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# Cloning, SequenceCharacterizationandPhylogenetic Analysis on Full-LengthcDNA of GrowthHormonefromSouthernFlounder (*Paralichthyslethostigma*)

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**Abstract**

The full-length cDNA encoding growth hormone (PLGH) of Southern flounder (*Paralichthyslethostigma*) was cloned by Switching Mechanism At 5' end of the RNA Transcript (SMART) RACE. The sequence was 902 nucleotides long, coding a polypeptide of 190 amino acids and a signal peptide of 17 amino acids. The 5' and 3' UTR of the messenger RNA were 131 and 198 nucleotides long respectively. After alignment of the deduced amino acid sequence with other Pleuronectiformes species, a consecutive absence of 14 amino acids segment in the C-terminal was found in all three species ofParalichthyidae. It can be proved that GHs of Paralichthyidae have the shortest length in Pleuronectiformes species. Homology analysis of PLGH cDNA showed the highest score with *Paralichthysorbignyanu*and the lowest score with *Soleasenegalensis.* The phylogenetic analysis on complete GH ORF sequences was performed with the maximum parsimony method. In the topology founded, the species fell into three groups: Soleoidei,Paralichthyidae and Pleuronectidae. The exclusive species ofSoleoidei selected in this work,*Soleasenegalensis* formed an independent branch and showed relatively high mean difference with Paralichthyidae and Pleuronectidae. The results of phylogenetic analysis based on the GH ORF sequences were consistent with the earlier results based on 12S, 16S rDNA and mitochondrial DNA sequences within Pleuronectiformes species, which would suggest the potential credible application of GH ORF sequences in Pleuronectiformes phylogeny.

*Keywords*: Southern flounder (*Paralichthyslethostigma*), growth hormone, cDNA, sequence characterization, phylogenetic analysis.

**GüneyPisiBalığının (*Paralichthyslethostigma*) BüyümeHormonundakicDNA (Tamamlayıcı DNA) ÜzerindeKlonlama, SekansTanımlanmasıveFilogenetikAnalizler**

**Özet**

Pisibalığının(*Paralichthyslethostigma*) DNA'sından sentezlenen (SMART) RACE RNA'nın 5' ucunda büyüme hormonundan (PLGH) kodlanan cDNA, anahtar mekanizması ile klonlanmıştır. Sekans, 190 amino asitli polipeptid kodlayan 902 nükleotidli, 17 amino asitli tek bir peptidtir. Messenger RNA'nın dönüşmeyen bölgesinin (UTR; untranslatedregion) 5' ve 3' uçları sırasıyla 131 ve 198 nükleotidlidir. Çıkarılabilir amino asit sekanslarının diğer Pleuronectiformes türleri ile birlikte sıralanmasından sonra, Paralichthyidae'nın üç türünün tümünde de C ile sonlanan ardışık 14 amino asit segmentinin olmadığı bulundu. Pleuronectiformes türleri arasında Paralichthyidae'nın büyüme hormonunun (GH) en kısa boya sahip olduğu kanıtlanabilir. Benzer analizler, PLGH'nincDNA'sının*Paralichthysorbignyanu*'da en yüksek skora, *Soleasenegalensis*'de en düşük skora sahip olduğunu göstermiştir. Büyüme hormonunun ORF (açık okuma çerçevesi) sekansının tamamında filogenetik analizler maksimum sadeleştirilmiş metot kullanılarak yapılmıştır. Topolojide türler üç gruba ayrılır: Soleoidei,Paralichthyidae ve Pleuronectidae. Bu çalışmada Soleoidei'nin en seçkin türü seçilmiş, *Soleasenegalensis*, bağımsız bir sınıf oluşturmuş ve Paralichthyidae ve Pleuronectidae'dan nispeten yüksek bir ortalama fark göstermiştir. Büyüme hormonunun (GH) ORF sekansına dayalı filogenetik analizlerin sonuçları, Pleuronectiformesfilogenide büyüme hormonunun (GH) ORF sekansındaki güvenilir potansiyel uygulamaları öneren Pleuronectiformes türlerinin 12S, 16S rDNA ve mitokondrial DNA sekanslarının daha önceki sonuçları ile örtüşmektedir.

*AnahtarKelimeler*: Güney pisi balığı (*Paralichthyslethostigma*), büyüme hormonu, cDNA, sekans karakterizasyonu, filogenetik analizler.

## Introduction

Southern flounder, *Paralichthyslethostigma*, a representative member of the Paralichthyidae family, is found in rivers and estuaries along the Atlantic Coast from North Carolina to Northern Florida, and from Tampa Bay, Florida along the Gulf coast into Southern Texas (Benetti, 2000), occurring from

freshwater-tidal riverine systems to full strength seawater (Wenner*et al*., 1990). Owing to its nutritionally rich (low contents of fat and calories, high protein and vitamin contents), delicious flavor, the adaptability of salinities in wide range and high market price, Southern flounder has been cultured widely in many countries. Southern flounder was introduced into China in 2001, after a few years' domestication, now it has been an important economical species of Chinese fishery.

Growth hormone (GH) is a single-chain polypeptide secreted by the anterior pituitary gland in vertebrates. Systemic administration of GH induces positive nitrogen balance and stimulates protein synthesis in muscle and longitudinal bone growth (Forsyth and Wallis, 2002). GH plays a role in several important physiological functions, including promotion, regulation of somatic growth and sexual maturation in fishes (Cavari*et al*., 1993; McLean*et al*., 1993; Tsai*et al*., 1994). The recombined GH was produced through gene engineering and had showed the function of accelerating growth rate in fishes (Acosta *et al*., 2008; Agelon*et al*., 1998; Li *et al*., 2003; Zang*et al*., 2007). Besides of application of recombined GH in aquaculture, coding gene and cDNA of GH have been cloned, sequenced and characterized from several species of teleosts, and the GH gene might serve as a potential natural marker to clarify theevolutionary relationships of various teleost groups(Almuly*et al*., 2000; Bernardi*et al*., 1993; Byrappa and Sydney, 1997; Chen *et al*., 2004; Marins,2003; Rubin and Dores, 1994, 1995; Rubin *et al*., 1996). Although flatfishes in the order Pleuronectiformes and families have been investigated by phylogeneticists (Saitoh*et al*., 2000; Suzuki *et al*. 2002), to our knowledge only a few representatives were compared simultaneously by using molecular techniques based on DNA which could help to elucidate some controversial aspects of flatfish systematic. So, analysis of growth hormone, including cDNA and DNA sequences, have potential ability to be applied to the establishment of phylogenetic relations between flatfishes belonging to different families. The aim of this work was to clone the GH cDNA of the Southern flounder, *Paralichthyslethostigma*, an introduced species of flatfish,and analyze relationships of species belonging to Pleuronectiformes. The results of the molecular analysis have been evaluated in relation to others previously published and them will help to clarify evolution of flatfish GH genes. The cDNA sequence of GH we obtained will also help to study the GH gene expression pattern through out the development of Southern flounder, the similar work had been reported in Milkfish (de Jesus, 2002), and the recombinant growth hormone produced by transgenetic technology will have potential application in aquaculture in the future.

## Materials and Methods

### Pituitary Collection and Total Rna Isolation

Three 6-month old Southern flounder*Paralichthyslethostigma* were collected from the culture field in Qingdao, China and temporarily sustained in the laboratory. After the live specimens were anesthetized and killed rapidly, pituitary glands were immediately isolated. Total RNA was extracted using Trizol Total RNA Extraction Kit (Sangon, China). RNA integrity was verified by ethidium bromide staining of 28s and 18s ribosomal bands on a denaturing agarose gel.

### Cdna Library Construction

The cDNA library of Pituitary was carried out using SMART cDNA Library Construction Kit (ClonTech, USA). The ds-cDNA was examined on an agarose/EtBr gel under UV lamp and a smear band of total ds-cDNAs appeared from 0.5 kb to 2 kb. According to the user manual of the kit, the quality of the constructed cDNA library was satisfiable for cloning.

### PCR Primer Design

Three antisense primers including one degenerate primer and 3 sense primers for 5'-RACE and 3'-RACE were designed based on the conserved sequences of GH gene of other flounders (Genbank accession No: AF086787, AB079553, DQ112550, X15055). The positions of all primers on cDNA sequence were shown in Figure 1. The sequences of the primers were listed as follows:

Ping3 (5'-CGGGATCCATGCAGCCAATCACACAGAGAACC-3'),
Ping4 (5'-ATAAGAATGCGGCCGCCTACAGGGTGCAGTTAGC-3'),
Gen1 (5'-GTTCA(A,G)(C,T)A(C,T)CT(G,C,T)CACCTG-3'),
Gen2 (5'-CA(C,T)TTGGC(G,C)ACGGTCAG-3'),
GSP1 (5'-CACGAGACACAAGGCAGCTCAGTTC-3'),
and GSP2 (5'-CAGCCTCATGAGTCCCGTCTTCAGTTC-3').

### Hemi-Nested Polymerase Chain Reaction (Hemi-Nested PCR) and Rapid Amplification of the cDNA Ends (RACE)

After the cDNA library of pituitary was constructed, the designed specific primers were applied into three rounds PCR to clone the full-length GH cDNA. After the first round of PCR, two rounds of Hemi-nested PCR were performed for amplification of 5'end and 3'end. Before each round, the ds-cDNA sample or PCR sample was diluted 1:1000 in ddH2O for amplification and 1 µl of the diluted sample was used as template in each Hemi-nested PCR reaction.

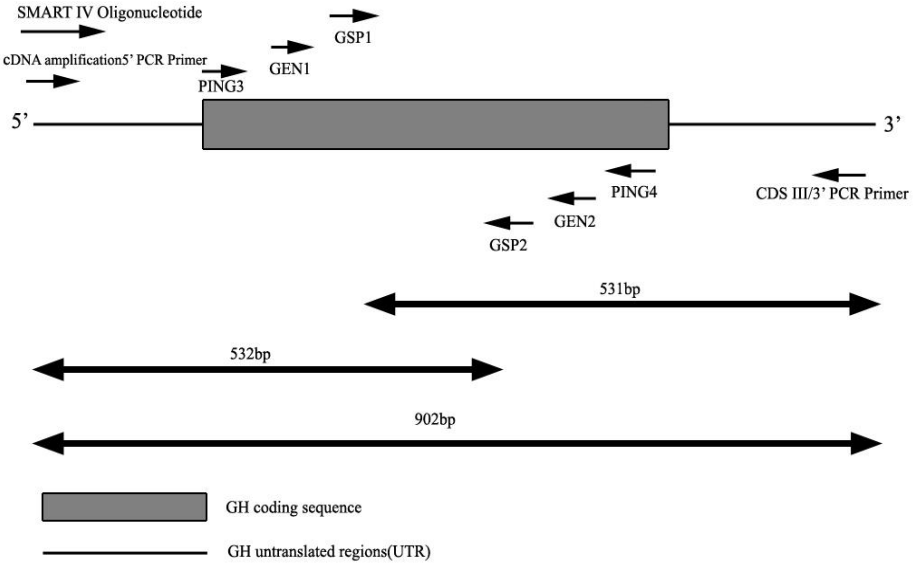


**Figure 1.** The position of primers applied in 5'- and 3'-RACE and 423 cDNA fragments amplified by PCR were marked. The arrows indicated the amplification direction of primers in the PCR.

**Amplification of cDNA 5' Ends**

In the first round of PCR, 1 µl of the diluted ds-cDNA sample and 2*Pfu PCR MasterMix (TianWei, China) were used. The PCR was carried out in a reaction volume of 20 µl for 5 min at 94°Cfor initial denaturing, followed by 30 cycles of 94°C for 1 min, 52°Cfor 1 min, and 72C for 1 min, and a final extension at 72°Cfor5 min. The primer sequences used for the amplification were cDNA amplification 5' PCR Primer (The SMART cDNA Library Construction Kit) and Ping4. The second PCR was undertaken using cDNA amplification 5' PCR Primer and an antisense specific primer Gen2 with the first PCR diluted products as the template, the programwas as follows: 94°C for 5 min and 30 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min, followed by a final extension at 72 for 5 min. In the third round, the combination of primers were the SMART IV Oligonucleotide (The SMART cDNA Library Construction Kit) and GSP2, the program was as follows: 94°C for 5 min and 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 5 min. After each round of PCR, the PCR product was analyzed by electrophoresis and UV light.

**Amplification of cDNA3' Ends**

Like the procedure of amplification of cDNA 5' end, one round of PCR and two rounds of Hemi-nested PCR were also applied to amplification of cDNA 3' ends. In the first round, 1 µl of the diluted ds-cDNA sample and 2*Pfu PCR MasterMix were added into a reaction volume of 20 µl. The program was as follows: 94°C 5min for initial denaturing, 30 cycles of 94°C for 1 min, 52°Cfor 1 min, and 72°C for 1 min, and a final extension at 72°C for 5 min. The primers were CDS III/3' PCR Primer (The SMART cDNA Library Construction Kit) and Ping3. In the second round, 1 µl diluted PCR sample of first reaction and 2*Pfu PCR MasterMix were used, the combination of primers were CDS III/3' PCR Primer and Gen1, the program was as follows: 94°C for 5 min and 30 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min, final extension at 72°C for 5min. In the third round, 1 µl diluted PCR sample of second reaction and 2*Pfu PCR MasterMix were used, the combination of primers were the CDS III/3' PCR Primer and GSP1, the program was as follows: 94°C for 5 min and 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, final extension at 72°C for 5 min. The 3' PCR product was analyzed by electrophoresis and UV light like amplification of cDNA 5' ends. After identification of the PCR products under UV, the amplified DNA bands were excised from the agarose gel and purified using AxyPrep DNA Gel Extraction Kit (Axygen, USA). The purified 5'- and 3'-RACE products of PLGH cDNA were cloned into pMD18-T vector (Takara, Dalian, China) and were then transformed into E. coli DH5α. Five positive clones of 5'-RACE and five positive clones of 3'-RACE were selected randomly and were sequenced using ABI 3730 sequencer.

**Sequence Analysis**

The full-length of PLGH cDNA sequence was obtained by comparing the same overlapping of 5'- and 3'-RACE products . Homology analysis was used to confirm specificity of the sequence by Mega Blast Search (www.ncbi.nlm.nih.gov/BLAST/mmtrace.shtml). The deduced amino acid sequence was respectively

analyzed using DNASTAR 5.0 (DNASTAR Inc., Madison, WI, USA).

### Phylogenetic Analysis

The complete GH ORF sequences were aligned with ClustalX 1.8 using the Gonet 250 similarity matrix with a gap opening penalty of 10.0 and a gap extension penalty of 0.1 for the pairwise alignment stage, and a gap opening penalty of 10.0 and a gap extension penalty of 0.2 for the multiple alignment strategy. Phylogenetic analysis was performed with PAUP software (version 4.0 b10)(Swofford, 2000) which had been applied in teleost growth hormone phylogeny (Rubin and Dores, 1995) and characterization analysis of growth hormonecDNAs from the tetraploid smallmouth buffalofish (Clements, 2004) and a prion protein cDNA from the gilthead sea bream (Favre-Krey, 2007). Species of Pleuronectiformes used for comparison and their Genbank accession numbers were as follows: *Paralichthyslethostigma*(southern flounder) DQ990918, *Paralichthysolivaceus*(Japanese flounder) CAA33155, *Paralichthysorbignyanus* AAZ16489, *Hippoglossushippoglossus*(Atlantic halibut) BAC07253, *Soleasenegalensis* (sole) AAA60372, *Veraspervariegatus*(spotted halibut) AAC36716 and *Veraspermoseri* (Puniaet al., 2000). *Micropterussalmoides* (largemouth bass),*Pagrus major* (red tail),and *Percaflavescen*(yellow perch) were collected as outgroup species,GenBank database accession numbers were as follows: ABG57074, CAA30033 and AAG09621. The cladogram was constructed by maximum parsimony algorithm. The MP analyses were performed using the heuristic search option with 100 random stepwise addition sequence replicates and 500 bootstraps to assign confidence levels to the nodes in the trees.

## Results

### Structure of GH cDNA Sequence from ParalichthysLethostigma(PLGH)

Before the third round of hemi-nested PCR of 5'-RACE, the band of PCR product on 1.0% agarose gel was a smear ranging from 200bp to 700bp. The same thing occurred in the 3'-RACE. After three rounds of PCR (including two rounds of hemi-nested PCR), 532 bp and 531 bpcDNA fragments were amplified by 5'- and 3'-RACE respectively using similar approaches (Figure. 1). The full-length cDNA of PLGH was obtained by overlapping the two cDNA fragments. Homologous analysis of the PLGH cDNA was performed by Mega Blast Search in GenBank database. The result of mega blast revealed that the PLGH cDNA had high homology with other GH cDNA sequences of Pleuronectiformes such as *Paralichthysolivaceous* (94.38%), *Verasper variegates* (92.52%), *Hippoglossushippoglossu* (92.92%) and *Paralichthysorbignyanus* (93.20%). After homologous analysis, the cDNA sequence of PLGH was deposited to the NCBI GenBank and the obtained accession number was DQ990918.

The complete sequence and the deduced amino acid sequence were presented in Figure 2. The length of the cDNA sequence was 902 bp, including a 131 bp fragment of 5'UTR and a 198 bp fragment of 3'UTR. The start codon ATG was located at positions 131 and the termination codon TAA was at positions 702. A potential polyadenylation signal AATAAA was at positions 862 and a poly A tail was from position 884 to 902. The open reading frame (ORF) was 570 bp long, beginning at position 132 and ending at position 701. The calculated molecular mass of the corresponding protein was 21.65 kD. Based on the deduced polypeptide sequence, the open reading frame of GH cDNA was found to be composed of 190 amino acids, including a 17 amino acids signal peptide sequence in the N-terminal region of the polypeptide chain (Figure. 2). The signal peptide sequence was predicted by alignment GH cDNA sequences with other flounder fishes (Pendonet al., 1994, Puniaet al., 2000;Watahikiet al., 1989, 1992). The signal peptide sequence of PLGH showed significant similarity to other species ofPleuronectiformes in GenBank database. It was completely identical with the signal peptide sequences of GH of Japanese flounder (*Paralichthysolivaceous*) and *Veraspervariegatu*s. Within the mature protein sequence of PLGH, 4 Cys residues (at position of 69, 163, 180 and 188) were identified (Figure. 2), which was different from GHs of Cyprinidae containing 5 Cys residues. For widely conserved residues in vertebrate GHs, 4 Cys residues formed two disulfide bond linkages (Somers *et al*., 1994). Only one potential N-glycosylation site (Asn-Cys-Thr) in C-region was identified in the PLGH amino acid sequence (Figure. 2), which was also different from GHs of Cyprinidae containing two N-glycosylation site (Asn-Glu-Ser and Asn-Cys-Thr) (Figure 2).

Comparison of GHs from seven Pleuronectiformes species was performed by ClustalX1.81 and DNASTAR 5.0 software. A high homologous region in the C-terminal was found in all aligned sequences A consecutive 14 amino acids segment in the C-terminal was found absent in GH of *Paralichthyslethostigma*, *Paralichthysorbignyanus* and*Paralichthysolivaceous* (Figure 3). 5 Cys residues were in GHs of *Veraspervariegatus*and*Veraspermoseri*and4 Cys residues were in other five GHs(Figure. 3). The same potential N-glycosylation site (Asn-Cys-Thr) in C-region was identified in all GH protein sequences of seven Pleuronectiformes species(Figure 3). PLGH showed the highest score of homology as 97.6% with*Paralichthysorbignyanus*and94.8% with *Paralichthysolivaceous*, followed by 83.4%, 86.7% and 86.7% identity with *Hippoglossushippoglossu*, *Veraspervariegatus*and*Veraspermoseri*respectively,

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gacactgaagaactgaaccagaaccagaccaagaacctgaacttgaacctgaaccat  57
caccagaacctgagcctgaacctgaacctgaacctgaacctgaacctgaactagaat 114
ctgaaccagagccagccATGAACAGAGTCATCCTTCTGCTGTCAGTCATGTGTGTGG 171
                   M  N  R  V  I  L  L  L  S  V  M  C  V     13
GCGTGTCCTCTCAGCCAATCACAGAGAACCAGCGCCTGTTCTCTATCGCTGTTGGTC 228
G  V  S  S  Q  P  I  T  E  N  Q  R  L  F  S  I  A  V  G     32
GAGTTCAGTATCTTCACCTGGTTGCTAAGAAACTCTTCAGTGACTTTGAGAACTCTC 285
R  V  Q  Y  L  H  L  V  A  K  K  L  F  S  D  F  E  N  S     51
TACAGTTGGAGGATCAACGTCAACTCAACAAAATCTTTTTAAAAGATTTTTGTCATT 342
L  Q  L  E  D  Q  R  Q  L  N  K  I  F  L  K  D  F  C  H     70
CAGATTATTTCTTGAGTCCAATCGACAAACACGAGACACAAGGCAGCTCAGTTCAAA 399
S  D  Y  F  L  S  P  I  D  K  H  E  T  Q  G  S  S  V  Q     89
AGCTTTTATCGATCTCTTATCGATTGATTGAGTCCTGGGAGTTTTCGAGTCGCTTCC 456
K  L  L  S  I  S  Y  R  L  I  E  S  W  E  F  S  S  R  F    108
TGGTTGCAAGTTTTGCTGTAAGGACCCAGGTTACATCCAAACTGTTAGAACTGAAGA 513
L  V  A  S  F  A  V  R  T  Q  V  T  S  K  L  L  E  L  K    127
CGGGTCTCATGAAGCTGATAGAGGCCAATCAGGATGGAGCAGGTGGATTCTCTGAGA 570
T  G  L  M  K  L  I  E  A  N  Q  D  G  A  G  G  F  S  E    146
GTTCGGTGCTCCAGCTCACGCCGTACGGAAATTACGAACTGTTTGCCTGCTTTAAGA 627
S  S  V  L  Q  L  T  P  Y  G  N  Y  E  L  F  A  C  F  K    165
AGGATATGCACAAGGTGGAGACATACCTGACCGTGGCCAAATGCCGACTCTTTCCAG 684
K  D  M  H  K  V  E  T  Y  L  T  V  A  K  C  R  L  F  P    184
AAGCTAACTGCACCCTGTAAccccacctctccgccaagaagtacctccccgcagatg 741
E  A  N  C  T  L  *                                        190
ccatcatatgcattctgtagccccctgtggttgccaaatctgctaactagcattaat 798
gttagcatctgttggttctgcattccaaacttatgatgtcattgtgatgtcacactg 855
tcagcaataaagagttcattcagttttcaaaaaaaaaaaaaaaaaa            902
```

**Figure 2.** Nucleotide sequence and deduced amino-acid sequence of the PLGH cDNA. The coding nucleic acid sequence was shown in upper case while the non-coding sequence was shown in lower case. Nucleotides were numbered from the first base at the 5' end. The start codon (ATG), the stop codon (TAA) were single-underlined and the polyadenylation signal (AATAAA) was doubly underlined. The predicted N-terminal signal peptide was denoted by a dashed underline. The 4Cys and the potential N-glycosylation site (Asn-Cys-Thr) in C-region were marked with rectangular boxes.

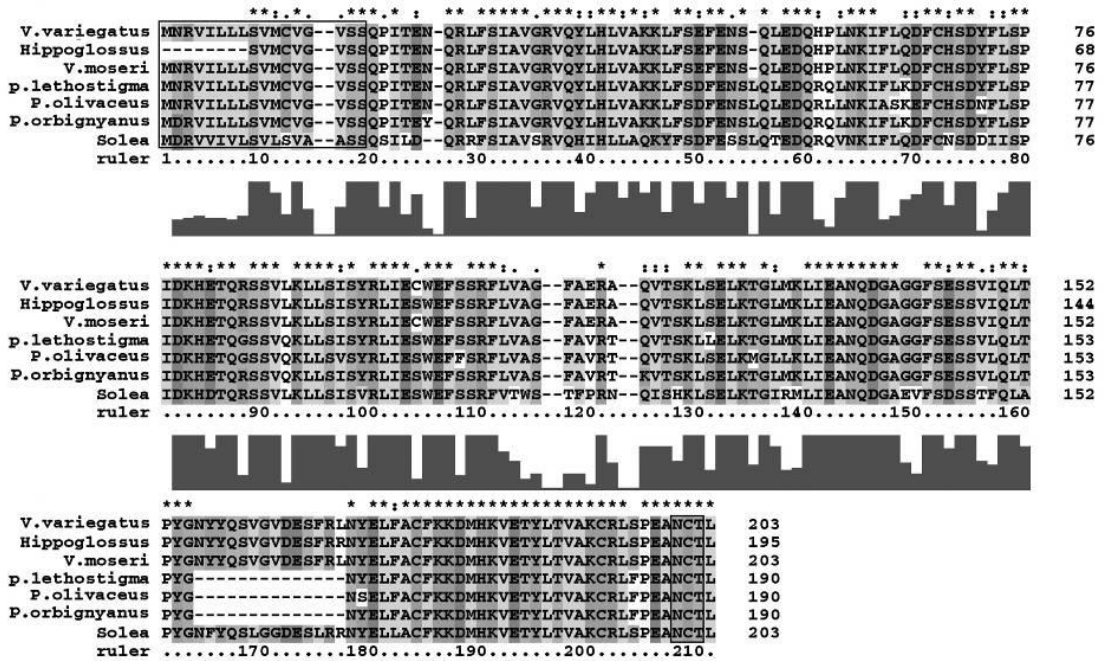


**Figure 3.** The alignment of the GH deduced amino acid sequence. The same potential N-glycosylation site (Asn-Cys-Thr) in C-region and the 17 amino acids signal peptide sequences in the N-terminal region were marked with rectangular boxes.A consecutive 14 amino acids segment in the C-terminal was identified absent in *Paralichthyslethostigma, Paralichthysorbignyanus*and *Paralichthysolivaceous*.

and only showed 68.2% identity with*Soleasenegalensis* (Figure 4). As a whole, GHs from Pleuronectiformes showed close distance with each other except GH from *Soleasenegalensis*, which showed the highest divergence and the lowest identity with other Pleuronectiformes species. In the residue substitution analysis, the highest frequency was 5 times which appeared respectively in the directional substitutions: from Ile (I) to Leu (L), Asp (D) to Glu (E) and Leu (L) to Val (V) (Figure 5). The above five residues showed high activity in evolution of GHs in Pleuronectiformes, which should be associated with functional region of GH (Figures 3,4 and 5).

**Phylogenetic Analysis**

In this study,*Micropterussalmoides*,*Pagrus major* and *Percaflavescen* as species from Perciformswere selected as outgroup based on the conclusion by Bernardi*et al.*(1993). In the topology of the MP tree, Pleuronectidae, Paralichthyidae, and Soleidae were separately grouped which was supported by high bootstrap values. Three genera ofPleuronectidae formed a monophyletic group with high value (>95%). *Veraspervariegatus* and *Veraspermoseri* showed much closer evolutionary relationship than with *Hippoglossushippoglossu* (Figure 6). InParalichthyidae, the analysis showed that closer evolutionary relationships was between *Paralichthyslethostigmas* and*Paralichthysorbignyanus*(distance value was 0.02632), whereas*Paralichthysolivaceous* was solely divergent from those two. *Soleasenegalensis* formed an independent cluster and showed far distance (mean values ranging from 0.27 to 0.31) related to Pleuronectidaeand Paralichthyidae (Figure 6, Figure 7). Besides Pleuronectiformes species, we chose some other representativespecies as outgroups to clarify the evolutional position of Pleuronectiformes. The outgroup species (*Micropterussalmoides*,*Pagrus major* and *Percaflavescen*) were selected from Perciforms families and formed one group with very high bootstrap value (100%) (Figure 6).

Percent Identity

| Divergence | 1 | 2 | 3 | 4 | 5 | 6 | 7 | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | 94.8 | 97.6 | 86.7 | 86.7 | 83.4 | 68.2 | 1 | P.lethostigma |
| 2 | 6.0 | | 93.4 | 82.9 | 82.9 | 79.6 | 64.9 | 2 | P.olivaceus |
| 3 | 2.7 | 7.8 | | 86.3 | 86.3 | 83.4 | 69.2 | 3 | P.orbignyanus |
| 4 | 7.2 | 12.1 | 7.8 | | 100.0 | 95.3 | 72.0 | 4 | V.variegatus |
| 5 | 7.2 | 12.1 | 7.8 | 0.0 | | 95.3 | 72.0 | 5 | V.moseri |
| 6 | 7.0 | 12.0 | 7.0 | 1.0 | 1.0 | | 71.1 | 6 | Hippoglossus |
| 7 | 34.3 | 40.3 | 32.6 | 35.4 | 35.4 | 32.4 | | 7 | Solea |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | | |

**Figure 4.** Homology analysis of the GH cDNA from *Paralichthyslethostigma, Paralichthysolivaceous, Verasper variegates, Hippoglossushippoglossu, Veraspermoseri, Paralichthysorbignyanus* and*Soleasenegalensis* (The identity and divergence of GH cDNAs were showed above-diagonal and below-diagonal respectively).

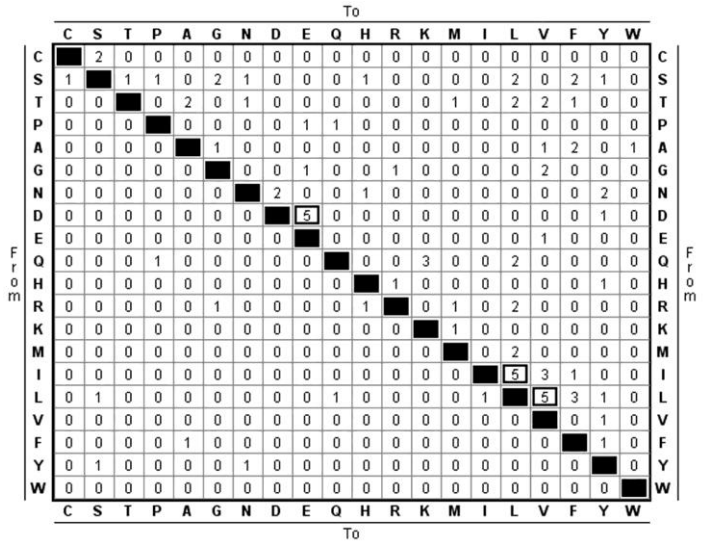


**Figure5.**The result of residue directional substitution. The highest frequency: 5 times was marked with rectangular boxes.

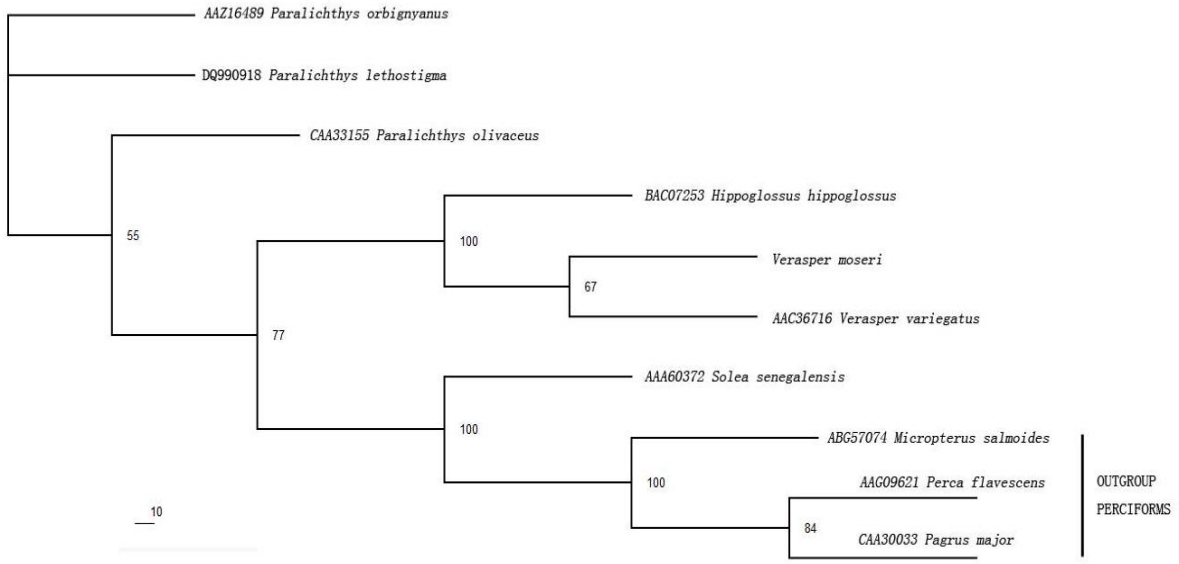


**Figure. 6.** Bootstrapped maximum parsimony dendrogram based on the complete GH ORF sequences of seven flatfish species. Numbers at the node of branches indicated bootstrap values for 500 replicates.

## Discussion

As an introduced species of flatfish, Southern flounder, *Paralichthyslethostigma* has some advantages just as the adaptability of salinities in wide range and fast growth compared to other flatfishes in China. In order to study the particularites of *Paralichthyslethostigma*in genetic or culture pattern, the growth hormone gene, PLGH was used toanalyze its relationship with other species of Pleuronectiformes. In the comparison of seven amino acid sequences among Pleuronectiformes, *Paralichthyslethostigma* showed significant similarity to species of Paralichthyidae (97.6% with*Paralichthysorbignyanus*and94.8% with *Paralichthysolivaceous*) and GH of *Soleasenegalensis* showed far distance with other six species of Pleuronectiformes. All aligned seven GH amino acid sequences from Pleuronectiformes were homologous highly at the C-terminal, which indicated high selective pressure on evolution of this functional region. The highly conserved residues of seven GH amino acid sequences were also revealed, which were probably essential for tertiary folding, hormone binding or hormone receptor interaction(Watahiki*et al*., 1989). The directional substitutions with the highest frequency were all happened between two amino acids of the same attribute, so these substitutions were all conservative, which would play more important roles in maintaining protein function than the stochastic substitutions. At the same time, some group-specificities were found, for example, 5 Cys residues was in GHs of *Veraspervariegatus*and*Veraspermoseri*; consecutive 14 amino acids segment was absent in GHs of three Paralichthyidae species. The absence of consecutive 14 amino acids was more probably a sign that GHs of Paralichthyidae have the shortest length in all Pleuronectiformes species. It seems reasonable to speculate that this deletion occurred in species of Paralichthyidae after the divergence of Pleuronectoidae, probably by a one-step mutation rather than one-by-one deletion.

The large proportion of informative sites and the considerable range of the sequence variations of the genetic distances, both suggested that GH ORF sequence might be suitable for reconstructing relationships of teleosts (Zhang *et al*., 2005). The result of this work was consistent with the earlier results based on 12S and 16S rDNA within Pleuronectiformes (Berendzen and Dimmick 2002; Pardo*et al*., 2005). Paralichthyidae , Pleuronectidae and Soleidae separated with each other, Paralichthyidae and Pleuronectidaehave closer relationship than with Soleidae, which according with the phylogenetic tree made by 5'mtDNAcontrol region fragment (Fausto*et al*., 1999). Similar to 5'mtDNA control region fragment, the GH ORF sequencesof Pleuronectoidaeformed a monophyletic group, whereas the species involved in the tree were different. Difference of GH ORF sequences was minor among Pleuronectoidaespecies (mean character difference<0.011), which showed more conservative evolution than among Paralichthyidae species (mean differences from 0.027 to 0.074). In outgroup, species from Perciforms group into a single cluster with 100% bootstraps value, both of them showed great difference with other species of Pleuronectiformes (distance value ranging from 0.24 to 0.31), the result proved the selection of outgroup wasappropriate.

Through the phylogenetic analysis of GH ORF

sequences, the data and topology of MP tree suggested that GH ORF might be reliable in resolving phylogenetic relationships among Pleuronectiformes species and potential application for other teleost species. Similar to the conclusion of phylogenetic relationships seen among *Sole* species based on mitochondrial DNA sequences (Carlos *et al*., 2004; Infante*et al*., 2004), longer GH sequences, for example, complete genome DNA or intronic sequences, will help to clarifyambiguities and result in more accurate phylogenetic relationships.

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