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Cloning and Sequence Characterization of Key Genes in the Growth Hormone/Insulin-like Growth Factor Axis of Quasipaa boulengeri Postprint

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Abstract

The growth hormone (GH)/insulin-like growth factor (IGFs) axis in organisms consists of the GH system and IGFs system, which promotes cell proliferation, regulates growth and development, controls physiological metabolism, and plays a crucial role in regulating organismal growth and development. To elucidate the functional structure and evolutionary characteristics of the GH/IGFs axis in the spiny-bellied frog (Quasipaa boulengeri) and provide a theoretical basis for research on the regulation of growth and development in this species, the present study cloned GH, insulin-like growth factor-I (IGF-I), and insulin-like growth factor-II (IGF-II) from the spiny-bellied frog and analyzed their sequence characteristics. The results revealed: 1) Multiple sequence alignment with amphibian model animals revealed that the functional domains of GH, IGF-I, and IGF-II in the spiny-bellied frog were strictly conserved and exhibited certain genetic polymorphism; the N-terminus of IGF-II displayed a trend of evolutionary reduction. 2) Phylogenetic clustering analysis showed that IGFs of the spiny-bellied frog clustered with amphibians and were relatively closely related to teleost fish, indicating that IGF-I and IGF-II occupy a relatively basal evolutionary position; GH of the spiny-bellied frog, however, was relatively closely related to aquatic animals such as frogs and fish, suggesting that this gene has undergone convergent evolution. 3) To further identify specific functional sites in the aforementioned genes, SWISS software was employed to analyze their protein structures, ultimately identifying THR52, LEU53, PHE72, PHE73, and SER74 of IGF-I as potential functional differentiation sites, TYR81, LYS82, and LYS83 of IGF-II as potential functional differentiation sites, and PHE208 of GH as a potential functional differentiation site. Therefore, the main genes of the GH/IGFs axis in the spiny-bellied frog are relatively conserved, but compared with known model species, there exist potential functional differentiation

sites that can serve as molecular targets for subsequent functional studies of the GH/IGF axis and analysis of genetic evolutionary characteristics in this species.

Full Text

Cloning and Sequence Characteristic Analysis of Growth Hormone/Insulin-Like Growth Factor Axis Key Genes from *Paa boulengeri*

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Abstract

The growth hormone (GH)/insulin-like growth factor (IGFs) axis, composed of the GH system and IGFs system, promotes cell proliferation, regulates growth and development, and controls physiological metabolism, playing a crucial role in organismal growth regulation. To clarify the functional structure and evolutionary characteristics of the GH/IGFs axis in *Paa boulengeri* and provide a theoretical basis for research on its growth and development regulation, this study cloned GH, insulin-like growth factor-I (IGF-I), and insulin-like growth factor-II (IGF-II) from *P. boulengeri* and analyzed their sequence characteristics. The results revealed: (1) Multiple sequence alignment with amphibian model animals showed that the functional domains of GH, IGF-I, and IGF-II from *P. boulengeri* were strictly conserved yet exhibited certain genetic polymorphisms; the N-terminus of IGF-II showed an abbreviated evolutionary trend. (2) Phylogenetic clustering analysis indicated that *P. boulengeri* IGFs clustered with amphibians and were relatively closely related to bony fish, suggesting that IGF-I and IGF-II occupy a relatively primitive evolutionary position; *P. boulengeri* GH, however, was more closely related to aquatic animals such as frogs and fish, implying a convergent evolutionary trend for this gene. (3) To further identify specific functional sites in these genes, SWISS software was used to analyze their protein structures, ultimately identifying THR52, LEU53, PHE72, PHE73, and SER74 in IGF-I, TYR81, LYS82, and LYS83 in IGF-II, and PHE208 in GH as potential functional differentiation sites. These findings demonstrate that while the major genes of the GH/IGFs axis in *P. boulengeri* are relatively conserved, potential functional differentiation sites exist compared with known model species, which can serve as molecular targets for future functional studies and genetic evolutionary characteristic analysis of the *P. boulengeri* GH/IGF axis.

Keywords: *Paa boulengeri*; insulin-like growth factor; growth hormone; sequence analysis; structural characteristics

Introduction

Growth hormone (GH) is a peptide hormone secreted by adenohypophyseal cells and represents the primary endogenous factor affecting animal growth and development. Insulin-like growth factors (IGFs) are classified into two types: insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II). These are plasma-resident peptides that possess both growth-promoting and insulin-like effects. Current research has established that organismal growth is controlled by the GH/IGFs axis. After secretion from the pituitary, GH stimulates the synthesis and secretion of IGFs in the liver and other tissues through GH receptor mediation, with IGFs subsequently exerting their biological functions via IGF receptor mediation [?].

Paa boulengeri, also known as the spiny-bellied frog or stone frog, belongs to the family Dicroglossidae, genus *Quasipaa*, order Anura. It is a rare and endemic amphibian species found in mid-to-high altitude regions of western China [?, ?]. Due to environmental pollution and ecological destruction, wild populations of *P. boulengeri* have been declining steadily and are now listed in the *China Red Data Book of Endangered Animals* [?] and *China Species Red List* [?]. Market demand for *P. boulengeri* remains strong, with artificially bred second-generation offspring commanding prices of 45–70 yuan/kg, making artificial cultivation an important pathway for poverty alleviation among mountain farmers. To better protect wild resources while meeting economic demands, interest in artificial breeding of *P. boulengeri* has been increasing annually.

Our research group previously explored artificial rearing conditions for *P. boulengeri* and found that both excessively high and low temperatures affect its growth and development, with temperature exerting particularly pronounced effects on the GH/IGFs axis. Genetic characteristic studies revealed that *P. boulengeri* populations belong to a monophyletic branch [?, ?] and are relatively independent during evolution [?, ?], with no relevant data from other model species available for reference. This study aims to clone key genes of the GH/IGFs axis (IGF-I, IGF-II, and GH) using information obtained from transcriptome sequencing and conduct bioinformatics analysis to deeply understand their genetic characteristics and identify species-specific potential functional sites, thereby laying a theoretical foundation for exploring the physiological responses and growth regulation mechanisms in amphibians represented by *P. boulengeri*.

Materials and Methods

Experimental Animals and Reagents

Healthy two-year-old *P. boulengeri* were reared at the amphibian flow-through culture system of the Chongqing Research Centers of Conservation and Development on Rare & Endangered Aquatic Resources. Trizol and diethyl pyrocarbonate (DEPC) were purchased from Shanghai Sangon Biotech Co., Ltd. cDNA

synthesis kits, Taq polymerase, and PCR purification kits were obtained from Promega Corporation. Gel extraction kits were purchased from OMEGA Bio-tek, and DL2000 DNA Marker was obtained from TaKaRa Bio (Dalian) Co., Ltd.

Primer Design and Synthesis

Gene sequences were primarily based on the Illumina Solexa high-throughput transcriptome database of *P. boulengeri* tadpoles previously established in our laboratory. Full-length gene sequences of GH, IGF-I, and IGF-II from *P. boulengeri* were obtained using real-time quantitative PCR (RT-qPCR) and verified to be consistent with transcriptome data through sequence alignment. Finally, gene-specific primers were designed using Primer 5.0 software and synthesized by Genewiz Biotechnology Co., Ltd. (Suzhou).

Table 1 Primer sequences for gene amplification

Gene names	Primer sequences (5’ $→$3’)	Amplification length (bp)
IGF-I	F: ATCCTTCTTCT-GTTTGCTAAATCTGR: CTCTAGGGGACACAGGC-TATTA	-
IGF-I	F: GCAACATCCAGCAAT-ACCACAGCGAR: CTTTGGT-GTCTCAGTTTGCTCGTTT	-
IGF-II	F: TGGAACCCGAACATA-CATCAGGAACR: AGACACAAAAAGCA-CAAAGCAAAAT	-

cDNA Preparation

Liver tissue was collected from *P. boulengeri* euthanized by double pithing and immediately snap-frozen in liquid nitrogen. Total RNA was extracted according to the kit protocol, and single-stranded cDNA (ss cDNA) was prepared using Promega’ s cDNA synthesis kit following the manufacturer’ s instructions.

Gene Cloning and Sequencing

High-fidelity DNA Taq polymerase was used for PCR amplification. The RT-qPCR reaction mixture contained: 1 L cDNA template, 2.5 L 10×Ex Taq PCR buffer, 0.65 U Ex Taq, 1 L dNTP (100 mol/L), 1 L $MgCl_2$, 1 L forward primer, 1 L reverse primer, and ddH_2O to a final volume of 25 L. The PCR program consisted of: (1) initial denaturation at 94°C for 4 min; (2) denaturation at 94°C for 90 s; (3) annealing at 58°C for 90 s; (4) extension at 72°C for 90 s; (5)

steps 2–4 repeated for 30 cycles; (6) final extension at 72°C for 10 min; and (7) storage at 12°C. PCR products were detected by 1% agarose gel electrophoresis, and fragments corresponding to the expected gene lengths were excised. After purification using the TaKaRa gel extraction kit, products were ligated into the pMD 19-T vector and transformed into *E. coli* JM109 competent cells. Positive plasmids were selected through blue-white screening, verified by double restriction enzyme digestion, and sent to Genewiz Biotechnology Co., Ltd. (Suzhou) for sequencing.

Bioinformatics Analysis

Genetic Polymorphism Analysis Homologous sequences of GH, IGF-I, and IGF-II were retrieved from the NCBI database. Multiple sequence alignment and Boxshade Server alignment were performed using ClustalX 1.83 software, and final results were processed using Photoshop CS6.

Phylogenetic Analysis Sequences from closely related species were retrieved from the GenBank database, aligned using ClustalW, and phylogenetic trees for GH, IGF-I, and IGF-II homologous genes were constructed using the neighbor-joining method in MEGA 5.0 software with 1,000 bootstrap replicates to estimate branch confidence values.

Protein Structure Analysis Protein models were initially searched using Phyre2 software (http://www.sbg.bio.ic.ac.uk/). The three-dimensional structures of target proteins were then predicted using the Swiss-model server (https://swissmodel.expasy.org/), and images were processed and protein structures rendered using Swiss-PdbViewer software.

Results

Sequence Characteristics of IGF-I

Using liver tissue cDNA from two-year-old *P. boulengeri* as template, IGF-I gene amplification [Figure 1: see original paper]-A and sequencing [Figure 1: see original paper]-B were performed. Comparison of *P. boulengeri* IGF-I with published homologous genes from two toad species revealed numerous amino acid mutations in the conserved functional domains [Figure 1: see original paper]-C.

Phylogenetic analysis of IGF-I from various representative species [Figure 2: see original paper] showed that IGF-I divided into two major clades: one comprising higher mammals, and the other including reptiles, birds, and amphibians. *Paa boulengeri* IGF-I clustered with anuran amphibians and some aquatic reptiles, showing close affinity with other terrestrial vertebrates, indicating substantial genetic divergence from amphibian model species such as *Xenopus*. Notably,

the Chinese alligator IGF-I has three copies: two copies clustered with avian species, while the third copy formed a separate clade with the Chinese soft-shelled turtle, clustering with the Burmese python and turtle-headed sea snake, suggesting that vertebrate IGF-I may have originated from primitive reptile-amphibian ancestors.

To deeply analyze the protein structure of *P. boulengeri* IGF-I, the Swiss-model server was used for structure prediction [Figure 3: see original paper]. Compared with the human IGFs protein structure 3lri.1A [?], the sequence similarity reached 84.6%. The amino acid sequence contained one transmembrane domain at positions 31–48 and an IGF-I-like superfamily functional domain at positions 51–109. The IGF-I protein structure primarily consisted of two β-sheets and four α-helices, with unknown functional regions at both termini. THR52, LEU53, PHE72, PHE73, and SER74 represented species-specific amino acid mutation sites, suggesting functional differentiation of this protein.

Sequence Characteristics of IGF-II

The IGF-II gene from *P. boulengeri* was amplified and sequenced, and genetic polymorphism was analyzed using ClustalX 1.83 software. Position 204 represented the C-terminal functional domain of IGF-II, implying that this gene may be primarily involved in immune defense in *P. boulengeri*. Compared with two known amphibian model animals (*Xenopus laevis* and *Xenopus tropicalis*), *P. boulengeri* IGF-II showed significantly reduced copy number, an abbreviated N-terminal evolutionary trend, and insertion mutations in its functional domain, indicating pronounced functional differentiation.

Phylogenetic analysis of *P. boulengeri* IGF-II homologous sequences [Figure 5: see original paper] revealed three major clades: higher mammals, amphibians, and a group comprising bony fish, terrestrial animals, and some birds, suggesting relatively conserved evolution of the IGF-II gene.

Swiss-model server was used to simulate the three-dimensional structure of IGF-II protein. The results showed a structure similar to *P. boulengeri* IGF-I protein, primarily composed of six α-helices. Specific amino acid mutations at TYR81, LYS82, and LYS83 suggested functional differentiation of this protein during species evolution.

To compare the differences in binding sites and interaction modes between IGF-I and IGF-II, PDB-Viewer was used to reconstruct the protein backbones of both proteins. As shown in [Figure 3: see original paper]-A and [Figure 6: see original paper]-A, the yellow regions represent identical interaction sites between IGF-I and IGF-II, with only three regions showing significant differences. Comparison of protein modification patterns revealed that all differential sites were located in functional binding regions [Figure 3: see original paper]-B and [Figure 6: see original paper]-B. Subsequent alignment of functional differentiation sites showed that two of the differential regions were conserved IGFs functional domains, with one region being specific to *P. boulengeri* IGF-I. These findings

indicate that in-depth investigation of IGFs functions is crucial for studying species-specific physiological metabolism in *P. boulengeri*.

Sequence Characteristics of GH

The GH gene sequence from *P. boulengeri* was obtained through cloning and sequencing [Figure 7: see original paper]-A and [Figure 7: see original paper]-B. Genetic polymorphism analysis revealed few amino acid mutations between *P. boulengeri* GH and known amphibian homologous genes, most of which were synonymous mutations with minimal impact on protein function [Figure 7: see original paper]-C.

Phylogenetic clustering analysis with GH homologous genes [Figure 8: see original paper] showed that *P. boulengeri* GH was closely related to bullfrog GH and formed a separate clade with aquatic animals including grass carp, zebrafish, and freshwater sturgeon, suggesting that aquatic animal GH genes share a common ancestor. Birds and terrestrial animals clustered together, while higher mammals formed a separate clade, confirming relatively conserved evolution of the *P. boulengeri* GH gene.

Protein structure analysis of *P. boulengeri* GH [Figure 9: see original paper] revealed a typical Hormone_1 domain [Figure 9: see original paper]-C, characteristic of 4-helix cytokines [Figure 9: see original paper]-A. PHE208 represented a mutation site compared with model vertebrates [Figure 9: see original paper]-B and [Figure 9: see original paper]-C but was relatively conserved compared with amphibian model animals, indicating that the functional domains of *P. boulengeri* GH are relatively conserved.

Discussion

In the GH-IGFs axis, GH occupies an upstream position while IGFs are downstream [?]. The GH-IGFs axis functions through a complex regulatory network composed of multiple factors and regulatory modes controlling GH synthesis and secretion [?]. The GH/IGF-I axis serves as the main regulator of organismal growth and development [?], with signal transduction occurring primarily through two pathways: the phosphatidylinositol-3-kinase (PI3-K) activation pathway and the mitogen-activated protein kinase (MAPK) activation pathway. These pathways transmit mitogenic and metabolic signals to the nucleus, thereby initiating IGFs secretion, promoting cell proliferation and differentiation, and inhibiting apoptosis. Upon binding to its receptor, IGF-I first induces phosphorylation of insulin receptor substrate-1 (IRS-1). Phosphorylated IRS-1 then recruits PI3-K and growth factor receptor-bound protein 2 (Grb2), initiating two signaling cascades. One pathway involves PI3-K activation and formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which serves as a signal for cell growth and represents the most classical pathway for inhibiting apoptosis. The other pathway activates extracellular signal-regulated kinase

(ERK), which transmits signals to the nucleus to initiate mitosis [?]. Thus, IGF-I promotes organismal growth both by increasing cell mitosis and by inhibiting apoptosis.

Current reports on GH function in amphibians are limited. Early studies found that exogenous GH before metamorphosis could stimulate growth in amphibian larvae—tadpoles—and played a special role in larval growth and development, particularly limb growth [?]. Exogenous GH can promote growth in toad larvae and hindlimb development in bullfrog larvae [?]. During late tadpole development, endogenous GH levels in blood continuously increase [?]. However, the precise role of endogenous GH in amphibian larval growth, development, and metamorphosis remains poorly documented.

This study analyzed endogenous GH, IGF-I, and IGF-II from *P. boulengeri*, revealing numerous amino acid mutation sites compared with known amphibian model organisms, suggesting a certain degree of functional differentiation. Phylogenetic analysis showed that *P. boulengeri* IGF-I and IGF-II exhibited species-specific differentiation trends compared with known amphibian model animals, while GH evolution remained relatively conserved. Protein structure analysis revealed five functional differentiation sites in *P. boulengeri* IGF-I and three in IGF-II compared with human IGFs, whereas *P. boulengeri* GH showed only a single amino acid mutation at PHE208 compared with model vertebrate GH, indicating this site has important research value for functional differentiation in *P. boulengeri*. Notably, this site is relatively conserved among amphibians, suggesting that PHE208 represents an amino acid site for functional differentiation between amphibians and higher mammals.

In summary, although functional domains and genetic evolutionary characteristics are relatively conserved, *P. boulengeri* IGF-I sites THR52, LEU53, PHE72, PHE73, and SER74, IGF-II sites TYR81, LYS82, and LYS83, and GH site PHE208 represent potential functional and binding sites. Given our previous findings that the GH/IGFs axis is susceptible to temperature effects, leading to failed metamorphosis and even death in *P. boulengeri* tadpoles and abnormal growth in adult frogs, this study has identified molecular targets for revealing how temperature mediates GH/IGFs axis metabolic mechanisms to regulate *P. boulengeri* growth and development, and provides a theoretical basis for future breeding of superior *P. boulengeri* varieties.

The major genes of the *P. boulengeri* GH/IGFs axis are relatively conserved, but potential functional differentiation sites exist compared with known model species, which can serve as molecular targets for future functional studies and genetic evolutionary characteristic analysis of the *P. boulengeri* GH/IGFs axis.

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