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RESEARCH PAPER

Insulin-Like Androgenic Gland Hormone Gene in the Freshwater Chinese Mitten Crab *Eriocheir sinensis*：cDNA Cloning, Expression Pattern and Interaction with EsIGFBP7

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Abstract

Androgenic gland hormone has an insulin-like structure and it is the male-specific hormone found in crustaceans. Androgenic gland hormone controls male sexual differentiation and maintains male sexual characteristics. In this study, the full-length cDNA sequence of the androgenic gland hormone gene in *Eriocheir sinensis* (*EsIAG*) was cloned. The total length of the *EsIAG* cDNA was 1,495 bp, encoding 151 amino acids. EsIAG has the typical conserved structure of other reported IAGs but had low sequence similarity with other crustacean IAGs. *EsIAG* was expressed specifically in the androgenic gland. Insulin-like growth factor binding protein 7 from the Chinese mitten crab *E. sinensis* (*EsIGFBP7*) was highly expressed in the male sex-related organs among nine crab tissues. Interactions between EsIAG and EsIGFBP7 were verified using a yeast-two hybrid assay. These results obtained in this study provide basic information about the regulatory mechanism of male sex differentiation in *E. sinensis.*

Keywords: *Eriocheir sinensis*, insulin-like androgenic gland hormone, EsIGFBP7, male sexual differentiation.

Introduction

The androgenic gland (AG) is found specifically in crustaceans and plays an important role in controlling male sexual differentiation and maintaining male sexual characteristics. It was first found in blue crab in 1947 (Cronin, 1947), and its regulatory function of male sexuality was demonstrated a few years later in the amphipod *Orchestia gammarella* (Charniaux-Cotton, 1954). Since then, many experiments have been to verify that at a particular growth stage excision/transplantation of the androgenic gland causes sex reversal (Nagamine *et al.*, 1980; Katakura and Hasegawa, 1983; Suzuki and Yamasaki, 1991; Khalaila *et al.*, 2001).

Androgenic gland hormone (AGH) is the male-specific hormone found in crustaceans (Ventura *et al.*, 2011). In 1999, the AGH in the isopod, *Armadillidium vulgare*, was demonstrated to be a two peptide glycoprotein (Martin *et al.*, 1999). This AGH was characterized, and the cDNA was cloned, this is the first example in crustaceans (Okuno *et al.*, 1999). The structure of AGH is relatively similar to that of insulin or the so-called insulin-like androgenic gland factor (IAG) (Manor *et al.*, 2007). Since then, more than 20 different crustacean IAG genes have been cloned, such as: *Callinectes sapidus*, *Fenneropenaeus chinensis*, *Macrobrachium Nipponese*, *Sagmariasus verreauxi* and *Jasus edwardsii*, etc (Manor *et al.*, 2007; Chung *et al.*, 2011; Li *et al.*, 2012b; Ma *et al.*, 2013; Ventura *et al.*, 2014). Members of the insulin-like growth factor binding proteins (IGFBPs) can bind insulin/insulin-like growth factor, so it was speculated that an interaction between IAG and insulin-like growth factor binding protein (IGFBP) product had occurred (Manor *et al.*, 2007). In 2013, researchers discovered that IAG gene interact with IGFBP gene products in the crayfish, *Cherax quadricarinatus*, which provided new clues to study the function of the crustacean AG pathway (Rosen *et al.*, 2013).

Many decapod crustaceans are commercially important species, such as the freshwater Chinese mitten crab, *Eriocheir sinensis*. Because of its ability to adapt to the environment and high commercial profitability, *E. sinensis* has been widely cultured in most regions of China (Shen *et al.*, 2014). Male *E. sinensis* grow faster than females, although the females have a higher economic value than that of their males counterparts. Breeding of a unisexual crab will therefore be a major milestone in increasing the profitability of the crab industry. In this study, the full-length IAG gene was first cloned in *E. sinensis*, the *EsIAG* expression profile was investigated in different tissues, and its interaction with EsIGFBP7

was confirmed in a yeast two-hybrid assay. We believe this study will provide valuable information about the AG pathway of *E. sinensis* and thus provide a theoretical basis for unisexual aquaculture of *E. sinensis.*

Material and Methods

Ethical Statement

This study was approved by the Animal Care and Use Committee of the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences.

Animal Collection

Adult Chinese mitten crabs (weight, 100–200 g) were collected from a farm located in Yandu District, Yancheng City, Jiangsu Province, China. Crabs were reared in water tanks at 20 ℃ under a natural photoperiod and fed a commercial crab diet once a day. The androgenic gland was collected from ten male crab and all collected samples were immediately frozen in liquid nitrogen and then stored at –80ºC until use.

Preparation of Total RNA and cDNA Synthesis

Total RNA from various tissues of Chinese mitten crabs was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Total RNA was treated with RNase-free DNase I (Qiagen) to avoid contamination with genomic DNA.The integrity and quality of the extracted RNA was evaluated by 1.5% agarose gel electrophoresis. Total RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNA was synthesized from 2 µg total RNA using Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA), following the manufacturer's protocol. The cDNA was used as a template in subsequent ORF cloning and polymerase chain reaction (PCR) analyses.

Sequence Cloning of EsIAG and EsIGFBP7

One candidate sequence of 1,080 bp was identified as having high similarity with the IAG gene from other crustaceans using BLAST software from high-seq data of the androgenic gland in our lab. Based on this sequence, gene-specific 3' and 5' rapid amplification of cDNA ends (RACE) primers were designed. cDNA templates for 3' and 5' RACE were prepared from total RNA from the *E. sinensis* androgenic gland, using a 3'-Full RACE Core Set Ver. 2.0 Kit and a 5'-Full RACE kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Two rounds of PCR were performed to obtain 3' and 5' end fragments of *EsIAG*. The final PCR products were cloned into the pEASY-T1 vector (Transgen Biotech, Beijing, China) and used to transform *E. coli* TOP 10 competent cells. Positive clones were isolated and sequenced. The PCR program was as follows: denaturation at 94ºC for 3 min; 35 cycles of 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 1 min; and 72ºC for 10 min. All primers used are listed in Table 1.

A candidate sequence that was deduced to include the entire IGFBP7 coding sequence with high similarity to other crustac ean IGFBPs was identified using BLAST software in high-seq data of the *E. sinensis* AG from our lab. The sequence was submitted to the GenBank database (Accession no. KU724193). Based on these two sequences, specific primers were designed to amplify the ORF of IAG and IGFBP7 using the high fidelity Taq enzyme (Toyobo, Osaka, Japan), and the products were cloned into the pEASY-BLUNT Zero plasmid (Transgen Biotech) and verified by sequencing.

Sequence Alignments and Phylogenetic Analysis

The deduced amino acid sequences of *E. sinensis* IAG were aligned with the five known IAGs of other crustacean species, derived from the NCBI GenBank database: SpIAG from *Scylla paramamosain* (accession number: AIF30295.1), CqIAG from *C. quadricarinatus* (ABH07705.1*), PmIAG from Penaeus monodon (ADA67878.1)*, SvIAG from *Sagmariasus verreauxi* (AHY99679.1) and *MnIAG from Macrobrachium nipponense* (AHA33389.1). A likelihood tree was constructed from multiple sequence alignments with EsIAG, and 20 other IAG protein sequences derived from the GenBank database using the molecular evolutionary genetics analysis (MEGA) software, version 5.0 (Tamura *et al.*, 2011). The names and the accession numbers of the IAG proteins used are as follows: FcIAG from *Fenneropenaeus chinensis* (accession number: AFU60547.1), LvIAG from *Litopenaeus vannamei* (AIR09497.1), MjIAG form *Marsupenaeus japonicas* (BAK20460.1), AvIAG from *Armadillidium vulgare* (BAA86893.1), PdIAG from *Porcellio dilatatus* (BAC57013.1), PsIAG from *Porcellio scaber* (BAC57012.1), JeIAG from *Jasus edwardsii* (AIM55892.1), PpIAG from *Portunus pelagicus* (ADK46885.1), CdIAG from *Cherax destructor* (ACD91988.1), CsIAG from *Callinectes sapidus* (AEI72263.1), PpacIAG from *Palaemon pacificus* (BAJ84109.1), PpauIAG from *Palaemon paucidens* (BAJ84108.1), MlIAG from *Macrobrachium lar* (BAJ78349.1), MrIAG from *Macrobrachium rosenbergii* (ACJ38227.1), and MvIAG from *Macrobrachium vollenhovenii* (AHZ34725.1).

Table 1. Nucleotide sequences of the primers for the *EsIAG and* Es*IGFBP7* cloning and expression analyses

Primer	Sequence (5' to 3')	Primer description
EsIAG-3a-Outer	*GAAACCTTCCAGCCAGAGTATCAG*	*3' RACE primer for first round*
EsIAG-3a-Inner	*GTCAATAGGTCAACAGGAGGTCA*	*3' RACE primer for second round*
EsIAG-5a-Outer	*AGCATCGGATTCTCCTCGTCTA*	*5' RACE primer for first round*
EsIAG-5a-Inner	*GGTGTTCCCAATCACTCCCACT*	*5' RACE primer for second round*
Rt-EsIAG-F	*GCAGCCGTCGAGATGTTAGA*	*FWD primer for EsIAG expression*
Rt-EsIAG-R	*ACACAGCACTGAGTGTAGGC*	*RVS primer for EsIAG expression*
EsIAG-CDS-F	*TTCAACGAGGATGTCCCTGC*	*FWD primer for EsIAG cds cloning*
EsIAG-CDS-R	*TGCCGACTTAACCTTCTGTTGA*	*RVS primer for EsIAG cds cloning*
EsIGFBP7-CDS-F	CCTTCGAGAGACCTCAGGATGGC	FWD primer for *EsIGFBP7* cds cloning
EsIGFBP7-CDS-R	CTACTTAGGCCTTCTTCGGATATTGC	RVS primer for *EsIGFBP7* cds cloning
Rt-*EsIGFBP7*-F	CACTCTCTCACTTTCTCACGCCC	FWD primer for *EsIGFBP7* expression
Rt-*EsIGFBP7*-R	AGCTGTCCTGCAAATCCGTTCTT	RVS primer for *EsIGFBP7* expression
EsACTIN-F	GCATCCACGAGACCACTTACA	*FWD primer for β-actin* expression
EsACTIN-R	CTCCTGCTTGCTGATCCACATC	RVS primer for β-actin expression
EsIAG-F-EcoRI	TCAACGGAATTCCAGGACTGCAGCTT CTC	*FWD primer for EsIAG cloning for Y2H*
EsIAG-R- BamHI	GAGAGGGGATCCTTATGCGCAAGGAT TTC	RVS primer for *EsIAG cloning for Y2H*
EsIGFBP7-F-NdeI	GACCTCCATATGGCGGGGTCTAAGG	*FWD primer for EsIGFBP7 cloning for Y2H*
EsIGFBP7-r-BamHI	TTGCCCGGATCCTTAGGCCTTCTTCGG	RVS primer for *EsIGFBP7 cloning for Y2H*

EsIGFBP7, insulin-like growth factor binding protein 7 from the Chinese mitten crab *Eriocheir sinensis*; EsIAG, insulin-like androgenic gland factor from *E. sinensis*

Quantitation of *EsIAG* and *EsIGFBP7* Transcripts by Real-Time PCR

Quantitative real-time PCR was performed to measure *EsIAG* and *EsIGFBP7* mRNA expression levels using the ABI 7500 system (Applied Biosystems, Foster City, CA, USA). The Rt-*EsIGFBP7*-F and Rt-*EsIGFBP7*-R primers were designed to detect *EsIGFBP7* expression. The real-time PCR program steps were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 34 s. Reactions were performed in triplicate for each sample. Expression of the *E. sinensis* β-actin gene (Accession no. HM053699.1) was selected as an endogenous control using the *EsACTIN*-F and *EsACTIN*-R primer pair. All primers used for quantitative real-time PCR are listed in Table 1. Student's *t*-test was used to detect differences between groups.

Yeast Two-Hybrid Assay

The Matchmaker™ Two-Hybrid System 3 (Clontech, Palo Alto, CA, USA) was used for the yeast two-hybrid assay. The activation domain (AD) vector was pGADT7 and the DNA-binding domain (BD) vector was pGBDT7. YH109 was used as the yeast strain. The experiments were performed following the manufacturer's protocols.

The coding sequences of the EsIAG and EsIGFFBP7 genes were amplified with primers containing BamHI and EcoRI enzyme loci, respectively (Table 1). The PCR products and pGADT7 and pGBKT7 plasmids were digested with the BamHI and EcoRI enzymes for more than 2 h at 37°C. The target bands were isolated and purified by gel electrophoresis, and four recombinant plasmids, such as IGFBP-pGADT7, IGFBP-pGBKT7, IAG-pGADT7, and IAG-pGBKT7 were constructed using T4 DNA ligase (Promega, Madison, WI, USA).

Four plasmid combinations, such as IGFBP7-pGBKT7 and IAG-pGADT7, IAG-pGBKT7 and IGFBP7-pGADT7, IGFBP7-pGBKT7 and pGADT7 (control), and IAG pGBKT7 and pGADT7 (control) were transformed into yeast on SD/-Trp-Leu medium using the Matchmaker™ Gold Yeast Two Hybrid System according to the manufacturer's protocol (Clontech). The plates with the transformed yeast strain were cultured at 30°C for 2–4 days until colonies appeared. The transformants were transferred to SD/-Trp-Leu-Ade-His medium and cultured at 30°C for 4–6 days. The transformation assay was repeated at least three times.

Results

Cloning of the Full-Length cDNA from the *E. sinensis* Androgenic Gland Gene

The full-length 1,495 bp cDNA sequence was obtained using 3′ and 5′ RACE method, and the sequence has been submitted to the GenBank database (accession no. KU724192) (Figure 1). The *EsIAG* cDNA consisted of a 210 bp 5' untranslated region (UTR), a 829 bp 3' UTR with a poly(A) tail, and a 456 bp ORF, which encoded a deduced 151 amino acid protein.

Sequence Comparisons and Phylogenic Analysis of EsIAG and IAGs from Other Species

The *E. sinensis* androgenic gland hormone is a

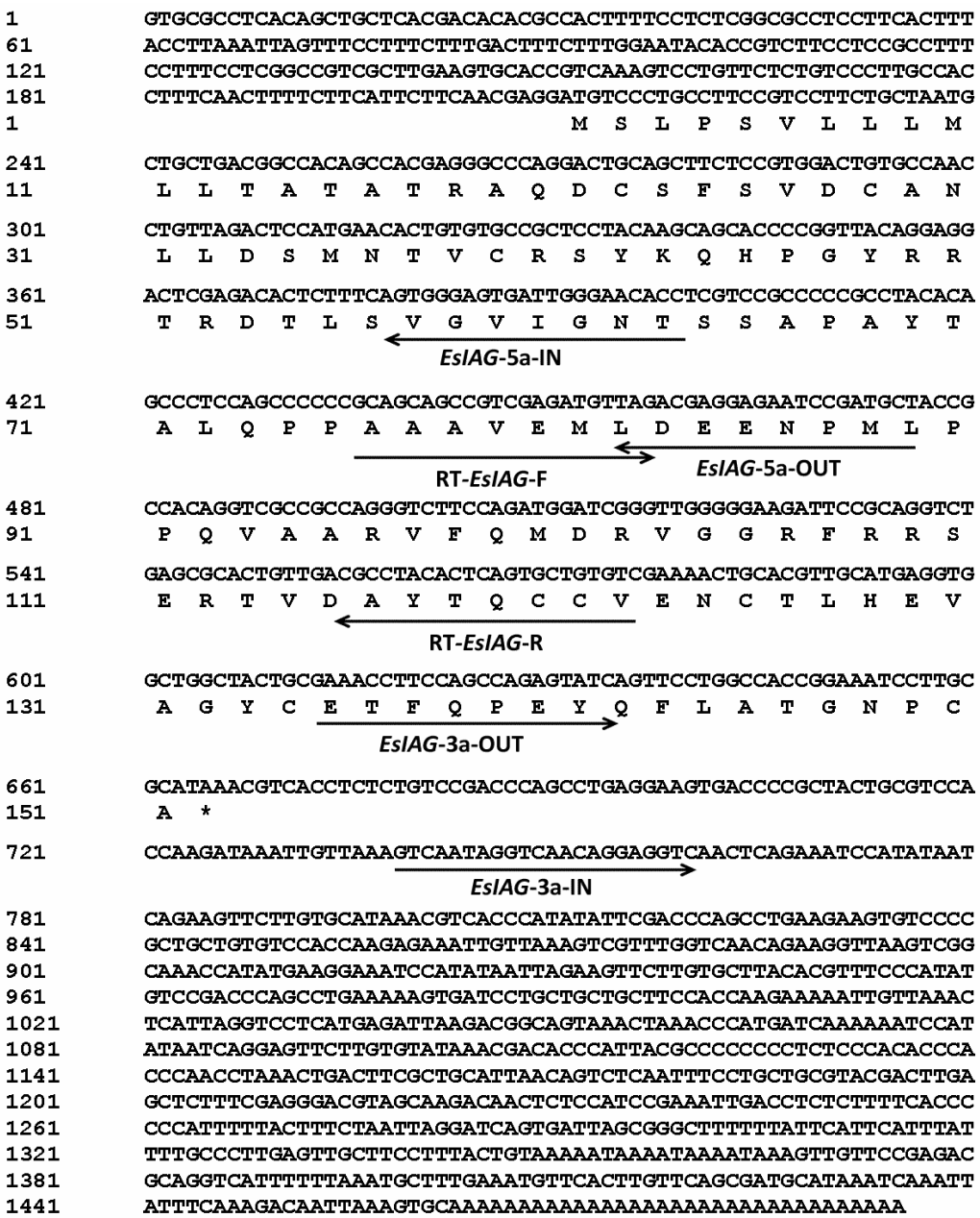

Figure 1. Full-length cDNA of *EsIAG* and its encoded amino acid sequence. The position of primer pairs for RACE PCR and RT-PCR are indicated by arrows. Asterisk represents the termination codon.

peptide, and its structure is shown in Figure 2A. The EsIAG precursor was comprised of A and B chains, a C peptide, and a signal peptide, but the mature IAG contained only the A and B chains, of 44 and 31 amino acids, respectively. The two chains were connected by disulfide bridges between Cys121 in the A chain and Cys28 in the B chain and between Cys134 in the A chain and Cys39 in the B chain. A disulfide bridge was also found between Cys120 and Cys125 in the A chain. The C peptide contained 57 amino acids, and the signal peptide contained 19 amino acids. The amino acid sequence alignment of six crustacean IAGs is shown in Figure 2B. The amino acids between EsIAG and the other five IAGs shared low sequence similarity, of which EsIAG had the highest similarity (40.9%) with CsIAG and the lowest similarity (20.9%) with PmIAG. The main chain had six characteristic cysteine residues (deep blue highlight), which produced a bridge between the two peptides (red line mark). A predicted glycosylation site (NxS/T sequence) occurred at amino acid 124 in the A chain. Two typical R-X-X-R proteolytic cleavage motifs were found at the C-terminal end of the C peptide (RFRR) and the C-terminal end of the B chain (RRTR).

A total of 21 IAGs, including EsIAG, were collected from the GenBank database, and an IAG evolutionary tree was built using MEGA software and the likelihood method. The phylogenetic tree (Figure 3) clearly showed that the IAGs of the mud crab

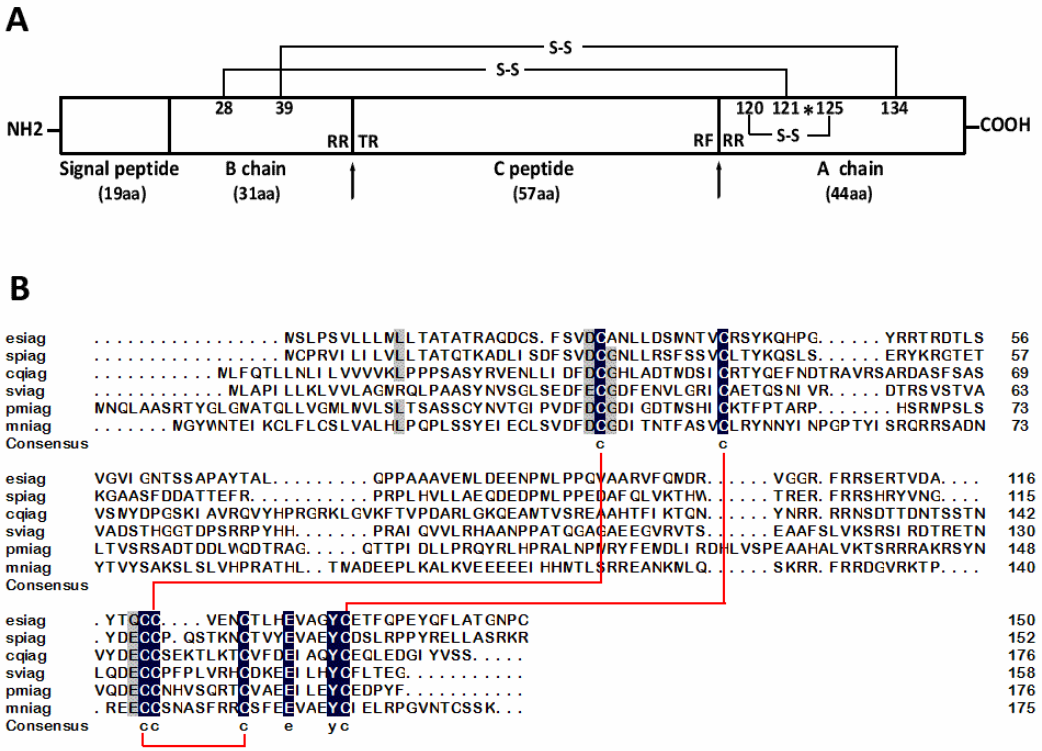

Figure 2. Character of deduced amino acid sequences of EsIAG. (A) Linear model of EsIAG. Linear model describing the deduced sequence of the four components of precursor of EsIAG: the signal peptide, B chain, C peptide and A chain. Two arrows indicate predicted Arg C proteinase cleavage sites at the C-terminal end of the C peptide (RFRR) and at the C-terminal end of the B chain (RRTR). Three disulfide bridges are indicated by lines, in which the B and A chains that are interlinked by two disulfide bridges; a third disulfide bridge is an intrachain bridge which is found within the A chain. Asterisks represent one predicted glycosylation sites (NxS/T sequence) at aa 124 in the A chain. (B) Comparison of deduced amino acid sequences of EsIAG with other five IAGs. Amino acid residues that are identical or similar between all sequences are highlighted. The most conserved feature is the backbone consisting of six cysteine residues, which gives rise to disulfide bridges (red lines).

(*Scylla paramamosain*), blue crab (*Callinectes sapidus*), and *E. sinensis* shared the closest genetic relationship, as they were clustered in one branch. On the whole, the phylogenetic tree showed that the IAGs formed two major clades: one with three isopods and the other containing 18 IAGs from the decapods, which constituted three subclades.

EsIAG and EsIGFBP7 Transcript Levels in Different Tissues

Real-time fluorescent quantitative PCR was used to analyze the expression levels of EsIAG in nine tissues, including the hepatopancreas (HE), intestine (IN), gill (GI), heart (HEA), ovary (OV), muscle (MU), testis (TE), androgenic gland (AG), and accessory sexual gland (AS). The results obtained indicated that the EsIAG expression level in the androgenic gland was the highest among all nine tissues (Figure 4A), whereas a low expression level was observed in testis. EsIAG was not detected in any of the other seven tissues, which is consistent with the function of IAG as regulator of male sexual differentiation.

EsIGFBP7 expression levels were also detected in nine tissues by real-time fluorescent quantitative PCR. Results obtained showed that *EsIGFBP7* was highly expressed in AS, TE, AG, and MU; the highest expression level was detected in AS, whereas low *EsIGFBP7* expression was observed in the IN, GI, HEA, HE, and OV (Figure 4B).

Interaction between EsIAG and EsIGFBP7

Four plasmid combinations, IGFBP7-pGBKT7 and IAG-pGADT7, IAG-pGBKT7 and IGFBP7-pGADT7, IGFBP7-pGBKT7 and pGADT7 (control), and IAG pGBKT7 and pGADT7 (control) were transformed into yeast and grown on SD/-Trp-Leu medium using the Matchmaker™ Gold Yeast Two Hybrid System in accordance with the manufacturer's protocol. When the transformants were transferred to SD/-Trp-Leu-Ade-His medium, only the IAG-pGBKT7 and IGFBP7-pGADT7 combination grew on SD/-Trp-Leu-Ade-His medium (Figure S1). In contrast, the other two combinations of IGFBP7-pGBKT7 and pGADT7 (control) and IAG-pGBKT7 and pGADT7 (control) did not grow on SD/-Trp-Leu-Ade-His medium, indicating that the results were reliable. These results indicate that ESIAG can

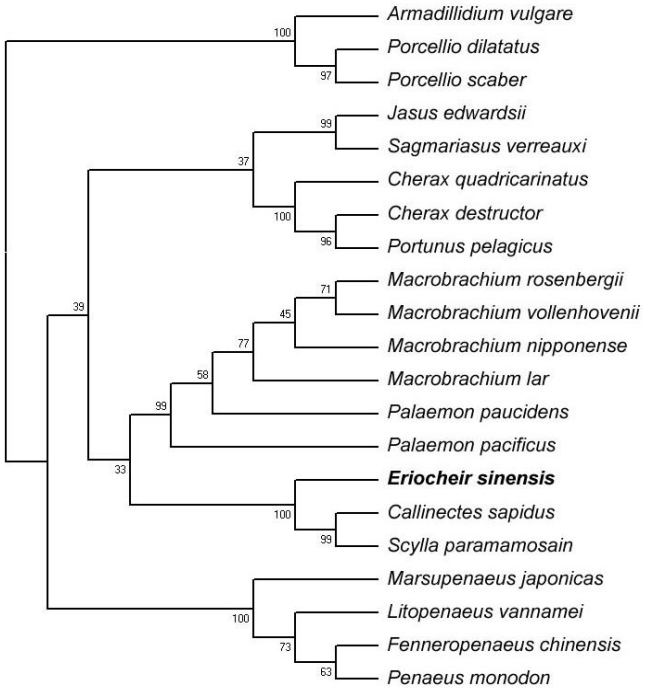

Figure 3. Phylogenetic tree of 21 IAGs. The tree was constructed using the likelihood method.
The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The amino acid sequences of the IAG proteins used for phylogenetic analysis were as follows: Armadillidium vulgare (BAA86893.1), Callinectes sapidus (AEI72263.1), Cherax destructor (ACD91988.1), Cherax *quadricarinatus* (ABH07705.1*), Eriocheir sinensis (*KU724192*),* Fenneropenaeus chinensis (accession number: AFU60547.1), Jasus edwardsii (AIM55892.1), Litopenaeus vannamei (AIR09497.1), Macrobrachium lar (BAJ78349.1), *Macrobrachium nipponense* (AHA33389.1), Macrobrachium rosenbergii (ACJ38227.1), Macrobrachium vollenhovenii (AHZ34725.1), Marsupenaeus japonicas (BAK20460.1), Palaemon pacificus (BAJ84109.1), Palaemon paucidens (BAJ84108.1), *Penaeus monodon (ADA67878.1)*, Porcellio dilatatus (BAC57013.1), Porcellio scaber (BAC57012.1), Portunus pelagicus (ADK46885.1), Sagmariasus verreauxi (AHY99679.1), and Scylla paramamosain (accession number: AIF30295.1).

interact with EsIGFBP7.

Discussion

The androgenic gland hormone in crustaceans has been demonstrated to be the main hormone controlling sexual differentiation and maintaining male sexual characteristics (Ventura *et al.*, 2011). In the present study, we cloned the full-length *E. sinensis* IAG cDNA sequence based on hi-sequence data from the *E. sinensis* androgenic gland. The amino acid sequence similarity between EsIAG and other reported crustacean IAGs was low, but the highest similarity was found between EsIAG and CsIAG (40.9%), and SpIAG (38.3%), and they clustered on one branch of the phylogenetic tree. The similarities between EsIAG and the other 18 IAGs were 15.9–24.1%. Consequently, we failed to clone the *E. sinensis* IAG gene using degenerate primers designed based on known IAG sequences. A similar negative result was reported when attempting to identify the IAG gene from *C. quadricarinatus* with degenerate primers designed based on isopod sequences (Manor *et al.*, 2007).

Although the similarities in the deduced amino acid sequences between EsIAG and other reported crustacean IAGs were low, the structure and organization of all IAGs, including EsIAG, were conserved, such as the two disulfide bridges observed in EsIAG, suggesting that these bridges are necessary for IAG functioning. The deduced amino acid sequence of the EsIAG precursor was comprised of 151 amino acids, with a 19 amino acid message peptide, 31 amino acid B chain, 62 amino acid C peptide, and a 42 amino acid A chain in a row from the N terminal to the C terminal. This organization is the same as that reported for other IAGs (Manor *et al.*, 2007; Chung *et al.*, 2011; Li *et al.*, 2012b; Ma *et al.*, 2013). Similar to IAGs reported in other crustacean species, EsIAG also had two typical R-X-X-R proteolytic cleavage motifs at the C-terminal end of the C peptide (RFRR) and at the C-terminal end of the B chain (RRTR). The relative positions of the six cysteine residues were conserved among the 21 IAGs considered. These six cysteine residues formed two putative disulfide bridges between the A and B chains, and a putative disulfide bridge in the A chain, suggesting that these are conserved structures indispensable for IAGs to perform their functions.

All 21 IAG sequences were collected from the GenBank database; three (AvIAG, PdIAG, and PsIAG) were from isopods, and the remaining 18

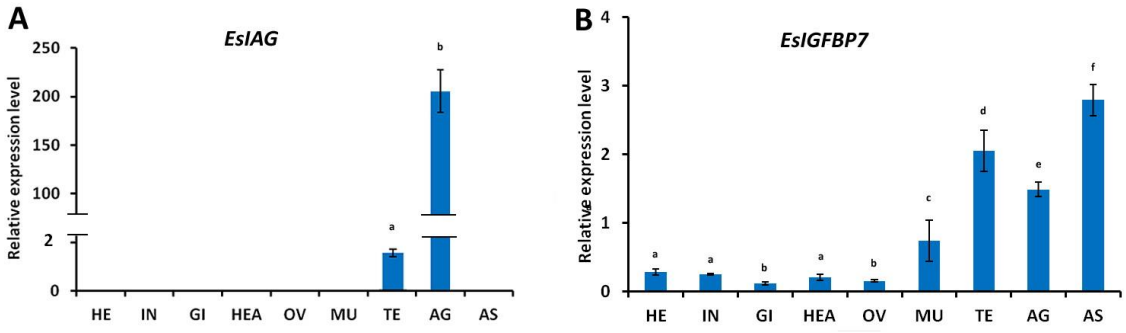

Figure 4. Quantitative polymerase chain reaction analysis of relative *EsIGFBP7* expression levels in nine *E. sinensis* tissues. TE: testis; OV: ovary; HEA: heart; HE: hepatopancreas; MU: muscle; IN: intestine; AG: androgenic gland; AS: accessory sexual gland. The data is reported as means and standard deviations (n = 3 samples). Different letters indicate significant differences (P<0.05).

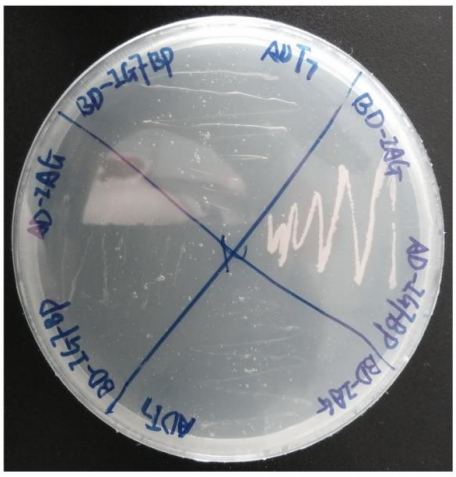

Figure S1. Different transformants growing on SD/Leu-Trp-His-Ade plates. Only the transformants in the binding domain-androgenic gland hormone (BD-IAG) and activation domain-insulin-like growth factor binding protein (AD-IGFBP) groups grew on SD/Leu-Trp-His-Ade plates.

sequences were from decapods. Two branches were observed from the root of phylogenetic tree in our analysis. One branch was comprised of three IAGs (EsIAG, CsIAG, and SpIAG) and the other branch was comprised of the other 18 IAGs, including the three isopod IAGs. These results show that the evolutionary divergence of IAGs in crustaceans is not fully consistent with their traditional classification, indicating that the classification of some crustacean species could be improved.

IGFBP7 binds strongly to insulin in vertebrates (Li *et al.*, 2012a). Although the IGFBP family has many members in vertebrates, only one member has been identified in crustacean species, until now (Rosen *et al.*, 2013; Li *et al.*, 2015). Crustacean IGFBPs are most homologous with vertebrate IGFBP7, and they share a common structural organization of an IB domain in the N-terminus, a KAZAL domain in the middle, and an IG domain in the C-terminus. IGFBP family members are cysteine-rich proteins and include 16–20 conserved cysteines in their peptide sequences. EsIGFBP7 similar to vertebrate IGFBP7s have 18 cysteines. In particular, the 12 cysteines in the N-terminal IB domain are highly conserved. Moreover, the CGCCxxC motif in the N-terminal of the IB domain is well-conserved among vertebrate IGFBPs and is thought to be important for interactions with insulin-like peptides (Hwa *et al.*, 1999; Daza *et al.*, 2011). The same motif was found in the N-terminal domain of EsIGFBP7 and other crustacean IGFBPs, which is consistent with their role as insulin-like peptide-binding proteins.

The interaction between crustacean IGFBP and IAG was first demonstrated in *C. quadricarinatus* (Rosen *et al.*, 2013). In this study, we verified the interaction between EsIGFBP7 and EsIAG using a yeast-two hybrid assay. A yeast-two hybrid system is comprised of a DNA BD and a transcriptional AD (Fields and Song, 1989). The BD is identified by specific DNA sequences, making the AD a target of upstream regulatory genes with a transcriptional role. Here, EsIAG and EsIGFBP7 were linked to the AD and BD in the yeast-two hybrid system, respectively. If these two proteins interact, the AD and BD are in close proximity to perform their functions,

downstream genes are expressed, which allows the yeast to grow in nutrient- deficient medium. Our experimental results fully verified our hypothesis because the yeast grew in nutrient-deficient medium when EsIAG was linked to the BD and EsIGFBP7 was linked to the AD, suggesting that EsIAG and EsIGFBP7 interacted. However, no growth occurred when EsIGFBP7 was linked to the BD and EsIAG was linked to the AD, indicating a potential spatial structural obstacle between the combination of BD-EsIGFBP7 and AD-EsIAG.

EsIAG and EsIGFBP7 expression levels were investigated in nine *E. sinensis* tissues by real-time fluorescent quantitative PCR. The results showed that EsIAG was dominantly expressed in the androgenic gland, which is consistent with other reported IAGs. EsIGFBP7 expression levels in male gonad-related tissues were higher than those in other tissues, indicating that EsIAG requires EsIGFBP7 to regulate and maintain the male gonad and that EsIGFBP7 plays a regulatory role in male sexual differentiation.

In summary, we cloned the cDNA sequence of EsIAG and established transcriptional patterns of *EsIAG* and its partner EsIGFBP7 in different *E. sinensis* tissues. We also verified interactions between EsIAG and EsIGFBP7. These results provide basic information about the regulatory mechanism of male sexual differentiation in *E. sinensis*.

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