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foxl2 and *foxl3* are two ancient paralogs that remain fully functional in teleosts

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ABSTRACT

FOXL2 is a forkhead transcription factor involved in mammalian development and regulation of reproduction. Two *foxl2* paralogs, *foxl2a* and *foxl2b*, have been described in various teleost species and were considered as fish-specific duplicates. Here, we report the isolation and characterization of *foxl2a* (*foxl2*) and *foxl2b* (*foxl3*) in European sea bass (*Dicentrarchus labrax*), together with the identification of these two genes in non-teleost genomes. Phylogenetic and synteny analyses indicate that these paralogs originated from an ancient genome duplication event that happened long before the teleost specific duplication. While *foxl2*/*foxl2a* has been maintained in most vertebrate lineages, *foxl2b*, which we propose to rename as *foxl3*, was repeatedly lost in tetrapods. Gonadal expression patterns of the sea bass genes point to a strong sexual dimorphism, and the mRNA levels of *foxl2* in ovary and *foxl3* in testis vary significantly during the reproductive cycle. When overexpressed in cultured ovarian follicular cells, *foxl2* and *foxl3* produced functional transcription factors able to control the expression of reproduction-related genes. Taken together, these data suggest that Foxl2 may play a conserved role in ovarian maturation, while Foxl3 could be involved in testis physiology.

1. Introduction

The Fox genes code for a family of transcription factors characterized by a winged helix forkhead box DNA-binding domain. The family is organized into subfamilies (A–S) based on the amino acid sequence of this domain. Fox genes have been identified in animal and fungal genomes with different numbers of members depending on the species (reviewed in Benayoun et al., 2011). FOXL2 was first reported by Crisponi et al. (2001), who established that Blepharophimosis Ptosis Epicantus inversus Syndrome in humans, a genetic disease involving eye lid malformation that can lead to premature ovarian failure, was related to a loss of function mutation in FOXL2. Since then it was established that in mammals FOXL2 is an essential transcription factor in ovarian sex differentiation and identity, but also follicle development and maintenance (for review see Caburet et al., 2012; Pisarska et al., 2011). In mammals, this transcription factor is crucial for reproductive success at different levels. In mouse (*Mus musculus*) pituitary, a role for FOXL2 in FSH production has been suggested (Corpuz et al., 2010; Justice et al., 2011). It also maintains the ovarian phenotype throughout adulthood by repressing *Sox9* expression, a crucial gene for testis development (Uhlenhaut et al., 2009) and by regulating granulosa cell differentiation, maintenance and function (Schmidt et al., 2004). FOXL2 also has been described as a repressor of several genes coding for steroidogenic enzymes, such as the steroidogenic acute regulatory protein (*STAR*), the mitochondrial cholesterol side-chain cleavage enzyme P450scc (*CYP11A1*) and the steroid 17-alpha-hydroxylase/17,20 lyase (*CYP17A1*), by directly binding to their promoters (Bentsi-Barnes et al., 2010; Kuo et al., 2011; Park et al., 2010; Pisarska et al., 2004). However, the role of FOXL2 in cytochrome P450 aromatase (*CYP19A1*) regulation is still unclear as contradictory cases have been described (Bentsi-Barnes et al., 2010; Pannetier et al., 2006).

In various teleost species two *foxl2* paralogs, *foxl2a* and *foxl2b*, have been described or predicted (Baron et al., 2004; Jiang et al., 2011). Most studies on Foxl2 in teleosts have focused on the role of Foxl2a during ovarian differentiation (Baron et al., 2004; Dong et al., 2011; Hale et al., 2011; Sridevi and Senthilkumaran, 2011; von Schalburg et al., 2011; Yamaguchi et al., 2007) or sex change in protandrous (Wu et al., 2008) and protogynous (Alam et al., 2008; Kobayashi et al., 2010) species. Another set of studies has investigated the relationships between Foxl2a and aromatase/*cyp19a1*, a key molecular marker in teleost ovarian differentiation. Foxl2a directly binds to the gonadal and brain aromatase promoters of tilapia (*Oreochromis niloticus*), catfish (*Clarias gariepinus*) and sea bass (*Dicentrarchus labrax*) to upregulate their transcription (Navarro-Martín et al., 2011; Sridevi et al., 2012; Wang et al.,

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2007; Yamaguchi et al., 2007). Furthermore, *foxl2a* and *cyp19a1* expression levels are positively correlated in rainbow trout (*Onchorhynchus mykiss*) and female catfish (Sridevi et al., 2012; von Schalburg et al., 2010). In turn, some reports also describe an upregulation of ovarian *foxl2a* and *foxl2b* expression by estradiol, the enzymatic product of aromatase activity (Baron et al., 2004; Jiang et al., 2011; Wang et al., 2012; Wu et al., 2008). It has been suggested that these processes may be involved in a steroidogenic positive feedback loop activated by gonadotropins (Guiguen et al., 2010). In fact, in catfish ovarian follicles and brain, *foxl2a* expression was seen to be enhanced after hCG stimulation during preparatory and prespawning phases but not during the spawning period (Sridevi and Senthilkumaran, 2011). All these data demonstrate the involvement of Foxl2a in ovarian function of teleosts both by acting directly in the ovary and indirectly at other levels of the brain-pituitary-gonad axis. As regards *foxl2b* in teleosts, the few studies available suggest that it either plays a role in the onset of oocyte meiosis or in the regulation of male specific genes during late testis development and testis maturation (Baron et al., 2004; von Schalburg et al., 2010, 2011), but its exact function is still unclear.

In this study we report the isolation of *foxl2a* and *foxl2b* genes in the European sea bass, a gonochoristic marine teleost that is an important aquaculture species. Data from the available genome databases show that the two *foxl2* paralogs emerged before the ray-finned fish-specific whole genome duplication, as they are also present in other species outside the teleost lineage. In fact, during the revision of this manuscript an evolutionary study has been published (Geraldo et al., 2013) that leads to the same conclusion. Therefore, following the recommended Fox nomenclature (Kaestner et al., 2000) we propose renaming *foxl2b* as *foxl3*, and to keep the name *foxl2* for teleost *foxl2a* genes. In addition, we have found that two copies of *foxl2* originated from the teleost-specific genome duplication (TGD) are still present at least in one teleost species, and following the naming for fish-specific gene copies they should be named as *foxl2a* and *foxl2b*. The *foxl2* and *foxl3* paralogs show different expression patterns in sea bass tissues and in different stages of adult gonadal development. Finally, both genes code for functional transcription factors that may recognize similar DNA binding sites.

2. Material and methods

2.1. Animal and tissue samplings

Sea bass were raised and maintained at the Instituto de Acuicultura de Torre la Sal (Castellón, Spain, 40°N) facilities. All animals were sacrificed in accordance with Spanish legislation concerning the protection of animals used for experimentation or other scientific purposes. Different tissues from adult males and females were extracted, snap frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Ovary and testis cDNA samples were the same as used in a previous work (Rocha et al., 2009). These gonad samples are from animals in their first year of sexual maturation and were extracted monthly (n = 5 fish/month) during a complete reproductive cycle. For each animal, ovarian and testicular stages of development were determined by histological analysis following previously established criteria (Asturiano et al., 2002; Begtashi et al., 2004).

2.2. Isolation of sea bass foxl2 (foxl2a) and foxl3 (foxl2b)

Medaka (*Oryzias latipes*) Foxl2 (GenBank accession number BAF42653) and rainbow trout Foxl2b (AAS87039) amino acid sequences were used as queries in TBLASTN (Altschul et al., 1997) searches against the sea bass genome database (Kuhl & Reinhardt, unpublished). Two partial sequences were identified as sea bass *foxl2a* (414 bp) and *foxl2b* (618 bp). The complete coding sequences of these intronless genes were isolated using four sea bass genomic DNA (gDNA) libraries constructed with the Universal GenomeWalker™ kit (Clontech Laboratories, Inc. CA). Specific primers (sfoxl3 and 4 for *foxl2a* and foxl2b3 and 4 for *foxl2b*) (Table 1) were used in combination with the adaptor primers of the kit in two rounds of PCR amplification according to the manufacturer's instructions. For each gene, the largest fragment amplified was cloned in the pGEM-T Easy vector (Promega Corp., Madison, WI) and sequenced on an automatic ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

The complete coding sequences of both genes were amplified from sea bass gDNA using specific primers flanking the corresponding open reading frames (sfoxl8/9 for *foxl2a* and foxl2b9/10 for *foxl2b*) (Table 1) and a high fidelity polymerase (*PfuTurbo*® DNA polymerase, Stratagene, La Jolla, CA). The amplified products were inserted into the pcDNA3 expression vector (Invitrogen Corp., Carlsbad, CA) as *Eco*RI/*Xba*I (*foxl2*) or *Eco*RI/*Bam*HI (*foxl3*) fragments to produce pcDNA3-foxl2 and pcDNA3-foxl3 plasmids. Both plasmids were verified by sequencing, as described above.

The 5′ and 3′ untranslated regions (UTRs) of *foxl2a* and *foxl2b* were amplified from cDNA directional libraries from sea bass ovary and brain, respectively, constructed in the UNI ZAP-XR vector. Gene specific primers combined with primers annealing to the UNI ZAP-XR vector were used to obtain the 5′ (sfoxl3 and foxl2b2 primers, Table 1) and 3′ UTRs (sfoxl1 and foxl2b4 primers, Table 1). All amplified fragments were cloned in the pGEM-T Easy vector (Promega Corp., Madison, WI) and sequenced as described above to identify sequences.

Touchdown-PCR was used for DNA amplifications with the following general conditions: an initial denaturation step at 94 °C 3 min or 95 °C 30 s, followed by 10 or 20 cycles of 94 °C 30 s or 95 °C 10 s, the highest annealing temperature (Table 1) for 30 s, and 72 °C for 30 s to 2 min. The annealing temperature was decreased 0.5 °C per cycle. These same conditions were used for 15–20 additional cycles using the lowest annealing temperature achieved in the touchdown.

2.3. Sequence analysis

Forkhead domains were identified through HMMER searches against the Pfam (Punta et al., 2012) database using the predicted amino acid sequences from sea bass Foxl2 and Foxl3 as queries. Nuclear localization signals were predicted using NLStradamus (Nguyen Ba et al., 2009).

Amino acid sequences were aligned with the CLUSTALW multiple alignment software (Thompson et al., 1994) using the Gonnet protein weight matrix and default parameters. Phylogenetic analysis was performed with the Molecular Evolutionary Genetics Analysis (MEGA 5) software (Tamura et al., 2011). The phylogenetic tree was constructed with the neighbor-joining, minimum-evolution and maximum likelihood methods and 1000 bootstrap permutations. All amino acid sequences used for alignments and phylogenetic analysis were extracted from GenBank (http://www.ncbi.nlm.nih.gov/genbank), RefSeq (http://www.ncbi.nlm.nih.gov/RefSeq/), Ensembl (http://www.ensembl.org) or Pre-Ensembl (http://pre.ensembl.org). Salmon sequences were searched at http://genomicasalmones.dim.uchile.cl/ and http://www.asalbase.org/.

Synteny analyses were carried out using information extracted from the Ensembl (http://www.ensembl.org) genome assemblies of human (*Homo sapiens*) GRCh37, opossum (*Monodelphis domestica*) BROAD05, chicken (*Gallus gallus*) WASHUC2, Chinese softshell