# At least two expressed genes for transcription factors Pitx2 and Rpx are present in common carp and are upregulated during winter acclimatization.

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ABSTRACT

The mechanisms of seasonal acclimatization in eurythermal fish such as common carp are not fully understood. Here, we concentrate on the regulation of pituitary factors, as this organ was shown to be highly affected by seasonal changes. We cloned and sequenced two different cDNAs for each of the transcription factors Pitx2 and Rpx, known to play a role in pituitary development. We show that these genes are conserved throughout evolution, to different degrees depending on the specific domain considered. Finally, we show that the cDNAs for both factors are clearly up-regulated during the winter season, in sharp contrast to other regulators such as Pit1 or pituitary hormone genes such as prolactin (*prl*) and growth hormone (*gh*). Our results suggest that increased expression of Pitx2 and Rpx contributes to seasonal adaptation of common carp to winter conditions.

Key words: Pitx2; Rpx; gene expression; carp fish; seasonal control; acclimatization;

## INTRODUCTION

Eurythermal fish rearrange their molecular and cellular functions to compensate for the circannual environmental seasonal changes of their habitat. This acclimatization process occurs through a cyclical reprogramming of molecular processes, involving in common carp (*Cyprinus carpio*) the modulation of transcriptional and translational events in various tissues (Krauskopf *et al.*, 1988; Figueroa *et al.*, 1994; Goldspink et al., 1995; Kausel *et al.*, 1999; Molina *et al.*, 2002). Likewise, although much less complex than the process of acclimatization, long-term adaptation to different temperatures also involves reprogramming of gene expression in different tissues (Gracey *et al.*, 2004), including carp pituitary gland (Figueroa *et al.*, 1997; Arends *et al.*, 1998). In fish, the pituitary seems to function as a central node controlling the adaptive compensatory response to stressors or environmental changes. Increased expression of prolactin (Prl) mRNA, the most versatile pituitary hormone, occurs in the *rostral pars distalis* (RPD) of summer-acclimatized carp, as compared to the negligible level of transcription detected in winter-acclimatized fish (Figueroa *et al.*, 1994). Photoperiod appears to be a particularly relevant factor for modulation of *prl* transcription in carp (Figueroa *et al.*, 1997). Another pituitary hormone of the Prl family that is up-regulated during the summer season is growth hormone (Gh) (Figueroa *et al.*, 2005). In good correlation with these observations, expression of the main regulator of these genes, the pituitary-specific transcription factor Pit1 is strongly increased during acclimatization of the common carp (*Cyprinus carpio*) from winter to summer (Kausel *et al.*, 1999).

During embryogenesis, various other transcription factors were shown to be involved in morphogenesis of the primordial pituitary and in specification and differentiation of the specific cell types in mammals (Zhu *et al.*, 2007). In zebrafish similar functions

are played by *pit1* (Herzog *et al.*, 2003; Nica *et al.*, 2004), *eya1* (Lopez *et al.*, 2006; Nica *et al.*, 2006) or *asc1* (Pogoda *et al.*, 2006), suggesting a good conservation of the regulation of pituitary ontogenesis from fish to man (Pogoda and Hammerschmidt, 2009). Rpx1 and Pitx2 are two members of the "paired-like" homeobox domain gene family that are expressed in mouse at early stages in development (Olson *et al.*, 2003; Chou *et al.*, 2006). Pitx2 is required for formation of various organs, including palate, heart, lung, muscle and tooth (Amendt *et al.*, 1998). It is mutated in the human Rieger syndrome and has been well studied for its role in determination of left-right asymmetry during embryogenesis (Essner *et al.*, 2000). In the pituitary, Pitx2 deficiency leads to defects in cell proliferation (hypoplasia) as well as to expansion of the Pit1 lineage and differentiation of gonadotrope cells. It binds to and activates the promoters of the *prl*, *gh*, *gsua*, *fshb*, *lhb*, *pomc* genes in combination with other, neighboring factors (Quentien *et al.*, 2002). Different mutations affecting human PITX2 DNA-binding or trans-activation have been described in Rieger patients (Quentien *et al.*, 2006). Besides its N-terminal, conserved DNA binding homeodomain, the Pitx2 factor contains a C-terminal domain (OAR, **o**tp-, **a**ristaless-, **r**ax-domain) that is involved in regulation of DNA-binding and transcriptional activation (Amendt *et al.*, 1999).

Rpx1 (Hesx1, Anf1) is a transcriptional repressor transiently expressed in the mouse pituitary primordium and playing a role in early determination and differentiation (Hermesz *et al.*, 2003). It is among the first factors expressed specifically in the pituitary primordium and is maintained until e13.5. Mouse embryos lacking Rpx1 display pituitary displasia, reduced proencephalon, anophtalmia or microphtalmia, defects in the olfactory tract and hypothalamus (Dattani *et al.*, 1998). Maintenance of Rpx1 expression in the pituitary beyond e13.5 in transgenic mouse embryos results

in loss of lineages depending on the related factor Prop1 indicating that a tight control of the opposing actions of these two factors is required for normal development in mouse. Prop1 is required for onset of Pit1 expression and thus for differentiation of the entire Pit1 lineage (Zhu *et al.*, 2007).

The two factors Pitx2 and Rpx are thus involved at different levels of pituitary development. Here, we obtained the cDNAs for their homologs in common carp (*Cyprinus carpio*) and we investigated their expression during seasonal acclimatization. We show that two different homologs are present for each gene, that all are expressed in adult carp pituitary and that their expression is clearly up-regulated during winter season, in contrast to other regulatory genes such as *pit1*.

## MATERIALS AND METHODS

### Animals and tissue preparation

Adult male carp (*Cyprinus carpio*) weighing about 1000 - 1500g were caught during winter and summer and maintained in a fixed 3 x 4 m cage submerged 2 m in an affluent of the same river. The water temperatures in winter and in summer were 8 - 10°C and 18 - 20°C, respectively.

Pituitary glands from winter- and summer-acclimatized carp were dissected and either fixed immediately for *in situ* hybridization in cold 4% paraformaldehyde or frozen in liquid nitrogen and stored at -80°C for RNA extraction.

### RT-PCR amplification of Pitx2 and Rpx cDNAs

Total RNA was isolated according to Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). RNA was treated with RNase-free DNase I (Invitrogen) and reverse transcribed using SuperScript II (Invitrogen) and oligo $dT_{15}$ (Invitrogen). PCR was carried out with 2.5 units of Taq DNA Polymerase (Invitrogen) on 0.5 µg cDNA in a solution containing 20 mM Tris/HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, and 0.5 mM forward and reverse primer. Amplification was performed for 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and elongation for 1 min at 72°C. Amplification products were subcloned into pGEM-T-Easy (Stratagene) and sequenced from both sides.

Two different carp *pitx2* cDNAs were cloned from a single individual, clone $pcPitx2_{110}$ (Pitx2-10s: 5'GAAGAGACAAAGGCGGCAACGAAC-3' / zPitx2-4a: 5'-TCTTACACCGGTCTATCCAC-3') corresponds to gene-I deposited in GenBank

accession number EF051103, and pcPitx2$_7$ (Pitx2-s: 5'-TGGTTCAAGAATCGACGGGCAAAATGG-3' / Ptx3a: 5'-GTTACAAGTGTCCCGGTAGAC-3') to gene-II accession number EF051104 (subscript numbers refer to laboratory clone numbers). The carp *rpx* 5'-cDNA sequence was amplified by 5'-RACE (RML-RACE Kit, Ambion) and antisense cRpx3a 5'-GCAAGTTCTTCACGTATATC-3' yielding pcRpx$_{46}$. From the same individual, two different cDNAs, pcRpx$_{64}$ and pcRpx$_{60}$, were obtained with anf1s 5'-TGAACTGGTACATCGGGCGCAGGCC-3' / zRpx4a 5'-CTCTCAGTGTTCTTCTCTGC-3'. Clearly overlapping sequences from pcRpx49 and pcRpx64 were combined and deposited as cRpx under accession number EF051105.

For quantitative real-time RT-PCR experiments, amplification was performed by denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and elongation for 30 s at 72°C. The primers used were: carp *ß-actin* derived from sequence M24113: cbeta-acts 5'-GGACCTGTATGCCAACACTG-3' and cbeta-acta 5'-GTCGGCGTGAAGTGGTAACA-3' (amplicon size in cDNA 281bp); carp *pitx2*: cPitx2-11s 5'-GAGAGGAGATCGCTGTTTGG-3' and cPitx2-12a 5'-CAGCCCAGTTGTTGTACGTG-3' (amplicon size 198bp); carp *rpx*: cRpx-11s 5'-CTGGATCTCCAGATGGCTTC-3' and cRpx-12a 5'-TCCACTGAAGCACCACTGTC-3' (206bp). The obtained Ct values were first normalized relative to ß-actin and the log-fold ratio between winter and summer samples was obtained by using summer values as calibrator.

***In situ* hybridization**

Carp pituitary sections were obtained using a cryostat, placed on gelatinized slides and stored at -80°C. *In situ* hybridization was performed on sections of different specimens (summer- and winter-acclimatized carp) in parallel as previously described (Figueroa *et al.*, 1994), except that the washing step was carried out at 42°C.

As probe, the carp *pitx2* specific antisense oligonucleotide cPitx2-2a 5'-CCGAATTTAGAGAGGGGTTGC-3' was used as described (Kausel *et al.*, 1999). Control hybridization was performed with sense oligonucleotide Pitx2-1s 5'-AGCCCTACGATGACATGTATC-3'. A zebrafish specific *rpx* riboprobe was utilized as described previously (Kausel *et al.*, 1999). Sections from four individual winter carp and four individual summer carp were processed in parallel. Quantification of the label in the tissue sections was performed using an automated image digitizing system Image-Pro-Plus 3.0 as described earlier (Kausel *et al.*, 1999). Three to five sections from each individual were analyzed in this way and mean optical densities were calculated. Differences were assessed using the Student's t-test. $P<0.08$ for *pitx2* and $P<0.09$ for *rpx* were considered significant.

## RESULTS

### Characterization of two distinct *pitx2* cDNAs in carp pituitary

We obtained total RNA from adult carp pituitary and synthesized cDNA by reverse transcription using an oligo(dT) oligonucleotide as primer. This cDNA was used to perform PCR reactions using primer pairs designed against various parts of the zebrafish *pitx2* sequence. Several amplified fragments were obtained, corresponding to overlapping regions of the carp *pitx2* cDNA. Sequence analysis and assembly of overlapping clones with identical sequences allowed us to obtain two different partial cDNA sequences coding for Pitx2 in carp. Both sequences start in exon III, the longer one at position 471 (cPitx2-I, GenBank Accession EF051103) and the shorter one at position 646 (cPitx2-II, GenBank Accession EF051104) relative to the coding sequence of the aligned zebrafish sequence (Fig. 1). Only the cPitx2-I cDNA covers the homeo-domain at the N-terminus. The carp sequences are highly conserved and also present a high degree of similarity to the zebrafish sequence. Alignment of the amino acid sequences deduced from the cDNA clones reveals the same high conservation between the two carp sequences, the zebrafish sequence and even to the human and mouse sequences (Fig. 2). Only four positions differ between the two carp sequences and only three between each carp sequence and zebrafish. A very high degree of similarity is also observed relative to mammalian sequences, human or mouse.

### Characterization of two distinct *rpx* cDNAs in carp pituitary

The same cDNA from adult carp pituitary was used to perform PCR reactions using primers designed against the zebrafish *rpx* sequence (GenBank accession NM131349). Several overlapping fragments were obtained, corresponding to various regions of the carp *rpx* cDNA. Assembly of overlapping clones with identical sequences allowed us to obtain two different cDNA sequences covering the entire coding region for carp *rpx* (Fig. 3). These two cDNAs differ by several single base pair changes, but importantly the shorter sequence (cRpx-II) presents a deletion of 21 nucleotides relative to the longer sequence (cRpx-I, GenBank Accession EF051105). In the rest of the sequence, the two carp cDNAs display a similarity of 95% at the nucleotide level. Relative to the zebrafish sequence, cRpx-I presents 15 silent substitutions in the C-terminal region, coding for the homeo-domain, while several non-synonymous substitutions and 2 insertions are present in the N-terminal region (Fig. 3). Alignment of the amino acid sequences deduced from the cDNA clones reveals a very high similarity between the two carp sequences, while conservation is very high to other species such as zebrafish or human only in the homeo-domain (Fig. 4). In the N-terminal region, similarity to the zebrafish sequence is still high while the conservation to the human and mouse sequences is low (Fig. 4). A striking observation is the putative extension of the N-terminal region in the carp Rpx sequences, due to the presence of an additional, more upstream ATG translation initiation codon giving rise to an open reading frame in frame with the rest of the coding region. This extension would be a unique feature for carp Rpx. Although supported by the perfect codon usage in carp, we cannot at this stage decide whether the upstream ATG is really used. The carp sequences present three deletions relative to mammalian Rpx genes in the N-terminal region consistent with the ones in the zebrafish.

## Pitx2 and Rpx expression during acclimatization

To evaluate modulation of *pitx2* and *rpx* genes expression during seasonal acclimatization and to map the cellular distribution of their mRNAs, *in situ* hybridization was performed on successive sections of pituitaries from male adult winter and summer carp. Using antisense riboprobes corresponding to the 5'-region of carp *rpx* cDNA or antisense oligonucleotides for *pitx2*, specific transcripts were detected in carp pituitary sections (Fig. 5). No signal was obtained after incubation of the samples with digoxigenin labeled sense probes (Fig. 5). In striking contrast to increased *prl* and *gh* signals in summer carp (Figeroa et al., 1994; 2005), the signals observed with *pitx2* and *rpx* probes were much stronger in pituitaries from winter carp, especially in the *proximal pars distalis* (Fig. 5, A and B). This became evident with the semi-quantitative analyses obtained upon digitalization of the hybridization signals (Fig. 5). In addition, when *pitx2* and *rpx* mRNA levels were compared in total RNA from 3 different winter and summer carp pituitaries by quantitative real-time RT-PCR, a clear induction was observed in winter (log-fold = 4.4 for *pitx2* and 3.5 for *rpx*) (Fig. 5C).

## DISCUSSION

We have cloned and sequenced the cDNA for the carp *pitx2* and *rpx* genes. In both cases, we obtained at least two different cDNAs. The fact that most of the sequences derive from overlapping identical clones argues against reverse transcription or sequencing errors, moreover, for *rpx*, one cDNA displays a 21 nucleotide (7 codon) deletion. The presence of multiple differences, especially in view of the outstanding conservation of *pitx2*, strongly suggests that these cDNAs do not represent different alleles of the same gene, but rather duplicated gene copies. In teleosts, the presence of duplicated genes is relatively common (Ferris and Whitt, 1977). Particularly in tetraploids such as carp, many occurrences of duplicated genes have been described (Kausel *et al.*, 2006). In some of these duplicate loci, only one copy is effectively transcribed (Ferris and Whitt, 1977). The duplicate *rpx* and *pitx2* cDNA sequences identified here from one individual pituitary show that both genes are expressed in the same organ.

Analysis of the Pitx2 amino acid sequences revealed an outstanding conservation, between the two carp sequences, between carp and zebrafish and between fish and mammalian. No substitution occurred in the homeo-domain or in the C-terminal OAR domain, only minor differences are found in the central part of the sequence (Furukata et al., 1997). These observations confirm the importance of these two conserved regions, which are involved in DNA-binding and/or interaction with other transcriptional cofactors (Olson et al., 2003; Amendt et al., 1998, 1999). One interesting feature is the doublet substitution SA in cPitx2-I to PT in cPitx2-II, compared to the zebrafish PA sequence at position 1013 (Fig. 2). This appears as an example of divergent evolution of two duplicated gene copies, where possibly an “ancestral”, zebrafish sequence PA diverged to SA in one copy and to PT in the

other. The functional significance of these changes remains to date unclear. None of the mutations in the carp *pitx2* sequences corresponds to a mutation described in a Rieger patient at the corresponding position in the human sequence, suggesting that the two encoded proteins in carp are likely to be functional (Tümer and Bach-Holm, 2009).

The Rpx amino acid sequence appears to be less conserved, only the homeo-domain displays an important similarity between fish and mammals (Fig. 4). Interestingly, the deletion in cRpx-II results in deletion of the two C-terminal amino acids of the generally recognized 60 amino acid long homeo-domain. Similarly, the R at position 446 is mutated to C in the cRpx-I sequence, although this was not the case in all the other clones that we obtained. Although these amino-acids are part of the extensive homology domain, these observations might indicate that these particular residues are not crucial for Rpx function. The two carp Rpx factors share with the zebrafish factor the deletions in the central region relative to mammals. These regions might be specifically involved in the action opposing Prop-1 during pituitary development in mammals, as there seems to be no homolog for this factor in teleosts (Olson et al., 2003; Mantovani et al., 2006). In contrast, both carp Rpx sequences retain the two N-terminal regions deleted in zebrafish and present an additional putative N-terminal extension. The importance of these regions is not clear to date.

We obtained the cDNAs for Pitx2 and Rpx from RNA extracted from one adult carp pituitary, indicating that both genes are expressed in this gland in adults. This observation is confirmed by the *in situ* hybridization experiments, where we detect both mRNAs mainly in the PPD of adult pituitaries. Expression of these transcription factors was mainly studied during embryogenesis, thus it is interesting that we

observed their expression in adults (Chou *et al*., 2006; Pogoda and Hammerschmidt, 2009). In rats, the three Pitx2 variants were found to be expressed in adult brain and pituitary in overlapping patterns (Smidt *et al.*, 2000). *Pitx2* expression was also detected in pituitaries of adult rat and human, as well as in pituitary cell lines and in certain prolactinomas, where it was shown to be required for full expression of *Prl* (Quentien *et al.*, 2002). Rpx is not detected in adult mice (Tümer and Bach-Hohn, 2009), however its expression was found in adult human normal pituitaries and in all types of pituitary adenomas (Mantovani *et al.*, 2006). Thus, our observation in carp is consistent with the notion that in most vertebrates, these two factors play a role in adult pituitary function.

During the seasonal acclimatization of carp, photoperiod appears to act as a relevant modulator of pituitary gene expression (Figueroa *et al.*, 1997). In particular, pituitary hormones Prl and Gh are highly expressed in summer, as well as one of their important regulators, the transcription factor Pit1 (Figueroa et al., 1994; 2005; Kausel et al., 1999). When we investigated the expression levels of *pitx2* and *rpx* by *in situ* hybridization and quantitative RT-PCR, it clearly appeared that both cDNAs are significantly induced in winter relative to summer carp, in sharp contrast to all other genes that were previously studied. At present, we do not know whether both genes coding for each factor are induced in winter, but the steady-state level of the cDNAs for each factor were clearly increased in winter. Only two genes up-regulated in winter have been described before, the gene for nucleolin, which is concomitant to a severe reorganization of nucleoli, nucleolar segregation and inhibition of ribosome synthesis (Alvarez *et al.*, 2003) and the gene for macroH2A-1 (Pinto *et al.*, 2005), which was proposed to be involved in DNA methylation and chromatin remodeling (Buschbeck *et al.*, 2009). While these genes' up-regulation represents an attractive

model for down-regulation of general transcription and protein synthesis in winter, our observation of an up-regulation of the Pitx2 and Rpx transcription factors hints at a more specific action on defined genes. Although expression of both genes is not restricted to pituitary during development and in the adult, the observed modulation in this central regulatory gland might be part of a general endocrine mechanism to down-regulate the metabolism of the entire organism in winter (Alvarez *et al.*, 2003). Rpx is known as a transcriptional repressor interacting with several cofactors such as the Groucho factor Tle1 (Olson *et al.*, 2003) and the DNA methylase DNMT1 (Sajedi *et al.*, 2008). During mammalian development, its down-regulation is required for formation of the Prop-1 and Pit1 dependent lineages (Olson *et al.*, 2003). Prolonged Rpx expression in mouse leads to pituitary hypoplasia (Olson *et al.*, 2003). Similarly, in the adult, it is conceivable that Rpx up-regulation could lead to the observed repression of Pit1 expression and down-regulation of its target genes. In contrast, Pitx2 is considered to be a transcriptional activator, controlling the expression of its target genes in combination with other factors such as Pit1 (Quentien *et al.*, 2002). It is interesting to note in this context that Pitx2 was recently shown to interact with nucleolin, one of the two factors up-regulated in winter (Huang *et al.*, 2009). The authors describe a DNA microarray study where 868 genes were up-regulated and 191 were down-regulated by Pitx2 in human cells (Huang *et al.*, 2009). Moreover, its transcriptional activity is controlled by phosphorylation (Espinoza *et al.*, 2005). It will certainly be interesting in the future to determine the target genes of these two regulators and to investigate their effects during seasonal acclimatization. Candidate target genes are the Pit1 dependent pituitary hormones, such a *prl, gh*, *sl* or *tshb*, all of which are potentially involved in the control of general metabolism (Pogoda and Hammerschmidt, 2009).

In conclusion, we cloned two cDNAs coding for each carp Pitx2 and Rpx and we show that both of these factors are clearly up-regulated in winter. Our results suggest that these two transcription factors play a role in adaptation of common carp to seasonal environmental changes.

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Fig.1: Alignment of *pitx2* cDNAs sequences from carp and zebrafish. Z: *Danio rerio* transcription factor *pitx2a* (pitx2a) mRNA, complete cds, 1820bp AF156906; I: *Cyprinus carpio pitx2* gene I EF051103; II: *Cyprinus carpio pitx2* gene-II EF051104. Grey shading highlights the homeodomain; in bold divergent amino acids and corresponding codons, slash indicates an amino acid change between the two carp sequences; the sequence of the degenerated oligonucleotides is underlined; arrows indicate exon-intron sites.

Fig.2: Very high conservation of Pitx2 protein domains in vertebrates. An alignment of amino acid sequences derived from mammalian and fish Pitx2 cDNAs is shown. Asterix indicates identical amino acids; colon indicates conserved amino acid substitutions and single point semiconserved amino acid substitutions; carp sequences are in bold; grey shading highlights the homeobox DNA binding domain; OAR domain in italics.

Fig.3: Sequence alignment of *Rpx* nucleotide sequences.
Z: *Danio rerio rpx* (GenBank accession NM131349ZF); I: *Cyprinus carpio rpx I* cDNA (GenBank accession EF051105); II: *Cyprinus carpio rpx II* cDNA (GenBank accession GU585761). The derived carp amino acid sequence is shown above the carp *rpx II* cDNA sequence, in bold the amino acids different relative to the zebrafish sequence are shown; slash indicates amino acid changes between the two carp sequences; grey shading highlights the homeodomain; the sequence of the oligonucleotides derived from zebrafish is underlined.

Fig.4: Alignment of amino acid sequences derived from mammalian and fish Rpx cDNAs. Asterix applies to identical amino acids; colon to conserved amino acid substitutions, single point to semiconserved amino acid substitutions, in bold carp sequences, grey shading highlights homeobox DNA binding domain.

Fig.5: Increased *pitx2* and *rpx* expression in somatotrophs of winter- compared to summer-acclimatized carp. (A) *pitx2* and (B) *rpx* specific transcripts by *in situ* hybridization in *rostral pars distalis*, region of somatotrophs, of summer- and winter-acclimatized carp pituitary sagittal sections (anterior to the left; dorsal to the top). The inset presents control hybridizations with the respective sense probes. Graphs represent differences of signals quantified in digitalized pictures of four individuals (n=4) from each season. Columns represent mean integrated optical density (IOD) with standard deviation indicated by bars. Student's t-test (A) $P<0.08$; (B) $P<0.09$ was considered significant. (C) Real-time RT-PCR quantification of *pitx2* and *rpx* mRNA from individual carp pituitaries. The columns represent the log-fold ratio between the means of three winter-acclimatized relative to three summer-acclimatized individuals +/- standard deviation.

**Figure1**

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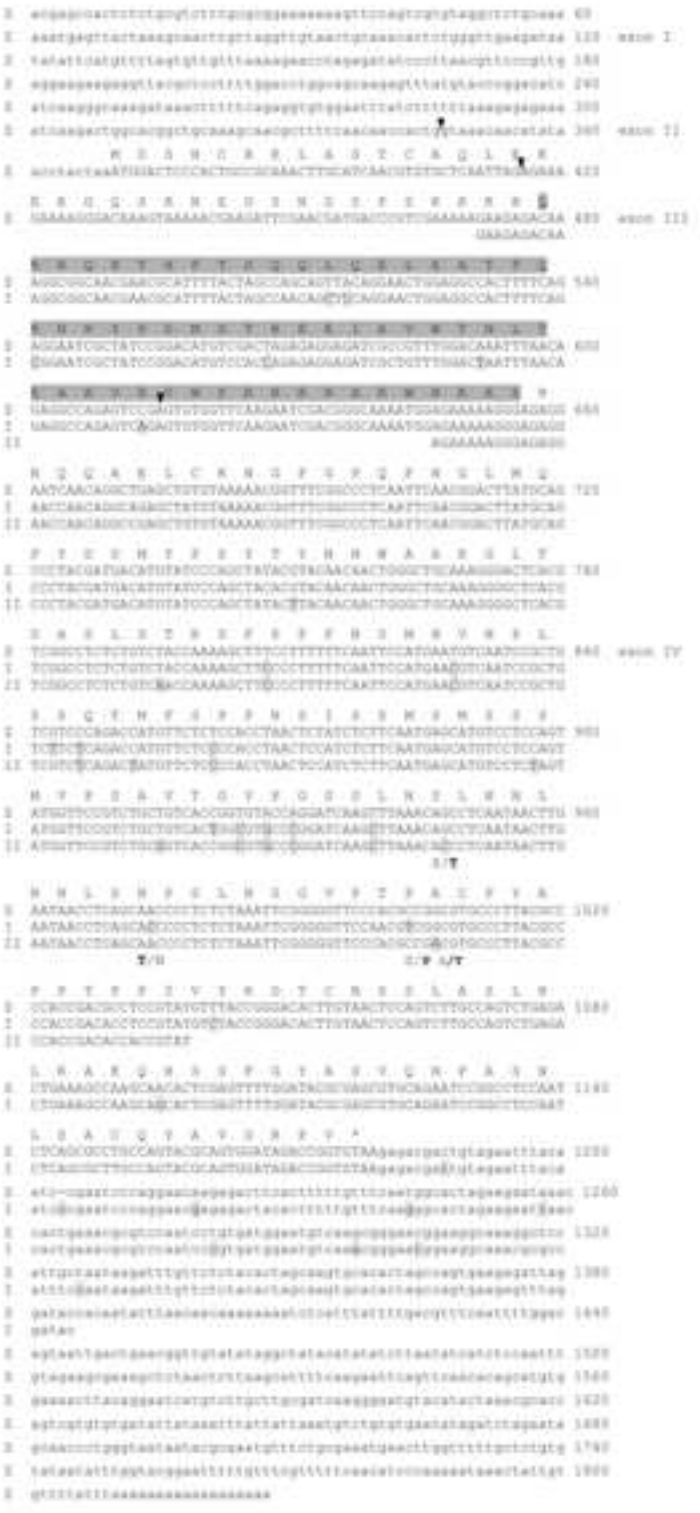

Figure 1

**Figure2**

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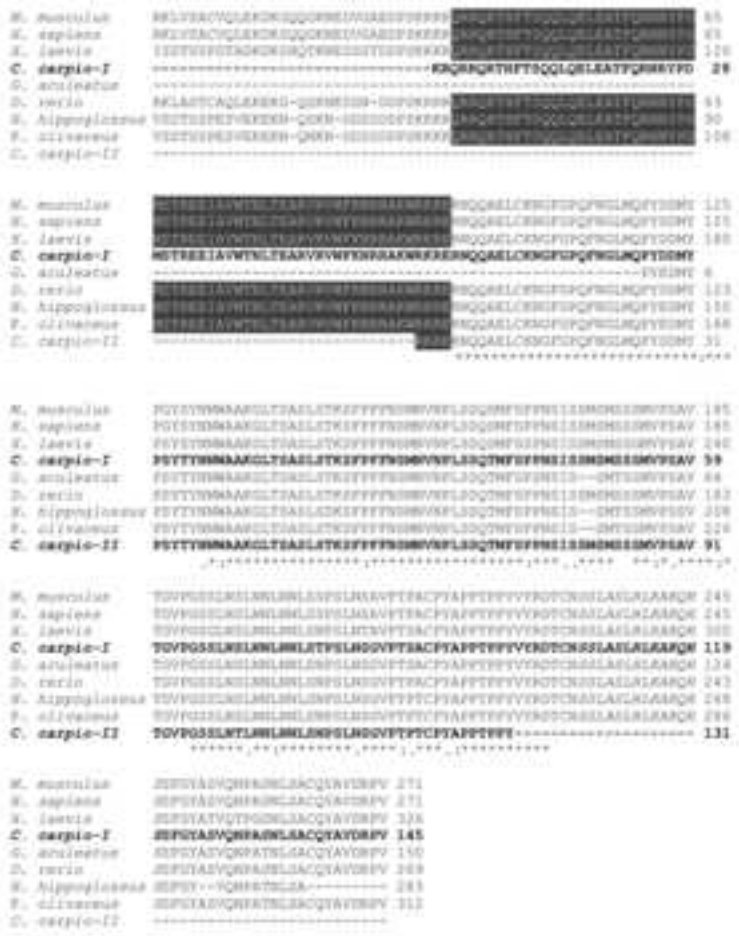

Figure 2

**Figure3**

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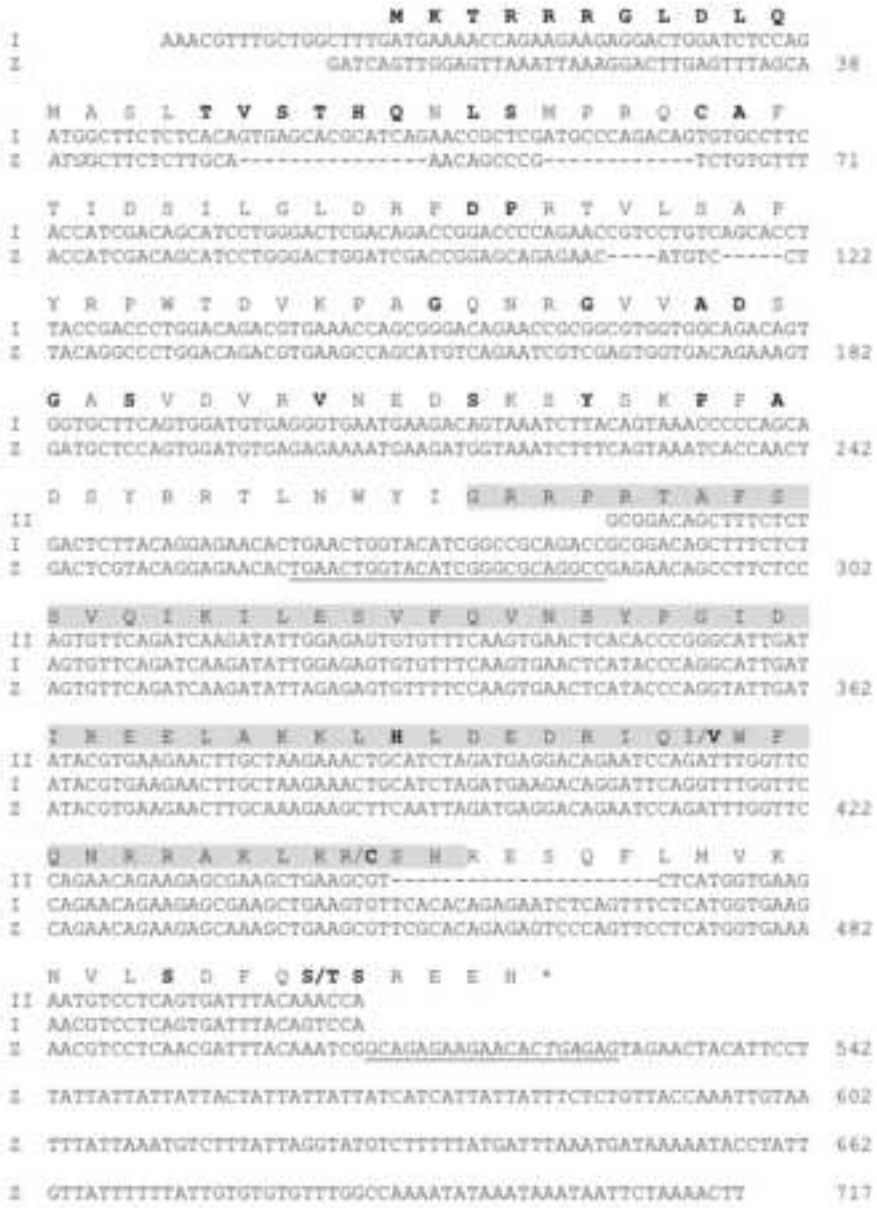

Figure 3

**Figure4**

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Figure 4

**Figure5**

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