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ANALYSIS OF SEQUENCE VARIATIONS OF CALPASTATIN GENE OF INHIBITORY REGION IN CYPRINUS CARPIO VAR. COMMUNIS

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ABSTRACT

Calpastatin gene, an endogenous inhibitor of calpain enzyme, is important for maintaining muscle texture and postmortem degradation of myofibrils. Present study was carried out for the identification of SNPs in the inhibitory region of calpastatin gene in common carp *Cyprinus carpio* var. *communis* for genetic improvement of genotypes. Primers were designed from cDNA sequences of Atlantic salmon (AFV08638.1), Rainbow trout (NP_001118010.1) and cDNA as well as genomic DNA sequences of Zebra fish (NM_001130591.2) to amplify the CAST gene in Common carps. Four exons (13, 14, 19 and 20) and intervening introns demarcated by zebrafish genomic data base of 316 and 311 base pairs were amplified. The sequencing and insilico translation was done for the identification and characterization calpastatin. The results showed that *Cyprinus carpio* var. *communis* have two highly conserved sequence motifs DTLPP, GYR repeats in the inhibitory domain which corresponds to the exon-14 and exon-20 of Zebra fish. The single amino acid change in the inhibitory motif G (GGT)/X (X = Q 394aa (CAA) in *Danio rio*, Sequence ID: ref|NP_001124063.2, E 491aa (GAG) in *Salmo salar*, Sequence ID: ref|NP_001167162.1, K 223aa (AAG) in *Onchorhyncus mykiss* long isoform, Sequence ID: ref|NP_001118010.1, and E 54aa (GAG) in *Onchorhyncus mykiss* short isoform, Sequence ID: ref|NP_001118117.1) have role in the diversification of inhibitory activity of the protein due to species diversification. Another interesting characteristic feature of the inhibitory motif of exon-20 in Common carps having Glysine (GGT) amino acid and rest instead of Glutamic acid (GAG) at 548th aa in *Danio rio* and 689th aa *Salmo salar*. It was also observed that Common carp shear (94-95%) CAST gene sequence with Zebra fish while as only (52-54%) sequence homology was observed with Atlantic salmon and Rainbow trout due to species divergence. Phylogenetic analyses of different species revealed telosts are more primitive than tetrapods. Variations observed in inhibitory domain play important role in postmortem proteolysis. Development of markers for meat tenderness and fillet quality based on the mutations in the inhibitory region will help in selecting genotypes of better fillet/texture quality.

KEYWORDS: Calpastatin gene; Inhibitory region; Identification; Characterization; Telosts; Mutation.

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1. INTRODUCTION

Fish forms cheapest source of quality of protein with proportionate essential amino acids and well balanced omega -3 polyunsaturated fatty acids. For this reason it finds its place only next to the proteins present in milk and eggs. Protein content in fish varies between 16-21 g per 100 g meat with high biological value of 0.75 to 8.0 [1]. Postmortem changes in fishes influence the characteristics of structural myofibrillar proteins which undergo a rapid deterioration leading to changes in their solubility. Moreover, aggregation of water soluble proteins leads to exposure of hydrophobic groups and decrease in myofibrillar proteins and unfolding of their tertiary structure. Post-rigor flesh progressively turns tough due to the development of permanent cross-bridges form between myosin and actin [2] and formation of acto-myosin complex. The possible protein - lipid interactions ensure alteration in the functional property as well.

Studies have shown calpastatin gene is known to have a characteristic property of keeping fish muscles tender by inhibiting calpain, a protein responsible for postmortem fillet gappinhg in Atlantic salmon. The gene is represented by 106 expression sequence tag (EST) from 16 cDNA libraries, codes for 937 amino acids with two inhibitory domains (www.ncbi.nlm.nih.govt/UniGene/org.) similar to CAST-L and CAST-S isoforms present in rainbow trout [3]. Softening of postmortem trout muscle could be accelerated by activation of calpains with exogenous calcium [4]. Moreover, net increase in calpain/CAST mRNA ratio with a corresponding increase in calpain catalytic activity under conditions of muscle breakdown is induced by starvation [5]. These researches elaborate the importance of CAST gene in controlling fish protein turnover, and suggest that CAST-L may be a good candidate as a biomarker for fish protein accretion. Therefore the present study was carried out for identification of novel SNP's in common carp (*Cyprinus carpio* var. *communis*) using cDNA sequences of calpistatin gene isolated from Atlantic salmon (*Salmo salar*), Rainbow trout (*Onchorhyncus mykiss*) and Zebra fish (*Danio rio*).

2. MATERIAL AND METHODS

2.1 Extraction of genomic DNA, qualitative and quantitative analysis

Tissue samples were collected from the fishes reared under captivity and the fishes captured from natural waters. Samples were collected in normal saline, transported in icepacks and stored at -20^{o} C for further processing. For the isolation of genomic DNA, phenol-chloroform method with some modifications was used [6]. The eluted DNA was stored at 4^{0}C for a short period and then kept at -20^{o}C for further analysis. The integrity of the genomic DNA was examined by gel electrophoresis using 0.8 percent agarose gel. Quantity of the DNA was determined by measuring optical density at 260 nm and 280 nm by double beam spectrophotometer (Htachi-U-1800). Ratio of 260/280 nm was calculated and DNA samples depicting ratio of 1.7-1.9 were considered for future use.

2.2 Gene databases and phylogenetic analysis

Gene databases from National Centre for Biotechnology Information (NCBI, http://www.ncbi.nih.gov), the Ensembl genome browser (http://www.ensembl.org/index.html), expressed sequence tag (EST) databases including the Salmon Genome Project (http://www.salmongenome.no/), the Gene Indices Project http://compbio.dfci.harvard.edu/tgi/) and Basic Local Alignment Search Tool (BLAST) [7] were used for the study. The sequences amplified and retrieved from NCBI of teleosts and tetrapods were used for constructing the phylogenetic tree. Bootstrap neighbor-joining method using CLUSTAL X (version 1.83) in PHYLIP format was used and visualized by Tree View [8].

2.3 Gene specific primer designing

The primers 5-CGCTGGATGCTCT-3, 5-TTAGGAGGAGGATA-3 and 5-CTCAATGCTTTGGGC-3, 5-TTAGGAGGAGGATATTTCA-3 for region 13, 14 & 19, 20 were designed by retrieving cDNA sequences of Calpastatin gene. cDNA sequences (NM-001124538.1, JX-489501.1 and gl/AA162761.1) of Rainbow trout, Atlantic salmon were aligned with the genomic DNA of Zebra fish by Clustal-W alignment software for primer designing by primer-5 software.

2.4 Polymerase chain reaction and purification.

TABLE 1: volume and concentraion of different reagents used in PCR

Reagent	Concentration	Final volume
PCR Master mix		12.5 µl
Forward primer	10pm/µl	1.0µl
Reverse primer	10pm/µl	1.0 µl
Template DNA	100ng/ µl	2.0 µl
Nuclease free water		8.5 µl
Total volume of reaction mixture		**25 µl**

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Amplification of the Calpastatin gene was carried out in Eppendorf mastercylcer in a 25µl reaction mixture as shown in table 1. Reactions were hot-started at 95^{o}C for 5 min, 95^{o} C for 30 sec, 55/60^{o} C sec, 72^{o} C for 30 sec and72^{o} C for 7 mints respectively. Purification of PCR product was done using PCR purification kit (Qiagen) using standard procedures.

3. RESULTS

3.1 Genetic results

Genomic DNA isolation showed unusual banding pattern both by manual and kit method. High RNA contaminations rendered decrease in the quality of Genomic DNA. Modified phenol chloroform method (SDS >10%) resulted in satisfactory quantified Genomic DNA observed under spectrophotometer by several folds (figure 1).

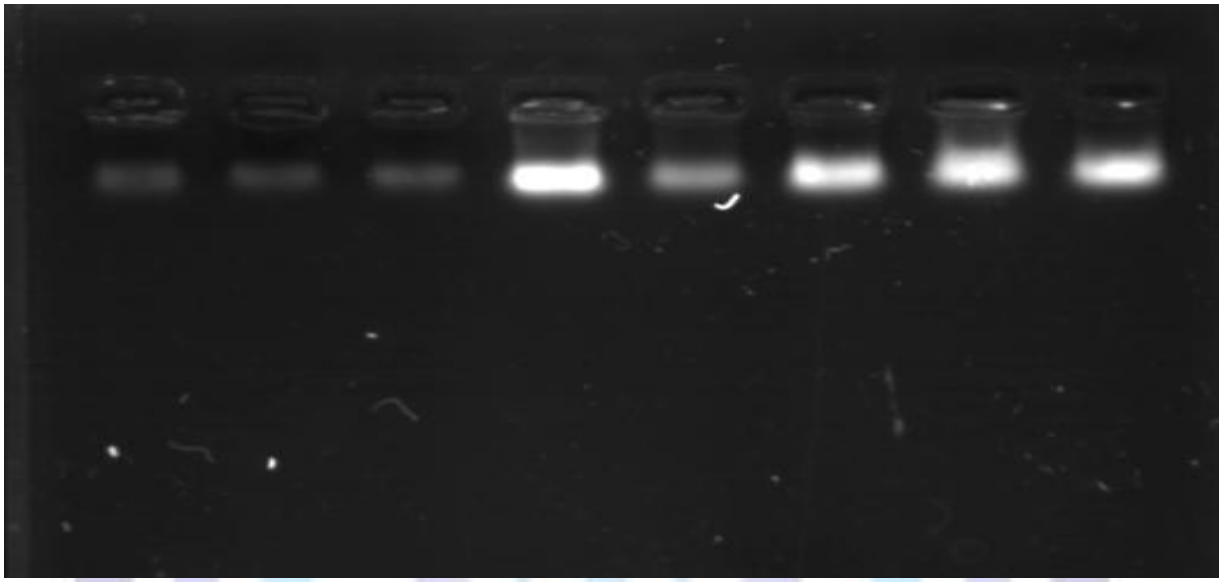

Figure. 1: DNA extraction of fishes of Cyprinidae family.

In the present study the region corresponding to inhibitory activity of calpistatin gene of 316 base pairs and 311 base pairs was amplified and used for identification under in- silico (figure 2a & 2b).

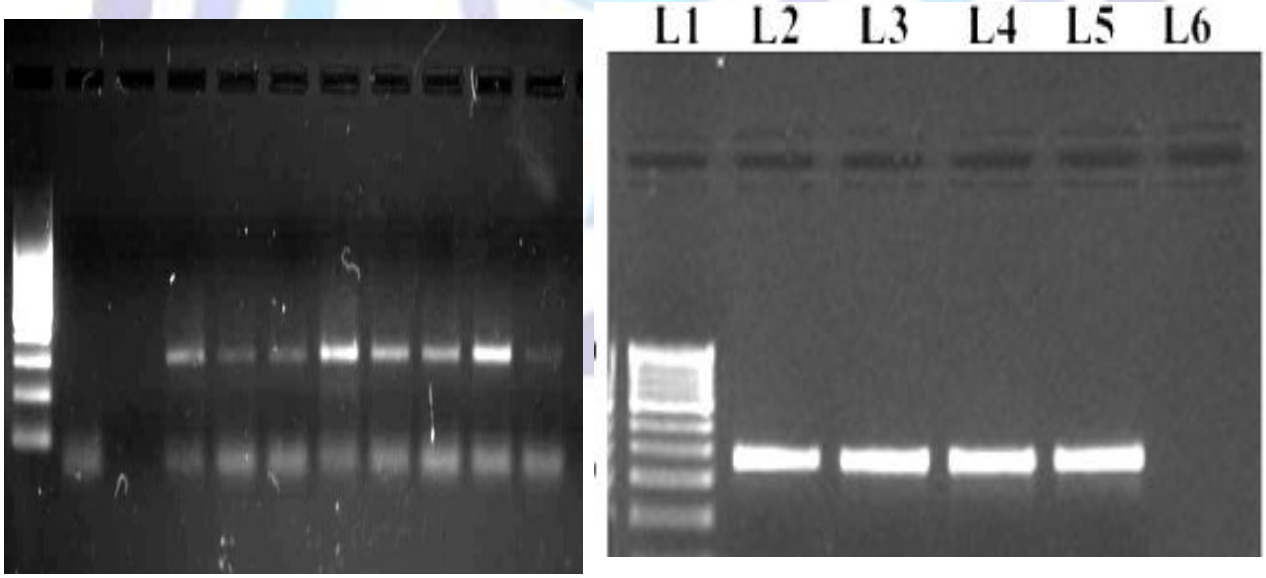

Figure 2 (a &b): Inhibitory domain of calpastatin gene in common carp for both regions in 2% agarose gel (documented by Cell Biosciences gel doc)

Amino acid sequences of Common carp shows highest degree of similarity with Zebrafish, Atlantic salmon and least similarity with Rainbow Trout long and short variant of Calpastatin protein. The LDAL**N**ALGDTL**G**APEP and VGER**E**DTLPP**G**YRF**S**E sequences at N-terminal and C-terminal are highly conserved and help in identification of exon-intron-exon boundaries. Mutations shown in red/bold color presumed to play important role in governing the muscle texture development (figure 3-7).

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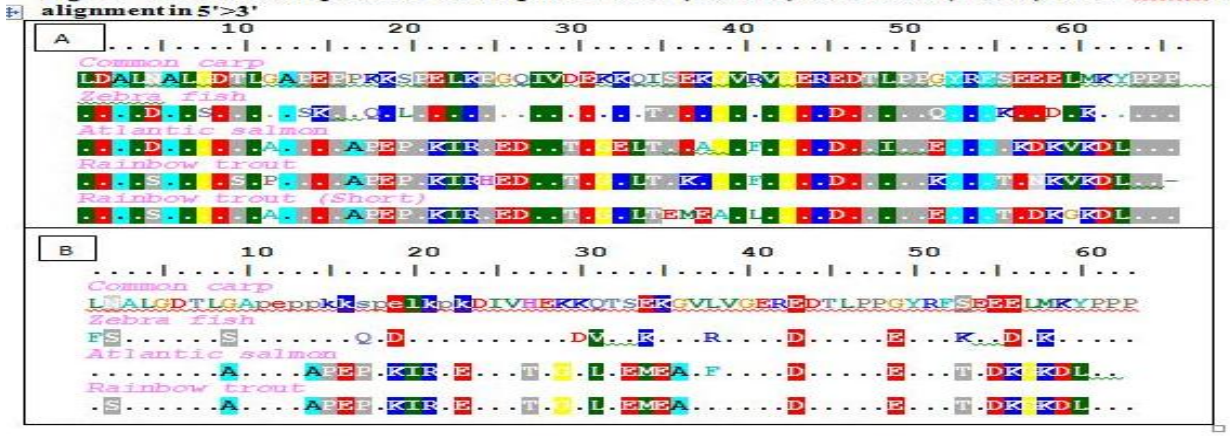
Fig. 3: Translational products of amplified exon (13, 14A) and exon (19,20B) after clustal-W alignment in 5'>3'



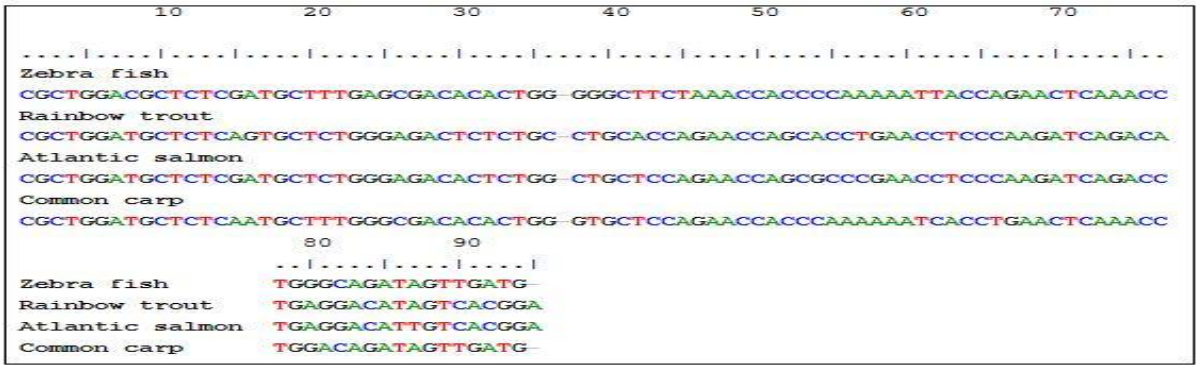


Figure 4: Sequence alignment of exon-13 for Cyprinidae and Salmonidae fishes.

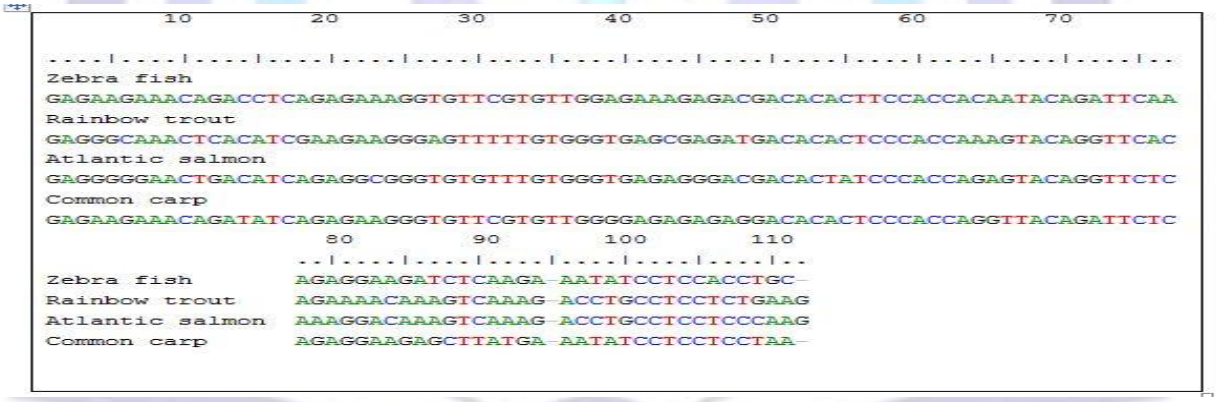


Figure 5: Sequence alignment of exon-14 by using clustal-W program of Bio-edit software

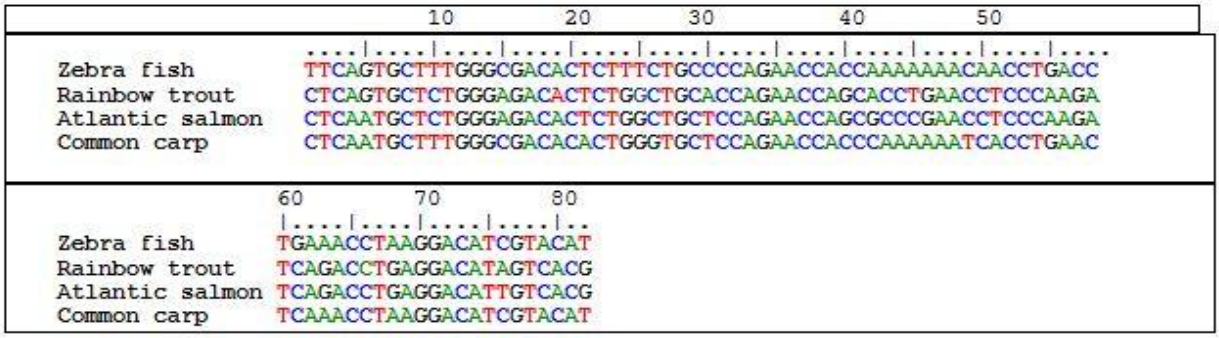


Figure 6: Sequence alignment of exon-19 between Salmonidae and Cyprinidae family by using clustal-W program of Bio-Edit software.

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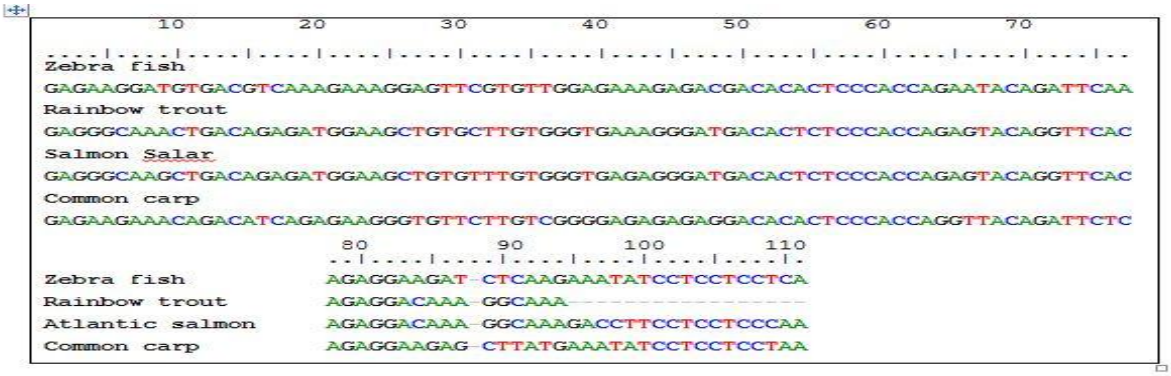

Figure 7: Sequence alignment of exon-20 between Salmonidae and Cyprinidae family by using clustal-W program of Bio-Edit software

Sequence alignment of CAST gene of Cyprinidae and Salmonidae showed that all mutations do not lead to amino acid change. The mutations which resulted in change of amino acids as given in table 2 and 3.

Table 2: Variability of amino acid and their codons within Common carp, Atlantic salmon and Rainbow trout in exon-13 and exon-14.

Position of Amino Acid	*Cyprinus carpio*	*Danio rio*	*Salmo salar*	*Onchorhyncus mykiss*
5th	N=AAT	D = GAT	D = GAT	S =AGT
10th	T =ACA	S = AGC	T = ACT	S =TCT
12th	G = GGT	S = TCT	A =GCT	A=GCT,P = CCT
17th	P =CCC	K = AAA	A = GCG	A = GCA
38th	K =AAG	Q = CAA	A =GCG	-
41st	R = CGT	L = TTA	F = TTT	F = TTT
46th	E = GAA	T = ACC	D =GAC	D = GAT
49th	L = CTC	D = GAC	I=ATC	-
52nd	G = GGT	Q = CAA	E =GAG	K = AAG
56rd	S = TCA	K = AAA	-	T = ACA

Table 3: variability of amino acid and their codons within Common carp, Atlantic salmon and Rainbow trout of both exons of 3rd region (19-20) in the conserved domain.

Position of amino acid	Common carp	Atlantic salmon	Rainbow trout
2nd	N = AAT	S = AAT	S= AGT
9th	G = GGT	A = GCT	A = GCT
14th	P = CCC	A= GCG	A = GCA
23rd	K = AAG	E = GAG	E = GAG
27th	H =CAT	T = ACG	T = ACG
38th	L = CTT	F = TTT	L = CTT
43rd	E = GAG	D = GAT	D = GAT
49th	G = GGT	E = GAG	E = GAG
53rd	S = TCA	T = ACA	T = ACA
55th	E = GAA	D = GAC	E = GAC

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PHYLOGENETIC ANALYSIS

Phylogenetic analysis was conducted for the partial Calpastatin gene in avian, tetrapod and teleost species. Sequences used for construction of tree (figure 8)

Figure 8: phylogenetic tree constructed by sequence alignment of study sample with that of sequences available in NCBI as shown in table 8.1

showed that *Danio rio* and *Cyprinus carpio* depicted a different banding pattern when compared to *Onchorhyncus mykiss* and *Salmo salar*. *Cyprinus carpio* was found phylogenetically more close (with 83 % similarity) to *Danio rio* than its mammalian counterpart *Gallus gallus* (with 47 % similarity) (Table 4).

Table 4: Calpastatin sequence similarity between even toed odd toed birds and rodents with that of bony fishes and for two inhibitory domains.

Description	MAX. SCORE	TOTAL SCORE	Query Cover	E-value	Identity	Accession number
Zgc:194249 protein [Danio rerio]	109	291	98percent	5.00E-26	83percent	AAI62761.1
PREDICTED: calpastatin-like [Oryzias latipes]	75.9	196	100percent	3.00E-14	62percent	XP_004068913.1
PREDICTED: calpastatin-like [Oreochromis niloticus]	70.5	162	98percent	1.00E-12	57percent	XP_0034428181
Calpastatin short variant 2 [Oncorhynchus mykiss]	60.1	60.1	100percent	7.00E-10	56percent	AAY18570.1
Calpastatin 2 [Salmo salar] >gb|AFV08638.1	61.2	61.2	98percent	1.00E-09	57percent	NP_001265997.1
Calpastatin long [Oncorhynchus mykiss]	62	103	98percent	1.00E-09	57percent	NP_001118010.1
Calpastatin [Rattus norvegicus]	37.4	72	81percent	0.29	42percent	CAA73916.1
Calpastatin [Gallus gallus]	37	72.8	86percent	0.52	47percent	ABP68381.1
Calpastatin type IV [Bos taurus]	35.4	35.4	81percent	1.4	41percent	AAV88518.1
PREDICTED: Calpastatin [Equus caballus]	34.7	34.7	81percent	2.4	41percent	XP_001503744.3
Calpastatin isoform III [Ovis aries]	34.7	67.8	84percent	2.8	39percent	ACO72573.1
Calpastatin isoform II [Ovis aries]	34.7	67.8	84percent	2.8	39percent	ACO72571.1
Calpastatin transcript variant 2 [Capra hircus]	34.3	100	84percent	2.9	41percent	ADI24334.1

4. DISCUSSION

4.1 Genetic analysis

Deterioration of muscle by autolysis is an unavoidable problem in post-rigor fish. It renders softening of muscles leading to fillet degradation. Calpastatin (CAST), an endogenous inhibitor inhibits the ability of calpains to destroy cellular proteins which in association with each other determine how the muscle tissue grows or wastes away. Rainbow trout CAST-L & CAST-S V1 has two inhibitory domains and CAST-S V2 has a single inhibitory domain[9]. In Atlantic salmon two peaks of CAST (Calpastatin) was detected after anion exchange chromatography as reported by Gaarder *et al.,* (2012). In present study only one CAST gene having two inhibitory regions was observed which is in agreement with the reports of [10]. These studies indicate Cyprinidae to be more close to mammals (having single CAST gene) than Salmonidae (with two CAST variants), hence justifies higher inhibitory activity of calpastatin in Cyprinidae and mammals than Salmonidae. Mouse CAST type IV has one repeat of the highly conserved sequence motif (TIPPXYR) characteristic of each inhibitory domain and is able to inhibit calpain specifically [11]. RBT CAST-L, CAST-S V1 and Zebrafish CAST have only two motif repeats whereas RBT CAST-S V2 and CAST of the Pufferfish have a single motif repeat indicating a single inhibitory domain [12,9]. In the present study, it was observed that, *Cyprinus carpio* have two highly conserved sequence motif repeats DTLPP, GYR in the inhibitory domain which corresponds to the exon-14 and exon-20 of Zebra fish. Consequently, fish CASTs are unique in possessing the fewest number of inhibitory domains, one or two out of four, reported so far. Further, a four-inhibitory domain containing CAST molecule inhibits 4 calpain molecules [13] and a three-inhibitory domain containing CAST molecule inhibits 3 calpain molecules [14]. Therefore, fish CASTs theoretically may have less potential per molecule to control calpain-dependent proteolysis than their mammalian counterparts which is probably due to selection pressure during evolution. The single amino acid change in the inhibitory motif Glycine (G) (GGT)/X (X = Q 394aa (CAA) in zebra fish, Sequence ID: ref|NP_001124063.2., Glutamic acid (E) 491aa (GAG) in Atlantic salmon, Sequence ID: ref|NP_001167162.1., Lysine (K) 223aa (AAG) in Rainbow trout long isoform, Sequence ID: ref|NP_001118010.1., and Glutamic acid (E) 54aa (GAG) in Rainbow trout short isoform, Sequence ID: ref|NP_001118117.1). Thus, the observation indicates that mutation in CAST inhibitory motif might have role in the diversification of inhibitory activity of the protein and requires further analysis. Another interesting feature of inhibitory motif of exon-20 was observed that only Common carp have G (GGT) amino acid and rest of the fishes so far studied have E (GAG) 548th aa in Zebra fish and 689th aa in Atlantic salmon are in the substitution, which needs further verification and analysis for validation. This divergence may also explain the higher Ca^{2+} requirement for half-maximal activity observed in calpains isolated from compared to mammalian counterparts.

4.2 Phylogenetic analysis

During present study, phylogenetic studies revealed that Common carp shear 84-95% CAST gene sequence with Zebra fish while as only 52-54% sequence homology was observed with Salmonids. This justifies the species divergence during the evolution of teleosts. Further, Common carp showed highest sequence similarity of regulatory and inhibitory domain with zebra fish than with Rainbow trout and Atlantic salmon. With an evolutionary separation of less than 150 million years, the Cyprinids are still closer to the aquacultural fish species than any mammalian model organism such as *Rattus norvegicus* and *Gallus gallus* (fig. 8) whose common ancestor with the lived around 400 million years ago [15]. In the present study, it was also observed that Calpastatin inhibitory domain of cyprinids shear 39-47% sequence homology with *Gallus gallus*, *Rattus norvegicus, Bos taurus*, *Ovis aries* and *Capra hiricus.* this identification signifies the fact that cyprinids are shearing maximum identities with that of mammals and is further evolving as due to the presence of single and two CAST variants in the teleosts as per the literature is accessed.

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