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The evolution and functional divergence of the *beta-carotene oxygenase* gene family in teleost fish—Exemplified by Atlantic salmon

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ABSTRACT

In mammals, two carotenoid cleaving oxygenases are known; beta-carotene 15,15′-monooxygenase (BCMO1) and beta-carotene 9′,10′-oxygenase (BCO2). BCMO1 is a key enzyme in vitamin A synthesis by symmetrically cleaving beta-carotene into 2 molecules of all-*trans*-retinal, while BCO2 is responsible for asymmetric cleavage of a broader range of carotenoids. Here, we show that the Atlantic salmon *beta-carotene oxygenase* (*bco*) gene family contains 5 members, three *bco2* and two *bcmo1* paralogs. Using public sequence databases, multiple *bco* genes were also found in several additional teleost species. Phylogenetic analysis indicates that *bco2a* and *bco2b* originate from the teleost fish specific genome duplication (FSGD or 3R), while the third and more distant paralog, *bco2 like*, might stem from a prior duplication event in the teleost lineage. The two *bcmo1* paralogs (*bcmo1* and *bcmo1 like*) appear to be the result of an ancient duplication event that took place before the divergence of ray-finned (*Actinopterygii*) and lobe-finned fish (*Sarcopterygii*), with subsequent nonfunctionalization and loss of one *Sarcopterygii* paralog. Gene expression analysis of the *bcmo1* and *bco2* paralogs in Atlantic salmon reveals regulatory divergence with tissue specific expression profiles, suggesting that the *beta-carotene oxygenase* subtypes have evolved functional divergences. We suggest that teleost fish have evolved and maintained an extended repertoire of beta-carotene oxygenases compared to the investigated *Sarcopterygii* species, and hypothesize that the main driver behind this functional divergence is the exposure to a diverse set of carotenoids in the aquatic environment.

1. Introduction

The mammalian family of carotenoid cleaving oxygenases contains two members; beta-carotene 15,15′-monooxygenase (BCMO1) and beta-carotene 9′,10′-oxygenase (BCO2) (Lobo et al., 2011). BCMO1 serves a key function in vitamin A synthesis by cleaving beta-carotene, at the 15,15′ position, into 2 molecules of retinal (von Lintig and Vogt, 2000; Wyss et al., 2000). The substrates of BCMO1 are pro-vitamin A carotenoids containing at least one unsubstituted beta-ionone ring, such as beta-carotene and beta-cryptoxanthin (Lindqvist and Andersson, 2002). BCO2, also named BCDO2, is responsible for asymmetric cleavage at the 9′–10′ double bond of the polyene backbone of its substrates (Kiefer et al., 2001), and is suggested as an alternative pathway to retinoic acid synthesis (Simões-costa et al., 2008). BCO2 displays broader substrate specificity than BCMO1, and is shown to provide oxidative cleavage of both carotenes, including lycopene, and xanthophylls like zeaxanthin, lutein and beta-cryptoxanthin in mammals (Kiefer et al., 2001; Kim et al., 2011; Mein et al., 2011). In contrast to BCMO1 which is a cytoplasmic protein (Lindqvist and Andersson, 2002), BCO2 was recently found to be expressed in the mitochondria where it acts as a carotenoid scavenger, providing protection from carotenoid-induced mitochondrial dysfunction (Amengual et al., 2011; Lobo et al., 2012).

Two genome duplications are hypothesized to have occurred early in the vertebrate lineage (the 2R hypothesis). Analysis of the sea lamprey genome and comparisons to genomes of jawed vertebrates

Abbreviations: aa, amino acid(s); ANOVA, analysis of variance; BCMO1, beta-carotene 15,15′-monooxygenase; bco, beta-carotene oxygenase; BCO2, beta-carotene 9′,10′-oxygenase; bp, base pair(s); cDNA, DNA complementary to RNA; ef1a, elongation factor 1-alpha; EST, expressed sequence tag; FSGD, fish-specific genome duplication; ICSASG, International Collaboration to Sequence the Atlantic Salmon Genome; MAFFT, Multiple Alignment using Fast Fourier Transform; MEGA, Molecular Evolutionary Genetics Analysis; ML, maximum likelihood; MYA, million years ago; NCBI, National Center for Biotechnology Information; NIVA, Norwegian Institute for Water Research; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR; RPE65, retinal pigment epithelium-specific 65 kDa protein.

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indicates that the two genome duplications likely took place in a common ancestor, before the divergence of jawed vertebrates (Smith et al., 2013). Several studies provide evidence for a fish-specific genome duplication (FSGD or 3R) estimated to have occurred approximately 350 Ma ago (Christoffels et al., 2004; Jaillon et al., 2004). Finally a whole genome duplication event occurred in the salmonid lineage 25–100 MYA, rendering salmonids partially tetraploid (Allendorf & Thorgaard, 1984; Lien et al., 2011).

In the present study, we have identified and characterized five *beta-carotene oxygenase* (*bco*) genes in Atlantic salmon. In addition, we have used public genome databases to identify other piscine beta-carotene oxygenases. The piscine *bco* sequences were used to investigate the evolutionary relationships within the teleost family of *bco* genes and in relation to *Sarcopterygii* orthologs.

2. Material and methods

2.1. Animals

Six healthy Atlantic salmon with an average weight of 1250 g were sampled from a research fish farm run by the Norwegian Institute for Water Research (NIVA). The fish were fed ad libitum commercial feed (Skretting), containing 50 mg/kg astaxanthin. The muscle, liver and intestinal tissue from the midgut were flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from salmon muscle tissue using TRIzol Reagent (Invitrogen) and purified with the RNeasy soft tissue Mini Kit (Qiagen). RNA was treated with Invitrogen DNase and RNaseOUT™ Recombinant Ribonuclease Inhibitor, protocol according to the manufacturer. Sufficient quantity and quality of the total RNA were confirmed using NanoDrop (Thermo Scientific) and Bioanalyzer (Agilent Technologies). cDNA was synthesized using SuperScript® III Reverse Transcriptase (Invitrogen), a poly dT-primer for sequencing or a 1:1 mix of random hexamers and poly dT for qRT-PCR, and 500 ng RNA, protocol according to the manufacturer.

2.3. In silico bco sequence mining

Zebrafish *bco* reference sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/), NM_001040312 and NM_131798, were queried using BLASTN against the NCBI BLAST EST database, The Gene Index Project and salmon trace archives, a *de novo* draft salmon genome based on sequences provided by the International Collaboration to Sequence the Atlantic Salmon Genome (ICSASG) (Davidson et al., 2010), to identify salmon *bco* sequences. Obtained salmonid *bco* sequences were continuously included as query to extend the search. *Bcmo1* and *bco2* sequences from additional species were identified in the NCBI, UCSC and Ensembl databases. Chromosomal positions of the medaka, *Tetraodon* and zebrafish *bco2* and *bcmo1* orthologs were obtained from the UCSC Genome Bioinformatics Site.

2.4. Amplification and sequencing of Atlantic salmon bco2 and bcmo1 genes

Primers were designed in Primer3Plus (Untergasser et al., 2007) based on sequences obtained from trace archives and the following ESTs: TC184762, BT026919.1, EV366861.1, CA041600, CB516394, and TC109507. A cDNA sequence for the *bco2 like* gene was kindly provided by Dr. Ben Koop (University of Victoria, Canada). The coding region of the salmon *bco2a* gene was amplified with primers P1 + P2 and P3 +

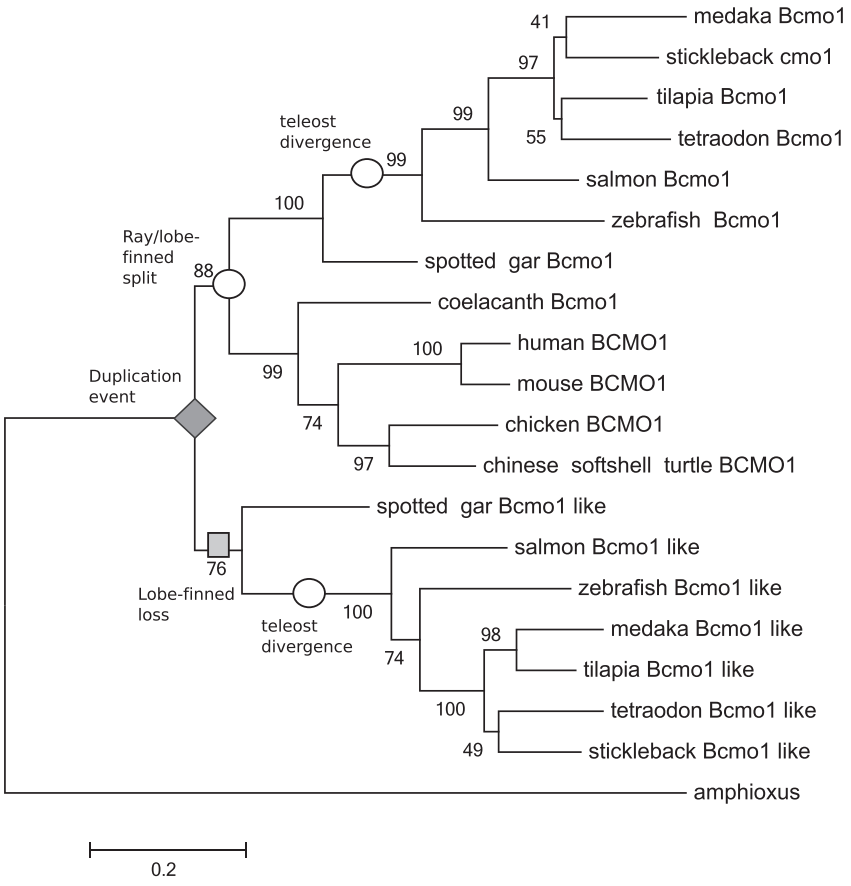

Fig. 1. Phylogenetic tree of Bcmo1 proteins in ray-finned and lobe-finned species, based on amino acid alignment. Amphioxus (*Branchiostoma floridae*) is included as outgroup.