STRUCTURE COMPARISON OF β GLOBIN GENE IN COMMON, CRUCIAN AND GRASS CARP

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Keywords: *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Carassius auratu*s, β globin gene, intron

Abstract: A pair of consensus degenerate primes was designed basing on N-terminal and C-terminal conservative amino acid of β chain. Using RT-PCR method, β globin cDNA was amplified and cloned from total RNA that was extracted from blood of three fishes, common carp (*Cyprinus carpio*), grass carp(*Ctenopharyngodon idellus*) *and crucian* (*Carassius auratus*). The result shows that all of the cDNA translation amino acid sequence extends for 441bp. Compared with the β globin amino acid sequences of three fishes, it can be concluded that despite they belong to *Cypriniformes* but the composition of amino acid of three fishes have a great difference. The special primers flanking the coding region were made based on the cDNA sequence and used in a PCR on genomic DNA to determine the presence, site, and size of introns in the three fishes β globin gene. The fragments of *Cyprinus carpio; Ctenopharyngodon idellus; Carassius auratus* β globin gene from initiation codon to ending codon is 667bp, 629 bp, 667 bp respectively. The genomic DNA has 3 extrons and 2 introns which insertion positions are B12.2 and G7.0. As far as length is concered, the length of intron 1 and 2 are completely identical. But compared with *Ctenopharyngodon idellus,* there have great difference in base pare.

INTRODUCTION

Fish is primordial aquicolous vertebrate and accounts for a half of existing vertebrate. Contrary with mammalian and bird, there exist diversity of Mb in fish, reptile and amphibian. The fishes of *cyprinoid* that widely distribute in Europe, Asian, north American and Africa are not only the larger fresh fishes but also an important target of capture. In Chain the product of fishes of *cyprinoid* which have important economic value account for 1/4 to 1/3 total gross and is one of the mainly protein resources for human. Fish has many specific character 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 about growth of fish. These sensitivity of oxygen is determined by amino acid sequence of globin gene. So it is important for fishery to try to elucidate globin gene structure.

MATERIALS AND METHODS

Material

Cyprinus carpio; Ctenopharyngodon idellus; Carassius auratus Linnaeus were bought in Xinxiang fair

Preparation of total RNA

Total RNA was extracted from blood using Promega RNAgents total RNA Isolation System kits. 0.2ml-0.3ml blood were taken by tail amputation and pellet the cells by centrifugation at 300g. Wash the cell pellet with sterile 1×PBS and repeat the centrifugation. Decant the supernatant, adding pre-chilled Denaturing solution. Homogenizing ensure that no fragments of clumps of cells are visible, adding 60μl of Sodium Acetate (pH4.0) mix thoroughly, adding 600μl Phenol:

chloroform: Isoamyl Alcohol to the tube carefully mix by inversion for 10s. Chill on ice for 15min and centrifuge at 12000rmp for 20min at 4°. Carefully remove the top aqueous phase to a fresh DEPC-treated tube and adding an equal volume of Isopropanol incubated the sample for 30min at -20°. Centrifugation at 12000rmp for 10min at 4°. Decant the supernatant and wash the pellet by adding 1ml of ice-cold 75% ethanol and centrifugation at 12000rmp for 10min at 4°. Air-dry the pellet and dissolve the RNA in 30µl Nuclease-free water.

Cloning of β globin cDNA

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 10mmolL dNTP mix, 5µl $MgSO_4$, 10µl 5× AMV/Tfl reaction buffer, 5U AMV Reverse Transcriptase, 5 U Tfl DNA Polymerase. Thermocycling was performed with 1 cycle of reverse transcription at 48°for 45min, 40 cycles of denature at 94° for 30s, annealing at 54°for 1min, extension at 72° for 2min. After 40 cycles, a final extension reaction was carried out at 72° for 10 min.

Upper primer:

5' GT_GTAC_GA(ATGC)TGGAC(ATGC)GA(CT)(CG)(AC)(ATGC)GA 3'

Down primer:

5' TG((AG)TA(CT)T(CG)(CT)CT(ATGC)CA(ATGC)A(AG)(ATGC)GC 3'

Amplified genomic DNA

PCR reaction were performed in a 20µl reaction volume containing 1µl DNA, 50pmol/L of each primer, 2mmol/L dNTP 2µl, 25 mmol/L $MgCl_2$ 2.0µl, 10×buffer 2µl TaqDNA Polymerase 1U. Thermocycling was performed with 1 cycle of reverse transcription at 95° for 5min, 35 cycles of denature at 94° for 30s, annealing at 58° for 1min, extension at 72° for 1min. After 35 cycles, a final extension reaction was carried out at 72° for 10 min.

Cyprinus carpio;

Primer 1 (amplification 5′part of globin gene)

Upper primer: 5′ATGATGGTGGAGTGGACGGA 3′

Down primer: 5′GGCCTTGATGTTGTCCATGT 3′

Primer 2 (amplification 3′part of globin gene)

Upper primer: 5′AGAGCCATCAAGAACATGGA 3′

Down primer: 5′CTAATGGTACTGTCTGCAGA 3′

Ctenopharyngodon idellus;

Upper primer: 5′ATGATGGTGGAGTGGACGGA 3′

Down primer: 5′CTAATGGTACTGTCTGCACA 3′

Carassius auratus

Primer 1 (amplification 5′part of globin gene)

Upper primer: 5′ATGATGGTGGAGTGGACGGA 3′

Down primer: 5′GGTGGCCTTGATGTTATCCA 3

Primer 2 (amplification 3′part of globin gene)

Upper primer: 5′AGAGCCATCAAGAACATGGA 3

Down primer: 5′CTAATGGTATTGTCTGCACA 3′

Subcloning and cDNA and DNA sequencing

PCR products were detected by running electrophoresis in a 1.5% agarose gel followed by ethidium-bromide (EB) staining. Purify the interest bands using DNA Gel Extraction Kits. The PCR products were directly ligated into pUCm-T vectors overnight at 4°, before transfomation, incubated it at room temperature for 1h. Adding 10μl ligated products into 200μl JM109 competent cells chilled on ice 30min, heat-shocked at 42°, adding 800μl SOC culture medium, incubated at 37° for 45min at 180rmp. 100μl spread on an LB solid plate which contain IPTG, X-Gal, Amp overnight at 37□. Put white clone in 3ml liquid LB culture medium overnight at 37°. Extracted of plasmid DNA using alkaline lysis. Detect positive clone. Sequencing DNA using dideoxy chain termination method. Sequence similarity searches were done using the BLAST algorithm in GenBank.

RESULTS

RT-PCR amplification

A pair of degenerate primers were used to amplify the total RNA and a about 450 nucleotide band was obtained in three fishes which length were consistent with designed (Fig.1). In *C.carpio* the band which length were about 3000bp was non-specific amplification.

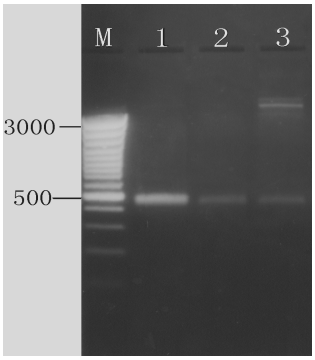

Fig.1 The RT–PCR amplification result of β globin cDNA in three fishes
M. 100bp ladder marker, 1.*C.idellus,* 2.*C.auratus,* 3. *C.carpio*

Sequncing of positive clone

The results of the positive clone show that the nucleotide are 441 in *C.carpio, C.idellus ,C. auratus* (accession number in GeneBank is AF528161_F528160_AF528159). the A+T residues of β globin coding sequence of *C.idellus*, *C.carpio*, *C.auratus* is 44.52--44.29--45.45

respectively.

Hb mode NA ←-----A-----→←-------B-----→←--C-→ CD ←

C.idellus

VEWTDDERTAILGLWGKLNIDEIGPQALSRCLIVYPWTQRYFATFGNLSS 1-50

C.carpio

VEWTDEDRSAIIGLWGKLNPDELGPQALARCLIVYPWTQRYFASFGNLSS

C.auratus

VEWTDAERSAIIGLWGKLNPDELGPQALARCLIVYPWTQRYSATFGNLSS

Hb mode --D-→←-------E-------→ EF ←--F--→ FG

C.idellus

PAAIIGNPKVAAHGKTVMGGLERAIKNLDNIKATYSALSVMHSEKLHVD 51-99

C.carpio

PAAIMDNPKVAAHGRTVMGGLERAIKNMDNIKATYAPLSVMHSEKLRVD

C.auratus

PAAIMGNPKVAAHGRTVMGGLERAIKNMDNIKATYAPLSVMHSEKLHVD

Hb mode ←-------G------→ GH ←--------H---------→ HC

C.idellus

PDNFRLLADCITVCAAMKFGPSGFNADVQEAWQKFLSVVVSALCRQYH 100-147

C.carpio

PDNFRLLADCITVCAAMKFGPSGFSANVQEAWQKFLSVVVSALCRQYH

C.auratus

PDNFRLLADCITVCAAMKFGPSGFNADVQEAWQKFLCVVVSALCRQYH

Fig.2 The β globin amino acid sequences of three fishes

Amplification of β globin gene

In order to character the structure of β globin gene at DNA lever, genomic DNA were amplified using special primers based on cDNA sequence. The results show that 350bp band we obtained in *C.carpio* and *C.auratus* whereas 620bp band were obtained in *C.idellus*. The other bands were non-specific amplification.

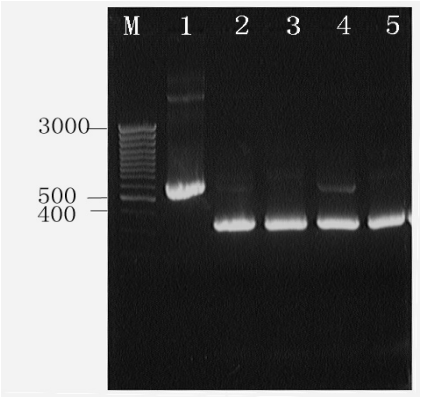

Fig.3 The amplification result of β globin intron 1and 2 in three fishes
M.100bp ladder, 1. *C. idellus*, 2. *C. carpio* gene 5′, 3. *C. carpio* gene 3′, 4. *C. auratus* gene 5′, 5. *C. auratus* gene 3′

DNA Sequence Analysis

Fig. 4 shows that the 441bp coding sequence is interrupted by two intones, the first splitting codon 30 and the second separating codons 105 and 106. Both introns are extremely rich in A+T residues. In *C.carpio*,*C.auratus* and *C.idellus* intron 1 is 102bp,102bp,1102bp in length and 65.59 , 65.69 , 62.83 A+T respectively, whereas intron 2 is 121bp,121bp, 73bp long and 67.77 , 71.07 , 63.89 A+T respectively. The splice junctions of both introns conform to the GT/AG rule, containing GT at their 5′donor junctions and AG at their 3′acceptor sites.

amino acid sequence----intron 1-------

C.idellus VEWTD……QALAR--- gtacaactgcatcagattctttatagacgcatcctataat 1-40
C.carpio VEWTD……QALAR--- gtatcattgcatctcagtctctaatagacacattctccgt
C.auratus VEWTD……QALAR--- gtatcattgcatcacattctttaatagacacaatcttcgt

C.idellus gactccctctgttacatgatgtcagctactcagtttatttaatgccgttcttaatgctt 41-99
C.carpio gttagatgatgtcagttgcgcagtttatttaattctgttcttaatgattctgttcattt
C.auratus gttagatgatgtcagctgcgcagtttatttaatgctgttcttcatgattctgtctattt

intron 1------amino acid sequence ---intron 2-----

C.idellus ctgatttttttaag---CLIVY……PDNFR-----gtaggttgtgctcatattatgtt 100-112
C.carpio aag----------CLIVY……PDNFR-----gtaggtttgagcaaataaaacta 1-23
C.auratus aag----------CLIVY……PDNFR-----gtaggtttgagaaaataaaacta

C.idellus gaagaatcatcaccgaaaaacagcaccttaatagatctctttctctacag ---------

C.carpio aaatttgcgtgatgtaaatatagaaaactgttgtgctcatgttatgtcaaatcaacttc 24-82
C.auratus atatttgcggattgtaaatatagaaaacgattgtgctcatattatgttaaatcaacttg

Intron 2------amino acid sequence

C.idellus ----------LLADC 83-121
C.carpio atcgaaaaacacacacctaatagatctcatgttctacag----LLADC
C.auratus atcgaaaaacacccactaaatggatctcatgttttacag----LLADC

Fig.4 The β globin intron sequences of three fishes

DISSCUSION

Globin Genes have so far been analyzed in many vertebrates, but the study of globin genes in fishes have been limited, to date, to the protein level. Fish is primordial vertebrate and it has many specific character adapted for variety physiological needs.

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)(Barra D, *et al.* 1983). 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 *Notothenііdea* (Antarctic rockcods) generally possess a major hemoglobin, Hb1, and a second, minor hemoglobin, Hb2, that differ in theirαchains (α1 and α2 respectively)(Fago,A.,*et al*. 1992). The more phyletically derived harpagiferids and bathydraconids have a 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(Ennio C. *et al*. 1995). The three species in this paper belong to *cyprinoid*. 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 β globin is different not only in suffocate point which in *C. carpio; C. idellus; C. auratus* is 0.34-0.3mg/L, 0.51-0.3mg/L, 0.13-0.11mg/L respectively but in amino acid sequence(). The position CD1Phe is vital to the ability of affinity for oxygen in *C. carpio; C. idellus* but in *C. auratus* it is replaced by a Ser residue which has a shorter side chain. This substitution is unprecedented. Phe is hydrophobic by virtue of its aromatic rings but Ser is polar amino acid due to the reactive hydroxyl group in the side-chain, and can also participate in hydrogen bonding. This key residue may concern with the low oxygen resistance of *C. auratus*.

The position of NA2βGlu is located in the organicphosphate binding pocket. The presence of a

Glu which has a shorter side chain in this position allows GTP to establish an additional hydrogen bond. But in *M.helena* HbI which the effect of GTP is significantly higher than that of ATP at the same concentration shows Glu in NA2β. The same dose not apply to *M.helena* HbIII which shows Glu in NA2β despite its similar respose to ATP and GTP (Mariagiuseppina P. *et al*. 1995). This point that there may exist a more complex mechanism than ever reckon.

The coding sequence of three fishes are relatively A+T-poor and quite similar in composition in their taxa. By contrary, the two introns are similarly A+T-rich residues. This result is identical with the A+T content of α globin in notothenioid *N.coriiceps*(Yuqiong Zhao, *et al*. 1998). A+T-rich genomes may facilitate DNA strand separation during transcription and replication in low temperature regimes[7]. This selective dominance is important to its survive in those cold area.

CONCLUSIONS

The ORF of β globin cDNA in *Cyprinus carpio*, *Ctenopharyngodon idellus* and *Carassius auratus* extends for 441bp and codes 147 amino acids. The composition of amino acid of three fishes have a great difference. In the genomic DNA, The fragments of β globin gene from initiation codon to ending codon is 667bp, 629 bp, 667 bp respectively. The genomic DNA has 3 extrons and 2 introns which insertion positions are B12.2 and G7.0. The length of intron 1 and 2 are completely identical. But the composition of them is very different.

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