CLONING AND COMPARISON OF HEMOGLOBIN α CHAIN CDNA SEQUENCES FROM FIVE SPECIES OF FISHES IN *CYPRINIFORMES*

QI YAN DU ZHONG JIE CHANG YING CHEN[1]

Key words: *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Carassius auratus*, *Misgurnus anguillicaudatus*, *Paramisgurnus dabryanus* , αglobin gene

Abstract: The complete cDNA sequences endcoding hemaglobin α chain subunit (or α globin) were cloned by RT-PCR amplification from five species of fishes, *Cyprinus carpio*, *Ctenopharyngodon idellus* ,*Carassius auratus* Linnaeus, *Misgurnus anguillicaudatus* ,*Paramisgurnus dabryanus*. Each of the five α globin cDNA contains full length of open reading frame that encode a 143 amino acid residues of α globin. The sequence comparison revealed that the α globin sequences among the five species are significantly divergent although they are all classified in *Cypriniformes*.

INTRODUCTION

Farm-grown fishes are one of the main protein sources in human protein supply. It has growing economic importance as the wild fishes were subjected to more strict protection from decreasing population. Studies to improve productivity and quality of farm fishes have been focused on selection and breeding of fish strains and/or species suitable for varieties of aquaculture purposes. Recent advances in transgenic technologies provided a powerful tool for generating fish strain that may bring about economic value to aquaculture production.

A number of genes, such as growth hormone (GH) and anti-freeze protein (AFP), were targeted to generate transgenic fish by heterologous integration[1]. The growth hormone transgenic fish were shown having a faster growth rate, whereas the AFP transgenic fish had little phenotypic change in tolerating to low water temperature. Extensive study was carried to explore the molecular structure and biochemical property of AFP [2]. Another tempt of transgenic fish study was to transfer hemaglobin gene from the fish that tolerates to low oxygen content in water to the fish sensitive to low oxygen condition. To explore the underline mechanisms for the variation of hemoglobin property, we have cloned hemaglobin a chain cDNAs from five species of fishes, *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Carassius auratus*, *Misgurnus anguillicaudatus*, *Paramisgurnus dabryanus* and compared their sequence similarity and phylogenetic relationship.

MATERIAL AND METHODS

Material

Fish samples, *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Carassius auratus*, *Misgurnus anguillicaudatus*, *Paramisgurnus dabryanus* were purchased from Xinxiang farmers’ market.

Preparation of total RNA

Total RNA was extracted from blood using Promega total RNA Isolation System according to the manufacturer’s protocol. Briefly, 0.2-0.3ml blood were collected by tail amputation, and the blood cells were precipitated by contrifugation at 300g, rinced with sterile 1×PBS, broken in lysis buffer by homogenization. The cell lysis suspension was extracted with 600μl of Phenol: chloroform: Isoamyl Alcohol after adding 60μl of Sodium Acetate (pH4.0). Total RNA was precipitated by centrifugation at 12000rmp and washed onec before air-drying. The RNA pellet was dissolved in 30μl of Nuclease-free water.

RT-PCR

Using Promega Access RT-PCR System kits, PCR reaction were performed in a 50μl reaction volume containing 1μl total RNA, 50pmol / L of each primer, 1μl 10mmol / L dNTP mix, 5μl $MgSO_4$, 10μl 5× AMV / Tfl reaction buffer, 5U AMV Reverse Transcriptase, 5 U Tfl DNA Polymerase . Thermocycling program was set with 1 cycle of reverse transcription at 48°C for 45min, 40 cycles of denature at 94°C for 30s, annealing at 54°C for 1min, extension at 72°C for 2min. After the final cycles, an extension reaction was carried out at 72°C for 10 min.

Upper primer:

5’ ATGAGTCT(G/C)(A/T)C(A/T/C/G/)G(C/A)(A/T/C/G/)(A/C)(A/C/G)(A/T/C/G)GA 3’

Down primer:

5’ TTATTA(T/G)C(T/G)GTA(T/C)TT(A/C/G)TCAG 3’

Cloning and analysis of PCR product

PCR products were detected by electrophoretic separation in a 1.5% agarose gel followed by ethidium-bromide (EB) staining. The bands of interests were purified using DNA Gel Extraction Kits. The PCR products were directly ligated into pUCm-T vectors, and transformed into JM109 competent cells. Positive clones were selected through X-Gal color indication. Plasmid DNA was prepared using alkaline lysis and sequenced using dideoxy chain termination method.

Analysis of αglobin sequence

Sequence similarity searches were done using the BLAST algorithm in GenBank.

RESULTS AND DISSCUSION

Cloning and sequences of globin cDNA of five fish species

Using degenerate primers, we amplified by PCR ~430 base pairs of cDNAs (Fig. 1) encoding full length of hemoglobin a chain subunit from five species of fishes, *Cyprinus carpio*, *Ctenopharyngodon idellus* ,*Carassius auratus* Linnaeus, *Misgurnus anguillicaudatus*. Each of the a globin cDNAs was cloned into pUCm-T vector and sequenced throughout its entirety. The results shown that they all encode a globin with 143 amino acid residues (Fig. 2) (GenBank accession number: AF528156, AF528157, AF528197, AF528198, AF528199). Sequence comparison with the existing hemoglobin a chain protein confirmed their identity.

Comparison of sequence similarity

Sequence alignment analysis shown that more than 81% of the amino acid residues are identical among the five globins. The highest sequence similarity, ~90% homology, was observed between the two closest species, *Misgurnus anguillicaudatus* and, *Paramisgurnus dabryanus*. (Table 1). The amino acid residues equivalent to those of mammalin a globin that are involved in interacting with heme and oxygen binding were identified by the sequence comparison, which include aa60 (His), aa62 (Lys), aa88 (Leu), aa89 (His), aa93 (Leu) and aa95 (Val). Although most of them are well conserved a number of intriguing variations exist in *Cyprinus carpio*, *Ctenopharyngodon idellus* ,*Carassius auratus* Linnaeus. Despite of the overall similarity, the α globin sequences are significantly diverged among these five species of fihes considering the conservative nature of hemoglobin in vertebrates. To explore the relationship between amino acid sequence similarity and species differentiation, we compared the above a-globin sequences plus five other fish globin sequences deposited in GenBank database by phylogenetic analysis (Fig. 3).

Hemaglobin Genes have so far been analyzed in many vertebrates, but the study of hemaglobin genes in fishes have been limited, to date, to the protein level. Fish is primordial vertebrate and it has many specific characteristics adapted for variety physiological needs. Oxygen content in water is not only one of the most important restriction factor but also one of the most important conditions affecting the growth of fish. The mobility of fish sharply reduced in hypoxic condition. When oxygen content remained at low level for an extended period of time, many symptoms may appear because of the declining of food intake, mobility, disease - resistance, etc. However, variation in hemablobin sequences among different species may render various tolerance levels to low oxygen content in water, which is attributed to changes of hemoglobin binding affinity and dissociation constant to O_2 and CO_2. The study of fish hemaglobin may help to understand the molecular mechanisms that under line the tolerance to low oxygen content in water.

Globin is species-specific. Different globin has different amino acid and different affinity of oxygen. The Hb components in adult fish are complicated. For instance, the rainbow trout shows three major Hb forms (HbIV, HbI, and HbII); HbIV and HbII display a strong Bohr effect, whereas HbI is insensitive to pH (i.e., the Bohr effect is completely absent)[4]. Rund published

the first systematic analysis of the “white” blood of an Antarctic icefish, *Chaenocephalus aceratus*. Furthermore, the oxygen-carrying capacity of *C.aceratus* blood was approximately 10% that of two red-blooded nototheniods. Adults of the family *Nototheniidea* (Antarctic rockcods) generally possess a major hemoglobin, Hb1, and a second, minor hemoglobin, Hb2, that differ in their αchains (α1 and α2 respectively) [5]. The more phyletically derived harpagiferids and bathydraconids have single hemoglobin. The trend toward reduced hemoglobin multiplicity in the notothenioid suborder, which reaches its extreme in the icefishes, probably results from evolutionary loss or mutation to transcriptional inactivity of globin genes[6]. The five species in this paper belong to *Cypriniformes*. They have close relationship. But they have different insensitive to low oxygen content in water due to they linked to the need for dealing with a mutable environment or different habitats. The αglobin is different not only in suffocate point which in *C. carpio*; *C. idellus*; *C. auratus* Linnaeus; *M. anguillicaudatus*; *P. dabryanus* is 0.34～0.3mg/L, 0.51～0.3mg/L, 0.13～0.11mg/L, 0.24mg/L, 0.16mg/L respectively but in amino acid sequence[7,8]. In *C. auratus* Linnaeus and *P. dabryanus* the suffocate point is close to each other but there are 25 differential amino acids. Q6, S22 ,I35, is special in *C. auratus* Linnaeus. D5 ,P6, G19, S48, is special in *P. dabryanus*. It is deduced that the change of these positions could enhance the affinity of oxygen. The suffocate point in *C. idellus* which specific amino acid is V65, A66, A79, R89, A112, A117 is lowest in five fishes. These amino acid may decrease or increase the affinity of oxygen. Tab1 shows that the similarity of α globin in five fishes is consistent with phylogenetic relationships from morphological. The result was discrepancy with previous study of β globin in our laboratory. This may be α globin experience less selective pressure.

The first case of transgenic fish was obtained in 1985 in China. Following the development of DNA cloning and recombinant technology, transgenic fish is likely to into the market first in China[9]. It is urgent for improved breeds of fish with traits of high quality, fast-growing and resistance to disadvantage environmental factor in cultivation industry. Therefore, more and more study on transgenetic fish will be done in order to improve the quality and resistance. Increasing the capacity of resistance low oxygen content can improve not only the cultivation density but the affinity of oxygen and then boost economic benefit. So breeding trans-globin genes fish that can resist low oxygen content has enormous economic benefit and will open a broader vista.

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1) College of life sciences, Henan Normal University, Xinxiang Henan, 453002

Fig.1 The RT-PCR amplification result of total RNA in five fishes

1. 100bp ladder marker

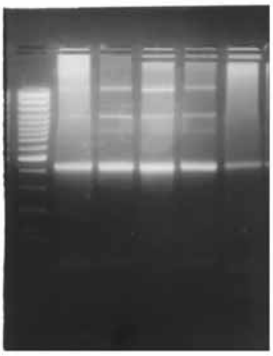
2. *Cyprinus carpio*

3. *Ctenopharyngodon idellus*

4. *Carassius auratus* Linnaeus

5. *Misgurnus anguillicaudatus*

6. *Paramisgurnus dabryanus*



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1    M S L S A R D K A A V K A L W A K I S S K S D D I G A E A L  C.carpio
1    M S L S D P D K A V V K A L W A K I G S R A D E I G A E A L  C.auratus
1    M S L T A R D K A V V K A L W S K I S S K A D E I G A E A L  C.idella
1    M S L S A R D K S V V K A L W G K I S S R A D D I G A E A L  M.anguillicaudatus
1    M S L S E Q D K S A V K A H W S K I S S R S D D I G A E A L  P.dabryanus

31   G R M L T V Y P Q T K T Y F A D W A D L S P G S G P V K K H  C.carpio
31   G R M L T V Y P Q T K T Y F S H W S D L S P G S G P V K K H  C.auratus
31   G R M L T V Y P Q T K T Y F S H W A D L S P G S G P V K K H  C.idella
31   G R M L T V Y P Q T K T Y F S H W A D L S P G S A P V K K H  M.anguillicaudatus
31   G R M L X V Y P Q T K T Y F S D W A D L S P G S A P V K K H  P.dabryanus

61   G K V I M G A V G D A V S K I D D L V G G L A S L S E L H R  C.carpio
61   G K T I M G A V G D A V S K I D D L V G A L S S L S E L H A  C.auratus
61   G K V I V A A V G D A V S K I D D L A G G L A A L S E L R A  C.idella
61   G K T I M G A V G E A E S K I D E V T G S L A A L S E L H A  M.anguillicaudatus
61   G K T I M G A V G E A V S K I D D L T G A L S A L S E L H A  P.dabryanus

91   S K L R V D P A N F K I F A H N V I V V I G M L S P G D F P  C.carpio
91   F N V R I D P A N F K I L A L N V I V V I G M H F P G D F T  C.auratus
91   F K L R V D P A N F K I L A H N L I V V I A M L F P A D F S  C.idella
91   F K L R I D P A N F K I L A T N L I V V I G M L F P G D F S  M.anguillicaudatus
91   F K L R I D P A N F K I L A T N L I V V I G M L F P G D F S  P.dabryanus

121  P E V H M S V D K F F Q N L A L A L S D K Y R  C.carpio
121  P E V H M S V D K F F Q N L A L A L S D K Y R  C.auratus
121  P E V H V S V D K F F Q N L A L A L S D K Y R  C.idella
121  P E V D V S V D K F F Q N L A L A L S E K Y R  M.anguillicaudatus
121  P E V H V S V D K F F Q N L A L A L S E K Y R  P.dabryanus
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Fig.2 The amino acid sequences of α-globin in five fishes

Tab.1 The similarity of α-globin amino acid sequence of five fishes

	C.idellus	*C.carpio*	*P.dabryanus*	*M.anguillicaudatus*	*C.auratus*
C.idellus	100%				
C.carpio	88.1%	100%			
P.dabryanus	83.2%	83.9%	100%		
M.anguillicaudatus	86.7%	85.3%	94.4%	100%	
C.auratus	86.0%	89.5%	86.7%	86.7%	100%

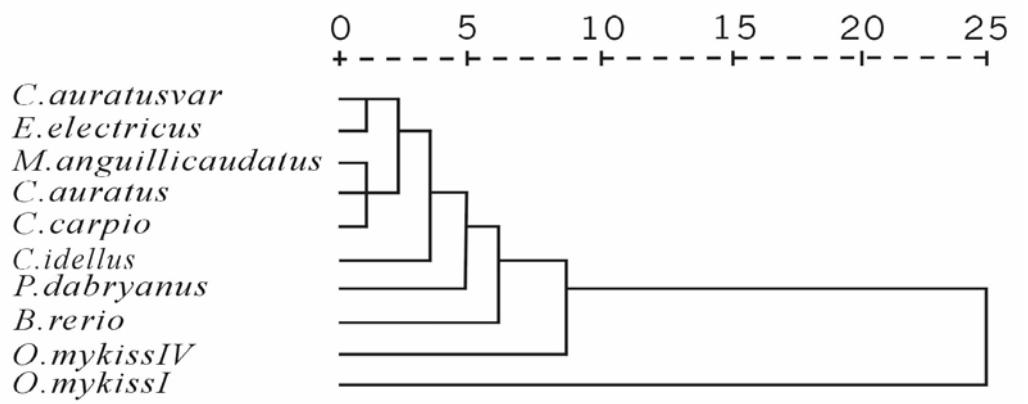

Fig.3 The dendrogram of ten fishes based on globin amino acid sequences similarity