Differential activation of kiss receptors by Kiss1 and Kiss2 peptides in the sea bass

Alicia Felip*, Felipe Espigares*, Silvia Zanuy and Ana Gómez

Abstract

Two forms of kiss gene (kiss1 and kiss2) have been described in the teleost sea bass. This study assesses the cloning and characterization of two Kiss receptor genes, namely kissr2 and kissr3 (known as gpr54-1b and gpr54-2b, respectively), and their signal transduction pathways in response to Kiss1 and Kiss2 peptides. Phylogenetic and synteny analyses indicate that these paralogs originated by duplication of an ancestral gene before teleost specific duplication. The kissr2 and kissr3 mRNAs encode proteins of 368 and 378 amino acids, respectively, and share 53.1% similarity in amino acid sequences. In silico analysis of the putative promoter regions of the sea bass kiss receptor genes revealed conserved flanking regulatory sequences among teleosts. Both kissr2 and kissr3 are predominantly expressed in brain and gonads of sea bass, medaka and zebrafish. In the testis, the expression levels of sea bass kisspeptins and Kiss receptors point to a significant variation during the reproductive cycle. In vitro functional analyses revealed that sea bass Kiss receptor signals are transduced both via the protein kinase C and protein kinase A pathway. Synthetic sea bass Kiss1–15 and Kiss2–12 peptides activated Kiss receptors with different potencies, indicating a differential ligand selectivity. Our data suggest that Kissr2 and Kissr3 have a preference for Kiss1 and Kiss2 peptides, respectively, thus providing the basis for future studies aimed at establishing their physiologic roles in sea bass.

Introduction

Soon after the Kiss/Gpr54 system had been demonstrated to play a role in the control of reproduction in mammals (Funes et al. 2003, de Roux et al. 2003, Seminara et al. 2003, Pinilla et al. 2012), further investigation was initiated in several vertebrate species, leading to the identification of multiple kiss and gpr54 paralogous genes in early osteichthyans (Biran et al. 2008, Felip et al. 2009, Lee et al. 2009, Akazome et al. 2010, Oakley et al. 2010, Um et al. 2010, Zohar et al. 2010, Kim et al. 2012, Pasquier et al. 2012a,b, 2014a,b, Tena-Sempere et al. 2012). To date, a single ligand (Kiss) and receptor (Gpr54 or Kissr) have been demonstrated to exist in placental mammals (human, opossum) and reptiles, while mammalian monotrems (platypus) possess two forms of Kiss and Kissr genes. Contrasting situations are found in other tetrapodes, such as amphibians, which have three kiss and kissr genes, whereas the kisspeptin system is absent in birds, although a recent investigation into the kisspeptin system in avian lineages provides molecular evidence of the presence of a kiss2-like gene that degenerated and lost its function (Pasquier et al. 2014b). All in all, advances in genome sequencing and comparative genomics in several groups occupying relevant phylogenetic positions have led to the identification of four kissr and three kiss genes in the coelacanth (Sarcopterygian), while in teleosts at least one kiss and kissr is known to be present in all the species investigated to date. Nevertheless, a second gene for kisspeptin and its receptor has been observed in the genomes of other fish species (Biran et al. 2008, Lee et al. 2009, Akazome et al. 2010, Oakley et al. 2010, Um et al. 2010, Zohar et al. 2010, Kim et al. 2012, Pasquier et al. 2012a,b, Tena-Sempere et al. 2012). So far, the occurrence of three kissr and two kiss paralogous genes has been reported in an early group of teleosts, the Elopomorphs (European eel, Anguilla anguilla) (Pasquier et al. 2012a,b). In the spotted gar, Lepisosteus oculatus (Actinopterygian, Ginglymodae), four kissr and two kiss have been identified, while four kiss but no kissr have been characterized in the elephant shark, Callorhinus milii (Chondrichthyan) (Pasquier et al. 2012a,b). Concerning cyclostomes, two kiss and one kissr genes have been identified in the sea lamprey (Petromyzon marinus) (Felip et al. 2009, Pasquier et al. 2012a,b). Furthermore, two forms of kiss receptor-like genes have been...
predicted in Ambulacrarians, such as the sea urchin, *Strongylocentrotus purpuratus* (Echinodermata) and acorn worm, *Saccoglossus kowalevskii* (Hemichordata) (Biran et al. 2008, Lee et al. 2009, Pasquier et al. 2012a). This situation demonstrates a complex functional and evolutionary scenario for this regulatory system in both vertebrates and invertebrates, which merits investigation.

Accumulating evidence has provided insight into the involvement of the kisspeptin system in the regulation of neuronal systems other than those involved in vertebrate reproduction, such as those related to complex behaviors, including sexual motivation and arousal states (Parhar et al. 2004, Kanda et al. 2008, Tena-Sempere 2010a, Servili et al. 2011, Shimizu et al. 2012, Zhao & Wayne 2012, Zmora et al. 2012, Escobar et al. 2013a,b). Expression levels of *kiss* and *kissr* in the brain and, to a lesser extent, in the gonad have been analyzed in several non-mammalian species, including amphibians (Chianese et al. 2013) and fish (Mohamed et al. 2007, Nocillado et al. 2007, Biran et al. 2008, Filby et al. 2008, van Aerle et al. 2008, Kitahashi et al. 2009, Mechaly et al. 2009, 2010, Migaud et al. 2012, Alvarado et al. 2013, Ohga et al. 2013), suggesting a putative role for kisspeptins in controlling reproduction. Moreover, in vivo studies in some teleosts have found that the systemic administration of kisspeptin forms induces detectable biological responses at brain and pituitary levels, with potencies depending on the species (Filby et al. 2008, Felip et al. 2009, Kitahashi et al. 2009, Li et al. 2009, Shi et al. 2010, Espigares et al. 2015). Furthermore, in vitro functional analyses have delineated ligand-receptor interactions in four fish species containing a duplicated Kiss system, namely zebrafish *Danio rerio* (Biran et al. 2008, Lee et al. 2009), goldfish *Carassius auratus* (Li et al. 2009), medaka *Oryzias latipes* (Kanda et al. 2013) and chub mackerel *Scomber japonicus* (Ohga et al. 2013), and in the orange-spotted grouper *Epinephelus coioides* with a single Kiss/Kissr pair (Shi et al. 2010). As is the case in mammals (Pinilla et al. 2012) for review), the activation of PKC-MAPKs is involved in the signaling pathways used by the fish Kiss receptors, but different levels of activation have been observed, depending on the ligand/receptor combination (Tena-Sempere et al. 2012).

The European sea bass, *Dicentrarchus labrax* L. provides an ideal model for studying aspects of fish reproduction, since important scientific achievements have been developed for this species. It is an economically important farmed fish that still exhibits reproduction-related problems, such as the appearance of early puberty, and the mechanisms underlying this process merit further investigation (Carrillo et al. 2009). Previous studies in this species have found that the sea bass has two distinct *kiss* genes, *kiss1* and *kiss2* (Felip et al. 2009), whose mRNA levels in the brain change significantly in relation to the reproductive status of the animals (Migaud et al. 2012, Alvarado et al. 2013). The distribution of *kiss1*- and *kiss2*-expressing cells and their receptors in the brain of male and female sea bass undergoing their first sexual maturation has revealed that no sexual differences in the localization of the kisspeptin systems exist in this species (Escobar et al. 2013a,b). Of note in this study was that the expression of *kissr3* presented an overlap with Kiss2 fibers in the central telencephalon and the lateral recess of the hypothalamus (Escobar et al. 2013b). Despite these new findings, the specifically reproductive function of the duplicated Kiss system in the sea bass still remains to be fully characterized. Here, we report on the complete cDNA identification and characterization of the two distinct Kiss receptors, namely *kiss2* and *kissr3*, and in silico analysis of their putative regulatory regions. We analyze the tissue distribution of both Kiss receptors in sea bass, medaka and zebrafish. In addition, the expression of *kissr* genes in brain, pituitary and gonads in adult and juvenile fish during the reproductive period is explored. The expression study of *kiss* and *kissr* genes was extended to the different stages of gonad maturation in adult male sea bass as recent findings have given evidence for a differential involvement of the two kisspeptin systems in controlling gametogenesis in this species (Migaud et al. 2012, Alvarado et al. 2013). Furthermore, melatonin administration has been shown to elicit changes in the expression of kisspeptin/gnRH system genes in the brain that appear to mirror disturbances in spermatogenesis (Alvarado et al. 2015). In addition, searching for the preferred activation of each sea bass Kiss receptor, a cell culture assay was used to delineate ligand-receptor interactions and signaling. Finally, we also studied the chromosomal location of the sea bass Kiss receptors and placed them in an evolutionary scenario.

### Materials and methods

#### Fish and sampling

Male and female sea bass and medaka (Carbio strain) were obtained from stocks at IATS, whereas zebrafish were obtained from a local pet shop. Brain and gonads were collected monthly (January–April) from prepubertal and adult sea bass (*n*=1–4 for each sex and developmental stage). In addition, different somatic tissues were collected from the samples in January for expression analyses using an RT-PCR approach. The quantitative gonadal expression level of *kiss* and *kissr* genes was analyzed by RT-qPCR in testis cDNA samples from animals in their first year of sexual maturation (*n*=5 fish/month) during a complete reproductive cycle (Rocha et al. 2009) where testicular stages of development were determined by histological analysis (Begtashi et al. 2004). Finally, different tissues of adult medaka and zebrafish (*n*=4–5 for each sex) were collected. All fish were anesthetized with an overdose of 2-phenoxyethanol (Merck Schuchardt OHG, Hohenbrunn, Germany) and killed by decapitation. All samples were frozen on dry ice and stored at −80°C until use. All animal experiments were conducted in accordance with the...
Molecular cloning of two kiss receptor genes in sea bass

The sequence of the human and tilapia kisspeptin receptors was used to query the medaka and zebrafish genome databases by TBLASTN searches. Two different sequences corresponding to kiss receptor-like genes were identified in each species. To amplify fragments of the sea bass receptors, degenerate PCR primers were designed based on conserved amino acids from the fish genes. PCR amplifications were carried out from four sea bass genomic DNA libraries constructed with the Universal GenomicWalker Kit (Clontech Laboratories, Inc., Mountain View, CA, USA). We will adopt the term kiss for kiss receptor genes in the sea bass (i.e., kissr2 and kissr3), also known as gpr54 according to the nomenclature referred to in Tena-Sempere et al. (2012) (i.e., gpr54-1b and gpr54-2b, respectively). Primary PCR amplifications for kissr2 were performed by using a primer (AP1) complementary to the adaptor sequence provided with the kit and degenerate kissr2 PCR primers (first PCR sense, kissr2-1: 5'-CCASYNSHNTTGYRNYRMNGCT-3'; antisense, kissr2-4: 5'-CTAHWNATTGGTACCGTCTTCAT-3'). Secondary and nested PCRs were performed by using a primer (AP2) complementary to the adaptor sequence provided with the kit and degenerate and nested kissr2 PCR primers (second PCR sense, kissr2-3: 5'-TTGRYNGACCGTGGTCCCHYN-3'; antisense kissr2-2: 5'- ACCGTCTTATYGRTGTYTNGTG-3'). Similarly, primary PCR amplifications for kissr3 were performed using AP1 primer and degenerate kissr3 PCR primers (first PCR sense, kissr3-1: 5'-CAYCCNNTCTYTSACNGAYGCMTG-3'; antisense, kissr3-4: 5'-TARTAARITNGTNCGNCTCTATYTG-3'). Secondary PCR amplifications for kissr3 were performed using AP2 primer and degenerate kissr4 PCR primers (second PCR sense, kissr3-2: 5'-TGSCCNYNTTYTTCTSCNATAHG-3'; antisense kissr3-3: 5'-GCCTCCTCA-TYTGYCTRtYNADT-3'). The primary PCR conditions were as follows: denaturation at 94 °C for 30 s, followed by seven cycles at 94 °C for 20 s and 76 °C for 3 min and 32 cycles at 94 °C for 20 s and 68 °C for 3 min, and 68 °C for 7 min after the final cycle. For the secondary PCR, the PCR product was diluted 1/50 and PCR conditions were as follows: 94 °C for 30 s, followed by seven cycles at 94 °C for 20 s and 76 °C for 3 min and 32 cycles at 94 °C for 20 s and 70 °C for 3 min, and a final step of 70 °C for 7 min. PCR products were cloned into the pGEM-T Easy Vector (Promega). One clone for each gene whose sequence corresponded to the consensus of the sequenced PCR product was selected as sea bass kissr2 and kissr3 cDNAs.

Sequence analyses

All amino acid sequences of different vertebrate species used for alignments and phylogenetic analysis were extracted from Ensembl (Ensembl Genome Browser, http://www.ensembl.org) and NCBI (http://blast.ncbi.nlm.nih.gov). Amino acid sequences were aligned using ClustalX (1.83) (Thompson et al., 1997), and phylogenetic analysis was performed with the Neighbor-Joining method using the Molecular Evolutionary Genetics Analysis Software (MEGA 4.1) (Kumar et al., 2008) and full-length amino acid sequences. The synteny analysis was performed using the Ensembl Genome Browser and PhyloView of Genomicus v67.01 web site (Muffato et al., 2010). In addition to the searches of vertebrate Kiss receptors, we used the Ensembl Genome Browser and NCBI to retrieve the kisspeptin receptor-like genes from the genomes of other chordates including urochordates (tunicates) and cephalochordates (amphioxus or lancelets) and invertebrates including echinoderms (sea urchin) and hemichordates (saccoglossus). In the case of sea bass, the AnnotationDraftV1 assembly from the sea bass genome database (Kuhl & Reinhardt, unpublished) was used.

The seven transmembrane (TM) helical segments, phosphorylation sites (NetPhos 1.0 Server; GPS 2.1), potential N-glycosylation sites (NetNGlyC 1.0 Server) and palmitoylation cysteine sites (CSS-Palm 2.0) in the C-terminus of the Kiss receptors were identified using prediction servers such as the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/) and HMMTOP Server of the Hungarian Academy of Sciences (http://www.enzim.hu/hmmtop). Two motifs, E/D/RWY and NpxXY, known to be conserved in these rhodopsin family G-protein coupled receptors (Gloriam et al., 2005), were also identified in the kiss receptors of sea bass.

In silico analysis of the putative promoter sequences of the sea bass kiss receptor genes was conducted to identify conserved regulatory elements in teleosts using bioinformatic tools (CHAO/DIALIGN at http://dialign.gobics.de/chao-dialign-submission; Brudno et al., 2004); Algen Promov 3 at http://alggen. lsi.upc.es/cgi-bin/promo_v3/promo/promoitn.cgi?dirDB= TF_8.3; Messegueur et al. (2002) and Farré et al. (2003); and Genomatix Software-MatInspector; Cartharius et al. (2005)).

RNA isolation, RT and cDNA synthesis: expression analyses

Total RNA was extracted from frozen tissues with TRI-REAGENT (Sigma) according to the manufacturer’s instructions. RT was performed on 2 μg of total RNA in the presence of 100 ng of random hexamers and using SuperScript II Reverse Transcriptase (Invitrogen). For expression analysis, two sets of gene-specific primers located in two different exons were used to amplify the full-length on sea bass brain cDNA using a proofreading DNA polymerase (Pfu DNA polymerase; Stratagene). An aliquot of the amplified fragments was directly used for sequencing and the remaining part was used for ligation into the pGEM-T Easy Vector (Promega). One clone for each gene whose sequence corresponded to the consensus of the sequenced PCR product was selected as sea bass kissr2 and kissr3 cDNAs.
Gene-specific primers used for RT-PCR analysis.

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All primers were designed between exon regions to exclude false positive bands in case of potential genomic DNA contamination. The elongation factor-1 alpha (ef1a) gene was used in the three fish species as reference gene (Felip et al. 2009). Primer direction: forward (F) and reverse (R).

Temperature (upper side) and number of cycles (at the bottom) in each touchdown PCR is indicated.

designed for sea bass, medaka and zebrafish kissr2 and kissr3 genes (Table 1). As previously described (Felip et al. 2009), RT-PCR analyses were performed using a touchdown PCR approach with the elongation factor-1alpha (ef1a) gene tested in the three fish species as reference gene using specific primers.

For testis samples from animals in their first year of sexual maturation, total RNA was isolated from ~100 mg of frozen gonads using the FastRNA Pro Green Kit (Qbiogene, Inc., Irvine, CA, USA) and homogenized on a FastPrep Instrument (Qbiogene, Inc.). One microgram of DNase-treated RNA was used for cDNA synthesis with the Superscript III reverse transcriptase (Invitrogen Corp.,) according to the manufacturer’s instructions and using random hexamers as primers. Gene expression was quantified by qPCR of the cDNA on a Realplex2 Mastercycler (Eppendorf AG, Hamburg, Germany) using 96 well optical plates. For each 25 µl of PCR reaction, optimized amounts of the corresponding primers and probes of kiss and kissr genes (Alvarado et al. 2013) were mixed with 1 µl of cDNA in 1× Abgene’s Absolute QPCR Mix (Abgene, Epsom, UK). The expression of the ribosomal protein L13a (rpL13a) gene was used as reference for testis samples (Mitter et al. 2009) using 1× Power SYBR Green PCR Master mix (Applied Biosystems) and cDNA samples were diluted 1:50. Male annual cycle data were normalized by dividing the input amount of the target gene by the amount of rpL13a RNA. Tenfold series dilutions of the plasmid containing the corresponding gene were included as standard curves.

Expression plasmids, cell culture and transfections

The complete coding sequences of sea bass kissr2 and kissr3 cDNAs were cloned into the pcDNA3 expression vector (Invitrogen) under the control of the cytomegalovirus (CMV) promoter to generate the pcDNA-kissr2 and pcDNA-kissr3 plasmids. Expression plasmids for human and mouse kisspeptin receptors were a generous gift from Robert Miller. pSRE-Luc and pCRE-Luc plasmids (BD Clontech, Palo Alto, CA, USA) contain the firefly luciferase gene under the control of promoters with Serum Response Elements (SRE) or CRE, respectively. The pRL-TK plasmid (Promega), which constitutively expresses a Renilla reniformis luciferase gene, was used to control transfection efficiency. Chinese Hamster Ovary (CHO) cells were maintained in DMEM supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C under 5% CO2 until confluence. Six hours before transfection, the cells were seeded in 24-well tissue-culture plates. The pSRE-Luc or pCRE-Luc reporter plasmids (1 µg), pcDNA-kissr2, pcDNA-kissr3 (160 ng) or human and mouse Kiss receptors (160 ng) and pRL-TK (320 ng) were co-transfected into the cells using Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. Two days after transfection, cells were replated in 96-well, flat, white-walled plates (Corning) and then stimulated with various concentrations (from 10⁻³ to 10⁻⁷ M) of sea bass Kiss1–10, Kiss2–10, Kiss1–15 and Kiss2–12 in OPTI-MEM medium (Invitrogen) for 6 h. In the case of transfections containing pSRE-Luc, cells were serum-starved for 2 h before stimulation. Two independent transfection experiments were performed, each conducted in duplicate. A parallel control transfection experiment was performed with pcDNA3, pCRE-Luc or pSRE-Luc and the internal control pRL-TK.

The kisspeptin peptides used in this study were synthesized by GenScript USA, Inc. (Piscataway, NJ, USA) and were as follows: Kiss1–10 (NH2-YLNNSFGLR-COH2), Kiss2–10 (NH2-FNFPFGGLRF-COH2), Kiss1–15 (pGLU)DVSSYN-LNSFGLRF-COH2) and Kiss1–12 (NH2-SKFNFPFGGLRF-COH2). Their sequences are based on the amino acid sequences deduced for sea bass kisspeptins. All peptides are amidated in C-terminal, and Kiss1–15 contains an N-terminal pyroglutamylation.

 Luciferase assays

Firefly and Renilla luciferase activities were determined using the Dual-Glo Luciferase Assay System (Promega) on an ULTRA Evolution Multi-Detection Microplate Reader (Tecan) and were expressed as relative light units. Final luciferase activity was represented as the ratio of firefly to Renilla luciferase activity, the latter acting as control for transfection efficiency.

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Table 1 Gene-specific primers used for RT-PCR analysis.

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*Temperature (upper side) and number of cycles (at the bottom) in each touchdown PCR is indicated.*
Data representation and statistical analysis

Gene expression levels in the testis during a complete reproductive cycle and receptor transactivation data are represented as the mean±S.E.M. All statistical analyses were carried out using a one-way ANOVA followed by a Tukey test. Before the analysis, values were log-transformed to meet normality and homoscedasticity requirements. Different letters in the figures represent significant differences between gonadal stages or the activation of Kiss receptors by the ligands. All analyses were conducted using SigmaStat version 3.5 (SYSTAT Software, Inc., Richmond, CA, USA). Differences were considered to be significant when \( P<0.05. \)

Results

Sea bass genome contains two kiss receptors

After using degenerate PCR primers and a genome walking approach, two PCR fragments were obtained. A PCR fragment of 140 bp corresponded to genomic sequence 5’ upstream from exon 1 of the sea bass kissr2 gene, whereas a PCR fragment of 200 bp corresponded to genomic sequence 3’ downstream from exon 1 of the kissr3 gene, respectively. Different rounds of genome walking in both genes revealed that kissr2 contains six exons, whereas kissr3 contains five exons. The full-length cDNA sequences of both genes were obtained from sea bass brain cDNA by using specific primers (GenBank accession number: kissr2 or gpr54-1b, JN202446 and kissr3 or gpr54-2b, JN202447). The kissr2 cDNA has a length of 1433 bp (Supplementary Figure 1A, see section on supplementary data given at the end of this article) and consists of a 5’UTR of 107 bp, a 3’UTR of 222 bp and an open-reading frame (ORF) of 1104 bp that encodes a protein of 368 amino acids (Fig. 1). The kissr3 cDNA is 2365 bp long (Supplementary Figure 1B) and contains an ORF of 1134 bp encoding a protein of 378 amino acids with 5’ and 3’ UTRs of 636 and 595 bp in length, respectively (Fig. 1). The two sea bass kisspeptin receptors share 53.1% amino acid sequence identity with each other, with 6.5% identity in the extracellular region, 60.7% identity in the TM regions and 6.3% in the C-terminus.

Kiss receptors have conserved structural elements

Kiss receptors are members of the rhodopsin-like or Class A family of G-protein coupled receptors, which contain conserved structural elements. The two sea bass receptor proteins show conserved residues and motifs similar to other vertebrate kisspeptin receptors. Both sea bass Kissr2 and Kissr3 are membrane proteins with an extracellular N-terminus, a cytoplasmic C-terminus and 7TM helices with three extracellular loops (EL) and three intracellular loops (IL) between the TM helices (Fig. 1). Several residues that are considered to be involved in ligand binding or formation of binding pockets are conserved in these receptors (Lee et al. 1999, Gloriam et al. 2005, Oh et al. 2006, Millar & Newton 2010), including the conserved asparagine (Asn, N) at position N51 for Kissr2 and N65 for Kissr3 in TM1, the conserved aspartate (Asp, D) at position D79 for Kissr2 and D92 for Kissr3 in TM2, the conserved aspartate (Asp, D) at position D103 for Kissr2 and D116 for Kissr3 in TM3, and some conserved prolines (Pro, P) in TM2–5 (P167, P211, P271, P326 for Kissr2 and P180, P224, P284, P323 for Kissr3). Cysteines (Cys, C) involved in a conserved N-terminal disulphide bond are located
in EL1 and EL2 for Kissr2 (C106 and C186) and for Kissr3 (C119 and C199). Almost all GPCRs are regulated by phosphorylation, which is the key to determining the signalling properties of this receptor family. The multi-site phosphorylation of GPCRs reflects the action of a number of protein kinase families and includes the presence of several serine (Ser), threonine (Thr) and tyrosine (Tyr) residues that were predicted to be phosphorylated. Thus, in the case of the Kissr2 four Ser, three Thr and two Tyr are potentially phosphorylated, while nine Ser, three Thr and five Try were predicted in the Kissr3. Interestingly, the results predicted for Kissr2 showed that phosphorylation in Ser192 (EL2) and Ser230 (IL3) might be mediated by the protein kinase C (PKC), while Thr146 (IL2) might be mediated by PKC and PKA. On the other hand, phosphorylation in Ser24 (N-terminus), Ser205 (EL2) and Ser336, Ser352 and Ser353 in the C-terminus might be mediated by PKC, while Ser262 (IL3) and Ser353 (C-terminus) might be mediated by both PKC and PKA in the Kissr3. Phosphorylation was also predicted on Thr159 via the PKA in the Kissr3. Further analysis predicted potential N-glycosylation sites for Kissr2 (17 NES) and Kissr3 (9 NTT, 17 NGS, 22 NFS). Conserved microdomains involved in receptor activation are also present in the two sea bass Kiss receptors, with the DrxY motif within TMIII, and NPyxY in TMVII. No palmitoylated cysteines in the C-terminus were predicted.

**Sequence analysis of the 5′-flanking regions of the sea bass kiss receptor genes**

The sequences of the 5′-flanking regions of the sea bass Kiss receptor genes were extracted from the Annotation-DraftV1 assembly from the sea bass genome database (Kuhl & Reinhardt, unpublished). 2.5 kb of 5′-upstream sequence from sea bass kissr2 and kissr3 putative promoter regions along with 2.5 kb of sequence upstream of the ATG start codon from available genome sequences of Kiss receptors in other teleosts were analyzed to ascertain potential conserved regulatory elements. Two conserved regions of 14 (Fig. 2A) and 15 bp (Fig. 2B) in size were identified in the 5′-upstream region of sea bass kissr2 at position −1050 and −19 with respect to the translation start codon. These conserved sequences were also found in the promoter regions of the kissr2 receptor.

**Figure 2** Putative conserved transcription factor binding sites on the promoter regions of kissr2 (A and B) and kissr3 (C and D) genes in the sea bass compared with other available genome sequences of teleosts. The numbers represent distance in bp from the putative translation initiation site. Regulatory element name abbreviations: GR-α, glucocorticoid receptor alpha responsive element; C/EBP, nuclear factor-interleukin 6; PR, progesterone receptor or nuclear receptor subfamily 3, group C, member 3; WT1, transcription factor Wilms tumor 1; LCR-F1, bZIP transcription factor; TFHIB, general transcription factor IIIB; GA-BF, GA-box binding factor; Pax-6, paired box 6; STE12, homeodomain transcription factor Ste12; XBP-1, X-box binding protein 1; HOXA5, homeobox protein Hox-A5; POU1F1α, POU class 1 homeobox 1; YY1, transcription factor Yin Yang 1; RFX1, regulatory factor X, 1 and CREM.
genes of zebrafish (−2517) and medaka (−210), respectively. Of particular note was the conserved location that these sequences had for GR-α, C/EBP, PR, WT1, LCR-F1, TFIIB, GA-BF, Pax-6 and STE12 binding sites (Fig. 2A and B). Similarly, the sea bass kissr3 promoter region was analyzed for conserved regions, including stickleback and tilapia. Two conserved regions of 17 bp (Fig. 2C) and 32 bp (Fig. 2D) identified in the sea bass genome (−1129 and −687 with respect to the translation start codon) were also present in the promoter region of the kissr3 receptor gene of stickleback (−1415 and −927) and tilapia (−1725 and −830). These sequences showed a marked degree of conservation as regards the location of potential binding sites for C/EBP, XBP-1, WT1, GR-α, YY1, CREM and RFX1. HOXA5 and POU1F1a showed overlapping binding sites in the sequence of all three teleost species (Fig. 2C).

**Phylogeny and synteny analyses of kiss receptors**

A phylogenetic tree highlighting piscine Kiss receptors was constructed based on the available protein sequences from the genome databases (Fig. 3). The results showed that vertebrate Kiss receptors separated into four groups, which were supported by significant bootstrap values (87, 64, 91 and 92%, respectively). Thus, according to the separation of the phylogenetic branches encompassing the Kiss receptor forms, they were named kissr1, kissr2, kissr3 and kissr4, where kissr1 was used to name the group including the mammalian receptors, as these were the first to be described. Present data demonstrate that the presence of these multiples forms could be the result of the two genome duplication events that constituted the segregation of the sequence of kissr1 from those of kissr2, suggesting that both emerged from a common ancestor and, in turn, these two closest forms could be sister groups after the 2R. On the other hand, we observed that the sequence of kissr3 segregated with those of kissr4, which was supported by significant bootstrap values (65 and 96%, respectively). Of note is the fact that the sequence of one Kiss receptor in the sea lamprey (*P. marinus*) could not be related to one of the four vertebrate Kiss receptors. In the case of the sea bass Kiss receptors, the two kissr sequences segregated into two different groups, namely kissr2 and kissr3 (Fig. 3). Searches in the available genome databases of Ambulacrarians pointed to the existence of Kiss receptor-like genes in the sea urchin and the acorn worm. Using NCBI Blast to search for the presence of Kiss receptor-like sequences in other invertebrate species such as *Branchiostoma floridae* (amphioxus or lancelets; Cephalochordate), we identified three sequences (Accession numbers: XP_002611682, XP_002591960 and XP_002608825) that produced significant alignments with medaka (Assembly MEDAKA1; Ch 9: 4484 331–4490 709 and Ch 17: 29 839 926–29 854 747) and zebrafish (EU047918, EU047917) Kissr amino acid sequences that were used as queries. All three blast hits were hypothetical proteins that presented the typical 7TM of the GPCR family and shared conserved residues and motifs with other vertebrate Kiss receptors. In tunicates (Ciona, Urochordate), the genomic searches failed to identify or predict Kiss receptor-like sequences.

Several vertebrate representatives were used to perform synteny analysis, the results of which revealed that the neighboring gene clusters surrounding the four kissr loci were highly conserved across vertebrate species and each presenting paralogs (Fig. 4). Furthermore, when multiple forms were identified in the same species, these genes were located in different chromosomes. The sea bass kissr2 and kissr3 genes were located at the chromosomes (i.e., linkage group, LG) LG20 and LG10, respectively.

**Kiss receptors show similar distribution in tissues of juveniles and adults**

The tissue expression patterns of each kissr mRNAs were analyzed by RT-PCR in sea bass, medaka and zebrafish, using gene-specific primers (Table 1). A comparison of the expression of kissr2 and kissr3 in different tissues of juvenile and adult male and female sea bass showed that it was predominately observed in the brain, pituitary and gonadal tissues (testis and ovary) (Fig. 5A). In adults, both genes were also highly expressed in the brain and gonads, with only faint levels of expression in pituitary. The presence of both mRNAs was also detected in a number of somatic tissues, the eye showing high expression levels of kissr3 in the juvenile males of sea bass (Fig. 5, upper right panel). In medaka, kissr2 and kissr3 were highly expressed in brain, while gonadal tissues showed weaker expression levels (Fig. 6, upper panel). In addition, kissr3 but not kissr2 was expressed in most tissues at low levels, although with a notable expression in the eye of both sexes. In zebrafish, kissr2 and kissr3 were clearly detected in the brain of males and females and testis, but not in ovary (Fig. 6, low panel). The presence of both genes was also observed in a wide range of somatic tissues of males and females, being particularly evident in the intestine, gill, eye and skin of both sexes.

**Temporal mRNA expression of kiss receptors**

Given the expression levels in the brain, pituitary and gonadal tissues at the beginning of the reproductive season (January; Fig. 5A) and the involvement of these organs in reproduction, sample collection from juvenile and adult fish was extended from February to April (Fig. 5B), the sampling period thus coinciding with the whole reproductive season of sea bass. The results showed that the expression of kissr2 and kissr3 in the brain was independent of the developmental stage or sex during the reproductive season (Fig. 5B, middle panel).
Figure 3  Unrooted phylogenetic tree of kisspeptin receptor sequences. The phylogenetic tree was generated with MEGA version 4.1 using the Neighbor-Joining bootstrap consensus tree (n=1000 bootstrap replicates). The number shown at each branch indicates the bootstrap value (%). Phylogenetic analysis was performed using the following sequences, which were retrieved from both the GenBank and the Ensembl Genome Browser database: human (Homo sapiens) (NM_012551), mouse (Mus musculus) (NM_053244), rat (Rattus norvegicus) (NM_023992), pig (Sus scrofa) (NM_0011044624), opossum (Monodelphis domestica) (XM_001374715), platypus (Ornithorhynchus anatinus) (XP_001507133.1) (XP_001515272.1 a second predicted form, is an incomplete sequence and was not included in this analysis), western clawed frog (Xenopus laevis), stickleback (Gasterosteus aculeatus) (assembly BROAD S1 group III), tetraodon (Tetraodon nigroviridis) (assembly TETRAODON 8.0; Chromosome 15_random), takifugu (Takifugu rubripes) (assembly FUGU 4.0; scaffold_80 at location 1 035 934–1 046 929), sea bass (D. labrax) (EU681171), lizard (Anolis carolinensis) (assembly AnoCar 1.0; scaffold_10 at location 6 382 138–6 392 509), goldfish (O. latipes) (FJ465139 for kissr3, EU853680 for kissr2) and ENSORLG00000017731 for kissr2), spotted sea bass (D. labrax) (EU681171), lizard (Anolis carolinensis) (assembly AnoCar 1.0; scaffold_10 at location 6 382 138–6 392 509), goldfish (O. latipes) (FJ465139 for kissr3, EU853680 for kissr2) and ENSORLG00000017731 for kissr2).
Characterization of sea bass kiss receptors

Figure 4 Conserved synteny and genomic organization of the kiss receptors in vertebrates, kissr1 (A), kissr2 (B), kissr3 (C) and kissr4 (D). Genomic organization of the four Kiss receptor loci was obtained from Ensembl Genome Browser. The schematic representations are not to scale. Numbers in brackets in each representation indicate on which chromosome (Ch), linkage group (LG), scaffold (S), group (G) or contig (Cg) the cluster is found. The gene searches were done at Ensembl against the genome assemblies of human (Hs) (assembly GRCh37), mouse (Mm) (M. musculus) (assembly NCBI m37), rat (Rn) (R. norvegicus) (assembly RGSC 3.4), opossum (Md) (M. domestica) (assembly MonDom 5), platypus (Oa) (O. anatinus) (assembly Ornithorhynchus_anatinus-3.0), lizard (Ac) (A. carolinensis) (assembly AnoCar1.0), spotted gar (Lo) (L. oculatus) (LepOcu1), Xenopus (St) (S. tropicalis) (assembly JGI 4.1), medaka (Ol) (O. latipes) (assembly Med8), zebrafish (Dr) (D. rerio) (assembly Zv8), takifugu (Fr) (T. rubripes) (assembly FUGU 4.0), Tetraodon (Tn) (T. nigroviridis) (assembly TETRAODON 0.0), stickleback (Ga) (G. aculeatus) (assembly BROAD S1) and sea bass (Dl) (D. labrax) (AnnotationDraftV1, unpublished) (illustrated here by a box). Gene name abbreviations: Palm, paralemmin; C19orf21, uncharacterized protein C19orf21; Kissr3, kiss receptor 3; Kissr4, kiss receptor 4; Kissr1, kiss receptor 1; kissr2, kiss receptor 2; Xab2, Xenopus A/B domain containing 2; Cfd, 25 kDa, C8orf55; Midn, delta subunit; N-methyl-D-aspartate 3B; protein; Atp6v08, ATPase, H+ transporting, lysosomal 21 kDa, V0 subunit b; Eef1d, eukaryotic translation elongation factor 1 delta; Trim47, tripartite motif-containing 47; Cbor55, chromosome 8 ORF 55; Trappc5, trafficking protein particle complex 5; Tecr, trans-2,3-enoyl-CoA reductase; Stxb2, syntaxin-Binding Protein 2; Xah2, XPA binding protein 2; Notch3, notch (Drosophila) homolog 3; Ephx3, epoxide hydrolase 3; Lppr3, lipid phosphate phosphatase-related protein type 3; Cld, complement factor D (adipsin); Atrd3a, AT rich interactive domain 3A; Grin3b, glutamate receptor, ionotropic, N-methyl-D-aspartate 3B; Abca7, ATP-binding cassette, sub-family A (ABC1), member 7; Polr2e, polymerase (RNA) II (DNA directed) polypeptide E; 25 kDa; Gpx4, glutathione peroxidase 4; Stkl1, serine/threonine kinase 11; Atp5d, ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit; Mdh2, mitochondrial; Isca1, iron-sulfur cluster assembly 1; Mcidas, multiciliate differentiation and DNA synthesis associated cell cycle protein; Mcc2, methylcrotonoyl-CoA carboxylase 2 (beta); Naa35, N(alpha)-acetyltransferase 35, NatC auxiliary subunit; Aggdp1, ATP/GTP binding protein 1.

Overall, the levels of expression of kissr3 were higher than those of kissr2 in adult and juvenile fish from both sexes, with kissr2 mRNAs from brain showing weaker expression levels in adults (Fig. 5B middle left panel) than juvenile fish for the same period of time (Fig. 5B middle right panel). In addition, the expression of kissr2 and kissr3 in the testis or ovary was moderate and it was independent of developmental stage or sex during the reproductive season (Fig. 5B upper panel). In this vein, moderate expression levels of kissr2 were observed in the pituitary, whereas faint expression levels of kissr3 were detected for the same period of time regardless of sex.
The post-spawning stage (Fig. 7B). The expression of (stages IV and V) and then significantly decreased during the post-spawning stage (Fig. 7A). The expression of kiss2 was higher than that of kiss1 in all the stages of testicular maturation. As regards the male yearly reproductive cycle, maximum values of kiss2 were observed in February (data not shown), coinciding with mid- and late recrudescence (stages IV and V) and then significantly decreased during the post-spawning stage (Fig. 7B). The expression of kissr2 showed a significant increase at the end of spermatogenesis/beginning of spermiation (stage IV) and then decreased during full spermiation (stage V) to levels that were not statistically different from the previous stage. During the post-spawning stage kissr2 levels remained low (Fig. 7C). The expression pattern of kissr3 gene showed that levels remained low throughout spermatogenesis (stages I–IV) (Fig. 7D). The levels of kissr3 increased significantly in stage V before falling during stage VI.

**Testicular expression of kiss/kissr genes in sea bass during an annual reproductive cycle**

The expression of kiss1 was low compared with that of the other genes analyzed, and remained constant during male spermatogenesis. Levels decreased during the full spermiation stage (stage V) and fell significantly during the post-spawning stage. As regards the male yearly reproductive cycle, maximum values of kiss2 were observed in February (data not shown), coinciding with mid- and late recrudescence (stages IV and V) and then significantly decreased during the post-spawning stage (Fig. 7B). The expression of kissr2 showed a significant increase at the end of spermatogenesis/beginning of spermiation (stage IV) and then decreased during full spermiation (stage V) to levels that were not statistically different from the previous stage. During the post-spawning stage kissr2 levels remained low (Fig. 7C). The expression pattern of kissr3 gene showed that levels remained low throughout spermatogenesis (stages I–IV) (Fig. 7D). The levels of kissr3 increased significantly in stage V before falling during stage VI.

**Ligand selectivity of sea bass Kiss receptors**

In order to delineate sea bass ligand-receptor interactions, we analyzed the transactivation of the luciferase gene placed under two different promoters, including SRE sequences as a reporter of PKC activation and CRE sequences as a reporter for the PKA pathway. In mock (pcDNA3)-transfected cells, sea bass Kiss1–10 and sea bass Kiss2–10 were not able to induce SRE- or CRE-driven luciferase activity (data not shown), indicating that CHO cells do not naturally express endogenous Kiss receptors. The use of the SRE reporter system revealed that sea bass Kiss1–15 was most able to activate Kissr2 with a ten-time increase of luciferase activity over the non-activated control, while Kiss1–10 elicited and increase of 4.84, in comparison to Kiss2–10 and Kiss2–12, which exhibited only 1.6- and 3.3-fold increase, respectively (Fig. 8A). Using the CRE reporter system, sea bass Kiss1–10 elicited an increase of luciferase activity of 9.4 compared with Kiss2–10, which showed no significant effect at all in CHO cells transfected with Kissr2. It is interesting to note that Kiss1–15 exhibited the highest potency in activating Kissr2 (24.7-fold increase in luciferase over control), whereas Kiss2–12 elicited only an increase of 2.49 times over control (Fig. 8B). On the other hand, cells transfected with kissr3 displayed the highest response to Kiss2–12, triggering similar activation via both SRE- and CRE sequences as a reporter of PKA activation. Using the CRE reporter system, Kissr3 exhibited the highest activity of luciferase over control, whereas Kiss2–12 elicited only an increase of 2.49 times over control (Fig. 8B). On the other hand, cells transfected with kissr3 displayed the highest response to Kiss2–12, triggering similar activation via both SRE- and CRE-reporter constructs, with 3.3- and 3.9-fold increase compared to basal levels, respectively (Fig. 8C and D). In addition, we tested whether sea bass decapeptide or...
longer kisspeptins differentially activated the human and rat Kiss1 receptors using the SRE-reporter system, a pathway commonly described for mammalian Kiss1 receptor. We found that for human GPR54 (or KISSR1), the peptides derived from sea bass Kiss1 efficiently activated the receptor (6.7 times over control for Kiss1–15 and 7.1 times over control for Kiss1–10), but Kiss2 dodecapeptide exhibited similar high potency (7.8 times over control) at a high peptide concentration (10^{-5} M). The sea bass Kiss2–10 showed very low potency (4.3 times over control) (Fig. 8E). In the case of mouse Kiss1 receptor, sea bass Kiss1–15 showed the highest potency (6.9 times over control) among the peptides examined, with Kiss1–10 and Kiss2–12 exhibiting significantly low potency (2.8 and 2.5 times over control, respectively) (Fig. 8F). The sea bass Kiss2–10 was not able to activate the mouse Kiss1 receptor.

Discussion

The present study reports the isolation and characterization of two Kiss receptor genes (kissr2 and kissr3 in our nomenclature; Supplementary Material Table S1, see section on supplementary data given at the end of this study). The results indicate that kisspeptins can differentially activate the human and rat Kiss1 receptors, and that the potency of activation can vary significantly depending on the species and receptor.

Figure 6 RT-PCR analysis of medaka and zebrafish kissr2 and kissr3 expression in tissues from male and female adult fish. Total RNA extracted from three to six pooled tissues was reverse-transcribed and amplified by PCR. The medaka and zebrafish ef1a were used as an internal control to verify the correctness of the RT reaction. C: negative control for PCR which were performed with sterile water as template.

Figure 7 Relative patterns of (A) kiss1, (B) kiss2, (C) kissr2 and (D) kissr3 expression in testis of male sea bass sampled during their first sexual maturation. Values are represented by stages of gonadal development as determined by histology. Stage I or immature (n=19), stage II or early recrudescence (n=6), stage III or mid recrudescence (n=3), stage IV or late recrudescence (n=6), stage V or full spermatiation (n=10) and stage VI or post-spawning (n=3). Statistically significant differences are indicated with different letters above the bars.

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internalization of GPCRs (Lee et al. 2005, Oh et al. 2006). The presence of glucocorticoid receptor (GR) and progesterone receptor (PR) elements, suggesting that the kiss receptors genes are regulated by steroids, as described in the yellowtail kingfish and zebrafish (Nocillado et al. 2013 and this study). Sea bass Kiss receptors may also be regulated by tumor-related genes such as transcription factor Wilms tumor 1 (WT1) and transcription factor Yin Yang 1 (YY1) (Roth et al. 2007, Nocillado et al. 2013), and it is also possible that photoperiod acts on the kissr genes at the level of expression as they contain C/EBP elements. In zebrafish, these elements correspond to the light-responsive D-box regulatory motif (Weger et al. 2011). These observations provide preliminary data that could offer new insights into kissr transcriptional regulation and function.

To date, multiple kissr genes have been described in vertebrate species. Recent findings pointed to the presence of four kissr genes in osteichthyan species and three forms in an early group of teleosts, thus demonstrating the existence of a triplicate Kiss system in fish (Pasquier et al. 2014a,b). Conversely, available data are scarce in invertebrates (Biran et al. 2008, Pasquier et al. 2012a,b, 2014a,b, Tena-Sempere et al. 2012). In addition to the characterization and/or prediction of Kiss receptor sequences in the sea urchin (Biran et al. 2008, Pasquier et al. 2012a) and the acorn worm (Pasquier et al. 2012b) and this study), we identify some

Figure 8: Differential ligand selectivity of sea bass Kiss receptors. SRE- (A, C, E, F) and CRE-driven (B and D) luciferase activities in CHO cells transfected with sea bass kissr2 (sb-kissr2) (A and B) or sea bass kissr3 (sb-kissr3) (C and D) and stimulated with Kiss1–10, Kiss1–15, Kiss2–10 or Kiss2–12. SRE-driven luciferase activities in CHO cells transfected with human KISSR1 (hKISSR1) (E) or mouse Kissr1 (mKISSR1) (F) and stimulated with Kiss1–10, Kiss1–15, Kiss2–10 or Kiss2–12. Results are mean values±S.E.M. from two independent experiments, each conducted in duplicate, and are expressed as the ratio of firefly luciferase activity to Renilla luciferase activity. The dashed line corresponds to basal levels of luciferase activity, in the absence of any stimulating peptide. Lowercase letters show significant differences with respect to the basal levels for each sample point (peptide concentration).
significant blast hits of kissr in the Branchiostoma genome (amphioxus or lancelets). This supports the hypothesis that at least one ancestral Kiss receptor might have existed before the emergence of vertebrates (Pasquier et al. 2014a), as Branchiostoma (Cephalochordata) reflects a primitive prevertebrate condition (Holland et al. 2008). On the other hand, the existence of more than one kissr gene in amphioxus, prior to the whole genome duplication (WGD) events of vertebrates (Dehal & Boore, 2005), is not strange, since it is known that Branchiostoma genome cannot itself be taken as the ancestral chordate genome, as it exhibited specialized features after its divergence from the rest of the chordate lineage (Holland et al. 2008, Louis et al. 2012). The current status and proposed evolutionary history of kissr genes among vertebrates points to the separation of Kiss receptors into four main clades (Pasquier et al. 2014a,b and this study). The sea bass Kiss receptors cluster into two different clades with the kissr2 being related to the forms found in a few teleosts such as medaka (Beloniformes), zebrafish and goldfish (Cypriniformes), the Elopomorph European eel, the Actinopterygian spotted gar and two Sarcopterygians (Xenopus and coelacanth) (Pasquier et al. 2012a). Thus, the sea bass kissr3 form is the ortholog of those forms found in most of previously described teleosts, including Mugiliforms, Pleuronectiforms, Tetraodontiforms, Gasterosteiforms and Perciforms. This clade also encompasses the Elopomorphes European eel, the Actinopterygian spotted gar and three Sarcopterygians (Xenopus, bullfrog and coelacanth) (Pasquier et al. 2012a). Synteny analysis shows that the four kissr neighboring genomic regions are highly conserved, and when multiple genes are identified in the same species, they are located in different chromosomes (Lee et al. (2009), Um et al. (2009), Akazome et al. (2010), Kim et al. (2012), Pasquier et al. (2012a,b) and this study). Our phylogenetic and synteny analyses agree with those data suggesting that this gene family might have arisen from a single ancestral gene – and genomic region – through the two successive WGDs (1R and 2R) early in vertebrate evolution, resulting in these four kissr paralogs. It seems that the occurrence of a third WGD event (3R) in the teleost fish lineage (~350 million years ago) had no impact on the number of kissr paralogs in current teleosts, as the potential existence of up to eight kiss receptor genes has not been reported in any species analyzed (Pasquier et al. 2014a,b). So far, the common ancestral kissr gene diversified through gene duplication, with subsequent gene losses, to display a large variability of Kiss receptors (and ligands) depending on the species (Akazome et al. 2010, Um et al. 2010, Kim et al. 2012, Pasquier et al. 2012a,b, 2014a,b). In this context, the impact of these events in Gnathostomos (chondrichthians) and Agnathos (cyclostomes) is still uncertain. Currently, only some partial Kiss receptor sequences have been identified but not published in chondrichthians (Pasquier et al. 2012b), while only one kissr has been identified in the sea lamprey, although it does not specifically correlate to any of the four osteichthyan kissrs (Pasquier et al. (2012a) and this study). However, it is known that the lamprey genome contains two kiss genes corresponding to the two rounds of whole genome duplication (Felip et al. 2009). In our study, we observed that the branch encompassing the kissr1 form separated from the kissr2 form, deducing that both emerged from a common ancestor. These findings are further supported by Pasquier et al. (2014b), who indicated that kissr1 and kissr3 (kissr2 in our nomenclature) could be sister groups after the 2R. In addition, the data presented in this study show that the branch encompassing the kissr3 form separated from the kissr4 form, thus concluding that kissr3 and kissr4 could be also sister groups. The Kissr form present in most teleost species and the amphibians, coelacanth and spotted gar has been tentatively referred to as kissr3 in our nomenclature after considering the distinct terminologies used to name Kiss receptors across species (see Supplemental Material Table S1 for comparison, see section on supplementary data given at the end of this article). Further characterization of Kiss receptor peptidic sequences in an increasing number of lineages will be crucial to providing new evolutionary insights into this receptor family in Deuterostomes.

The tissue expression of kissr genes, and those of their cognate ligands kiss1 and kiss2, in the brain and the gonads of sea bass, medaka and zebrafish is fully compatible with their putative roles in fish reproduction (Felip et al. (2009) and this study). In this respect, the mRNA expression profiles of kissr and kiss genes in the brain of sea bass suggested that these two kisspeptin systems might be involved in segregated functions of neuroendocrine signaling that control early or late events of gametogenesis in this species (Migaud et al. 2012, Alvarado et al. 2013). Moreover, the expression profiles of two kisspeptin genes and their receptors in the gonads of adult male sea bass support the role of kisspeptins in the regulation of late stages of spermatogenesis in this species, as reported in other teleosts. In the Senegalese sole (Solea senegalensis), the kiss2/kissr3 system is expressed in all germ cell types at different stages during spermatogenesis (Marín-Juez et al. 2013), while in sea bass the expression of kiss1 and kiss2 did not change across the progression of gonadal growth, kissr2 and kissr3 reaching a maximum at the initiation and completion of the spermatogenesis period. For the first time in a multiple group-synchronous spawner teleost, the sea bass, the present study describes the expression profiles of all four kisspeptin system genes during the first gonadal recrudescence in males. Interestingly, the elevated expression of Kiss receptors in spermatogenesis could be due to the group-synchronous nature of gonadal development in this species, in which these expression levels would need to be maintained in some clutches of developing gametes. On the other hand,
in vitro functional luciferase assays conducted in this study demonstrate that sea bass Kissr2 and Kissr3 signals can be transduced via both PKA and PKC pathways. These findings are consistent with data from zebrafish (Biran et al. 2008), medaka (Kanda et al. 2013) and chub mackerel (Ohga et al. 2013). In contrast, and as observed in mammals (Kotani et al. 2001, Muir et al. 2001), no PKA activation has been described in orange spotted grouper (Shi et al. 2010) and bullfrog (Moon et al. 2009). Thus, the sea bass Kiss1–15 exhibited the highest potency when activating the PKA pathway through Kissr2, as the CRE-driven luciferase activity was higher than that driven by the SRE promoter. Kissr3 was maximally activated by Kiss2–12. These findings indicate that long Kiss peptides are more potent activators of Kiss receptors than the corresponding Kiss10 peptides in sea bass as has also been described in zebrafish (Lee et al. 2009), medaka (Kanda et al. 2013) and chub mackerel (Ohga et al. 2013). Our results indicate that Kiss1 exhibits a preference for Kissr2, while Kiss2 exhibits a preference for Kissr3, although this receptor is also activated by the Kiss1 ligand. These findings are in close agreement with previous data in zebrafish, where it was demonstrated that synthetic Kiss1 and Kiss2 peptides activate Kiss receptors with different potencies, the Kissr2 form exhibiting higher sensitivity toward Kiss1-derived peptides than Kiss2-derived peptides (Lee et al. 2009). In the southern bluefin tuna (Thunnus maccoyii) and in yellowtail kingfish (Seriola lalandi), Kissr3 showed a higher response to Kiss2–10 than to Kiss1–10, with stronger transduction via the PKC than PKA pathway (Nocillado et al. 2012). Conversely, in goldfish, according to Li et al. (2009), Kiss1–10 enhances the PKC pathway through Kissr3 activation, while Kiss2–10 exhibits higher preference for Kissr2 and the ability to activate both PKC and PKA pathways. It is interesting to note that the concentration of ligands used for receptor activation in sea bass was higher than in medaka, zebrafish and yellowtail kingfish, while the concentration of ligands for receptor activation in yellowtail kingfish and southern bluefin tuna showed to be higher than those used in medaka, zebrafish and chub mackerel. This can be presumably due to different experimental conditions of transfection assays such as expression vectors, origin of the cells, plasmid concentrations and/or transfection reagents as well as the luciferase reporter assays tested. Taken together, these data point to the existence of different levels of activation for the Kiss/Kissr systems in teleosts. When two Kiss receptors coexist in a fish species, both receptors may be activated by the two Kiss ligands with different potencies, indicating differential ligand selectivity (Biran et al. 2008, Lee et al. 2009, Li et al. 2009, Kanda et al. 2013 and Ohga et al. 2013 and this study) and influencing physiological actions (Felip et al. 2009, Kitahashi et al. 2009, Li et al. 2009). Of note, our results show that the sea bass Kiss1-derived peptides exhibit high potency to activate the human KISSR1. Furthermore, our findings have demonstrated that Kiss2 dodecapeptide has a potency similar to that of Kiss1 peptides. In the case of mouse Kissr1, sea bass Kiss1–15 showed the highest potency while Kiss1–10 and Kiss2–12 exhibited a similar potency. Therefore, the ability of the sea bass Kiss2-derived peptides for the activation of human KISSR1 might support the initial evidence that provides the existence of a kiss2-like gene in the genome database of primates including humans (Osugi et al. 2013).

All in all, the neuroanatomical distribution of kiss- and kissr-expressing neurons in sea bass (Escobar et al. 2013a,b) and zebrafish (Servili et al. 2011) agree with the higher potency of Kiss2 for activating Kissr3 rather than Kissr2. While kissr2 mRNAs exhibit a modest expression in limited regions of the brain, kissr3 mRNAs present a very large distribution and a high level of expression in the fore-, mid- and hindbrain (Servili et al. 2011, Escobar et al. 2013b). As in the zebrafish, sea bass positive Kiss2 immuno-reactive cells are observed in the central telencephalon and the region surrounding the lateral recess of the hypothalamus that exhibits kissr3-expressing neurons (Escobar et al. 2013b). Therefore, it has been suggested that Kiss2 may be the physiological ligand of Kissr3 in these brain regions (Escobar et al. 2013b). This is in line with the observed enhancement of the expression of the kiss2/kissr3 system compared with kiss1 and kissr2 genes in the brain during different gonadal stages of adult male and female sea bass (Alvarado et al. 2013). In addition, the fact that many teleosts lack kissr2 but possess kissr3 suggests that the kiss2/kissr3 system may be the most robust one during the teleost evolutionary process, thus operating as the Kiss/Gpr54 system does in mammals. Nevertheless, it must be taken into account that zebrafish mutant lines of the ligands or receptor genes have demonstrated that the reproductive capability is not impaired in male and female knockouts, indicating that the kiss/kissr systems are not absolutely essential for reproduction in certain nonmammalian vertebrates (Tang et al. 2015). In this line, these results show that among vertebrates, fish have an exceptional range of reproductive strategies, and thus several scenarios might be favored in evolution. Further investigation into the comparative physiology of kispeptins and their receptors will be necessary to understand the functionality of these paralog genes.

Supplementary data
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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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