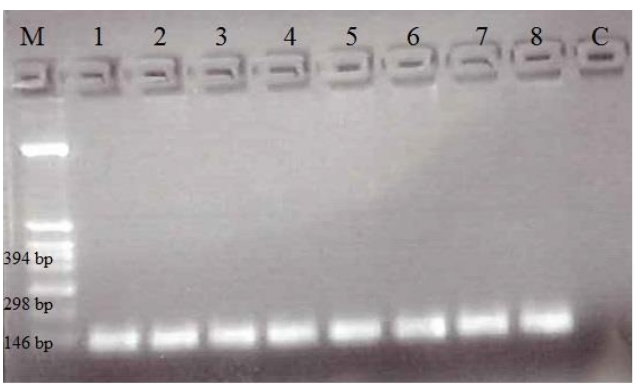

Figure 1 PCR amplification of IGF-I in pooled DNA of *P. gigas* (lane 1-4) and *P. hypophthalmus* (lane 5-8). Lane M represents for 100 bp markers and lane C for water negative control

1 and 3 of the same species revealed C allele indicating a heterozygosity of the locus in *P. gigas*. Amino acid prediction suggested that the single nucleotide polymorphisms (SNPs) is non-sense, both alleles code for Leucine. In *P. hypophthalmus*, all four clones sequenced identified only the C allele suggesting a non-polymorphic likelihood of the locus in striped catfish. However, genotyping in a large population is warranted. In addition, comparing a consensus sequence of *P. gigas* and *P. hypophthalmus* IGF-I with that of the *I. punctatus* identified two sequence variations at g.75A > G and g.78A > G positions (Figure 2). These two SNPs were synonymous substitutions and did not change the amino acid prediction for Alanine and Proline.

Phylogenetic analysis

Base on the genetic distances calculated with the Poisson correction model, a phylogenetic tree was constructed by the Neighbour-Joining method to investigate the phylogenetic relationships of the IGF-I gene in vertebrate species (Figure 3). The results showed that these species were separated into two groups, one is compose of the catfish together with other teleost species. While another group was composed of mammals, bird and teleost. In the branches of the *P. gigas* and *P. hypophthalmus* from consensus sequence of other species, however they showed closely related with *Ictalurus punctatus*, suggesting that this genomic region is highly conserved of IGF-I sequence between the catfish species. Comparing this consensus sequence with other species using CLUSTAL W alignments between *P. hypophthalmus* and other species also showed a wide range of sequence similarity including *Ictalurus punctatus* (24.4%), *Danio rerio* (7.6%), *Oreochromis niloticus* (18.9%), *Ctenpharyngodon idella* (14.1%), *Elopichthys bambusa* (13.4%), *Cyprinus carpio* (8.1%), *Procypris rabaudi* (19.6%), *Carassius auratus* (13%), *Larimichthys crocea* (5.1%), *Oncorhynchus mykiss* (12.7), *Gallus gallus* (13%), *Homo sapiens* (1.3%), *Bos taurus* (11.8%), *Sus scrofa* (17.8%) shown in Table 1.

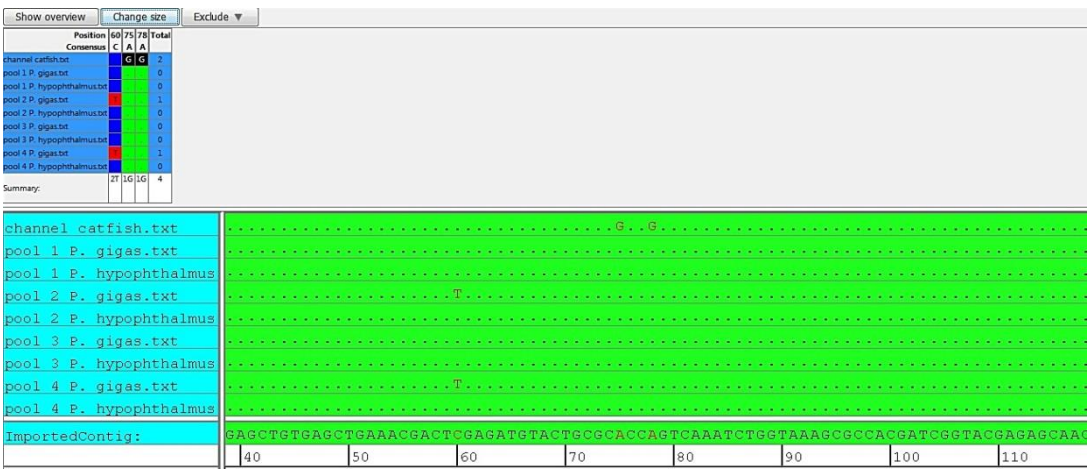

Figure 2 Sequence alignment and single nucleotide variation of a 146 bp fragment of IGF-I using BioEdit sequence alignment program. Pooled DNA sequencing in *P. gigas* and *P. hypophthalmus* showed the g.60C > T SNP. Comparing with the channel catfish (*Ictalurus punctatus*) reference identified two SNPs, g.75A > G and g.78A > G

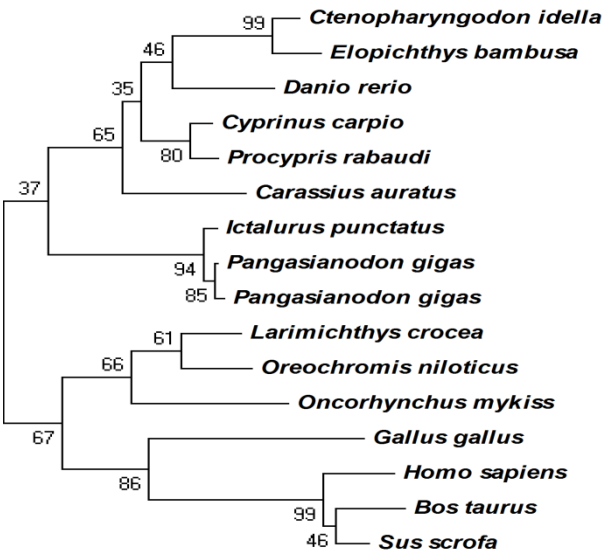

Figure 3 Phylogenetic tree of IGF-I gene between *Pangasianodon gigas, P. hypophthalmus, Ictalurus punctatus* (DQ 088971), *Danio rerio* (BC114262), *Oreochromis niloticus* (NM_001279503), *Ctenopharyngodon idella* (EU051323), *Elopichthys bambusa* (JF712623), *Cyprinus carpio* (D83271), *Procypris rabaudi* (EU787399), *Carassius auratus* (GU583648), *Larimichthys crocea* (NM_001303334), *Oncorhynchus mykiss* (NM_001124696), *Gallus gallus* (NM_001004384), *Homo sapiens* (NM_000618), *Bos taurus* (NM_001077828), *Sus scrofa* (NM_214256) using Neighbor-Joining method with 1000 bootstrap procedure

Discussion

In this study, the conserved sequences were characterized and phylogenetic tree analyses. The partial sequences of IGF-I of *P. gigas* and *P. hypophthalmus* were isolated and sequenced. The results suggested that this genomic region is highly conserved between *P. gigas*, *P. hypophthalmus* and *I. punctatus* (Figure 3). The comparison of sequence similarity between *I. punctatus*, *P. gigas* and *P. hypophthalmus* was 24.4% as shown Table 1. In addition, the phylogenetic tree was constructed, the comparison with other vertebrate clearly separated of two distinct groups. It has been shown that the catfish IGF-I gene was quite different from other fishes, avian, and mammalian species (Clay *et al*., 2005). The genome evolution and biodiversity in teleost fish showed that catfish closely related with *Danio rerio* (Volff, 2005), however, our partial sequence of IGF-I gene in *P. gigas*, *P. hypophthalmus* similarity with *Danio reio* only 7.5%. Whereas, *I. punctatus* showed high closely related with *Danio rerio* (32.6%) (Table 1). The low of sequence similarity percentage between Pangasius catfish and *Danio reio*, it might be from the short sequences (146 bp). The high potential will appear if we found the full length of this gene. In the conserve amino peptide of IGF-I, it was found that 87% amino acid identity of walking catfish (*Clarius macrocephalus*) when compare with Black seabream (*Acanthopagrus*

Table 1 The genetic similarity of IGF-I gene between catfish, teleost, mammals and bird species

species	*P.hypophthalmus*	*B. taurus*	*C. auratus*	*C. Idellus*	*C. carpio*	*D. rerio*	*E.bambusa*	*G.gallus*	*H. sapiens*	*I.punctatus*	*L. crocca*	*O. mykiss*	*O.niloticus*	*P. gigas*	*P. rabaudi*	*S.scrofa*
P.hypophthalmus	1.000															
B. taurus	0.118	1.000														
C. auratus	0.134	0.334	1.000													
C. Idellus	0.141	0.48	0.543	1.000												
C. carpio	0.081	0.212	0.309	0.336	1.000											
D. rerio	0.076	0.274	0.295	0.488	0.525	1.000										
E.bambusa	0.134	0.479	0.529	0.954	0.343	0.495	1.000									
G. gallus	0.131	0.693	0.355	0.463	0.175	0.247	0.451	1.000								
H.sapiens	0.013	0.106	0.039	0.059	0.078	0.086	0.059	0.086	1.000							
I. punctatus	0.244	0.37	0.354	0.543	0.243	0.326	0.553	0.347	0.055	1.000						
L. crocca	0.051	0.162	0.163	0.189	0.318	0.287	0.191	0.144	0.100	0.158	1.000					
O. mykiss	0.127	0.437	0.389	0.586	0.246	0.372	0.59	0.392	0.060	0.417	0.272	1.000				
O. niloticus	0.189	0.359	0.580	0.437	0.244	0.240	0.42	0.353	0.042	0.307	0.250	0.497	1.000			
P. gigas	0.993	0.118	0.133	0.14	0.081	0.075	0.133	0.131	0.013	0.242	0.051	0.126	0.19	1.000		
P. rabaudi	0.196	0.404	0.741	0.696	0.397	0.362	0.676	0.353	0.046	0.408	0.188	0.42	0.588	0.196	1.000	
S. scrofa	0.178	0.622	0.422	0.374	0.216	0.201	0.358	0.432	0.07	0.275	0.153	0.32	0.462	0.178	0.512	1.000

Catfish = *Pangasianodon gigas, Pangasianodon hypophthalmus, Ictalurus punctatus.*

Teleost = *Danio rerio, Orcochromis niloticus, Ctenphanyngodon Idella, Eloplchthys bambusa, Cyprinus carpio, Procypris rabaudi, Carassius auratus, Larimichthys crocca, Orcochromis niloticus, Oncorhynchus mykiss.*

Mammals = *Homo sapiens, Bos taurus, Sus scrofa.*

Bird = *Gallus gallus*

schlegeli) (Chen *et al*., 1998). However, the high conserve sequences that produce only in the brain (McRory and Sherwood, 1994).

The polymorphism of IGF-I gene in *P. gigas* and *P. hypophthalmus*, a synonymous SNP was identified at the position g.60C > T in *P. gigas*. Although the SNP does not change the amino acid sequence and is unlikely to change functions of the protein, the SNP may be closely linked to yet uncharacterized causative SNPs. Therefore, the SNP may be useful as a valuable genetic marker for association study in a segregated population. The IGF-I gene information in Pangasius catfish are still limited. A polymorphic CT/GA microsatellite close to the end of intron 1 of IGF-I has been previously reported in catfish. Other characterization of a relevant growth performance gene, growth hormone (GH) in *P. hypophthalmus* suggests that the gene is highly conserved compared to vertebrates. Moreover, a high degree of similarity of the GH amino acid sequence between *P. gigas* and *I. punctatus* has been demonstrated (Poen, 2009). The GH in *P. hypophthalmus* and *P. gigas* are closely related based on the similarity of amino acid sequence. A substitution of asparagine and serine at the position 163 has been previously reported between *P. gigas* and *P. hypophthalmus* (Poen, 2009). In this study, partial sequences of the *P. gigas* and *P. hypophthalmus* IGF-I gene were high similarity, but nucleotide sequence was difference, it showed SNP at position g.60C > T (Figure 2). From this result, it might be cause of the variation on body weight in *P. gigas* and *P. hypophthalmus*. However, growth performance trait was depended on genetic background and environment.

For functional study of the gene, an expression of IGF-I has been reported in multiple fish species including embryos and larvae of sea bream (*Sparus aurata*) (Radaelli *et al*., 2003), rainbow trout (Perrot and Funkenstein, 1999), and common carp (*Cyprinus carpio*) (Tse *et al*., 2002). Interestingly, the IGF-I transcript has been detected as early as in unfertilized eggs in development stage of seabream (Fukenstein *et al*., 1996) and rainbow trout (Greene and Chen, 1997). However, it is only detected after hatching and during embryogenesis in rabbitfish (*Siganus guttatus*) (Ayson *et al*., 2002). A study of the IGF-I gene in Chilean flounder (*Paralichthys adspersus*) has been suggested a high degree of conservation of the protein family during vertebrate evolution (Elies *et al*., 1999; Nakao *et al*., 2002). In catfish, IGF-I mRNA expression on channel catfish is expressed in liver higher than in muscle, brain, heart, and kidney (Clay *et al*., 2005). However, it is few reported about IGF-I gene expression between *P. gigas* and *P. hypophthalmus*. Therefore, a future functional study should be start with liver tissue, and validate the relationship is promising.

Conclusions

Using degenerate primers designed from highly conserved region of *I. punctatus* IGF-I, partial genomic DNA sequence (146 bp) of the *P. gigas* and *P. hypophthalmus* IGF-I were cloned and sequenced. A sequence comparison showed 99.3% of similarity. The Neighbour Joining clustering analyses separated the *P. gigas* and *P. hypophthalmus* from consensus sequence of other species; however, they showed closely related with *I. punctatus* (24%). A comparative sequencing identified a SNPs at g.60C > T position between *P. gigas* and *P. hypophthalmus*.

From this result, the variation of growth performance may cause by this SNPs. Comparing the isolated sequence and the *I. punctatus* reference showed two synonymous substitution, g.75A > G and g.78A > G suggesting a high conservation, at least at this genomic location between these species. This is the first report on the partial sequences of the *P. gigas* and *P. hypophthalmus* IGF-I gene and identified SNPs. The further study may be used as a genetic marker for a growth trait-association in segregating populations. And the relationship of gene expression between difference species need to validation.

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