



## Characterization and expression of the calpastatin gene in *Cyprinus carpio*

W.X. Chen<sup>1,2</sup> and Y. Ma<sup>1</sup>

<sup>1</sup>School of Food Science and Technology, Harbin Institute of Technology, Harbin, China

<sup>2</sup>Animal Institute of Science and Technology, Northeast Agricultural University, Harbin, China

Corresponding authors: Y. Ma / W.X. Chen

E-mail: maying@hit.edu.cn / weixing9951@126.com

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**ABSTRACT.** Calpastatin, an important protein used to regulate meat quality traits in animals, is encoded by the *CAST* gene. The aim of the present study was to clone the cDNA sequence of the *CAST* gene and detect the expression of *CAST* in the tissues of *Cyprinus carpio*. The cDNA of the *C. carpio CAST* gene, amplified using rapid amplification of cDNA ends PCR, is 2834 bp in length (accession No. JX275386), contains a 2634-bp open reading frame, and encodes a protein with 877 amino acid residues. The amino acid sequence of the *C. carpio CAST* gene was 88, 80, and 59% identical to the sequences observed in grass carp, zebrafish, and other fish, respectively. The *C. carpio CAST* was observed to contain four conserved domains with 54 serine phosphorylation loci, 28 threonine phosphorylation loci, 1 tyrosine phosphorylation loci, and 6 specific protein kinase C phosphorylation loci. The *CAST* gene showed widespread expression in different tissues of *C. carpio*. Surprisingly, the relative expression of the *CAST* transcript in the

muscle and heart tissues of *C. carpio* was significantly higher than in other tissues ( $P < 0.01$ ).

**Key words:** *Cyprinus carpio*; Real-time PCR; Tissue expression; Calpastatin

## INTRODUCTION

China is not only the world's biggest fish producing nation; it also consumes more fish products than any other nation in the world. The quality and consumer acceptance of fishery products are largely affected by meat tenderness, water-holding capacity, and composition. Hence, meat quality has become the primary target of breeding programs (Harris and Newman, 1994).

Rather than increasing growth strictly by measuring weight gain as a trait, it is of greater benefit to select a production trait, such as efficient muscle accretion, maintenance of protein turnover, and meat tenderness (Overturf and Gaylord, 2009). Regulation of protein turnover in muscle most likely plays a significant role in the variation of growth rate. The imbalance of increased protein synthesis and control protein degradation will lead to muscle growth (Klasing et al., 1987). Muscle growth in vertebrates is the synergistic response of both muscle synthesis and degradation. However, digestion through the action of nonlysosomal intracellular calcium-dependent calpains is an important pathway of protein degradation and cellular turnover (Overturf and Gaylord, 2009). The calpain system includes a large family of calcium-dependent cysteine proteases and the endogenous calpain inhibitor calpastatin (CAST) (Goll et al., 2003; Wendt et al., 2004). Presently, calpains have been extensively studied because of their association with meat quality and it is widely accepted that proteolytic calpain activity contributes to meat tenderization (Sentandreu et al., 2002; Koohmaraie and Geesink, 2006).

CAST is a specific endogenous inhibitor of calpain, regulating calpain activity *in vivo*, and is known to be restricted to vertebrates (Carragher et al., 2002; Goll et al., 2003). CAST inhibits both the *CAPN1* and the *CAPN2* genes, a process that requires calcium concentrations that are reported to be close to or below those that are required to activate calpain (Goll et al., 2003). A consistent observation of the calpain system's involvement in meat tenderness is that high levels of CAST are associated with poor quality meat. The model being that high levels of CAST reduce the activity of calpain thereby reducing the proteolysis required for tender meat. Some studies have reported that increased activity of CAST in muscles was accompanied with reduced protein degradation (Morgan et al., 1993; Killefer and Koohmaraie, 1994).

To date, a few studies have been performed in some animals to quantify the expression of the *CAST* gene. *CAST* was expressed at the highest levels in the muscles of rabbit and chicken from younger animals and expression decreased with increasing age (Ou and Forsberg, 1990; Sams and Birkhold, 1992). In chicken, *CAST* expression may be related to muscle fiber development (Zhang et al., 2012). Lepage and Bruce (2008) reported the characterization and comparative expression of calpain in zebrafish. Overturf and Gaylord (2009) compared the expression of the *CAST* gene in the muscle of fish fed fishmeal with plant protein diets and suggested that diets replacing fishmeal with plant material can have some effects on protein turnover in the muscle of rainbow trout. However, no work has been conducted to date regarding the molecular characterization and expression of the *CAST* gene in *Cyprinus carpio*. The aim of this study was to characterize the *CAST* gene in *C. carpio* and to compare expression levels among different tissues.

## MATERIAL AND METHODS

### Animal material

Six *C. carpio* from the fisheries technology extension station in Heilongjiang Province were used in this study. Tissue samples (including brain, hepatopancreas, mesonephros, muscle, heart, spleen, intestines, and gonad) were snap-frozen in liquid nitrogen and then stored at -80°C for total RNA isolation.

### RNA isolation and cloning of the cDNA fragment

Total RNA was isolated from the brain, hepatopancreas, mesonephros, muscle, heart, spleen, intestines, and gonad tissues using the TRIzol reagent (TaKaRa Biotechnology (Dalian) Co., China). The quality of RNA was determined via a 1% formaldehyde agarose gel. Isolated RNA was treated with 8 µL DNase (TaKaRa Biotechnology (Dalian), Co., for 20 min at 37°C and stored at -80°C.

cDNA was synthesized using PrimeScript Reverse Transcriptase (TaKaRa Biotechnology (Dalian) Co) according to the manufacturer protocol. The first reaction was performed in a 10-µL volume containing 5 µL total RNA, 1 µL oligo-dT, 1 µL of each dNTP, and 3 µL ddH<sub>2</sub>O. The reverse transcription was maintained at 65°C for 5 min, and in ice for 2 min. The second reaction was performed in a 20-µL volume containing 10 µL mixture from the first reaction, 4 µL PrimerScript buffer, 0.5 µL RNase inhibitor, 1 µL PrimeScript reverse transcriptase, and 4.5 µL ddH<sub>2</sub>O. The reverse transcription was maintained at 30°C for 10 min, 42°C for 60 min, 70°C for 10 min, and ended with ice for 2 min. The cDNA product was stored at -20°C.

Based on the conserved region of the *CAST* gene in zebrafish (accession No. NM\_001130591), three pairs of primers (Table 1) were designed to amplify a specific coding region of *CAST*. PCR was carried out in a 50 µL reaction system containing 2.0 µL first strand cDNA, 0.5 µL TaKaRa LA Taq, 1.0 µL 10 pmol forward primers, 1.0 µL 10 pM reverse primers, 8.0 µL 2.5 mM dNTP mixture, 5.0 µL 10X PCR buffer, and 32.5 µL ddH<sub>2</sub>O. The PCR cycling conditions were pre-denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products obtained were separated on an agarose gel, and then purified using an agarose gel purification kit. The purified PCR products were ligated to a pMD18-T vector, followed by sequencing by the BGI Biotechnology Company, China.

### 3'RACE and 5'RACE

Based on the sequence of the cDNA fragment cloned above, four specific primers (Table 1), two specific outer primers (CAST5R1, CAST3R1) and two specific inner primers (CAST5R2, CAST3R2), were designed to clone the complete cDNA sequence of *CAST* by rapid amplification of cDNA ends (RACE) methods. The 3'RACE-PCR includes an outer PCR and an inner PCR. In 3'RACE-PCR, the PCR was performed with the 3'RACE outer primer, the 3'RACE inner primer, CAST3R1, CAST3R2, and the adaptor primer (Table 1). The PCR profile was as follows: 94°C for 3 min, 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min (20 cycles for outer PCR and 30 cycles for inner PCR) and a final step of 72°C for 10

min. For 5'RACE-PCR, according to the 5'-Full RACE kit, the experimental process includes dephosphorylation treatment, reaction to the hat, connection of the 5'RACE adaptor, the reverse transcription reaction, outer PCR, and inner PCR. The PCR products were subjected to electrophoresis, ligation transformation in a pMD18-T vector, and sequencing as described above. The resulting sequences were verified and subjected to cluster analysis.

**Table 1.** Primers used for cloning and RT-qPCR of the *CAST* gene in *Cyprinus carpio*.

Primer name	Nucleotide sequence (5'→3')	TM (°C)
CAST1	TCAACACCTGCATCTTCTGC TTGAGCTGGAGACACAAACG	55.3
CAST2	CGGATACAGAAAGGAAGACCTG AGTATGTCCATCGCATCATCTG	54.8
CAST3	GAACACAGATGATGCGATGG TGATCTGGTGGAAGGGAGTC	55.6
3'RACE Outer Primer	TACCGTCGTCCACTAGTGATTT	60.0
3'RACE Inner Primer	CGCGGATCCTCCACTAGTGATTCACTATAGG	62.0
CAST3R1	GTTTGTCTGCCTCTAAGTCT	60.0
CAST3R2	TTCAGTCTGCCGTCTCTGGCCCC	60.2
CAST5R1	GGTTGTGATGATGGTAAAGTGC	59.9
CAST5R2	CCTTATCTTTCAGAGGGTCCGT	59.8
CASTY	CATCTGTTGTCTGCCTCTAAG GATGAGGAACCTCTGACAACTG	60.1
β-actin	CGACATCCGTAAGGACCTGT GCTGGAAGGTGGACAGAGAG	60.1

TM = annealing temperature.

### Real-time quantitative PCR (RT-qPCR) assay for *CAST*

Expression of *CAST* was analyzed with the housekeeping gene *β-actin* (EU664997) as an endogenous control. Real-time PCR was carried out on a 96-well optical reaction plate and a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The amplifications were performed in 20 μL reaction mixtures containing 10.0 μL SYBR Premix Ex Taq™ II (TaKaRa Biotechnology (Dalian) Co.), 2.0 μL cDNA from the equivalent of 50 ng total RNA), 0.4 μL 1 μM forward primer, 0.4 μL 1 μM reverse primer, and 6.4 μL distilled water. The thermal cycling parameters were set as follows: initial activation of the DNA polymerase at 95°C for 30 s, 40 cycles consisting of denaturation at 95°C for 5 s, and primer annealing and extension at 60°C for 34 s. After real-time PCR, the products were immediately run through a dissociation curve assay to confirm specific amplifications. Relative expression of target genes was calculated using the  $2^{-\Delta\Delta C_t}$  method based on the threshold cycle ( $C_t$ ) values, as described by Livak and Schmittgen (2001).

### Sequence analysis

Nucleotides of the *C. carpio CAST* gene, as well as the derived amino acid sequences, were aligned with other reported *CAST* sequences from different species using the online BLAST search tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and DNAMAN 6.0 (Wang et al., 2011) software. The phylogenetic tree was constructed by the neighbor-joining (NJ) method using the programs CLUSTAL X1.83 (Lepage and Bruce, 2008) and MEGA4 (Tamura et al., 2007). The conserved domain of the *C. carpio CAST* protein was analyzed using the NCBI CD-Search tool, and the secondary structure was predicted using GOR IV (<http://gor.bb.iastate>).

edu/) and ExPASy ProtScale (<http://web.expasy.org/protscale/>) software. Phosphorylation loci were predicted using NetPhos 2.0 Server (<http://www.cbs.dtu.dk/services/NetPhos/>) and NetPhosK 1.0 Server (<http://www.cbs.dtu.dk/services/NetPhosK/>) software.

### Statistical analysis

Data are reported as means  $\pm$  SD and were analyzed using the SPSS 16.0 for Windows software (SPSS Integral Solutions Limited, Chicago, USA). Gene expression levels of the tissues were analyzed by one-way ANOVA and the Student *t*-test. A *P* value  $<0.05$  was considered as statistically significant, and a *P* value  $<0.01$  was considered as highly statistically significant.

## RESULTS

### Cloning and sequence analysis of the *CAST* gene

A 2834-bp nucleotide sequence representing the complete cDNA sequence of *CAST* was obtained by overlapping three fragments, and the sequence was deposited in GenBank (accession No. JX275386). The complete sequence of *CAST* cDNA contains a 5'-untranslated region (UTR) of 10 bp, a 3'-UTR of 190 bp with a canonical polyadenylation signal sequence AATAAA and a poly A tail, and an open reading frame of 2634 bp encoding a polypeptide of 877 amino acids with a predicted molecular weight of 91,186.79 Da and a theoretical isoelectric point of 5.23. Its chemical formula is  $C_{3986}H_{6419}N_{1079}O_{1340}S_9$  and the total number of atoms is 12,833.

### Secondary structure analysis of the *C. carpio* CAST protein

Sequence alignment revealed that the amino acid sequence of the *C. carpio* CAST protein was 88, 80, and 59% identical to the CAST sequence in grass carp, zebrafish, and other fish CAST (Figure 1). Multiple sequence alignments revealed that the *C. carpio* CAST contains four typical conserved domains (Figure 2) with a seven-peptide sequence composed of Thr-Leu-Pro-Pro-X-Tyr-Arg (T-L-P-P-X-Y-R) (Figure 3), which is a family-protein characteristic and a possible functional domain (Emori et al., 1987).

Prediction of the encoded *C. carpio* CAST protein using GOR IV and the ExPASy ProtScale software showed that the random coil, alpha helix, extended strand, and a Beta turn account for 65.11, 20.07, 8.78 and 6.04% of the secondary structure, respectively, and it contains rich hydrophobic regions and certain phosphorylation sites, including 54 serine, 28 threonine, and 1 tyrosine site (Figure 4). There are six specific protein kinase C (PKC) phosphorylation loci at sites 50, 68, 87, 167, 353, and 815.

### Tissue expression analysis of the *CAST* gene

The *CAST* gene was expressed in all tissues collected from *C. carpio* (brain, hepatopancreas, mesonephros, muscle, heart, spleen, intestines, and gonad) analyzed in this study. The *C. carpio* *CAST* transcript showed the highest expression in muscle, followed by heart, intestines, brain, gonad, and mesonephros, with the lowest expression observed in the hepatopancreas and spleen (Figure 5). In particular, the relative expression of the *CAST* transcript in muscle and heart tissue was significantly higher than the expression in other tissues ( $P < 0.01$ ; Table 2).

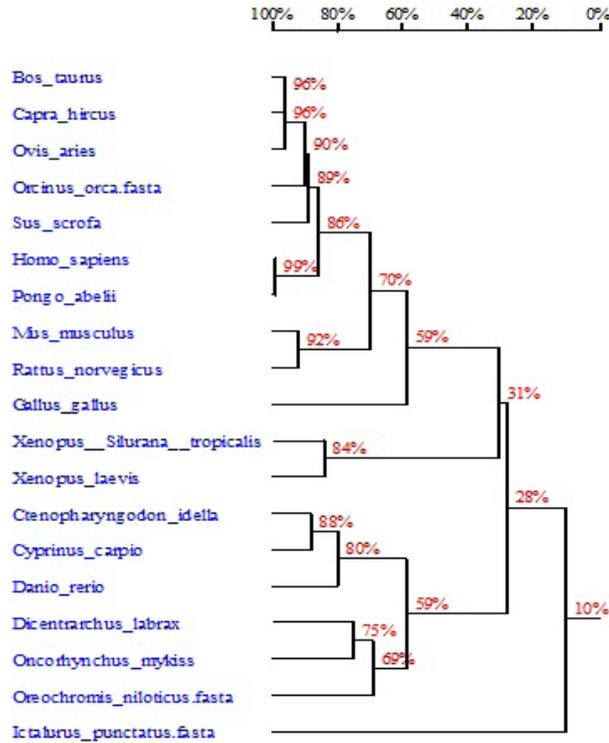


Figure 1. Homologous tree of the *CAST* gene in *Cyprinus carpio* and related species. Values represent level of similarity.

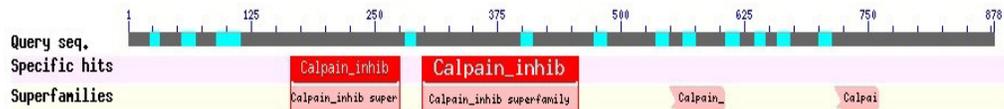


Figure 2. Four conserved domains within the *CAST* protein of *Cyprinus carpio*.

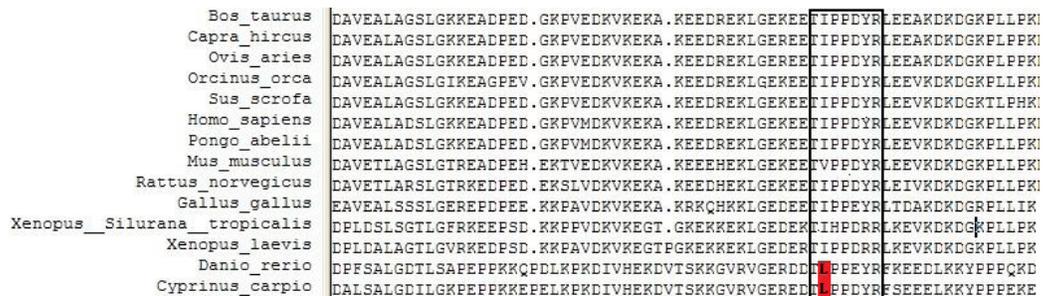
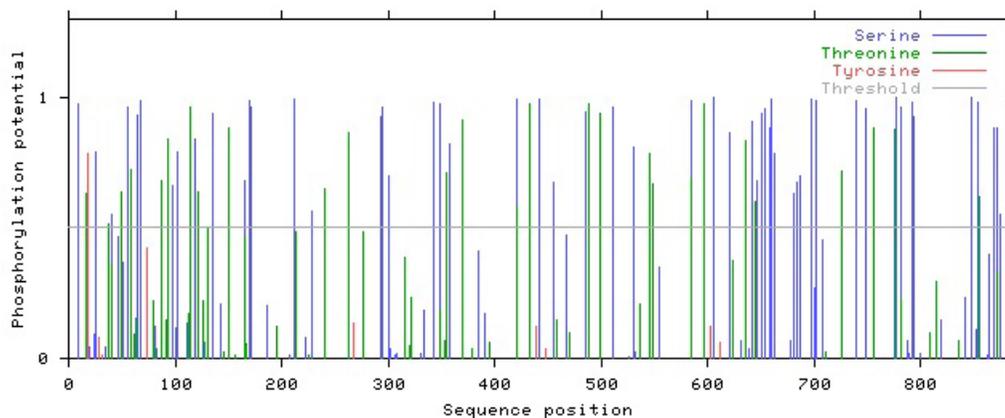


Figure 3. Conserved domains and seven-peptide sequences (within box) within the *CAST* protein of *Cyprinus carpio* and other species.

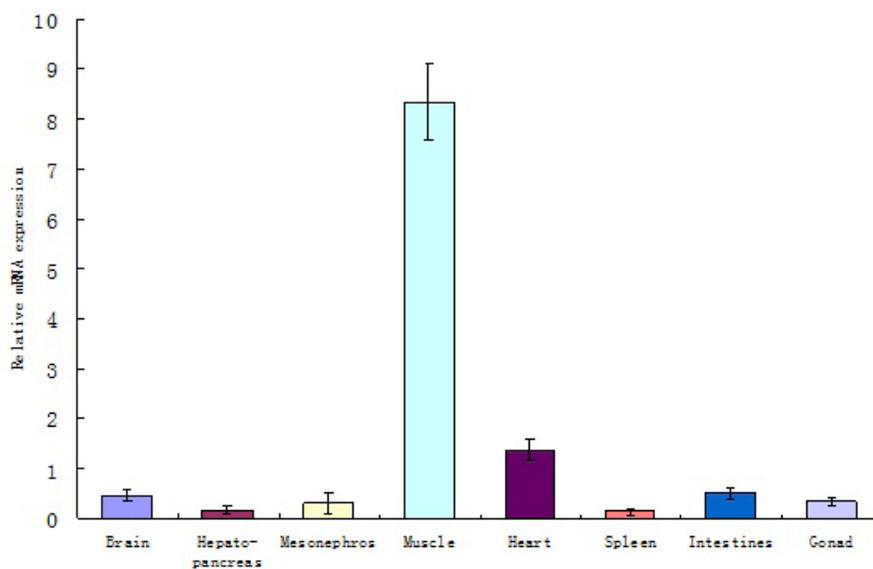


**Figure 4.** Phosphorylation sites in the sequence of the *Cyprinus carpio* CAST protein.

**Table 2.** Multiple comparison analysis of *CAST* gene expression in different tissues of *Cyprinus carpio*.

	Tissues							
	Brain	Hepato-pancreas	Mesonephros	Muscle	Heart	Spleen	Intestines	Gonad
Relative mRNA expression	0.4549 ± 0.1265 <sup>Abc</sup>	0.1728 ± 0.0810 <sup>Ba</sup>	0.3105 ± 0.2059 <sup>ABabd</sup>	8.3422 ± 0.7541 <sup>C</sup>	1.3762 ± 0.2202 <sup>D</sup>	0.1414 ± 0.0575 <sup>Ba</sup>	0.5114 ± 0.1280 <sup>Ac</sup>	0.3403 ± 0.0664 <sup>ABbcd</sup>

Data are reported as means ± SD. \*Superscript capital letters indicate highly significant difference ( $P < 0.01$ ) and small letters indicate significant difference ( $P < 0.05$ ).

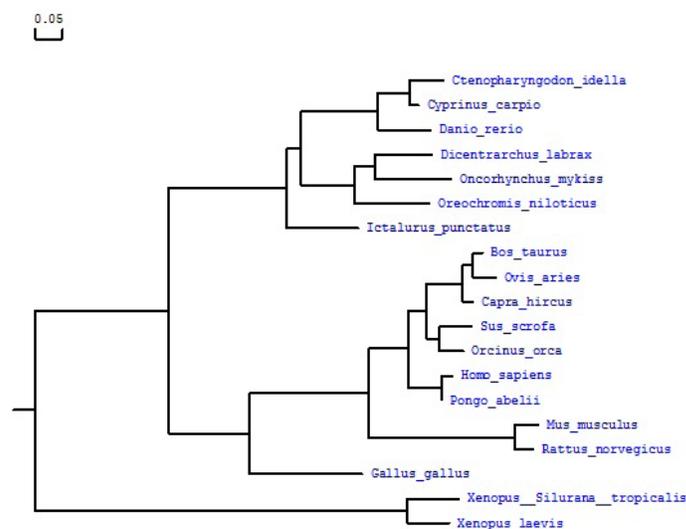


**Figure 5.** Relative expression of *CAST* in different tissues of *Cyprinus carpio*.

## DISCUSSION

The *CAST* gene plays a key role in meat quality traits, especially in regards to the tenderization of animal meat. In the last few years, the *CAST* gene has been widely studied as a candidate gene to regulate meat quality and growth traits. Smith et al. (2009) verified single nucleotide polymorphisms (SNP) in the *CAST* gene that were associated with major gene effects for meat tenderness in Brahman steers. Curi et al. (2010) evaluated carcass and meat traits in *Bos indicus* and *Bos taurus-Bos indicus* cross beef cattle using *CAST* SNPs. Furthermore, Li et al. (2013) detected the *CAST* gene and found 25 SNPs, of which the E1-1, E1-2, and C3-1 loci were correlated with meat tenderness height and were highly correlated with fatty acid and amino acid content. However, the E4-2 locus was not correlated with meat tenderness; it was correlated with cooking loss, brightness, and yellowness, among other traits (Li et al., 2013). The exon 28 genotypes of *CAST* showing allele frequencies of C (0.429) and T (0.571) were found to be significantly associated with calpastatin activity and Warner-Bratzler Shear Force (Chung and Davis, 2012). Zhang et al. (2012) detected the expression of *CAST* in two Chinese chicken breeds using RT-qPCR, and found that the *CAST* mRNA level exhibited a “rise-decline-rise-decline” developmental change in breast muscle and liver, with the highest expression observed in 2 week old chicken and the lowest expression in 8 week old chicken. Gandolfi et al. (2011) investigated the effect of *CAST* polymorphism on the activity of native and autolyzed  $\mu$ -calpain and m-calpain, measured from 1 to 72 h post-mortem in pig. Higher *CAST* expression was found in the longissimus dorsi with high shear force, confirming a direct role for calpastatin in meat tenderization, thus, the *CAST* gene affected post-mortem activation time of calpain and drip loss (Gandolfi et al., 2011).

In this study, according to the *CAST* mRNA sequence of zebrafish, the cDNA of the *C. carpio* *CAST* gene was successfully cloned using RACE technology, a rapid and efficient technology that has been successfully applied to gene cloning. Phylogenetic tree analysis based on the *CAST* sequence revealed that *C. carpio* was grouped with other fish into a single cluster with nearest distance (Figure 6).



**Figure 6.** Phylogenetic cluster of *Cyprinus carpio* and related species based on the *CAST* gene sequence.

Croall and DeMartino (1991) pointed out there are five structural domains in calpastatin, but the first domain does not exist in some cells and its function is as yet unclear. The remaining four domains are four similar structural repeating units with 20-35% homology of the amino acid residue, and each repeating unit contains three similar conserved regions (Croall and DeMartino, 1991). Within these domains, there are three regions, A, B, and C, that are predicted to interact with calpain, and a seven-peptide sequence consisting of Thr-Leu-Pro-Pro-X-Tyr-Arg is very conservative and may be a key functional area (Emori et al., 1987). The present study showed that there are also four conserved domains in *C. carpio* calpastatin, with a conserved seven-peptide sequence in domain C. However, the peptide "Ile" in the seven-peptide sequence transformed to the peptide "Leu" in fish (Figure 3). These results reveal the accuracy of the *CAST* gene cloning; however, its functional mechanism still requires further study.

In the process of the *CAPN-CAST* interaction, phosphorylation and dephosphorylation of *CAST* plays a very important role. PKC, as a multi-functional kinase of serine and threonine, can promote phosphorylation of serine and threonine, and affects the activity of calpastatin as well as cellular metabolism, differentiation, and proliferation (Nishizuka, 1986; Aversa et al., 1999). In this study, the results showed that there are six specific PKC phosphorylation loci; however, the mechanisms of *CAST* phosphorylation, PKC-mediated signal transduction pathways, and complex formation of *CAPN-CAST* are not clear yet.

A better understanding of the exact nature of the relationship between muscle protein synthesis and muscle protein degradation and net muscle growth is required. Wheeler and Koochmarai (1992) have reported that in living muscle, when protein degradation was reduced, the activity of calpastatin was increased. *CAST* gene expression has been shown to be regulated via several promoters (Meyers and Beaver, 2008). The difference between the transcriptional activity of the *CAST* promoters among species and their differing response to stimuli is probably, in part, responsible for the variation in *CAST* gene expression among species, breeds, and individuals, which contributes to variations in meat tenderness. It is known that a SNP may modify levels of gene expression because a polymorphism in the coding region may be in linkage disequilibrium with a polymorphism in the promoter region, affecting the efficiency of transcription or creating alternative splicing sites (Debeljak et al., 2000; Muráni et al., 2009). The strong correlation between calpastatin activity and tenderness has been well described in pigs (Kemp et al., 2010). The lower expression of the *CAST* gene observed in pigs was manifested by a marked improvement in meat quality, arising from the rate of glycolytic and energy metabolism, which in effect results in higher culinary and technological value (Sieczkowska et al., 2010). Parr et al. (1999) and Sensky et al. (1998), studying a random selection of commercially slaughtered pigs, demonstrated that high levels of calpastatin in the first few hours after slaughter were associated with an increased incidence of toughness.

In this study, the *CAST* gene showed widespread expression in different tissues of *C. carpio*; however, the specific differences observed among tissues may be due to the cell composition of the tissue. The relative expression of *CAST* was 80 times higher in muscle than in the other tissues. This issue is worth further investigation. The present study on the *CAST* gene and derived protein provides an important theoretical basis for further research on the function and application of *CAST* in *C. carpio*.

In conclusion, calpastatin has an important role in influencing meat tenderization and acts as a marker for meat quality. *CAST* expression is related to muscle fiber development (Zhang et al., 2012). These results are necessary to understand the effects of *CAST* on the regulation of muscle protein metabolism and for defining the biological significance of degradation

of the myofibrillar proteins in *C. carpio*. *CAST* gene expression could be one factor regulating calpastatin activity, and further analysis at the gene expression level may provide information on tenderness in the flesh of fish.

### Conflicts of interest

The authors declare no conflict of interest.

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