Molecular cloning, tissue distribution and daily expression of cry1 and cry2 clock genes in European seabass (Dicentrarchus labrax)

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A R T I C L E   I N F O

Article history:
Received 18 May 2012
Revised in received form 10 July 2012
Accepted 10 July 2012
Available online 25 July 2012

Keywords:
Circadian rhythm
cry
Cryptochrome
Molecular clock
Sea bass

A B S T R A C T

Biological rhythms are driven by circadian oscillators, which are ultimately controlled by the cyclic expression of clock genes. Cryptochromes (CRY), blue light photoreceptors, belong to the negative elements of the transcriptional feedback loop into the molecular clock. This paper describes the cloning and characterization of two cryptochromes (cry1 and 2) in European seabass, which is considered an interesting chronobiology model due to its dual (diurnal/nocturnal) behavior. The cloned cDNA fragments encoded for two proteins of 567 and 668 amino acids, which included the FAD-binding and the DNA-photolyase domains. Moreover, both proteins had a high homology with cryptochrome proteins (Cry) of other teleost species (only found in zebrafish (Danio rerio)) and in mammals. The Cry3 and 4 genes were expressed in several tissues of seabass (brain, liver, heart, retina, muscle, spleen, gill and intestine). In addition, the daily expression of cry1 was rhythmic in brain, heart and liver with the acrophase around ZT 03:15 h (after the onset of lights). Similarly, the cry2 daily expression was rhythmic in liver, peaking at ZT 03:28 h, whereas in brain the acrophase was at ZT 11:08 h (shortly prior to the offset of lights). These findings provide new elements to help understanding the functioning of the molecular clock of seabass.

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1. Introduction

All organisms are adapted to cyclic environmental changes, and are able to anticipate and predict them. Circadian rhythms are generated, regulated and maintained by interlocked autoregulatory feedback loops (positive and negative), whereby a group of circadian clock genes and their proteins interact (Wang, 2008). Cryptochromes (CRYs) belong to the DNA photolyase/cryptochrome flavoprotein family (Cashmore et al., 1999). These proteins are very much conserved across bacteria, plant and animal kingdoms, and are thought to act as blue light photoreceptors (Sancar, 2003). The first evidence of the existence of cryptochromes was found in Arabidopsis thaliana (Ahmad and Cashmore, 1993), where it is involved in specific light-dependent developmental and circadian entrainment. Later, two CRY proteins (CRY1 and CRY2) were identified in humans and mice (Hsu et al., 1996; Kobayashi et al., 1998), and another CRY protein in Drosophila (Emery et al., 1998). Recently, a larger number of cryptochrome (Cry) proteins have been described in fish, including Cry1a and b, Cry2a and b, Cry3 and Cry4 in zebrafish (Danio rerio) (Kobayashi et al., 1998) and Cry1, 2 and 3 in goldfish (Carassius auratus) (Velarde et al., 2009).

Despite the presence of cryptochromes during phylogeny, the photolyase function is only present in plant cryptochromes. Cryptochromes were defined as molecules with a protein sequence homologous to the photolyase sequence; they do not repair DNA but do have an enzymatic function that is activated by blue light (Sancar, 2003). Due to this function, cryptochromes were proposed as photoreceptors or phototranslators for blue light, mediating circadian photoreception (Hsu et al., 1996; Todo et al., 1996; Miyamoto and Sancar, 1998; Sancar, 2003). In vertebrates, CRY1 and CRY2 belong to the negative elements of the transcriptional feedback loop of the circadian clock, which drives the biological rhythms of the organisms. Vertebrate CRY protein joins Period protein (PER) to form a complex which binds to and blocks the protein complex formed by Circadian Locomotor Output Cycles Kaput and Brain and Muscle Aryl hydrocarbon receptor nuclear translocator (ARNT)-Like (CLOCK:BMAL1), inhibiting CRY transcription (Okamura et al., 2002; Iuvone et al., 2005). Among fish species, only the clock gene machinery of zebrafish is well known, differing from the mammalian mechanism in the larger number of cryptochrome (cry) and period (per) genes. Although both zebrafish cry1 and 2 have two subtypes (“a” and “b”), the role of their corresponding proteins is similar to that of their homologues in mammals. In contrast, the Cry3 and 4 proteins (only found in fish) do not bind to the CLOCK:BMAL1 complex, and their function is still unknown (Kobayashi et al., 2000).

Besides being found in the master clock, cryptochrome (Cry) genes are expressed in a long list of peripheral tissues from mammals (Balsalobre, 2002; Muhlauer et al., 2004; Peirson et al., 2006; Zvonic et al., 2006), and fish (Kobayashi et al., 2000; Velarde et al., 2009). Furthermore, the expression of several cry genes differs in zebrafish and goldfish and is tissue-dependent in the latter, where cry genes oscillate.

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1095-6433/$ - see front matter © 2012 Elsevier Inc. All rights reserved. http://dx.doi.org/10.1016/j.cbpa.2012.07.004
with different acrophases depending on the tissue. However, cry genes expression was rhythmic in all cases, fitting a cosinor function in most (Kobayashi et al., 2000; Velarde et al., 2009).

European seabass (Dicentrarchus labrax) has great aquaculture and scientific interest, so that several genes of key endocrine axis (i.d. reproduction and food intake control) have been cloned and characterized (Cerda-Reverter et al., 1998; Gonzalez-Martinez et al., 2004; Isorna et al., 2008; Sauzet et al., 2008). In addition, this fish species is a prominent chronobiological model because of its dual behavior (diurnal/nocturnal), which allows seabass to change their feeding phase during the year and points to a flexible circadian system (López-Olmeda and Sánchez-Vázquez, 2010). However, the components of the seabass molecular clock and its function have been little explored. Only per1 gene has been cloned, and its distribution and expression have been characterized in several tissues (Sánchez et al., 2010), including in brain and liver of diurnal and nocturnal seabass (del Pozo et al., 2012).

The aim of the present research was to clone two clock genes, cry1 and cry2, in European seabass, as well as to study their tissue distribution and daily rhythm of expression in brain, heart and liver (central and peripheral oscillators, respectively).

2. Materials and methods

2.1. Fish-rearing conditions and sampling

Thirty-two adult seabass of 400 g were supplied by a local fish farm (Culmarex, S.A., Aguilas, Spain). Fish were reared in an aquaculture laboratory at the Naval Station of Algama (Cartagena, Spain). Seabass were kept in two 500 L tanks with an open seawater system and constant water temperature of 20 °C, and they were randomly fed during the photophase. The photoperiod was set at 12 h light: 12 h dark (LD), with 300 lux illumination on the water surface during the photophase. Light onset and offset were at 9:00 h (zeitgeber time, ZT 00:00) and 21:00 h (ZT 12:00), respectively. After 2 weeks, 5 seabass were anesthetized and sacrificed every 3 h (ZT 00:00, ZT 03:00, ZT 06:00, ZT 09:00, ZT 12:00, ZT 15:00, ZT 18:00 and ZT 21:00) and brain, heart and liver samples were taken. The samplings were performed under light during the photophase and dim red light (<2.4 μW/cm²) during the darkphase. The fish were taken alternately from different tanks at every time-point to minimize stress. Moreover, retina, spleen, gill, muscle and intestine samples were collected at ZT 06:00 to analyze the tissue distribution of cry1 and cry2 expression. TRIZol® (Invitrogen) was immediately added to the samples, which were frozen in dry ice and stored at −80 °C for future analysis.

Fish were reared and manipulated following Spanish legislation on Animal Welfare and Laboratory Practices, the experimental protocol was approved by the National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare and the legislation concerning the protection of animals used for experimental purposes (Directive 2010/63/EU).

2.2. Cloning of seabass cry1 and cry2

RNA from the whole brain was isolated using a commercial kit, PureLink Micro-to-Midi Systems (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. A multialignment of cry mRNA from several species identified conserved regions, which was used to design a degenerate primer set (Forward primer: 5’ CARGA GGGVTGATCACCACCA 3’ and reverse primer: 5’ TACATYTAYGACBC BTGGA A 3’) to amplify the cry CDNA fragments. The cDNA was synthesized from 1 μg of total RNA from European seabass whole brain homogenates, using the Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. This fragment was amplified by 35 cycles of PCR (30 s at 94 °C for denaturing, 30 s at 55 °C for annealing and 1 min at 72 °C for extension). The cDNA fragments were identified as putative cry genes of seabass by nucleotide BLAST analysis (National Center for Biotechnology Information, NIH, USA, http://blast.ncbi.nlm.nih.gov/Blast.cgi), using the cry genes of different fish species. The cry specific primers and nested primers for 3’ and 5’-RACE were designed from these partial cDNA fragments. Then, these fragments were lengthened to obtain the full-length by Rapid amplification of cDNA ends (RACE), using the SMART RACE CDNA amplification kit (Clontech Laboratories, Mountain View, California, USA), according to the manufacturer’s protocol and the 3’ and 5’-RACE described above. The first PCR conditions for the RACE reaction were: (Ahmad and Cashmore, 1993) 5 cycles at 94 °C for 5 s, 72 °C for 3 min; (Amores et al., 1998) 5 cycles at 94 °C for 5 s, 70 °C at 10 s, and 72 °C for 3 min; and (Balsalobre, 2002) 25 cycles at 94 °C for 5 s, 68 °C for 10 s, and 72 °C for 3 min. The nested PCR involved 25 cycles at 94 °C for 5 s, 68 °C for 10 s, and 72 °C for 3 min. Finally, the resulting RACE products were cloned in TOPO-TA vector (Invitrogen) and then sequenced. Coding sequences were submitted in GenBank database (accession no. JX046479 for cry1 and JX046478 for cry2).

The amino acid sequences were deduced from the cDNA sequences and compared to counterpart sequences currently available in GenBank (http://www.ncbi.nlm.nih.gov), using the BLASTX program (National Center for Biotechnology Information, NIH, USA, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Furthermore, an alignment was performed from the cry protein sequence of seabass and other cry proteins of teleosts (zebrafish, Somalian cavefish: Phreatichthys andruzzii, and Atlantic salmon: Salmo salar) available in GeneBank using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The accession numbers of the proteins used in both analyses are listed in Table 1.

The protein domains were predicted with the simple modular architecture research tool (SMART) program version 4.0 (Letunic et al., 2006; Schultz et al., 1998) (http://smart.embl-heidelberg.de/) and ScanProsite (http://expasy.org/tools/).

2.3. Phylogenetic analysis

Another alignment of CRY protein sequences was performed by the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index).

Table 1

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2.4. Tissues distribution of cry1 and cry2 gene expression and their daily expression

All samples were homogenized inTRizol® (Invitrogen) by means of a tissue homogenizer (Polytron®, PT1200, Kinematica, Switzerland). RNA was extracted using PureLink Micro-to-Midi Systems (Invitrogen) according to the manufacturer’s protocol. Total RNA concentration was measured by spectrometry. DNase I amplification grade (1 unit/μg RNA, Invitrogen) was added to avoid genomic DNA contamination. cDNA synthesis was performed with Supercript III Reverse Transcriptase and random primers (Invitrogen) in a 20 μL reaction volume.

To analyze the distribution of cry1 and cry2 expression in different tissues, cDNA of cry1, cry2 and β-actin from brain, heart, liver, retina, spleen, gill, muscle and intestine samples at ZT 0:00 h were amplified by PCR with specific primers (cry1 forward: TCTTTGTGGTTGCCCCCTCAT; cry1 reverse: CCTGAAACCAGTGGTGCCTGT; cry2 forward: CGCCAACCCTTGGGAAGCT and reverse: cry1: CCTGAAACCAGTGGTACGT and cry2: TGCCAGGTGTTGCTTCTGA) specified primers were included according to the manufacturer’s protocol. All samples were run in triplicate. An ABI Prism 7500 apparatus (Applied Biosystems) supported the ABI Sequence Detection System 7000 software (Applied Biosystems), which reproduced a cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s and finally a cycle at 60 °C for 1 min. All specified primers were designed with Primer Express software (Applied Biosystems) and synthesized by Invitrogen. The standard curve method tested the amplification efficiency, specificity of primers, and the amount of cDNA/sample. The relative expressions of cry1 and cry2 were calculated by the Ct method with efficiency correction, using β-actin gene as the reference gene (accession no. AJ493428), which has been validated for seabass by Patruno et al. (2008). The β-actin specific primers were: forward 5′ ACC CAG TCC TGC TCA CAG AG and reverse 5′ GGG AGT CCA TGA CAA TAC CAG TG.

2.5. Statistical analysis

Differences in cry1 and cry2 relative expression for each tissue (brain, heart and liver) along 24 h were analyzed by one-way ANOVA (ANOVA1) with SPSS 15.0 software followed by a Tukey test. Moreover, the Cosinor (CSR 3.0.2) analysis was performed to determine whether the rhythmic expression of cry1 and cry2 in brain, heart and liver, fitted the cosine function (Y = M + A × [cos (Ω × t + ϕ)]) where Ω is mesor, A is amplitude, ω is angular frequency (24/24 for the circadian rhythms) and ϕ is acrophase. The significance level was fixed at p < 0.05 for all the statistical analyses.

3. Results

3.1. Cloning of seabass cry1 and cry2

For cloning, degenerated PCR primers were designed from conserved regions in Cry protein of several species. Thus, the seabass cDNA partial sequences were sequenced and compared to other fish crys by BLAST (National Center for Biotechnology Information, NIH, USA), identifying both sequences as putative cry genes. Primers were designed from these nucleotide sequences to lengthen them by RACE to obtain full-length cDNA sequences. These full-length cDNA sequences of both seabass cry genes were compared with the Cry protein sequences of different fish species from the NCBI databases by BLASTX. The seabass cry1 and 2 showed high homology with other Cry proteins from several teleost fish. The homology percentage and the accession number of the used database proteins are presented in Table 1.

Moreover, the complete cDNA sequences codified two proteins of 567 and 668 aminosacids, respectively. Two domains, FAD-binding and DNA-photolyase, were indentified within both proteins (SMART, p < 0.01). The FAD-binding domain was included between the 5–169 and 5–170 positions for the Cry1 and Cry2 protein sequences, respectively, whereas, the DNA-photolyase domain was included between the 212–488 positions in both Cry protein sequences.

Consequently, the ClustalW2 analysis aligned the deduced protein sequence of seabass Cry1 and 2 with other Cry1 and 2 fish proteins from the database, showing high homology (Fig. 1).

3.2. Phylogenetic analysis

Evolutionary relations of different Cry subtypes were found between seabass and other vertebrates. The phylogenetic analysis separated Cry1 and Cry2 of all species in two sides of the tree (Fig. 2). The
Cry1 and Cry2 of tetrapods were grouped at both ends of the phylogenetic tree. In the middle, the Cry1 and Cry2 of the teleost fish were divided into two different groups, in which both seabass (D. labrax) Crys were included. Thus, seabass Cry1 was closely related to zebrafish (D. rerio) Cry1b and Cry1a and clownfish (Amphiprion melanopus) Cry1, whereas seabass Cry2 was closely related to zebrafish Cry2a, followed by goldfish Cry2 and zebrafish Cry2b.

3.3. Tissues distribution of cry1 and cry2 gene expression and their daily expression.

Both cry1 and cry2 of seabass were expressed in all analyzed tissues (brain, liver, heart, retina, muscle, spleen, gill and intestine) (Fig. 3). Moreover, the cry1 expression showed a daily rhythm in brain, liver and heart, peaking in the tree tissues at ZT 03:00 h, which differed statistically from the expression levels observed during the rest of the day (ANOVA I, p < 0.01) (Fig. 4). This rhythm fitted a cosineur curve (COSINOR, r < 0.01), showing the acrophase around ZT 03:15 h, a similar mesor and amplitude. On the other hand, the cry2 expression was rhythmic in brain and liver (not in heart), fitting a cosineur curve (COSINOR, r < 0.01) (Fig. 5), although the acrophases differed among tissues, at ZT 11:08 h in brain and ZT 03:28 h in liver. The cosine parameters (mesor, amplitude and acrophase) and statistically significance of cry 1 and 2 genes expression rhythms in seabass brain, liver and heart have been represented in Table 3.

4. Discussion

In the present study, two cryptochrome genes (cry1 and cry2) were cloned and characterized in seabass. These cDNA sequences codified two Crys proteins, 1 and 2 (of 597 and 668 aminoacids, respectively) which included two typical domains of CRY proteins, DNA-photolyase and FAD-binding. Animal cryptochromes belong to the photolyase-like flavoproteins family, which, together with the photolyase DNA repair enzymes, compose the cryptochrome/photolyase protein family. Cryptochromes and photolyase proteins share sequence homology as well as FAD (flavin adenine dinucleotide) cofactor and DNA-binding activity, although cryptochromes lack DNA-repair activity (Brudler et al., 2003; Song et al., 2007; Oztürk et al., 2007).

The deduced protein from the cry1 and 2 cDNA sequences of seabass showed high homology (> 78%) with Cry1 and 2 proteins from other teleost fish species such as zebrafish, Somalian cavefish and Atlantic salmon. In mammals, only two Cry (Ahmad and Cashmore, 1993; Amores et al., 1998) genes have been discovered so far (Kobayashi et al., 1998), while in fish species several cry paralogues have been described. Thus, as many as 6 cry genes (cry1a, cry1b, cry2a, cry2b, cry3 and cry4) have been found in zebrafish (Kobayashi et al., 2000), 3 crys genes (cry1, cry2 and cry3) in goldfish (Velarde et al., 2009) and 5 crys genes (cry1a, cry2b, cry3, cry4, cry5) in cavefish (Cavallari et al., 2011). The existence of the Cry paralogues in vertebrates has been explained by the presence of two rounds of whole-genome duplication (2R hypothesis) during the emergence of vertebrates (Ohno, 1970; Panopoulou and Poustka, 2005). In addition, a third round of whole-genome duplication (3R hypothesis) would have generated a larger number of cry genes in fish than in mammals, which would have occurred in the teleost lineage after its divergence from the tetrapod lineage but before its radiation (Amores et al., 1998; Woods et al., 2005). However, the 3R hypothesis alone is not able to elucidate the total number of cry genes in zebrafish and cavefish, so more studies are necessary to clarify this point (Wang, 2008).

In the present study, the phylogenetic analysis of seabass crys showed that CRYs of all species are clustered together and separately from CRYs, which form another cluster. Furthermore, the fish Cry (seabass included) were gathered within both subclusters (for Cry1 and Cry2) and separated from the CRYs of other taxa, suggesting that they appeared later in evolution. Similarly, Kobayashi et al. (2000) performed a phylogenetic analysis of the photolyase/cryptochrome family, and described a subcluster within the animal CRY cluster composed of all CRYs and 2 from fish and mammals and CRY of zebrafish (zCry3). In this subcluster, zCry1s and CRY1 of mammals (mCRY1s) were closely related, whereas, zCry2s were closer to CRY1s of both fish and mammals species than to mCRY2s. However, zCry3 diverged previously and zCry4 would not be included in this subcluster. Therefore, cry genes could be classified in three groups: the first one shared by cry1s and cry2s, and other two groups only with one member: cry3 and cry4, respectively. Only Cry1 and 2 had the capacity to self-inhibit their own transcription by means of blocking the CLOCK:BMAL1 complex, as in mammal Crys. The function of Cry3 and Cry4 remains
unknown, but they could be classified as *Drosophila* type CRY or as a separated group (Kobayashi et al., 2000; Kume et al., 1999). Among all zCrys, the zCry4 structure is the closest to *Drosophila* CRY (dCRY), seeming the candidate for a zebrafish circadian photoreceptor with a similar function to that of dCRY. Nevertheless, more in vivo experiments should be performed to clarify the zCry3 and 4 functions (Kobayashi et al., 2000).

PCR screening showed that *cry1* and *2* gene expression was present in all the seabass tissues analyzed in this investigation (brain, liver, heart, retina, muscle, spleen, gill and intestine), which also expressed *per1* gene (Sánchez et al., 2010). To date, circadian clocks in peripheral tissues have been reported in *Drosophila*, zebrafish, mammalian cell lines and tissues, supporting the hypothesis on the existence of decentralized clocks (Tamai et al., 2005). Moreover, *Drosophila* and adult zebrafish tissues, as well as zebrafish cell lines and embryos, are directly light-responsive (Whitmore et al., 1998; Schibler and Sassone-Corsi, 2002). In this research, the *cry1* daily rhythms were similar in seabass liver, heart and brain, peaking shortly after the lights were switched on. Also, the *cry2* oscillated rhythmically in liver with a similar acrophase to *cry1*, while in brain the acrophase of *cry2* was at the end of the light phase. Similarly, the rhythmic expression of different *cry* genes displayed dissimilar acrophases in several tissues of zebrafish (Kobayashi et al., 2000) and goldfish, which also showed different acrophases for the same *cry* subtype in different tissues (Velarde et al., 2009). Sometimes the cry expression is not rhythmic in some tissues, as reported in *cry1* expression in goldfish gut and liver (Velarde et al., 2009), and as we observed in *cry2* expression in seabass heart. On the other hand, the maximum expression peak of *per1* in several tissues (brain, retina, liver and gut) has been observed close to light onset in several species (Vallone et al., 2004; Lahiri et al., 2005; Velarde et al., 2009; Sánchez and Sánchez-Vázquez, 2009), including seabass brain, liver and heart (Sánchez et al., 2010; Del Pozo et al., 2012). Therefore, seabass *per1*, *cry1* and *cry2* expressions in all tissues, where their rhythmicity has been reported (except *cry2* in brain), were in phase, and so both the proteins encoded by these genes (*Per1* and Cry1 and Cry2) could join together and form the Per-Cry complex, which, in turn, would inhibit the cry transcription, closing the negative loop of the molecular mechanism that directs the circadian clock.
In summary, two cry genes of seabass, cry1 and cry2, which were expressed in several tissues (brain, liver, heart, retina, muscle, spleen, gill and intestine), were cloned. Cry1 expression was rhythmic in brain, liver and heart, whereas cry2 displayed a daily rhythm in brain and liver. In all the tissues investigated, the acrophases occurred shortly after lights onset, except cry2 expression in brain with an acrophase at the end of the light phase. These findings provide the basis for understanding how the molecular clock of seabass is assembled. Actually, per1 expression differs in diurnal and nocturnal seabass (Del Pozo et al., 2012). However, further research will be required to test such differences in cry genes and the role of other clock genes (e.g. clock and bmal1) in the dual phasing behavior.

Acknowledgements

This research was supported by the Spanish Ministry of Science and Innovation (MICINN) through projects AGL2010-22193-C02-01 and AQUAGENOMICS (Consolider-Ingenio 2010 Program), and SENeca through project P658/PI/07 granted to Francisco Javier Sánchez Vázquez. Ana del Pozo Cano holds a predoctoral fellowship from the University of Murcia.

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