



Molecular characterization and expression analysis of two new C-reactive protein genes from common carp (*Cyprinus carpio*)

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ABSTRACT

C-Reactive protein (CRP) plays an important role in the acute phase response. Transcripts encoding two new CRP-like molecules (ccCRP1 and ccCRP2) from European common carp have been characterized which has enabled seven CRP-like genes to be identified in zebrafish. 79.3% (ccCRP1) and 74.5% (ccCRP2) identity to CRP from East-Asian common carp occurs and fish CRP genes form a distinct clade. ccCRP2 gene organization comprises four exons and three introns, in contrast to the two exons/one intron organization of mammalian CRP genes. Gene expression assays showed both ccCRP-like molecules are constitutively expressed in liver, skin, gill, gut, muscle, kidney, spleen and blood. Protein levels of ccCRP in serum and spleen were significantly different from other organs analyzed, and levels were greatest in the liver. It is proposed that the two carp CRP genes defined differ in their expression profiles which may suggest differences in their biological activities.

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1. Introduction

The innate immune system which is considered as the first line of the hosts' defense against exogenous pathogens assumes great importance in fish, in which the adaptive immune system is considered in a primitive state of evolution. One important component of the humoral innate immune system is a group of proteins, the pentraxins, which play a significant role in the immune response (Baltz et al., 1982; Bayne and Gerwick, 2001).

The pentraxins, C-reactive protein (CRP) and serum amyloid P-component (SAP), are a family of soluble pattern recognition proteins which are involved in the acute phase response (APR) to trauma, injury or infection which result in an increase in serum concentration of these so-called acute phase proteins (Baltz et al., 1982; Bayne and Gerwick, 2001; Gewurz et al., 1982; Pepys and Baltz, 1983; Steel and Whitehead, 1994; Volanakis, 2001). These pentraxins are characterized by their ability to bind to a wide range of specific ligands in a characteristic calcium dependent manner, for example CRP binds mainly to phosphorylcholine (PC), a pneumococcal C-polysaccharide, and phospholipids (Szalai et al., 1999; Tennent and Pepys, 1994; Volanakis and Kaplan, 1971). It has also been reported that these innate immune proteins bind to autogenous compounds, such as apoptotic nuclear components and other intracellular components released during cell death (Du Clos, 1989; Hicks et al., 1992), as well as exogenous harmful mol-

ecules, such as mercury (Agrawal and Bhattacharya, 1990). CRP molecules also increase clearing via phagocytosis (Bharadwaj et al., 2001; Gewurz et al., 1995; Mold et al., 2001) and trigger the activation of the complement system by the classical pathway (Baltz et al., 1982; Bickerstaff et al., 1999; Tennent and Pepys, 1994; Volanakis, 1982).

Since CRP is evolutionarily conserved in both vertebrate (Lund and Olafsen, 1998; Tennent and Pepys, 1994; Volanakis and Kaplan, 1971) and invertebrate organisms (Iwaki et al., 1999; Nguyen et al., 1986; Shrive et al., 1999; Tharia et al., 2002), it has been proposed that they may play a common and important role in host defenses (Ellis, 2001). In carp (*Cyprinus carpio*), previous studies (Cartwright et al., 2004; MacCarthy et al., 2008) have utilized a complex affinity chromatography protocol to isolate pure CRP-like proteins from the serum of European common carp. This has enabled the development of an ELISA which has revealed that the levels of CRP-like protein in carp serum increase in an acute phase manner when the fish are infected with the bacterial pathogen, *Aeromonas hydrophila* (MacCarthy et al., 2008). European and East-Asian common carp (*C. carpio carpio* and *C. carpio haematopterus*, respectively) diverged more than 500,000 years ago (Zhou et al., 2003) and both subspecies are commonly used in aquaculture and laboratory research. Although the isolation and sequence of transcripts encoding for a CRP molecule from East-Asian common carp has been reported (Fujiki et al., 2001) the full characterization of CRP genes has not been completed in carp, or indeed in any other fish species, and their associated expression levels in a range of tissues have not been ascertained.

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In the present study, the molecular properties of C-reactive protein are investigated and transcripts encoding for two new CRP-like molecules (ccCRP1 and ccCRP2) from European common carp are sequenced and characterized. Such information can be utilized using the zebrafish (*Danio rerio*) genome to further characterize the CRP gene repertoire in fish. In addition, the expression of ccCRP1 and 2 in several immune- and non-immune tissues was determined for the first time and the implications on the functional divergence of the CRP gene are discussed.

2. Materials and methods

2.1. Source and maintenance of fish

European common carp (10–12 g) were obtained from a commercial supplier (Fair Fisheries, Malpas, Cheshire, UK), and maintained in 250 l aquaria with a re-circulating dechlorinated aerated water system under a 12 h/12 h light/dark cycle. The ambient water temperature was 15 °C and fish were fed daily with a commercial diet (TetraMin®). Prior to experiments, fish were acclimatized to laboratory conditions for at least two weeks. Fish were killed by over-exposure to 0.2% 2-phenoxyethanol (BDH, UK) and organs of interest were collected under sterile conditions and immediately stored at –80 °C until use.

2.2. Database searching for ccCRP-like expressed sequence tags (ESTs)

To screen the cyprinid EST databases in GenBank (<http://www.ncbi.nlm.nih.gov>) for putative CRP sequences, known CRP-like protein amino acid residue sequences from carp (Cartwright et al., 2004) were used. The Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was applied through its tblastn option that searches translated nucleotide database using a protein query. Default algorithm parameters were used but selecting carp as the organism of interest. In this way, one putative partial ccCRP EST was obtained for ccCRP1 (GenBank accession number: DY655882) and two for ccCRP2 (GenBank accession numbers: EX881898 and EX880722), respectively. Using the sequences retrieved from the EST databases in GenBank, the full ccCRP1 and ccCRP2 RNA sequences were obtained.

2.3. Sequencing of ccCRP-like genes

Total RNA was purified from liver and head kidney of carps with the “RNeasy” kit (Quiagen) following manufacturer’s instructions.

Isolated RNAs were resuspended in diethylpyrocarbonate (DEPC)-treated water (Invitrogen) and stored at –80 °C.

RNA (500 ng) was used as template to obtain cDNA by using random hexamers (Applied Biosystems) and the Moloney murine leukemia virus reverse transcriptase (M-MLV) (Invitrogen) according to manufacturer’s instructions.

PCR amplification was performed on a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) using specific primers, first-strand cDNA synthesized from isolated RNA and GoTaq Flexi DNA polymerase (Promega) according to manufacturer’s instructions. All primers used (Eurofins MWG Operon, London, UK) are listed in Table 1.

The PCR products were analyzed by electrophoresis on a 1.5% agarose gel using ‘High-pure’ Low EEO Agarose (BioGene) and Tris-acetate-EDTA (TAE) buffer incorporating a 100-bp DNA ladder (Promega). The bands obtained were purified by using the “Isolate PCR and Gel” kit (Bioline) and then commercially sequenced (Eurofins MWG Operon, London, UK).

To check if the sequences retrieved from the EST databases in GenBank were present in sampled fish, specific primers were designed based on these sequences, (Table 1 – CRP1_4.FW and CRP1_6.RV for ccCRP1; CRP2_4.FW and CRP2_6.RV for ccCRP2) and PCR carried out (95 °C for 2 min, followed by thirty cycles of 95 °C for 0.5 min, 58 °C for 0.5 min, and 72 °C for 1 min, with a final extension of 72 °C for 7 min).

Rapid amplification of 5’ cDNA ends (RACE) method was performed using “5’ RACE System for Rapid Amplification of cDNA Ends” kit (Invitrogen), following manufacturer’s instructions and incorporating gene specific primers (Table 1 – 2CRP_3.RV (GSP1) and CRP1_4.RV (GSP2) for ccCRP1; 2CRP_3.RV (GSP1) and CRP2_4.RV (GSP2) for ccCRP2).

3’ RACE method was performed as described by Maniatis (Maniatis et al., 1982). Briefly, an oligo(dT) adaptor and the M-MLV reverse transcriptase were used to obtain cDNA according to manufacturer’s instructions. A PCR reaction was then performed using a mix of oligo(dT) adaptor and adaptor primer, and comprising one cycle of 94 °C for 5 min, 58 °C for 5 min and 72 °C for 40 min, then 20 cycles of 94 °C for 40 s, 58 °C for 1 min and 72 °C for 3 min, and finally one cycle of 94 °C for 40 s, 58 °C for 1 min and 72 °C for 15 min.

To ascertain the full length ccCRP2 gene, genomic DNA was extracted from dorsal flank muscle using the TRIzol reagent (Invitrogen), following manufacturer’s instructions. PCR amplifications were performed using, 94 °C for 2 min, then 35 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 2 min, with a final extension

Table 1
Oligonucleotide primers used in this study.

| Name | Sequence (5'–3') | Application | Reference |
|--------------|-------------------------------------|---------------------------------------|---|
| 2CRP_3.RV | GTGGAGAGAGGAGGCAGAC | 5' RACE | |
| CRP1_4.FW | AGCAATGCAACATTTTCCGTC | transcript sequence | |
| CRP1_4.RV | GACGGAAAAATGTTGCATTGCT | 5' RACE | |
| CRP2_4.FW | GATGCTGCAGCATTTTTCAGTC | transcript sequence, genomic sequence | |
| CRP2_4.RV | GACTGAAAAATGCTGCAGCATC | 5' RACE, genomic sequence | |
| CRP1_6.FW | GTGGGTGGCTTTGACGCAAGT | 3' RACE | |
| CRP1_6.RV | ACTTGGCTCAAAGCCACCCAC | transcript sequence | |
| CRP2_6.FW | ATTGAGCAACTTGTATGCGGAG | 3' RACE, genomic sequence | |
| CRP2_6.RV | CTCCGATCAAAGTTGCTCAAAT | transcript sequence, genomic sequence | |
| CRP1_7.FW | GCTAGTGACTCAAGATTAGATAACG | 3' RACE | |
| CRP2_7.FW | AAATGTGCTAGTGTGCAAGATAACT | 3' RACE, genomic sequence | |
| CRP2_7.RV | AGTTATCTTGCACCACTAGCACATTT | genomic sequence | |
| CRP2_8.RV | GCTGAGAAAAAATTATTGTTAGCC | genomic sequence | |
| CRP2_5gen.FW | ACCCGAAGTGATCTTCTCAT | genomic sequence | |
| 3p_adap.RV | GACTCGAGTCGACATCGAT ₍₁₇₎ | 3' RACE | Primers used previously by Maniatis et al. (1982) |
| 3p_pcr.RV | GACTCGAGTCGACATCG | 3' RACE | |
| B-actin.FW | GCTATGTGGCTCTTGACTTCGA | transcript sequence | From the carp b-actin gene sequence (M24113). |
| B-actin.RV | CCGTCAGGCAGCTCATAGCT | transcript sequence | Primers used previously by Huttenhuis et al. (2006) |

Nucleotide denomination: A (adenine); T (thymine); C (cytosine); G (guanine). FW, forward. RV, reverse.

of 72 °C for 7 min. The amplified genomic DNA segments were also purified, sequenced and gene organization of ccCRP2 established.

The nucleotide sequences of the ccCRP1 and ccCRP2 RNA and DNA are deposited in the GenBank (GenBank ID: JQ010977 and JQ010978 for CRP1 and CRP2 RNA respectively and JQ010979 for CRP2 genomic sequence).

2.4. Database searching for zebrafish CRP-like genes and locus tracing in the genome

To identify CRP-like orthologues in zebrafish, BLAST was applied through its tblastn option. Thus, ccCRP1 and ccCRP2 protein sequences were used as protein queries to find high sequence similarities with zebrafish by searching the genomic entries from NCBI's Reference Sequence database (refseq_genomic). Default algorithm parameters were used. Retrieved sequences were traced to ascertain their locus in the zebrafish genome version 9 (Zv9) and additional similar sequences were found in adjacent regions. Seven zebrafish CRP-like genes (Table 2) were found in the chromosome 24 and were named zfCRP and numbered from 1 to 7 accordingly to their positions.

2.5. In silico characterization of ccCRP and zfCRP sequences

To fully characterize the identified CRP sequences, multiple sequence alignments were performed and a phylogenetic tree drawn. CRP sequences from various species were gathered from GenBank and used in the analyses (Table 3). The putative common carp and

zebrafish CRP-like protein sequences were determined using the ExPASy web server and the Translate tool (<http://www.expasy.ch/tools/dna.html>). The multiple sequence alignments were created by ClustalW (Larkin et al., 2007) using the putative common carp and zebrafish CRP-like protein translations and known CRP protein sequences from various species that were retrieved from the Swiss-Prot database (Table 3). The phylogenetic tree was constructed using the MEGA 4.0 software (Tamura et al., 2007).

Signal peptides were predicted by the SignalP 3.0 program (www.cbs.dtu.dk/services/SignalP/) (Emanuelsson et al., 2007). The molecular weight of the proteins was calculated using the ProtParam tool (<http://www.expasy.org/tools>).

2.6. Expression analysis of ccCRPs by RT-PCR

Total RNA (0.5 µg per sample) prepared from several tissues (liver, head kidney, gut, gill, muscle, skin, blood and spleen) from three different fish were reverse transcribed with M-MLV and PCR-amplified with GoTaq Flexi DNA polymerase as described previously. Specific primers (Table 1 – CRP1_4.FW and CRP1_6.RV for ccCRP1; CRP2_4.FW and CRP2_6.RV for ccCRP2), which amplify the same regions for their corresponding genes and thus give products of the same size, were incorporated in a PCR reaction comprising 95 °C for 2 min, followed by thirty cycles of 95 °C for 0.5 min, 58 °C for 0.5 min, and 72 °C for 1 min, with a final extension of 72 °C for 7 min. A 89-bp cDNA fragment of carp β-actin protein was amplified as a positive control with the same PCR conditions. To confirm specific amplification PCR products were analyzed by electrophoresis and bands obtained purified and sequenced as described previously.

2.7. Quantification of ccCRP protein in carp tissues

ELISA was used to quantify ccCRP proteins in several tissues (liver, head kidney, gut, gill, muscle, skin, blood and spleen) from three different fish. Proteins were prepared in a ten times diluted standard RIPA buffer and quantified by Bradford (Bio-rad). Subsequently, 1 mg of protein per tissue from each fish was dissolved in 100 µL of Tris–NaCl buffer (50 mM Tris Base, 150 mM NaCl, pH 7.8) and used to coat individual wells of a polystyrene 96-well plate (NUNC). Plates were incubated for 18 h at 25 °C and blocked with 200 µL of 1% bovine serum albumin (BSA, Sigma) in Tris–NaCl buffer for 1 h at room temperature (20 °C). After washing in 3 × 200 µL Tween–Tris–NaCl buffer (Tris–NaCl buffer with 0.05% Tween-20), 100 µL of rabbit polyclonal antibody anti-ccCRP (Cartwright et al., 2004) at a concentration of 50 µg/mL in BSA–Tris–NaCl buffer (Tris–NaCl buffer with 0.5% BSA) was added to the wells and incubated for 2 h at room temperature. The unbound antibody was then removed by washing in 4 × 200 µL Tween–Tris–NaCl buffer, and 100 µL of the secondary antibody goat anti-rabbit IgG conjugate (Sigma) 1/4000 diluted in BSA–Tris–NaCl buffer was applied for 1 h at room temperature. After a final wash step (4 × 200 µL Tween–Tris–NaCl buffer), 100 µL of SigmaFast OPD in substrate buffer (prepared according to the manufacturer's guidelines), was added to each well and incubated in the dark. The reaction was stopped after 15 min with 25 µL per well of 2.5 M HCl and absorbance read at 492 nm using an ELISA plate reader (Labsystems Multiskan MS). Data are represented as the means of the absorbance values at 492 nm (subtracting the values corresponding to background) ± standard deviation for three independent individuals, each one performed in duplicate.

2.8. Statistical analysis

Data from ELISA were statistically analyzed by using one way analysis of variance (ANOVA) and Turkey's multiple comparisons

Table 2
CRP sequences from zebrafish.

| Name | Locus ^a | GenBank AC | Transcript length (bp) | Swiss-Prot AC | Protein length ^b (aa) |
|--------|--------------------|-------------|------------------------|---------------|----------------------------------|
| zfCRP1 | LOC570508 | XM_693995.4 | 621 | A3KPH3 | 225/206 |
| zfCRP2 | LOC327615 | BC097160 | 1296 | Q7S253 | 222/207 |
| zfCRP3 | LOC100141350 | BC154042 | 803 | A3KPG5 | 223/209 |
| zfCRP4 | LOC678538 | BC115188 | 1372 | Q1RM11 | 224/208 |
| zfCRP5 | LOC751795 | BC121777 | 795 | Q66I62 | 234/207 |
| zfCRP6 | LOC797437 | BC162745 | 735 | A3KPH6 | 225/209 |
| zfCRP7 | LOC100124600 | BC150371 | 1097 | A3KPH5 | 225/209 |

zfCRP, zebrafish CRP-like.

AC, accession number.

aa, amino acid residues.

^a Locus number on chromosome 24 from zebrafish.

^b With signal peptide/without signal peptide.

Table 3
CRP sequences from other organisms.

| Specie | Swiss-Prot AC | Protein length ^a (aa) |
|------------------------------|---------------|----------------------------------|
| <i>Cyprinus carpio</i> (EA) | Q90YD1 | 227/208 |
| <i>Homo sapiens</i> | P02741 | 224/206 |
| <i>Sus scrofa</i> | O19062 | 222/203 |
| <i>Bos taurus</i> | C4T8B4 | 224/205 |
| <i>Rattus norvegicus</i> | P48199 | 230/211 |
| <i>Mus musculus</i> | P14847 | 225/206 |
| <i>Oryctolagus cuniculus</i> | P02742 | 225/205 |
| <i>Cavia porcellus</i> | P49254 | 225/206 |
| <i>Mesocricetus auratus</i> | P49262 | 225/206 |
| <i>Gallus gallus</i> | Q2EJU6 | 227/211 |
| <i>Xenopus laevis</i> | Q07203 | 238/222 |
| <i>Limulus polyphemus</i> | P06205 | 242/218 |

AC, accession number.

aa, amino acid residues.

Homo sapiens, human; *Sus scrofa*, domestic pig; *Bos taurus*, domestic bull; *Rattus norvegicus*, brown rat; *Mus musculus*, common house mouse; *Oryctolagus cuniculus*, domestic rabbit; *Cavia porcellus*, guinea pig; *Mesocricetus auratus*, golden hamster; *Gallus gallus*, chicken; *Xenopus laevis*, African clawed frog; *Cyprinus carpio* (EA), East-Asian common carp; *Limulus polyphemus*, horseshoe crab.

^a With signal peptide/without signal peptide.

to determine significant differences between the different tissues. GraphPad Prism v4 software was used for creating the graphs and statistical analysis.

3. Results

3.1. Sequence analysis

The full sequences of ccCRP1 and ccCRP2 cDNAs (lower case; Fig. 1) comprised 857 and 853 bp, respectively. The ccCRP1 cDNA encloses an open reading frame (ORF) of 678 bp with a 73 bp 5'-untranslated region (UTR) and a 106 bp 3'-UTR. A polyadenylation

signal sequence AGTAAA was detected 16 bp upstream of the poly-A tail for ccCRP1 cDNA (Fig. 1A). In comparison, the ccCRP2 cDNA encloses an open reading frame (ORF) of 684 bp with a 57 bp 5'-UTR and a 112 bp 3'-UTR. A typical polyadenylation signal sequence AATAAA is detected 17 bp upstream of the poly-A tail for ccCRP2 cDNA (Fig. 1B).

The ccCRP1 and ccCRP2 predicted ORFs encode for two putative proteins of 225 and 227 amino acids in length (upper case, Fig. 1), respectively. The deduced amino acid sequences were predicted to have a signal peptide (Fig. 1A and B) of 19 amino acid length and a putative cleavage site located between Thr-19 and Glu-20 for ccCRP1 and between Ala-19 and Glu-20 for ccCRP2 (Fig. 1A and

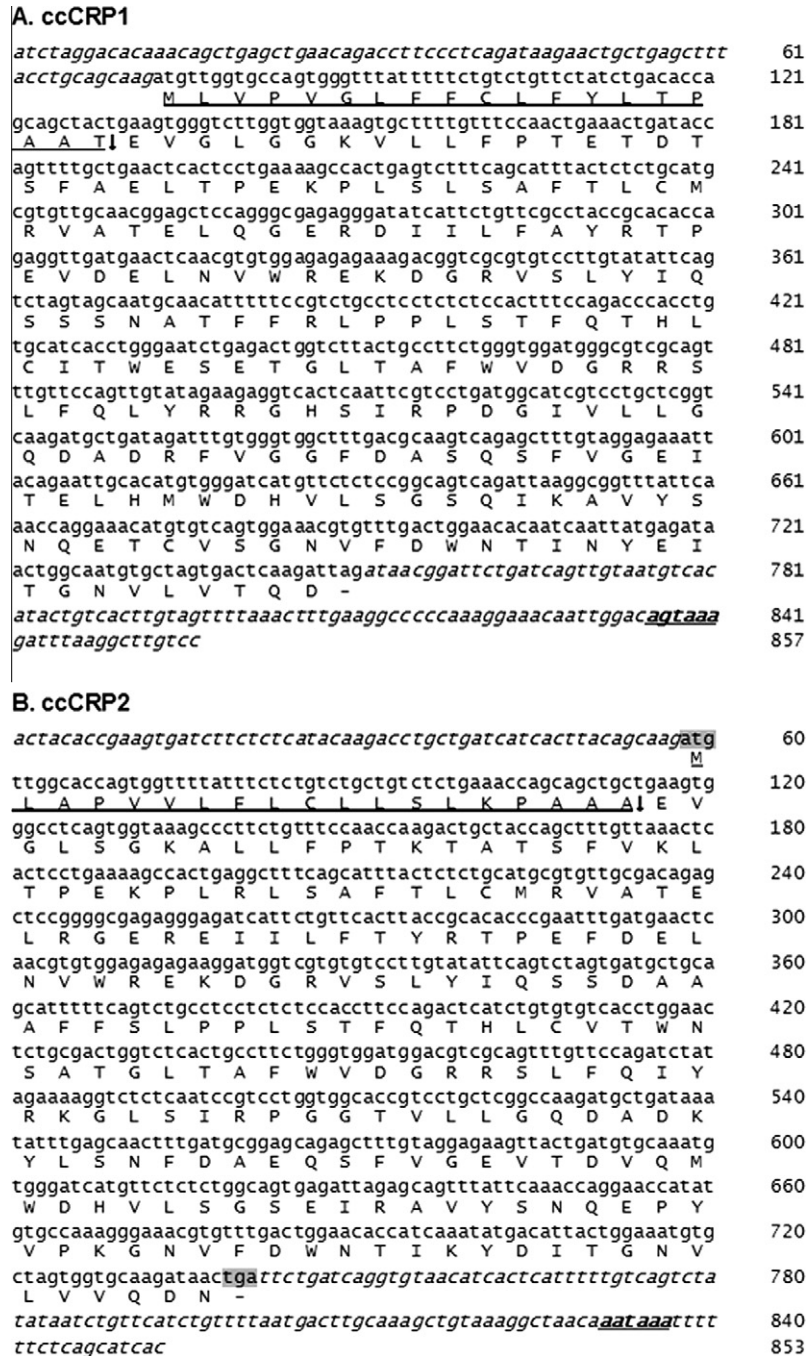


Fig. 1. Nucleotide (lower case) and predicted amino acid (upper case) sequences of ccCRP1 (A) and ccCRP2 (B). The putative signal peptide is underlined and the arrow indicates the presumed cleavage site for signal peptidase in the amino acid sequence. Within the nucleotide sequence, the start and stop codons are highlighted in gray and the polyadenylation signals are in bold and underlined. UTR sequences are indicated in italics.

B, respectively). The estimated molecular weight of the mature proteins was 23195 and 23460 Da for ccCRP1 and ccCRP2, respectively.

Analysis of nucleotide ccCRPs sequences carried out using ESTs databases in BLAST, revealed that orthologues occurred in other fish species. Highest similarity to the putative ccCRP1 was found with sequences from zebrafish (*D. rerio*, GenBank accession number: EV558462.1, 77% of identity, e-value $2e-87$) and fathead minnow (*Pimephales promelas*, GenBank accession number: DT256512.1, 71% of identity, e-value $2e-88$), whilst similarity to the putative ccCRP2 was obtained with zebrafish (GenBank accession number: EV558462.1, 84% of identity, e-value $5e-89$) and fathead minnow (GenBank accession number: DT256512.1, 68% of identity, e-value $3e-88$). Searching the nr (non-redundant) database, alignments showed highest similarity to the putative ccCRP1 to sequences from zebrafish (GenBank accession number: BC165153.1, 75% of identity, e-value $2e-94$), East-Asian common carp (GenBank accession number: AB028455.1, 74% of identity, e-value $2e-92$) and grass carp (*Ctenopharyngodon idella*, GenBank accession number: FJ547474.1, 72% of identity, e-value $1e-88$). The putative ccCRP2 sequences were similar to zebrafish (GenBank accession number: BX001030.7, 78% of identity, e-value $5e-91$), East-Asian common carp (GenBank accession number: AB028455.1, 76% of identity, e-value $2e-98$) and grass carp (GenBank accession number: FJ547474.1, 75% of identity, e-value $1e-96$).

3.2. Identification of zebrafish CRP-like sequences

The high similarities found between the carp CRP-like sequences and some zebrafish sequences retrieved from the database was perhaps expected as both fish species belong to the family *Cyprinidae*. Since CRP-like sequences have not been reported in zebrafish, in an attempt to locate zebrafish CRP-like orthologues and extend the range of CRP sequences in cyprinid fish, extensive systematic BLAST searches in the genomic entries from NCBI's Reference Sequence database were carried out which identified seven different CRP-like sequences in the zebrafish genome (Table 2).

3.3. Analysis of the syntenic regions of zebrafish CRP-like genes

As zebrafish is a model organism in scientific research and its genome has been extensively sequenced, the loci of the identified CRP-like genes can be determined. Fig. 2 shows the order and orientation of CRP-like and adjacent genes in zebrafish genome. All of them are located consecutively in the 3' region of the chromosome 24 and were named zfCRP and numbered from 1 to 7 accordingly to their positions. The gene order is: coiled-coil domain containing 78 (*ccdc78*, LOC100150366) and the glial cell differentiation regulator meteorin (*mtrn*, LOC494493) flanked the 5' region; the zebrafish CRP-like molecules (*zfcrp*) 1–7 (LOC570508, LOC327615, LOC100141350, LOC678538, LOC751795, LOC797437 and LOC100124600, respectively), and the immunoglobulin C1-set domain (LOC798043) and immunoglobulin light chain (LOC100149648) flanked the 3' region.

3.4. Comparison of common carp and zebrafish CRP-like sequences

Alignment of the deduced amino acid sequence (excluding the signal peptides) of ccCRP1, ccCRP2, East-Asian common carp CRP-like (GenBank accession number: AB028455, Table 3) and the identified zebrafish CRP-like molecules show they are highly conserved along the full sequence (Fig. 3). All of them occurred within the typical pentraxin domain (HxCxS/TWxS, in which x is any amino acid), except for zebrafish CRP5 in which Ala replaces Ser/Thr. Highest identity scores for the CRP molecules in carp and zebrafish

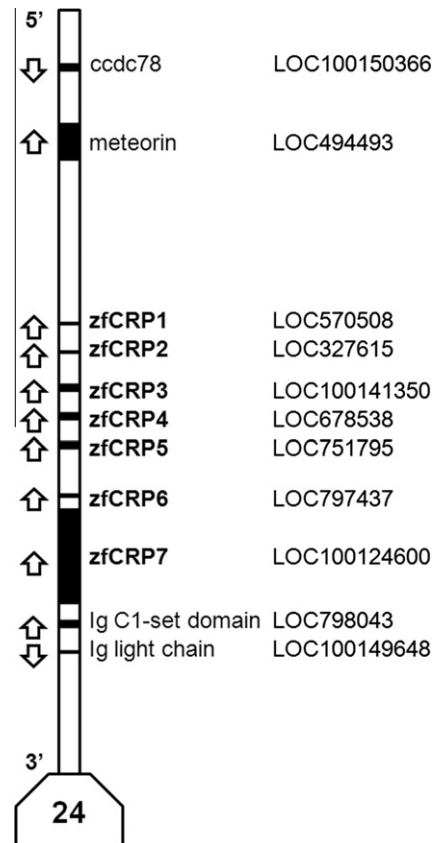


Fig. 2. Location diagram of the CRP-like genes in the chromosome 24 of the zebrafish genome. Genes are indicated with black boxes and orientation is indicated with arrows. Gene names and corresponding GenBank locus accession numbers are arranged in order. Gene sizes and chromosome distances are scaled. *ccdc78*, coiled-coil domain containing 78; *mtrn*, glial cell differentiation regulator meteorin; *zfcrp*1–7, zebrafish CRP-like molecules 1–7; Ig, immunoglobulin.

were found when comparing any of the carp sequences with zfCRP2 (Swiss-Prot accession number: Q7SZ53), which shared 75.8%, 78.8% and 71% identity with ccCRP1, ccCRP2 and East-Asian common carp CRP, respectively (data not shown). ccCRP1 and ccCRP2 exhibited identity scores of 74.5% and 79.3%, respectively with the East-Asian common carp CRP (Table 4).

3.5. Phylogenetic analysis

Phylogenetic analysis was performed based on amino acid sequences (excluding single peptide) of known CRP-like molecules in non-fish organisms (Table 3). Multiple sequence alignments of the deduced protein sequence indicated that ccCRP1 and ccCRP2 molecules are highly conserved in regions that are functionally important (Fig. 4, Table 4). For example, two cysteine residues and some amino acids involved in calcium binding (Asp-141, Gln-151 and probably Asp-60 and Asp-139) were conserved in ccCRP1 and ccCRP2 (Fig. 4) and acidic residue critical for C1q-binding in humans (Agrawal and Volanakis, 1994) was also present in ccCRP1 and ccCRP2 molecules (Asp-117).

Based on the pairwise alignment scores of the mature CRP sequences i.e. amino acid sequences excluding the signal peptide, (Table 4), the ccCRPs sequences showed 26.1–27.0% identity with the CRP protein sequences of *Xenopus laevis*, 28.4–29.4% identity with *Gallus gallus* and 30.3–34.6% identity with mammalian sequences (33.7–34.1% identity with *Homo sapiens* sequence). Lowest identity was found with *Limulus polyphemus* (25.2%).

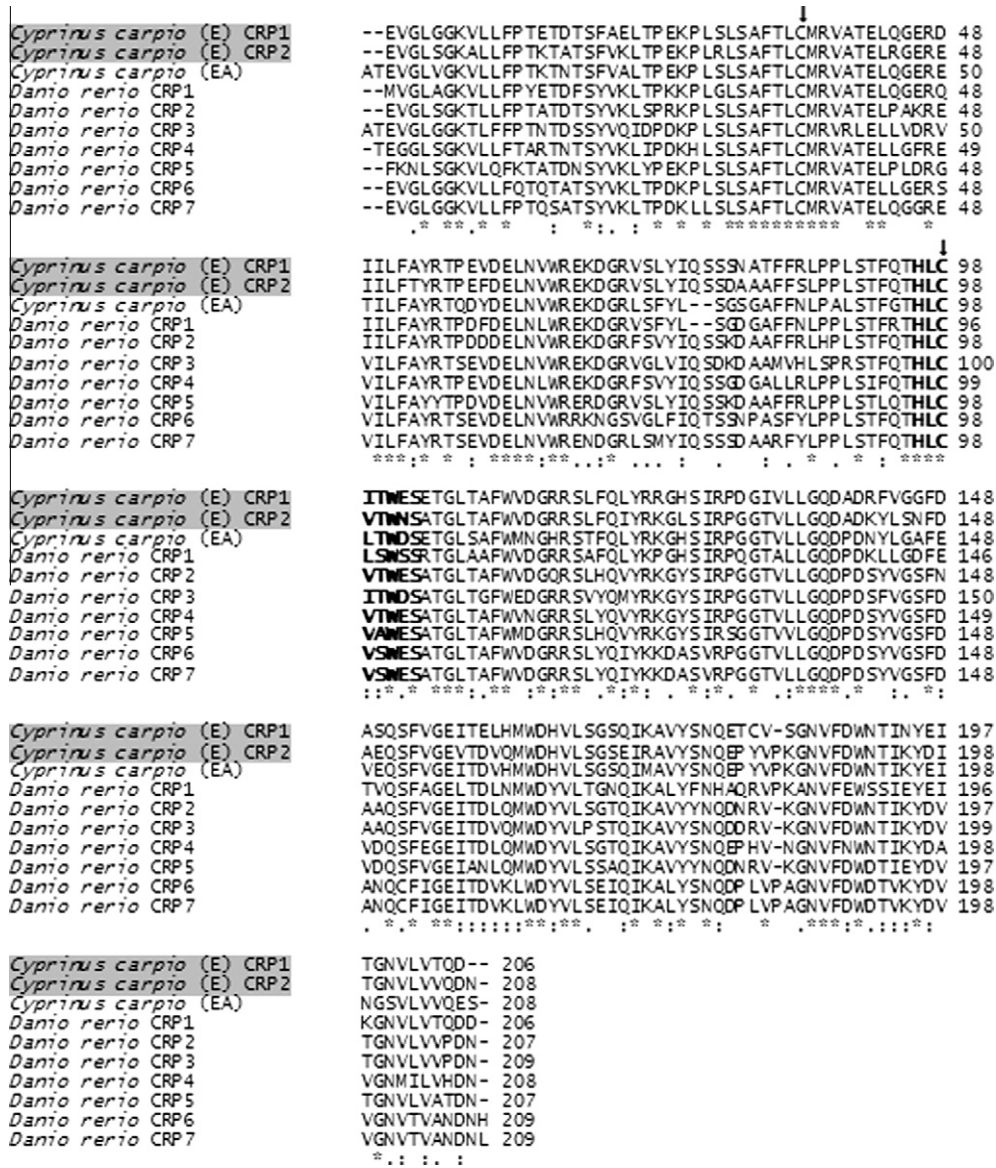


Fig. 3. Multiple alignment of amino acid sequences of European and East-Asian common carp and zebrafish CRPs. Alignment was performed using ClustalW v2.0 with the sequences without signal peptide of European and East-Asian common carp (Table 3) and zebrafish CRPs (Table 2). Pentraxin domain is indicated in bold. Conserved cysteine residues (C) are indicated by an arrow. Mismatches are marked by dashes in the amino acid sequences. Below the alignments, identical amino acids are indicated with asterisks, and conservative substitutions are indicated with “.” or “:.” The positions of the alignment are numbered. E, European subspecies; EA, East-Asian subspecies.

In a phylogenetic tree (Fig. 5) based on mature protein aligned sequences including carp, zebrafish (Table 2) and non-fish organisms (Table 3), CRP sequences from fish clustered together with a bootstrap percentage of 100%. The CRP sequence of *X. laevis* branched closely to the fish group (bootstrap percentage of 98%), whilst mammalian CRP proteins formed a distinct clade with a bootstrap percentage of 99%. CRP sequence from *L. polyphemus* proved to be a suitable comparative outgroup.

3.6. Gene organization

Gene organization of ccCRP2 comprised four exons and three introns (Fig. 6). Two out of three introns are located inside the 5'-UTR, the first one (63 bp long) is situated 45 bp downstream of 5'-terminal of the cDNA and the second one (92 bp long) is 50 bp downstream of 5'-terminal from the cDNA. Consequently, the exon present between them is short, 5 bp. The third intron (115 bp long) is situated just after the codon correspondent to

the first amino acid of the mature protein, 117 bp downstream of 5'-terminal of the cDNA. Interestingly, the sequences around the splice sites (Fig. 6A) in the first two introns are different to the typical consensus sequences for exon-intron limits (U2 and U12 introns) of eukaryotic genes.

In comparison with the gene organization of CRP from *X. laevis* and *Homo sapiens* (Fig. 6B), the first and second intron from ccCRP2 corresponded to the first intron from *X. laevis* (145 bp). All of these introns interrupted the 5' UTR in equivalent positions. These introns are not present in the human CRP gene. The position of the third intron is similar to those found in CRP genes from *X. laevis* and *Homo sapiens*, however, this intron (115 bp in ccCRP2) is longer in *X. laevis* (1819 bp) and human CRP (278 bp).

3.7. Tissue distribution of ccCRPs mRNA and protein

From each organ type the total RNA extracted from three common carp was pooled and used to investigate the distribution of

Table 4
Identity and similarity percentage between the CRP mature protein amino acid sequences from several species.

| % Similarity | % Identity | | | | | | | | | | | | | |
|--------------|---------------------------------|-----------------------------|---------------------|------------------------------|------------------------|-----------------------------|---------------------|--------------------------|-------------------|-------------------|----------------------|-----------------------|---------------------------|------|
| | <i>Cyprinus carpio</i> (E) CRP1 | 77.4 | 79.3 | 34.1 | 32.7 | 34.6 | 32.2 | 32.2 | 32.2 | 30.3 | 34.6 | 28.4 | 26.1 | 25.2 |
| 15.9 | <i>Cyprinus carpio</i> (E) CRP2 | 74.5 | 33.7 | 32.7 | 32.7 | 30.3 | 31.7 | 30.8 | 31.7 | 33.7 | 29.4 | 27.0 | 25.2 | |
| 15.9 | 18.8 | <i>Cyprinus carpio</i> (EA) | 33.5 | 33.0 | 35.4 | 30.6 | 33.0 | 30.3 | 32.0 | 36.9 | 26.5 | 28.4 | 27.1 | |
| 40.9 | 40.9 | 43.7 | <i>Homo sapiens</i> | 75.2 | 73.3 | 70.9 | 70.9 | 64.9 | 70.4 | 67.5 | 48.3 | 41.9 | 26.1 | |
| 39.4 | 41.8 | 41.3 | 19.4 | <i>Oryctolagus cuniculus</i> | 70.4 | 68.4 | 66.5 | 63.0 | 65.9 | 68.8 | 44.5 | 38.7 | 26.1 | |
| 35.6 | 38.9 | 37.9 | 18.9 | 21.8 | <i>Cavia porcellus</i> | 70.9 | 67.5 | 63.5 | 68.4 | 63.6 | 45.0 | 37.8 | 24.8 | |
| 43.3 | 45.2 | 45.6 | 24.3 | 22.8 | 21.8 | <i>Mesocricetus auratus</i> | 78.2 | 73.9 | 68.9 | 62.1 | 46.0 | 36.0 | 25.7 | |
| 41.3 | 42.8 | 41.3 | 23.3 | 25.2 | 24.8 | 18.0 | <i>Mus musculus</i> | 70.6 | 65.0 | 60.7 | 46.0 | 36.0 | 26.1 | |
| 39.3 | 40.3 | 39.8 | 24.6 | 25.6 | 26.1 | 18.5 | 20.4 | <i>Rattus norvegicus</i> | 61.6 | 60.2 | 44.1 | 37.8 | 26.6 | |
| 40.4 | 38.5 | 41.3 | 21.4 | 27.3 | 24.3 | 24.3 | 27.2 | 27.0 | <i>Bos taurus</i> | 67.3 | 50.7 | 34.2 | 26.1 | |
| 40.9 | 42.8 | 40.3 | 23.3 | 20.5 | 23.3 | 27.2 | 28.2 | 24.2 | 20.5 | <i>Sus scrofa</i> | 46.0 | 41.0 | 25.2 | |
| 31.3 | 34.6 | 37.4 | 27.0 | 31.8 | 30.8 | 30.8 | 31.8 | 33.2 | 25.1 | 30.3 | <i>Gallus gallus</i> | 38.7 | 27.1 | |
| 38.3 | 36.9 | 35.6 | 30.2 | 32.9 | 29.7 | 36.5 | 36.0 | 33.8 | 33.3 | 31.1 | 33.3 | <i>Xenopus laevis</i> | 22.1 | |
| 35.3 | 35.3 | 35.8 | 33.0 | 31.2 | 37.6 | 37.2 | 34.4 | 36.7 | 36.2 | 33.0 | 34.9 | 36.9 | <i>Limulus polyphemus</i> | |

Species names are highlighted in gray. Upper region, % identity measured as the percentage of identical amino acids in the same position following alignment of sequences with Clustal2. Lower region, % similarity measured as the percentage of amino acids sharing similar characteristics in the same position following alignment of sequences with Clustal2. *Cyprinus carpio* (E), European common carp subspecies; *Cyprinus carpio* (EA), East-Asian common carp subspecies.

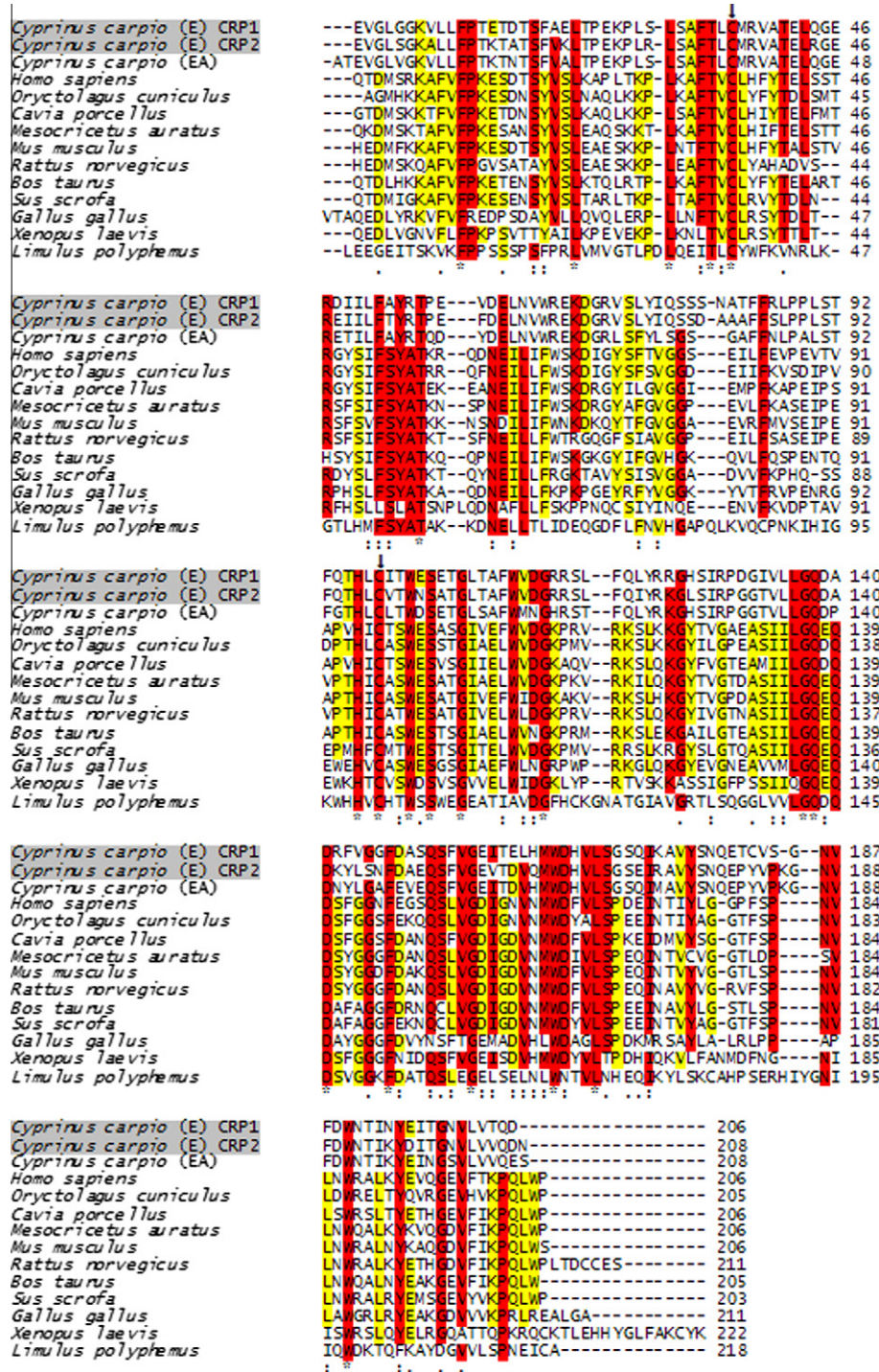


Fig. 4. Multiple alignment of amino acid sequences of CRPs from several organisms including ccCRP1 and ccCRP2. Alignment was performed using ClustalW v2.0 with the sequences without signal peptide compiled in Table 3 and *Cyprinus carpio* (European subspecies) CRP1 and CRP2 sequences. The alignment was edited manually. Positions with more than 50% of identity are highlighted in yellow and with more than 75% in red. Conserved cysteine residues (C) are indicated by an arrow. Mismatches are marked by dashes in the amino acid sequences. Below the alignments, identical amino acids are indicated with asterisks, and conservative substitutions are indicated with “.” or “:”. ccCRP1 and ccCRP2 names are highlighted in gray. The positions of the alignment are numbered. E, European subspecies; EA, East-Asian subspecies.

ccCRP1 and ccCRP2 in healthy fish using RT-PCR. In all samples analyzed a β-actin product was amplified in parallel to confirm the quality of cDNA used and to serve as an internal control. In all cases a strong PCR product was obtained. ccCRPs transcripts were observed in all tissues (skin, gill, gut, muscle, kidney, spleen, liver and blood) investigated (Fig. 7A). The highest expression of ccCRP1 and 2 was found in blood and in the gut tissue. Lower levels of expression of these two genes were observed in skin, gill, muscle

and spleen tissues. In contrast, in the head kidney whilst relative high level of expression of ccCRP2 occurred, only moderate expression of ccCRP1 was noted. In the liver however, whilst high level of expression of ccCRP1 was recorded, ccCRP2 expression levels in this tissue was the lowest found.

ELISA revealed that there was a significant difference ($F = 17.26$ and $P < 0.0001$) in the levels of CRP within the organs analyzed (Fig. 7B). Levels of ccCRP in the liver were significantly

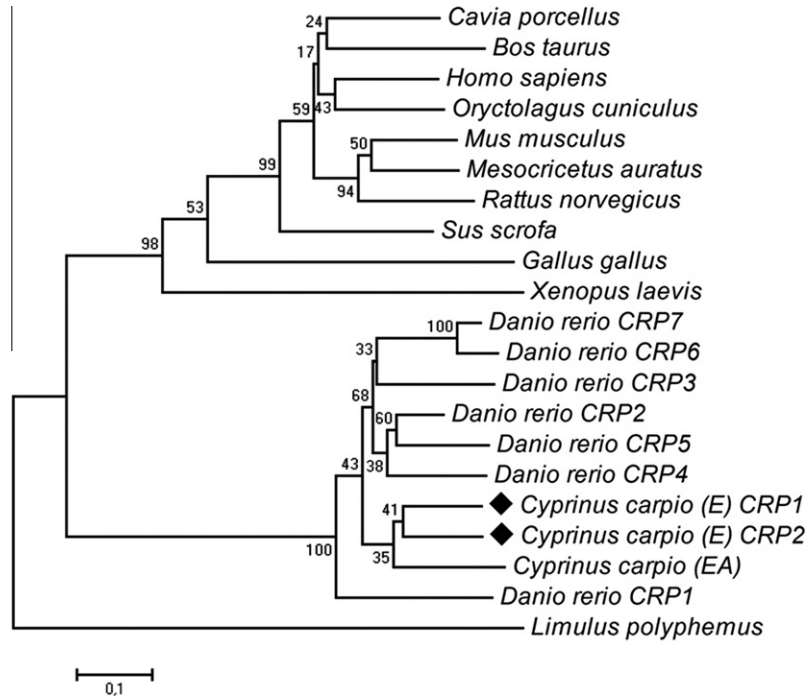


Fig. 5. Phylogenetic tree constructed using amino acid sequences of CRPs from several organisms including common carp and zebrafish CRP-like molecules. The tree was constructed using the Neighbour-Joining algorithm in MEGA version 4 and bootstrapped 10,000 times. Bootstrap percentages are shown next to the branches. Mature protein sequences used for the construction of the phylogenetic tree are provided in Table 2 and Table 3. *Limulus polyphemus* CRP sequence was used as an outgroup. The scale represents amino acids substitutions. Positions corresponding to ccCRP1 and ccCRP2 are indicated with (♦). E, European subspecies; EA, East-Asian subspecies.

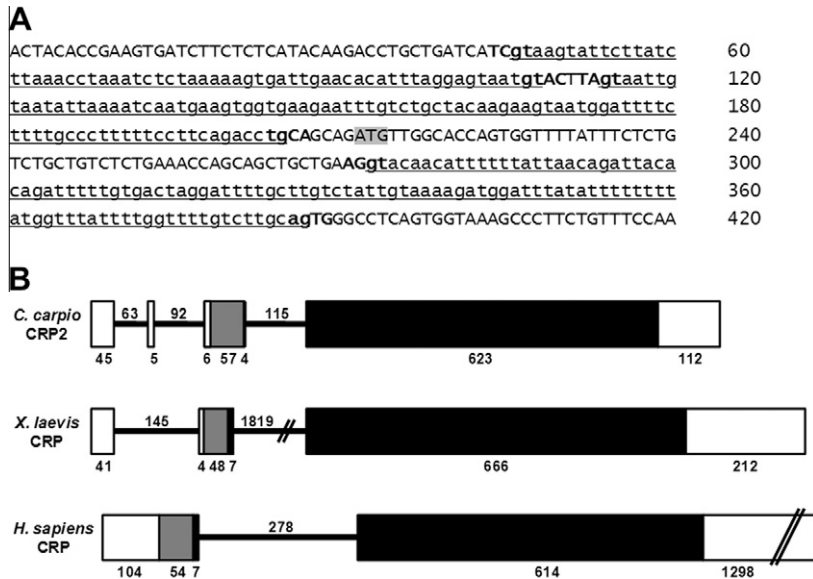


Fig. 6. Gene organization of ccCRP2. (A) First 400 bp of ccCRP2 gene sequence, where the three introns of the gene are located. Introns are indicated in upper case and exons in lower case and underlined. The splice sites are indicated in bold. The start codon is highlighted in gray. Nucleotides are numbered. (B) Diagram of *Cyprinus carpio* CRP2 gene and *Xenopus laevis* and *Homo sapiens* CRP genes. Exons and introns are represented by boxes and black lines, respectively. 5' and 3' Inside the boxes corresponding to exons, the UTRs are colored in white, the signal peptide in gray and the mature protein in black for each gene. Lengths are scaled and represented in base pair numbers on the top of the introns and in the bottom of the exons.

higher ($P < 0.05$) than those observed in the rest of tissues examined. The lowest level of CRP recorded was in the serum which was significantly different ($P < 0.05$) from all other tissues with the exception of the spleen. The levels of CRP in this organ was also significantly different ($p < 0.05$) to those found in the head kidney and liver.

4. Discussion

In the present study two new CRP-like genes from common carp have been identified and characterized showing that *C. carpio* possess multiple CRP genes. Since the pioneering work of Pepys et al. (1978) on the structure of CRP molecules in fish there has been few

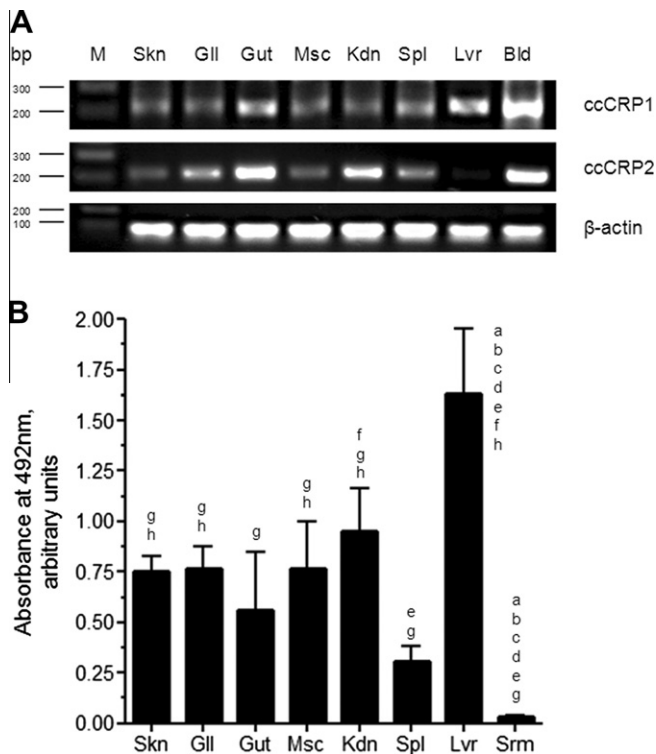


Fig. 7. Expression analysis of ccCRP1 and ccCRP2 in various tissues of healthy common carp by RT-PCR (A) and ELISA (B). (A) RT-PCR amplified from total RNA from pooled organ types from three individuals. Following PCR, products were visualized in 1.5% agarose gels. β -Actin mRNA was used as an internal control. Size marker is shown by M and size is represented in base pair numbers. bp, base pair. (B) ELISA was performed by duplicate with homogenized individual tissues of the same three fish and ccCRP was detected with a rabbit polyclonal antibody anti-ccCRP. Data are represented as the means arbitrary units of absorbance at 492 nm \pm standard deviation. Tissues include: Skin (Skn), gill (Gll), gut (Gut), muscle (Msc), head kidney (Kdn), spleen (Spl), liver (Lvr) and blood (Bld) or blood serum (Srm). Statistical differences ($P < 0.05$) between tissues are represented by: a (different to skin), b (different to gill), c (different to gut), d (different to muscle), e (different to head kidney), f (different to spleen), g (different to liver) and h (different to serum).

detailed studies on this important serum protein in lower vertebrates. Although the structure of carp CRPs cannot be reliably ascertain from the primary sequences identified in this work, the low identity and similarity in percentages (Table 4) found when comparing them to the human CRP sequence which structure is elucidated (Shrive et al., 1996), suggest that carp CRP molecules may not have a pentameric nature as happens with other pentraxins such as the *L. polyphemus* serum amyloid P-component-like “pentraxin” that displays two distinct molecular aggregations, heptameric and octameric (Shrive et al., 2009). However, multiple sequence alignment of both genes with CRP gene sequences from several different vertebrate species and one invertebrate (*L. polyphemus*) revealed twenty conserved amino acids (two cysteines, two threonines, three tryptophans, one serine, four glycines, one glutamine, three leucines, one histidine, one aspartic acid and two phenylalanines). In addition, highly conserve motifs that are considered important for the CRP function, such as the amino acid sequence for PC-binding and the surrounding regions (amino acids 28–56 from the human sequence) or the calcium-binding region (amino acids 149–171 from the human sequence) were noted. Another two key amino acids (Phe-66 and Glu-82) which in humans mediate the binding of PC (Agrawal et al., 2002; Black et al., 2003; Thompson et al., 1999), are substituted in carp CRP molecules by tryptophan and serine, respectively. In contrast, the presumed sites in human CRP for complement C1q binding and Fc γ receptors

(Asp-112 and Tyr-175) (Agrawal et al., 2001; Agrawal and Volanakis, 1994) are both present in the CRP sequences characterized in common carp.

Although partial pentraxin-like sequences have been described in several fish species including common carp (Cartwright et al., 2004), plaice (*Pleuronectes platessa*) (White et al., 1981), rainbow trout (*Oncorhynchus mykiss*) (Murata et al., 1995, 1994), channel catfish (*Ictalurus punctatus*) (Szalai et al., 1992), Atlantic salmon (*Salmo salar*), halibut (*Hippoglossus hippoglossus*), common wolfish (*Anarhichas lupus*), cod (*Gadus morhua*), Indian carp (*Catla catla*) (Lund and Olafsen, 1998) and striped catfish (*Pangasianodon hypophthalmus*) (Huong Giang et al., 2010), CRP-like sequences have not been reported previously in zebrafish. The discovery of the two new CRP sequences from common carp allowed the identification of seven different zebrafish CRP-like orthologs which share up to 75.8% and 78.8% of identity with ccCRP1 and ccCRP2, respectively. All these seven sequences are located consecutively in the same region of the chromosome 24 from zebrafish, indicating all of them may have evolved from the same CRP-like gene ancestor. This is supported by the phylogenetic tree produced which revealed that all the fish CRP-like genes group together into one main clade and that the CRP sequence of the amphibian, *X. laevis*, branched closely to the fish group.

The presence of seven CRP-like sequences in zebrafish may suggest the existence of more CRP-like sequences in carp than the two already reported in this paper. Indeed, transcripts encoding for the CRP molecule from East-Asian common carp reported by Fujiki (Fujiki et al., 2001) have not been detected in European common carp (data not shown). Further advances on the sequencing of the carp genome will shed light on the full CRP gene complex.

The expanded repertoire of CRP-like genes in common carp and zebrafish noted also reflects the situation in other members of the innate immune proteins such as those involved in the complement system (Nakao et al., 2003). Indeed, it has been reported that in several fish species multiple isoforms of innate immune proteins, which may be consider as redundant, are maintained because they display different functional activities (Nakao et al., 2003; Nonaka, 2001; Sunyer and Lambris, 1998). Pentraxins are considered as functional ancestors of antibodies (Garlanda et al., 2005) hence the presence of the multiple CRP-like isoforms in the common carp and zebrafish could increase the recognition repertoire against pathogens in animals where the adaptive immune system is evolutionary less developed (Sunyer et al., 1998). Further studies on both the structure and the function of these molecules, especially on their ligand-binding properties, are required to ascertain if this isoform complexity is translated into functionality. These studies will also help to answer the current controversy about the evolution and ancestral origin of CRP and SAP (Jensen et al., 1997) because, although it has been previously reported that the proteins derived from the carp CRP sequences described in the present study bind to PC and respond as an AP molecule (MacCarthy et al., 2008), they share comparable identity and similarity to mammal CRPs and SAPs (data not shown).

Gene organization is one of the most striking features of the CRP genes analyzed in this study. A four exon arrangement occurs, with two introns interrupting the 5' UTR with a third intron being located at the end of the sequence coding for the signal peptide. This means almost the entire protein is encoded within the fourth exon. This contrasts to the situation in mammals, where only two exons are present (Lei et al., 1985; Rubio et al., 1993) and there is only one intron just after the sequence coding for the signal peptide. This appears to correlate with the third intron found in ccCRP2 gene sequence. In *X. laevis*, apart from one intron in the same position described previously, a second intron is also present and divides the 5'UTR in a position that correlates with the first and second intron found in ccCRP2 gene, supporting the proposal that

the range of CRP defined have probably evolved from the same ancestor gene (Lin and Liu, 1993). The three/four exon organization may represent a more ancestral state, and if this is the case then an intron fusion and posterior deletion appears to have occurred in an ancestral amphibian CRP gene.

Analysis of the sites of CRP expression in carp revealed a widespread constitutive expression of both genes. Mucosal sites (skin, gut, gill) as well as systemic sites (muscle, spleen, head kidney, liver, blood) were positive for the two CRP genes characterized in common carp. However, different expression levels were observed with respects to each CRP gene. This is particularly obvious in the liver where only ccCRP1 is expressed in any substantial quantities and may suggest different biological roles of the two common carp CRPs characterized. The differential tissue expression of ccCRP1 and ccCRP2 could suggest a tissue-specific regulation of CRPs in fish. Further studies utilizing immunostimulants are needed to elucidate whether expression levels could be modulated and whether this translates into different functional activity especially for those organs where expression was relatively low. However, evidence from the current study does indicate that, while structural conservation of carp CRPs is marked throughout vertebrate evolution, its expression patterns appear to have evolved differently.

It is of interest that although blood was found to be the tissue with highest values of both ccCRPs gene expression, protein measurements clearly show that CRP serum levels are the lowest level recorded. Whilst this observation supports previous studies in several fish species in which 'normal' CRP levels in serum were relatively low e.g. in the range of 1–400 µg per mL (Huong Giang et al., 2010; Liu et al., 2004; MacCarthy et al., 2008; Paul and Mandal, 1998; Paul et al., 2001; Pepys et al., 1978; Robey et al., 1983; Sinha and Mandal, 1996; White et al., 1981; Winkelhake et al., 1983) our results do suggest that serum levels alone may not be an accurate reflection of the disease protection induced by this pentraxin.

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