The Bromodomain testis-specific gene (*Brdt*) characterization and expression in gilthead seabream, *Sparus aurata*, and European seabass, *Dicentrarchus labrax*

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Abstract

Multiple genes and transcription factors are involved in regulation and control of the complex process of sex determination and differentiation of fish species. Also more, several hormonal factors and some environmental conditions can also be adequate spawning strategies and stimuli for inducing reproduction of fish species. *Brdt* gene belongs to the bromodomain-extraterminal domain (BET) family of transcriptional coregulators. In mammals, *Brdt* gene is almost exclusively expressed in testis. Furthermore, Brdt protein is involved in elongating spermatids, and is required for proper spermatogenesis and male fertility. However, from our understanding of fish species, the role of this gene as key, during gametogenesis, still remains unknown. In this study, two *Brdt* mRNA transcripts were isolated from two teleostean fish species, gilthead seabream and European seabass. In both species the shorter form lacked a functional C-terminal domain, which may involve a different function as transcriptional regulator. The pattern of *Brdt* expression showed that the highest levels occurred in the gonads. Significantly lower levels of expression were detected in brain, pituitary and different organ systems (heart, kidney, gills, among other somatic tissues) from both studied species. *In situ* hybridization approach evidenced that *Brdt* mRNA expression was restricted to specific cell-types of the germ line, during both oogenesis and spermatogenesis processes.

Introduction

The gilthead seabream (*Sparus aurata*) and the European seabass (*Dicentrarchus labrax*) are two fish species currently farmed on a large scale. These marine teleostean species are common throughout the Mediterranean and are also found along the Eastern Atlantic coasts.[1,2] In aquaculture systems, understanding and control of reproduction is needed for synchronous and reliable maturation and spawning.[3] Although the basic mechanisms of reproduction of both fish species have been extensively studied,[4,5] application of genetic, molecular and cellular markers will allow for advancement in the understanding of the molecular mechanisms that can be involved in the genetic regulation of the reproductive process in fish. There are multiple genes and transcription factors (*cyp19a*, *amh*, *vasa*, *dmrt, etc.*) involved in the regulation and control of sex determination-differentiation and gametogenesis in fish species.[6,7] Currently, other genes are analyzed for their involvement in cell proliferation process, such as the Bromodomain testis-specific (*Brdt*) gene, which in mammals regulates spermatogenesis and it is particularly important for male fertility.[8] However, from our understanding, there are no studies of *Brdt* gene in fish.

The *Brdt* gene encodes a protein member of the bromodomain and extra terminal (BET) protein family.[9] The BET proteins are characterized by two N-terminal bromodomains that bind to acetylated histone tails, with the exception of plant BETs which contain a single bromodomain.[10] BETs also have an extra-terminal motif (ET) in the C-terminal region, often followed by a SEED motif which contains polyserine residues interspersed with aspartic and glutamic acids.[11] Members of the BET family of proteins are usually well conserved in many species from yeast to human,[11] and they seem to play an important role in the modulation of gene expression by epigenetic mechanisms, which regulate genome reprogramming during gametogenesis, early embryogenesis, cell differentiation, and maintenance of lineage commitment.[12]

In mammals, there are four BET genes: *Brd2*, *Brd3*, *Brd4*, and *Brdt*, which are differentially expressed during spermatogenesis,[8] and particularly, only *Brdt* is almost exclusively expressed in the testis.[13,14] The Brdt protein is an essential factor for proper spermatogenesis and for male fertility. Indeed, homozygous mice for a mutation $Brdt^{DBD1/DBD1}$ lacking the first bromodomain (BD1) of *Brdt* presented severe reduction in the spermatozoa number, and decreased motility and high abnormalities in the sperm, and $Brdt^{DBD1/DBD1}$ spermatids failed to elongate properly.[15,16] It has also been reported that BD1 was involved in chromocenter formation and maintenance, showing multiple heterochromatic foci in spermatids of $Brdt^{DBD1/DBD1}$ mutant mice instead of a single focus as was established in round spermatids of wild-type mice.[15]

Brdt protein acts as a transcriptional regulator, binding histone H4-diacetylated in K5 and K8 by BD1,[17] and it has been demonstrated that round spermatids of $Brdt^{DBD1/DBD1}$ mutant mice suffer a significant alteration in transcription of genes in comparison to normal mice.[18] Brdt also plays an important role in post-transcriptional processing during spermatogenesis, interacting with several spliceosome components for mRNA splicing in the testis, and it is involved in 3'-UTR processing in round spermatids.[18]

It is known that Brdt protein is required for the completion of meiosis I, since it is expressed from the pachytene to diplotene stages of spermatocytes in mice, acting as a transcriptional regulator.[19] However, $Brdt^{DBD1/DBD1}$ mutant mice completed meiosis successfully,[16] so it is thought that other domains but not BD1, possess an important role in meiosis. In this sense, only mutations in the second bromodomain (BD2) of the yeast BET gene *Bdf1* failed to sporulate, so it is thought that BD2 may be the key to completion

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Key words: *Brdt;* gene expression; *in situ* hybridization; oogenesis; spermatogenesis; seabream; seabass.

Contributions: JBOD, CS, study conception, participation in the experimental design; MUM, performing of experimental technical part and statistical analysis, manuscript writing. All authors have participated actively in the study and approved the final manuscript.

Conflict of interest: the authors declare no conflict of interest.

Acknowledgments: the authors are grateful to Mr. Juan Canales for technical assistance in this work, and to Dr. Martínez-Rodríguez for helping in some molecular technique-optimization.

Funding: this study was supported by funding of CSIC (Project-Germfish 2014-2015, and AGL2014-52906R; Spain).

Received for publication: 16 February 2016.
Accepted for publication: 12 April 2016.

European Journal of Histochemistry 2016; 60:2638
doi:10.4081/ejh.2016.2638

of meiosis.[20]

Brdt protein also shows the capacity for remodeling and compacting chromatin after induction of histone hyperacetylation during the critical post-meiotic stages of spermatogenesis.[13] Gaucher *et al*.[19] suggested that Brdt has a potential role in the replacement of hyperacetylated histones by transition nuclear proteins, which are later replaced by protamines to genome condensation during the elongation of spermatids.[21]

In this novel study we report, for the first time in two marine fish species, the identification, molecular cloning and characterization of *Brdt* in gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). A first molecular and cellular approach was performed using quantitative and qualitative methods (qPCR and *in situ* hybridization techniques, respectively) to determinate the gene expression patterns in different organ systems (gonads, brain, hypophysis, heart, kidney, spleen among other somatic tissues).

Materials and Methods

Biological samples

All specimens of gilthead seabream (*S. aurata*) and European seabass (*D. labrax*) used in this study were cultured in the facilities of the Institute of Marine Sciences of Andalusia (ICMAN.CSIC, Puerto Real, Spain). These aquaculture facilities were approved for animal experimentation (certificate number REGA-ES110280000311) in accordance with Spanish legislation (R.D. 53/2013) and the experimental protocol was approved by the Spanish National Research Council (CSIC) Ethics Committee from project CSIC-Germfish 2014-2015. Deep anesthesia was induced with 1500 ppm phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA) and six adult fish of each species were slaughtered by decapitation. Samples of different organ systems and tissues (gill, intestine, rectum, anterior kidney, posterior kidney, heart, spleen, liver, brain, muscle, ovary, testis, and hypophysis, plus stomach only in European seabass) were collected into RNAlater® (Sigma-Aldrich), incubated for 24 h at 4°C and stored at -20°C until RNA extraction (within two weeks). For *in situ* hybridization (ISH), gonads were sampled and fixed with 4% paraformaldehyde in diethylpyrocarbonate (DEPC) treated phosphate-buffered saline (PBS) and then processed according to Úbeda-Manzanaro *et al*.[22]

Nucleic acids extraction

Total RNA from hypophysis was extracted using the NucleoSpin® RNA XS kit (Machery-Nagel), whereas total RNA from other organs and tissues was isolated using NucleoSpin®RNA II kit (Macherey-Nagel) according to the manufacturer's protocol. Total RNA concentration was assessed by spectrophotometry (A260 nm/A280 nm ratio >1.8) and integrity verified with a Bioanalyzer 2100 and with the RNA 6000 Nano kit (Agilent Technologies).

Cloning and phylogenetic analysis

Testis and ovary cDNA templates were synthesized from 1 µg total RNA using the SMARTer™ RACE cDNA Amplification kit (Clontech) according to the manufacturer's instructions. Firstly, this cDNA was used to perform a PCR with degenerated pair of

Table 1. Primers used for the sequencing and quantification of *Brdt* mRNA levels in *Sparus aurata* (prefix Sa-) and *Dicentrarchus labrax* (prefix Dl-).

Primer	Sequence (5'→3')	Application	R^2	E
Brdt-degF	CCYGGGGATGAYATTGTTYTKATGGC	Obtaining a partial specific sequence		
Brdt-degR	CCMAGTTTGTCWCCAGGMAGCTTGTT	Obtaining a partial specific sequence		
Sa-Brdt-1345F	GAGGCAGAGGGTACATCAGAGGTGGTGGCC	3'-RACE		
Sa-Brdt-1617F	GCTCGCATCAATTCCAGTGACT	3 nested		
Sa-Brdt-1809F	GAGGTTTGTTGCAGCATGTC	3 nested		
Sa-Brdt-1401R	CCGCTCCTTCAGATCCGCAAGCTGTGTGGC	5'-RACE		
Sa-Brdt- 526F	CAATTGCCGAAGGAAGAGTTTGA	*In situ* hybridization		
Sa-Brdt-696R	TCAATTTGTGCTGAGAGCTGG	*In situ* hybridization		
Sa-Brdt-874F	AAAGACTTGCCGACCTTTGA	qPCR	0.9992	1.9992
Sa-Brdt-985R	GCCAGGCGTATGGATAGTGT			
Sa-18S-F	AACCAGACAAATCGCTCCAC	qPCR	0.9998	1.9817
Sa-18S-R	CCTGCGGCTTAATTTGACTC			
Sa-Beta actin-F	TCTTCCAGCCATCCTTCCTCG	qPCR	0.9995	2.0110
Sa-Beta actin-R	TGTTGGCATACAGGTCCTTACGG			
Dl-Brdt-1607F	GGTCCAATCAATACCAGTGACC	3'-RACE		
Dl-Brdt-1354R	AGCTGTGAGGCCACCTCTTCGGATGAAACTC	5'-RACE		
Dl-Brdt-691R	TCAATTTGTGCAGAGAGCTGG	5 nested & *in situ* hybridization		
Dl-Brdt-501F	CAAATGCCTAAGGAAGAGTGTGA	*In situ* hybridization		
Dl-Brdt-2265F	GAAACCAAAGGGCTCCCAAC	qPCR	0.9968	1.9873
Dl-Brdt-2392R	GCAGCCCAGTCCAGTGAAAC			
Dl-L13a-F	TCTGGAGGACTGTCAGGGGCA	qPCR	0.9987	2.0006
Dl-L13a-R	AGACGCACAATCTTGAGAGCAG			
Dl-Beta actin-F	CAGGGAGAAGATGACCCAGA	qPCR	0.9994	1.9823
Dl-Beta actin-R	CCGGAGTCCATGACAATACC			

100 % efficiency is E=2 (slope -3.32). To accept the standard curve the R^2 value must be >0.99. K=G/T; M=A/C; W=A/T; Y=C/T

primers Brdt-degF/Brdt-degR (Table 1) to amplified specific partial sequences from *Brdt* of both fish species. These primers were designed from sequence alignment of teleost fish orthologs using ClustalW (http://www.genome.jp/tools/clustalw/) and FastPCR 6.0.[23] PCRs were carried out in a Doppio thermocycler (VWR) using a total volume of 50 µL containing 2 µL of 5'-RACE-Ready cDNA, 5 µL complete reaction buffer (10x), 400 µM of dNTP mix, 200 pM of each primer, and 2.5 U of DFS-Taq DNA polymerase (Bioron). Thermocycler conditions were 1 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 56°C, and 1.5 min at 72°C, and a final 7 min extension at 72°C. The PCR products were purified and inserted into pGEM®-T Easy Vector System (Promega). The positive clones were sequenced at the Sequencing Service from Biomedal (Seville, Spain).

Secondly, gene specific primers (saBrdt-1345F, saBrdt-1401R, dlBrdt-1607F, dlBrdt -1354R, saBrdt-1617F, saBrdt-1809F, and dlBrdt-691R, Table 1) were designed using the obtained *Brdt* partial sequences for performing 3'- or 5'-RACE-PCR and nested reactions, and obtaining the full-length sequences of *Brdt* cDNA. The RACE-PCR reactions were performed according to the manufacturer's manual, adapting the annealing temperature to the corresponding primer. The obtained sequences were assembled in a single one using BioEdit 7.0.9.0.[24]

The *Brdt* sequences of gilthead seabream and European seabass were translated into amino acids using a translate tool (http://web.expasy.org/translate/) and a phylogenetic reconstruction was performed using the neighbor-joining method[25] with MEGA 6 software,[26] after a multi-alignment of selected BET-family proteins with ClustalW algorithm. Bootstrap resampling method[27] was applied to assess support for individual nodes using 10,000 replicates, and the evolutionary distances were computed using the Poisson correction method,[28] uniform rates among sites, and *pairwise deletion* option treatment of gaps and missing data.

Expression analysis: real-time qPCR

Total RNA (≈500ng) was reverse-transcribed using qScript™ cDNA Synthesis kit (Quanta BioSciences, Gaithersburg, MD, USA) according to the manufacturer's protocol.

For the relative quantification of gene expression using real-time PCR (qPCR) of *Brdt* genes, specific primer pairs (Table 1) were finally designed at a common region to both transcripts of each species, since the sequence of the short transcript (*saBrdt-b* or *dlBrdt-b*) differs from the long transcript (*saBrdt-a* or *dlBrdt-a*, respectively) in only about 200 bp, which limited the design of specific primers for the short isoform. qPCR reactions were performed on a Mastercycler® ep gradient S Realplex[2] with Realplex software version 2.2 (Eppendorf). Reactions were performed in a final volume of 10 µL with 400 nM of each primer, 4 µL of a 1/10 dilution of cDNA (≈10 ng), and 5 µL PerfeCTa™ SYBR® Green FastMix™ (Quanta BioSciences). qPCR was run with the following parameters: 95°C for 2 min, 40 cycles at 95°C for 15 s, 56°C for *S. aurata* genes or 60°C for *D. labrax* genes for 15 s, and 60°C for 15 s. All primers gave single distinctive melting peaks, demonstrating that no primer dimmers and unspecific amplification products were present. The real-time PCR efficiencies were calculated from the slope, according to the equation $E=10^{[-1/slope]}$. Relative gene quantification was performed using the method of Pfaffl,[29] using β-actin (primers from Mohammed-Geba *et al.*[30]) and 18S rRNA (Martos-Sitcha *et al.*[31]) as the internal control genes for *S. aurata* or β-actin [GenBank: AJ537421.1] and L13a (Mitter *et al.*[32]) for *D. labrax*, with mRNA from the lowest expression level as calibrator (gills in the case of *S. aurata*, or liver for *D. labrax*). Three biological replicates of each sample were analyzed and each PCR was performed in parallel with a technical duplicate. Negative qPCR controls using double-distilled water and RNA instead of cDNA were included in the assays for each primer pair.

Statistical analysis

The normality of data was checked with the Shapiro-Wilk's test and the homoscedasticity of variance with the Levene's test. Differences in tissue distribution for *Brdt* expression were evaluated by one-way analysis of variance (ANOVA) performed after logarithmic base 10 transformation. Pairwise comparisons were performed by the Student-Newman-Keuls (SNK) *post-hoc* test. Differences were considered statistically significant at $P<0.05$. Statistical analyses of data from qPCR were performed using SPSS 23.0.0.0 software (IBM).

In situ hybridization

The *Brdt* riboprobes for both fish species were generated from a 191 nt fragment from a region located between the first bromodomain and the motif A (primers in Table 1). Digoxigenin (DIG)-labeled antisense and sense riboprobes were synthesized by *in vitro* transcription with the DIG RNA labeling Kit (SP6/T7) according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). *In situ* hybridizations were performed according to Úbeda-Manzanaro *et al.*[22] on histological section of testes and ovaries. Sections were rehydrated and permeabilized for 15 min in 10 µg/mL proteinase K in phosphate buffered saline with Tween 20 (PBST: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 0.1% Tween-20) at 37°C. After postfixation in 4% paraformaldehyde-PBST for 30 min, sections were incubated for 2x 15 min in PBST containing 0.1% active DEPC, and equilibrated for 15 min in PBST. The histological sections were then pre-hybridized for 2 h at 57°C in the hybridization mix, and later, they were hybridized with the sense or antisense probes at 57°C overnight in a humidified chamber. After incubation, the sections were washed three times in 2x SSC at 52°C for 30 min, later they were washed twice at 52°C with 1.4xSSC/0.6% CHAPS (3-[3-cholamidopropyl)-dimethylamonio]1-propanesulfonate, and once with 1:1 PTW: maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5). Detection of the hybridized probe was carried out using alkaline phosphatase-coupled anti-digoxigenin antibody, and the hybridization signals were detected by NBT/BCIP system according to the manufacturer's instructions (Roche), adding 5 mM levamisole (Sigma-Aldrich) to neutralize the high endogenous alkaline phosphatase activity previously detected in gonad samples and particularly in ovaries of both species.

Results

Isolation and characterization of *Brdt* cDNA and phylogenetic analysis

Two full-length *Brdt* cDNAs from gonads of *S. aurata* were obtained using degenerated RT-PCR and 3'- and 5'-RACE, and the two transcripts were designated as *saBrdt-a* and *saBrdt-b*. The *saBrdt-a* [GenBank: KT734775] is the longest transcript (3,957 nt), and consisted of 132 nt 5'-UTR (untranslated region), an open reading frame (ORF) of 2,937 nt (corresponding to 978 deduced amino acids), and a 3'-UTR of 861 nt with a poly (A) tail. The *saBrdt-b* [GenBank: KT734778] is the shortest transcript (2,347 nt), showing an ORF of 1,989 nt (662 aa), followed by a shorter 3'-UTR of 198 nt ending in a poly (A) tail. Similarly, we also described two full-length *Brdt* cDNAs from testis of *D. labrax*: a longer transcript called *dlBrdt-a*, and a shorter one named *dlBrdt-b*. The *dlBrdt-a* transcript [GenBank: KT734772] is 3982 nt, with a 5'-UTR of 107 nt, an ORF of 2940 nt (979 aa), and a 3'-UTR of 911 nt with a poly (A) tail. The *dlBrdt-b* [Genbank: KT734773] is 2309 nt, with an ORF of 1995 nt (678 aa) and a 176 nt 3'-UTR with its poly (A) tail. Both *Brdt-a* and *Brdt-b* transcripts of both fish species differ in the 3'-UTR sequence and the 3'- end of the coding sequence. Figure 1

shows the putative protein sequences of these transcripts and the conserved motifs.

The phylogenetic tree analysis constructed with amino acid sequences for BET protein family (Figure 2) showed a strong evolutionary conservation of BET sub-family of proteins in fish. Both Brdt protein sequences from gilthead seabream and European seabass were clustered in the Brdt subfamily, with the other Brdt homologues from fish species of the order Perciformes.

Brdt mRNA expression analysis

The *Brdt* expression patterns in both fish species were analyzed by qPCR showing relative expression levels in all tested organ systems and tissues from adult specimens, particularly in gonads. In this study, qPCR was not performed taking into account the two different transcripts, because the differences between short and long forms were less of 200 bp, so the design of a primer pair for the short transcript was limited. For gilthead seabream, higher statistically significant expression levels were detected in the testis, followed by the ovary; moderate levels of *Brdt* mRNA expression were observed in the brain and muscle, whereas in the other organs and tissues analyzed, *Brdt* mRNA expression levels were lower (Figure 3A). Nevertheless in European seabass, the highest expression levels were similarly detected in ovary and testis, moderate expression level was observed in the brain, and very low levels of *Brdt* mRNA expression were detected in the rest of the organs and tissues (Figure 3B).

Spatial *Brdt* mRNA tissue expression was analyzed by using specific sense and antisense RNA probes for *in situ* hybridization approach on ovarian and testicular histological sections of *S. aurata* and *D. labrax* adult specimens. In testis of gilthead seabream, *Brdt* mRNA is specifically expressed in secondary spermatocyte cysts and in spermatids, whereas in the ovary the *Brdt* mRNA was expressed in the cytoplasm of late previtellogenic oocytes, and a weak *in situ* hybridization signal was detected in early vitellogenic oocytes. No hybridization signals were detected in primary spermatocyte cysts, spermatozoa, early previtellogenic oocytes and advanced vitellogenic oocytes from *S. aurata* (Figure 4 A,B). In European seabass, the *Brdt* mRNA expression in the ovary was restricted to cytoplasm of late previtellogenic oocytes, and in testis, *in situ* hybridization signals were detected in spermatids. No hybridization signals were detected in spermatozoa, spermatogonia, early previtellogenic and vitellogenic oocytes from the European seabass gonads (Figure 4 C-E).

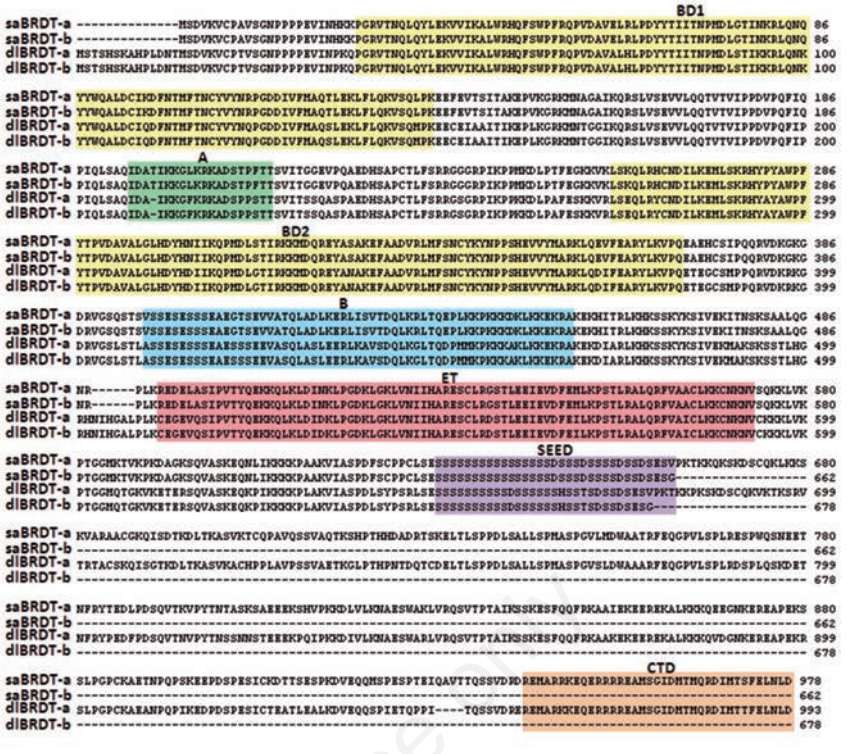

Figure 1. Amino acid sequence alignment of the *Brdt* transcripts from gilthead sebream and European seabass. The bromodomains 1 and 2 are shaded in yellow, the A motif in green, the B motif in blue, the ET motif in red, the SEED motif in purple, and CTD motif is shaded in orange. Number on the right denotes the amino acid position. Dashed line (--) indicate gaps inserted for improved alignment.

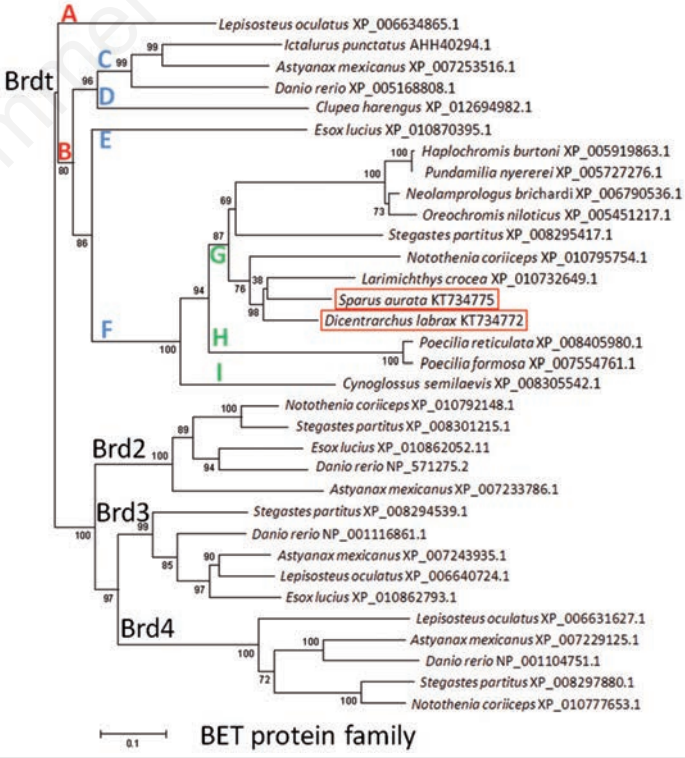

Figure 2. Phylogenetic tree of fish BET proteins using the neighbor-joining method with MEGA 6.06. The Genbank accession numbers of the sequences used are written beside the name of the fish species. Infraclass: Holostei (A), Teleostei (B). Superorders: Ostariophysi (C), Clupeomorpha (D), Protacanthopterygii (E), Acanthopterygii (F). Orders: Perciformes (G), Cyprinodontiformes (H), Pleuronectiformes (I), Beloniformes (J).

Discussion

This is the first study designed to understand the expression of the *Brdt* gene during gametogenesis of fish species and to assess the utility of *Brdt* mRNA expression as a marker for the presence of germ line cells in testes, as was largely pointed out for mammals. This study shows that in both gilthead seabream and European seabass species *Brdt* genes express two transcripts which encode proteins differing at their functional C-terminal end, which may suggest that the short *Brdt* transcript has a different function from the long *Brdt* form as a transcriptional regulator. Mammalian BET proteins show a conserved organization of their domains, which is also observed in homologues from other organisms like *Drosophila* Fsh and *Saccaromyces cerevisiae* Bdf1 and Bdf2 proteins.[33] The putative amino acid sequences of the transcripts described in *S. aurata* and *D. labrax* contain the characteristic domains of the BET protein family: BD1, BD2 and the ET domain. Other regions such as motifs B and SEED are also highly conserved, and to a lesser extent the A motif.[34] *Brdt* contains a CTD (C-terminal domain) which is not present in *Brd2* and *Brd3*, making it structurally similar to *Brd4* and to the long isoform of *Drosophila* female sterile homeotic [*Fs(1)hL*].[11,33] However, *saBrdt-b* and *dlBrdt-b* transcripts from seabream and seabass fish specimens, respectively, encode a truncated protein product lacking CTD, which is comparable to short isoforms of *brd4* and *fs(1)h*.[11,33] A similar short *Brdt* mRNA transcript lacking CTD has not been reported in humans or mice, although diverse isoforms of the *Brdt* generated by alternative splicing have been reported in *Drosophila*, mouse and human.[9,13,35] BET proteins containing CTD, such as Brd4, Brdt and Fs(1)hL specifically interact with positive transcription elongation factor b (P-TEFb) in mammalian cells by their C-terminal domains.[36] Therefore, Brdt is the unique BET protein that would be able to perform P-TEFb-mediated transcriptional regulation in spermatocytes or spermatids,[19] considering that Brd4 was not expressed in these cells in mice.[14]

Paillisson *et al*.[34] suggested that Brdt sequence is the least conserved among mammalian BET protein family because a phylogenetic analysis performed with five species of mammals showed that Brdt presents the highest evolutionary rate in comparison with Brd2, Brd3, and Brd4. The phylogenetic analysis performed with BET proteins from different fish species (Figure 2) also supports the hypothesis that Brdt evolves faster than other BET paralogs. In mammals, Brdt protein has a known role in germ cell differentiation,[16,19] and it has

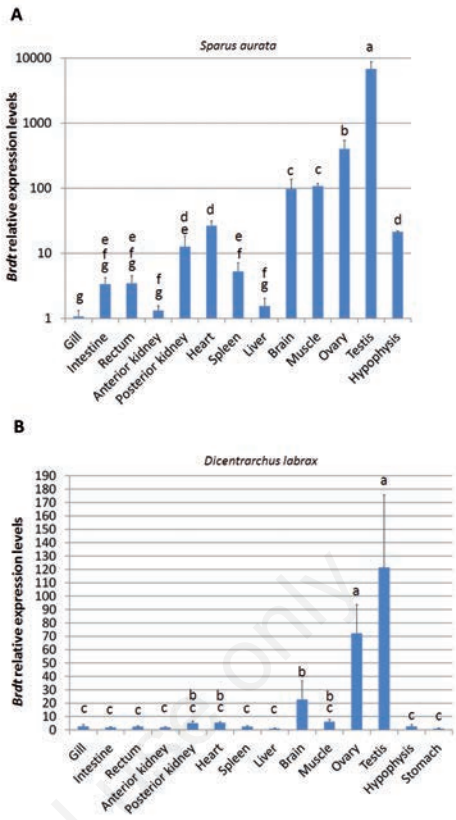

Figure 3. Relative expression profile of *Brdt* mRNA in adult gilthead seabream tissues (A), and in adult European seabass tissues (B). Small case letters (a-g) indicate statistically significant differences detected by ANOVA, SNK *post-hoc* test, P<0.05 (mean ±SEM, n=3).

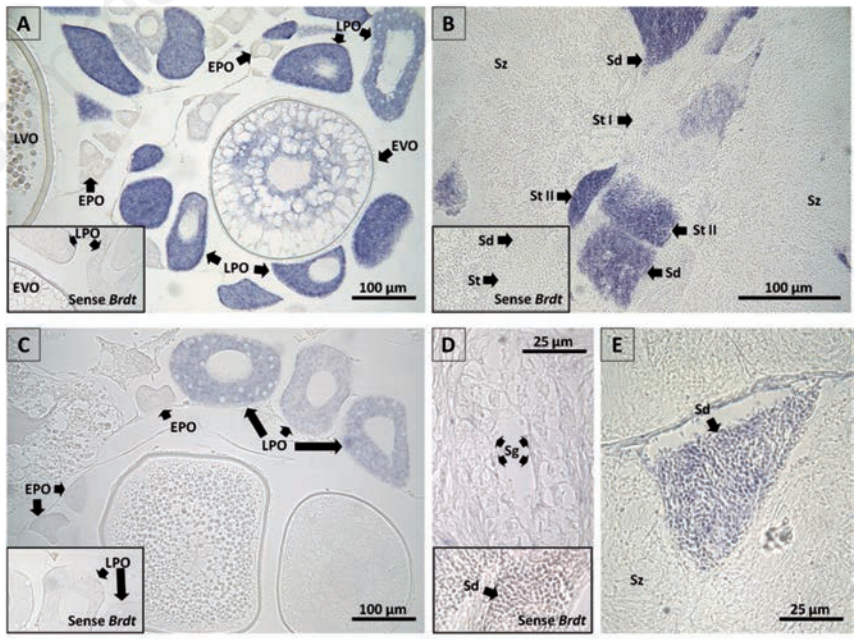

Figure 4. *In situ* hybridization analysis of *Brdt* mRNA expression in gonadal sections. A) Gilthead seabream ovary. B) Gilthead seabream testis. C) European seabass ovarian. D,E) European seabass testis. Sense probe controls in ovary and testis are included in the lower left corner of panels A, B, C, and D, respectively, with the same scale as for antisense riboprobe pictures. EPO, early previtellogenic oocytes; EVO, early vitellogenic oocytes; LPO, late previtellogenic oocytes; LVO, late vitellogenic oocytes; StI, primary spermatocyte cysts; StII, secondary spermatocyte cysts; Sd, spermatids; Sg, spermatogonia; Sz, spermatozoa.

been demonstrated that genes involved in sex determination-differentiation and reproduction events evolve faster than genes expressed in somatic tissues.[37]

In mammals, *Brdt* gene is almost exclusively expressed in the testis,[9,13,14,38] although some authors have also reported low expression levels in oocytes[34] and in the brain[14] of mice. This controversy in the *Brdt* expression patterns of mice may be due to the application of different methodology, using techniques of distinct sensitivity, such as northern-blot, reverse transcriptase-PCR, and ISH. The tissue expression profiles of the *Brdt* genes from gilthead seabream and European seabass were performed by qPCR. In both species, *Brdt* mRNA expression levels have been detected in many organ systems and tissues showing weak levels of expression, with some exceptions such as gonads or the brain. In *S. aurata* the highest expression level was observed in testis, whereas *Brdt* expression level in the ovary was high but statistically lower than in testis. In *D. labrax* the highest *Brdt* mRNA expression levels were reported in both ovary and testis, without statistical differences.

According to Shang *et al*.[14], in mice, *Brdt* mRNA was expressed at meiotic prophase of spermatocytes, especially at the pachytene stages, although Brdt protein was evidenced in several stages from pachytene and diplotene spermatocytes, and in the round spermatids.[16] Human Brdt protein was located in spermatocytes up to ejaculated spermatozoa, suggesting that there may be some interespecific differences in the functions of *Brdt* genes.[39] Our results confirm the *Brdt* mRNA expression in one type of spermatocyte cysts, suggesting that *Brdt* also plays a role in completion of meiosis I during fish spermatogenesis. We also reported *Brdt* expression in spermatids, which may be associated with the Brdt function in genome condensation or as a transcriptional regulator during this spermatogenic developmental stage. As it is largely known, the Brdt protein has two N-terminal bromodomains that bind to acetylated histone tails.[10] Interestingly, Kurtz *et al*.[40] reported that H4 histone acetylation occurs during spermiogenesis of both *S. aurata* and *D. labrax*, but in the first fish species (*S. aurata*) there was no displacement of histones by protamines or any other sperm nuclear basic proteins, whereas in the second fish species (*D. labrax*) the histone substitution by protamines during spermiogenesis was reported. These differences observed in the expression of *Brdt* gene between both studied fish species, could suggest a different role of Brdt protein during spermatogenesis as a transcription regulator and/or remodeling and compacting the chromatin depending on each species.[17,19]

In situ hybridization approach performed on ovarian histological sections from both *S. aurata* and *D. labrax* confirmed that *Brdt* mRNA is specifically expressed in oocytes, similar to results previously reported in mice oocytes.[34] However, the *Brdt* expression pattern observed in both gilthead seabream and European seabass differ slightly. The tissue-specific expression pattern of *Brdt* seems to denote a different role in male and female gametogenesis of both fish species, although it may be that in the gonochoric fish species, European seabass[4] Brdt plays a more important role as a key during female gametogeneis than in gilthead seabream, which is a protandrous hermaphroditic fish species with a bisexual gonad containing both spermatogonia and oogonia stem cells.[41]

A more exhaustive functional role of *Brdt* gene could be demonstrated by complementary analysis in parallel by using some molecular, cellular and physiological approaches, such as studies on variation patterns during the annual reproductive cycles in both males and females, as well as during sex determination-differentiation developmental stages in juveniles of both fish species.

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