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Identification and Distribution of Three Gonadotropin-Releasing Hormone (GnRH) Isoforms in the Brain of a Clupeiform Fish, *Engraulis japonicus*

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To gain a better understanding of the reproductive endocrinology of a primitive order clupeiform fish (Japanese anchovy, *Engraulis japonicus*), cDNAs encoding three gonadotropin-releasing hormone (GnRH) isoforms were isolated from the brain, and their distribution was analyzed using in-situ hybridization (ISH). The three GnRH isoforms include GnRH1 (herring GnRH), GnRH2 (chicken GnRH-II) and GnRH3 (salmon GnRH), and their full-length cDNAs encode 88, 86, and 89 deduced amino acids (aa), respectively. Alignment analysis of Japanese anchovy GnRH isoforms showed lower identities with other teleost fish. The major population of GnRH1 neurons was localized in the ventral telencephalon (VT) and nucleus preopticus (NPO) of the preoptic area (POA) with minor population in the anterior olfactory bulb (OB). GnRH2 neurons were restricted to the midbrain tegmentum (MT), specific to the nucleus of the medial longitudinal fasciculus (nMLF). GnRH3 neurons were localized in the olfactory nerve (ON), ventral OB, and transitional area between OB and ON. Interestingly, GnRH1 neurons were also localized in the olfactory bulb, in addition to its major population in the preoptic area. These results indicate the differential distribution of three GnRH isoforms expressed in the brain of the Japanese anchovy.

Key words: GnRH, Clupeiformes, Japanese anchovy, cDNA cloning, In-situ hybridization

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a neuropeptide that controls the cascade of hormones through the brain-pituitary-gonadal (BPG) axis, involved in the regulation of reproductive processes (Dubois et al., 2002; Kah et al., 2007; Matsuyama et al., 2013). GnRH neurons innervate anterior pituitary regions to stimulate the synthesis and secretion of the gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), which promotes sex steroid production responsible for the growth and maturation of gonads (Chang et al., 2009). Further, these sex steroids, in turn, feed back to the brain, including the pituitary, to complete the BPG axis (Shahjahan et al., 2010). In addition to the primary role of GnRH in the endocrine regulation, it is also known to play a neuromodulatory role in sexual behaviour and transducing olfactory signals (Dubois et al., 2002). It also modulates conventional synaptic transmission (Kinoshita et al., 2007), and electrical activity of vasotocin (VT) and isotocin (IT) neurons in the brain (Saito et al., 2003), and acts as a melatonin-releasing factor in the pineal of teleost fish (Servili et al., 2010). In addition, it directly regulates synthesis and release of somatolactin as well as growth hormone (GH)/prolactin (PRL) (Marchant et al., 1989; Weber et al., 1997; Kakizawa et al., 1997; Onuma et al., 2005).

To date, 30 isoforms of GnRH have been reported; representing 15 vertebrate and invertebrate species, respectively (Roch et al., 2011; Selvaraj et al., 2012a). Interestingly, eight out of 15 vertebrate GnRH isoforms have been isolated in teleost fish (Roch et al., 2011; Adams et al., 2002), and two or three unique GnRH isoforms have been identified in each representative species (Carolsfeld et al., 2000; Adams et al., 2002). Teleost fish expressing two GnRH isoforms in the brain include members from order anguilliformes (Japanese eel, *Anguilla japonica*; Okubo et al., 1999), salmoniformes (masu salmon, *Oncorhynchus masou*; Amano et al., 1991), siluriformes (African catfish, *Clarias gariepinus*; Bogerd et al., 1994), cypriniformes (goldfish, *Carassius auratus*; Kim et al., 1995), and osteoglossiformes (butterfly fish, *Pantodon buchholzi*; O'Neill et al., 1998). Fish with three GnRH isoforms include members from order perciformes (African cichlid, *Haplochromis burtoni*; White et al., 1995; gilthead sea bream, *Sparus aurata*; Gothilf et al., 1996; Atlantic croaker, *Micropogonias undulates*; Mohamed et al., 2005; chub mackerel, *Scomber japonicus*; Selvaraj et al., 2012a, b), beloniformes (medaka, *Oryzias latipes*; Okubo et al., 2000), pleuronectiformes (barfin flounder, *Verasper moseri*; Amano et al., 2002; Japanese floun-

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der, *Paralichthys olivaceus*; Pham et al., 2007), atheriniformes (pejerrey, *Odontesthes bonariensis*; Guilgur et al., 2007), salmoniformes (whitefish, *Coregonus clupeaformis*; Vickers et al., 2004), tetraodontiformes (grass puffer, *Takifugu niphobles*; Shahjahan et al., 2010) and clupeiformes (Pacific herring, *Clupea harengus pallasi*; Carolsfeld et al., 2000; American shad, *Alosa sapidissima*; Abraham, 2004). In the clupeiform fish (Pacific herring), a herring GnRH (hrGnRH) isoform was first detected by HPLC analysis as a novel isoform (Carolsfeld et al., 2000). Subsequently, Abraham (2004) cloned full-length cDNA encoding hrGnRH isoform in the brain of American shad. However, detailed information on the molecular cloning and distribution of multiple GnRH isoforms of any clupeiform fish is lacking.

Traditionally, GnRH isoforms have been named based on the species in which they were first identified (O'Neill et al., 1998). In recent years, GnRH isoforms are classified as GnRH1, GnRH2, and GnRH3 based on phylogenetic analyses and neuroanatomical distribution (Fernald and White, 1999). In this paper, we follow the recent classification system. Expression studies of three GnRH isoforms in fish have shown restricted regional distribution within the brain, suggesting distinct physiological roles and functions. GnRH1 is the species-specific hypophysiotropic and reproductively relevant isoform expressed by neuronal populations distributed in the ventral telencephalic-preoptic area and hypothalamus (HYP) of the forebrain regions. GnRH2 is highly conserved in all species studied to date; it is the chicken GnRH-II (cGnRH-II) isoform and expressed exclusively in the midbrain tegmentum (MT); their fibers project widely into different regions of the brain, suggesting a possible role in neuromodulation; however, its overall function is not fully understood. GnRH3 is a teleost-specific isoform, if present, it is salmon GnRH (sGnRH) isoform and expressed in the olfactory bulb-terminal nerve area and regulates sexual behaviour, including spawning migration (Wirsig-Wiechmann, 2001).

The Japanese anchovy, *Engraulis japonicus*, belongs to a relatively primitive order of fish, clupeiformes. In Japan, more than 10 clupeiform species are utilized as food resources (Ohkubo et al., 2010). Among the economically important clupeiform species, Japanese anchovy is one of the most important species in the commercial capture fishery in Japan. It is a multiple-spawning, small pelagic fish that spawns from early spring to late autumn; in addition, the life cycle is short that facilitates easy experimentation. The populations of economically important clupeiform species were shown to fluctuate extremely in response to climate regime shift (Watanabe, 2007), and in particular, population fluctuations of Japanese anchovy and sardine (*Sardinops melanostictus*) are closely linked; when Japanese anchovy becomes abundant, sardine becomes scarce, and after a few decades, for unknown reasons, the dominant species starts to alter (Mantua and Hare, 2002). However, it remains unclear whether climate regime shifts influence the reproductive and endocrinological processes underlying this shift. There is thus a need to understand the basic aspects of reproductive endocrinology of clupeid fishes. With this background, our laboratory is experimenting with Japanese anchovy and sardine.

In the present study, we cloned and sequenced cDNAs encoding three GnRH isoforms in the Japanese anchovy, and analyzed their distribution in the brain using in-situ hybridization (ISH) technique.

MATERIALS AND METHODS

Animal and tissue preparation

Adult Japanese anchovy were collected from Omura Bay, Nagasaki prefecture and reared at the Fishery Research Laboratory of Kyushu University, Fukuoka, Japan under natural day length and temperature supplied with seawater. Forty sexually mature fish of both sexes were sampled for the study. At each sampling point, fishes were sacrificed in accordance with the guidelines for animal experiments of the Faculty of Agriculture and Graduate Course of Kyushu University and according to the laws (No. 105) and notifications (No. 6) of the Japanese Government. Body length, body weight and gonad weight were measured, and the midsection of each gonad from individual fish was fixed in Bouin's solution for gonadal histology. The mean body length, body weight, and gonad weight were 9.30 ± 1.21 cm, 10.97 ± 0.42 g, and 0.43 ± 0.05 g, respectively.

The whole brains (n = 10–12 of each sex) were removed following decapitation, transferred immediately to liquid nitrogen, and stored at –80°C until total RNA extraction. A set of whole head (n = 12–14 of each sex) were fixed in 4% paraformaldehyde (PFA) at 4°C overnight for ISH analysis. Another set of whole head (n = 10) was stored in RNA later (Qiagen, Hilden, Germany) solution, immediately after sampling for RT-PCR analysis.

Histology

Bouin's solution-fixed gonad samples were dehydrated in a graded series of ethanol, embedded in paraffin, and sectioned at 5 μm using a rotary microtome (RM2155, Leica, Germany). The sections were stained with toluidine blue, and observed under a light microscope to confirm the gonadal stage; observation showed that male and female fish used in this study were in the spermiation and late vitellogenic stages, respectively (data not shown). A few brain samples were also processed and sectioned at 10 μm thickness and stained with Nissl stain (cresyl violet) to confirm that GnRH-mRNA expressing cells are neurons.

Cloning, sequencing and phylogenetic analysis of three GnRH isoforms

cDNA cloning and sequencing

Total cellular RNA was extracted from the whole brain using ISOGEN (Nippon Gene, Japan), following the manufacturer protocol. First-strand cDNAs were synthesized from 1 μg of total RNA using oligo (dT) primer (OdT) (Sigma) and superscript III reverse transcriptase (Invitrogen). The degenerate primers for amplification of 3′ ends of all three GnRH isoform cDNAs were designed from highly conserved decapeptide region of each GnRH cDNA sequence in other teleost fish. The list of primers is shown in Table 1. The cloning and sequencing of three GnRH isoforms were carried out following the protocol described by Selvaraj et al. (2012a) and Kitano et al. (2012) with slight modifications.

The temperature cycle profile for each GnRH isoform was same for 3′ and 5′ RACE (rapid amplification of cDNA ends) PCR: 95°C for 9 min, 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min. For amplification of the full-length sequences, temperature profile was set as 95°C for 9 min, 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min.

Phylogenetic and sequence identity analyses

The cDNA sequences encoding 40 different GnRH precursors of teleost fishes were obtained from GenBank (http://www.ncbi.nlm.nih.gov). Phylogenetic analyses were conducted by

pairwise alignment with ClustalW using BioEdit Sequence Alignment Editor, version 7.1.30 software (Hall, 1999) and a phylogenetic tree was generated using the neighbor-joining method with MEGA4 software (Tamura et al., 2007). Amino acid sequence identity between GnRHs of the Japanese anchovy and those of other teleost species were calculated by BioEdit Sequence Alignment Editor, version 7.1.3.0 (Hall, 1999).

In-situ hybridization of three GnRH isoforms

Riboprobe synthesis

The ORF (open reading frame) regions of the Japanese anchovy GnRHs were used to synthesize GnRH riboprobes. Single-strand anti-sense and sense RNA probes labelled with digoxigenin (DIG; Roche) were synthesized for non-fluorescent ISH, according to the standard protocol prescribed for DIG Kit. To prepare linear plasmid, 10 μg of pGEM-T Easy vector plasmid DNA containing either Japanese anchovy GnRH1, GnRH2, or GnRH3 cDNA was linearized with restriction enzymes, SalI or NcoI (Promega). The antisense and sense riboprobes were synthesized by using the Riboprobe In-Vitro Transcription system (Promega). One-microgram of the linearized plasmid DNA was incubated for 2 h at 37°C with 2.0 μL of T7 or SP6 RNA polymerase, in a volume of 20 μL, containing 4.0 μL of 5× optimized transcription buffer (Promega; #P1181), 2.0 μL of 100 mM DTT, 2.0 μL of DIG-RNA labelling mix, 1.0 μL of RNase inhibitor (RNase out), and 8.0 μL of RNase-free water. Following the transcription reaction, the plasmid DNA template was digested for 15 min at 37°C with 0.5 μL of RNase out and 2.0 μL of DNase I (DIG kit). The riboprobes were purified by ethanol precipitation with 1 μL of tRNA (10 mg/ml, Sigma) and stored at −80°C until used for ISH.

Table 1. List of primers used for cDNA cloning of GnRH1 (hrGnRH), GnRH2 (cGnRH-II), and GnRH3 (sGnRH), and RT-PCR analysis of GnRH1 and GnRH3 in the Japanese anchovy.

Primer	Nucleotide sequences	Purpose
Oligo dT Primer (OdT)	GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T	RACE-PCR
Oligo dT adaptor Primer (AP)	GGC CAC GCG TCG ACT AGT AC	RACE-PCR
hrGnRH-DP1	CARCACTGGTCNCAYGGNYT	3′ RACE
hrGnRH-DP2	CACTGGTCNCAYGGNYTNAG	3′ RACE (Nested)
hrGnRH-GSP1	CATTTGCTCTGCTCATCAGGCTG	5′ RACE
hrGnRH-GSP2	CCTTTCCTGATTGGCTGCATCTC	5′ RACE (Nested)
hrGnRH-GSP3	GAAAACCACAGAGCCAAGACC	Full length
hrGnRH-GSP4	AGCAGTGGTGACTTTCAGTGAAC	Full length (Nested)
cGnRH-II-DP1	CARCACTGGTCYCAYGGBTGG	3′ RACE
cGnRH-II-DP2	TCYCAYGGBTGGTAYCCBGGDGG	3′ RACE (Nested)
cGnRH-II-GSP1	CCAGCCAGTGATGTCACTTCCTC	5′ RACE
cGnRH-II-GSP2	ATTCTCTCGTCAGGGCTTCCAAC	5′ RACE (Nested)
cGnRH-II-GSP3	CAAATACAGCTCCAGTGATGGC	Full length
cGnRH-II-GSP4	TAGAGATCACTCGACCACTGACG	Full length (Nested)
sGnRH-DP1	CAGCAYTGGTCNTAYGGNTGG	3′ RACE
sGnRH-DP2	TAYGGNTGGCTDCCNGGNGG	3′ RACE (Nested)
sGnRH-GSP1	GCGTGCATCAGTCAAGCAATTC	5′ RACE
sGnRH-GSP2	TCTTCGTAATGGCCTTCGACCTC	5′ RACE (Nested)
sGnRH-GSP3	GAAAACCACAGAGCCAAGACC	Full length
sGnRH-GSP4	AGCAGTGGTGACTTTCAGTGAAC	Full length (Nested)
hrGnRH RT Fw	CGAATCACAAGTGATGGAGG	RT-PCR
hrGnRH RT Rv	CATTTGCTCTGCTCATCAGG	Tissue distribution
sGnRH RT Fw	GCATTGGAGGAGAACTGGAG	
sGnRH RT RV	ATGGCCTTCGACCTCTTCTC	
β-actin Fw	ATGACACAGATCATGTTCGAGAC	Internal control
β-actin Rv	TCACACCATCACCAGAGTCC	

Oligomix: N=A+C+G+T; B=C+G+T; D=A+G+T; R=A+G; Y=C+T.

Tissue preparation

PFA-fixed Japanese anchovy whole heads were trimmed to collect the brain. The brain was dehydrated using ethanol, embedded in paraffin, and sectioned at 5 μm thickness in sagittal orientation. Eight sections were mounted on each MAS-coated superfrost micro slide glass (Matsunami, Osaka, Japan). Two consecutive slides were prepared with alternative sections for hybridization of GnRH mRNAs using anti-sense and sense riboprobes, respectively.

In-situ hybridization (*ISH*)

ISH was carried out using TSA plus DNP AP system kit (PerkinElmer, USA) according to manufacturer protocol (http://www.perkinelmer.com/CMSResources/Images/44-73610MA NTSAPlus DNPChromoInSitu.pdf). The deparaffinized, dehydrated tissue sections were first washed with phosphate buffer (PB, pH 7.4), treated with proteinase K (10 μg/ml) for 10 min at 37°C, washed with PB for 1 min, followed by post-fixation in 4% PFA for 15 min, washed twice in phosphate buffered saline (PBS; pH 7.4) for 15 min each,

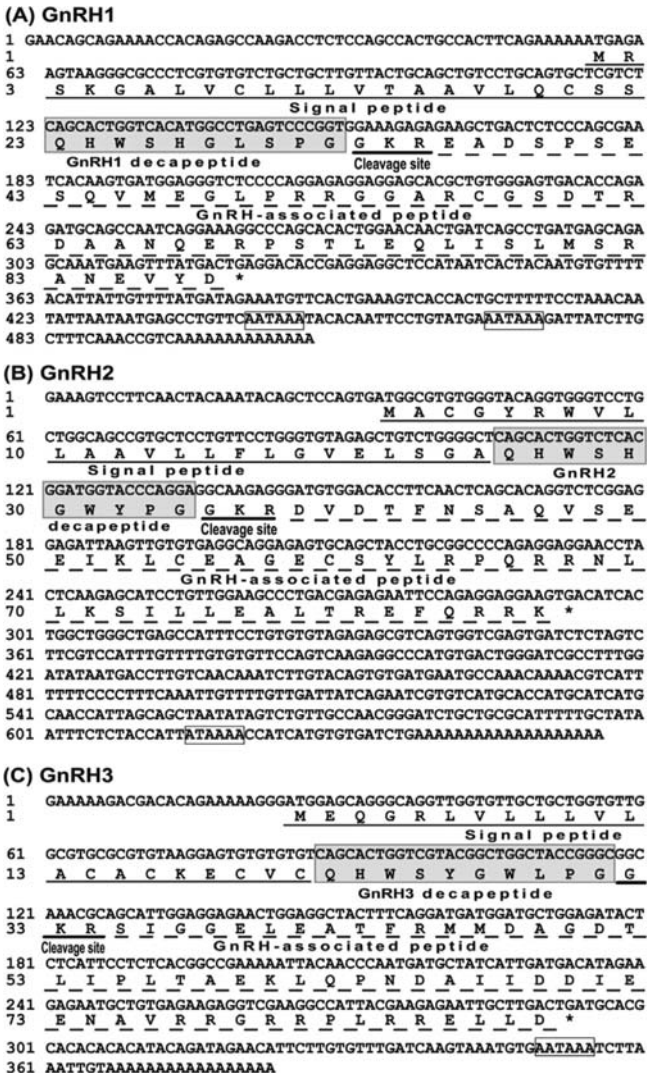

Fig. 1. Nucleotide and deduced amino acid sequences of Japanese anchovy GnRH1 **(A)**, GnRH2 **(B)**, and GnRH3 **(C)** cDNAs. The putative signal peptide, cleavage site, and GnRH-associated peptide are underlined and GnRH decapeptide region highlighted. The stop codon (TGA) is indicated by an asterisk (*), the polyadenylation signal is boxed. GenBank accession numbers are JX406273 (GnRH1), JX406274 (GnRH2), and JX406275 (GnRH3).

and in 5x standard saline citrate (SSC) buffer for 15 min. The sections were pre-hybridized in a wet humidified chamber (wet with 5x SSC) for 2 h at 58°C, and then hybridized with 100 ng/ml DIG-labelled GnRH riboprobes diluted with hybridization buffer [50 ml preparation: 100% formamide: 25 ml, 20x SSC: 12.5 ml, 50x Denhardt's (Nacalai): 5 ml, Baker's Yeast RNA (Sigma; 11.4 mg/ml): 175 µL, RNase-free water: 4.8 ml, and Sperm DNA (Nacalai; 10 mg/ml): 2.5 ml] at 58°C for 16–20 h. The hybridization was followed by washes in 2x SSC for 30 min at room temperature (RT), 2x SSC for 1 h at 65°C, 0.1x SSC for 1 h at 65°C, and Tris-NaCl-Tween (TNT) wash buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) for 5 min at RT. The sections were blocked with Tris-NaCl-blocking buffer (TNB; supplied with kit) for 30 min at RT and incorporated with anti-DIG-POD, Fab fragments (Roche, 1:1000 in TNB) for 30 min at RT, followed by three washes with TNT buffer for 5 min each, incubated in DNP amplification reagent working solution (supplied in kit; DNP stock solution diluted in 1× plus amplification diluent at 1:50) for 10 min at RT, three washes in TNT buffer for 5 min each, incubated with anti-DNP-AP (supplied in kit; diluted in TNB at 1:100) for 30 min at RT, three washes with TNT for 5 min each, and equilibrated with detection buffer, Sodium-Tris-Magnesium (NTM) buffer for 5 min. Alkaline phosphatase staining was performed by incubating the sections with Nitro-Blue-Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) for 15–20 min in the dark. After washing with ultrapure water (UPW), the sections were cover slipped using Aquatex aqueous mounting media (Merck, Darmstadt, Germany).

Nissl counterstain

Few sense probe hybridized ISH slides were counterstained with Nissl stain (cresyl violet) following the protocol described by Kádár et al. (2009) with slight modifications. Slides were dehydrated using ascending series of ethanol, dipped in xylene for 5 min twice and rehydrated in descending series of ethanol and

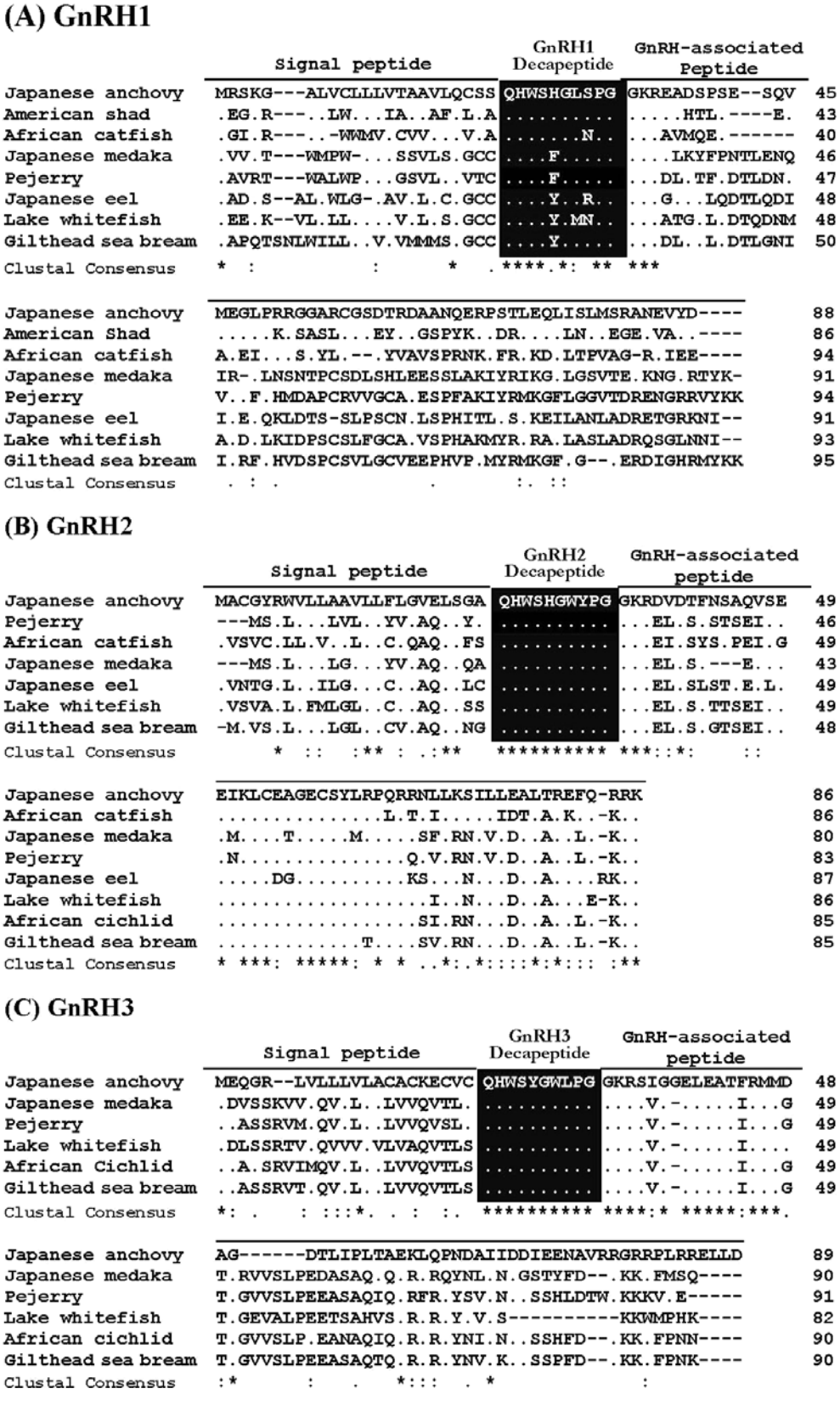

Fig. 2. Sequence alignment of the amino acid sequences of GnRH1 **(A)**, GnRH2 **(B)**, and GnRH3 **(C)**. Identical amino acids are indicated by dots. Black lines indicate the amino acids corresponding to signal peptide and GnRH-associated peptide; and GnRH decapeptide is highlighted. GenBank accession numbers of the GnRH sequences are presented in Fig. 3.

in water. The sections were then treated with 1% cresyl violet solution for 10 min followed by washing in water for 10 min and dehydrated in ascending series of ethanol, dipped in xylene and coverslipped using DPX Mounting medium.

Observation of slides

All slides were examined with an Olympus BX 51 microscope and photographed digitally. To achieve optimal images, all digitally captured images were adjusted for auto contrast in Adobe Photoshop 7. The images presented in results represent both male and female fish, as there was no major difference between the sexes.

Reverse transcription-polymerase chain reaction (RT-PCR)

The expression of GnRH1 and GnRH3 transcripts in olfactory bulb (OB) of adult spermiating male and late vitellogenic female fish was examined using RT-PCR. Total RNAs were extracted from OB of 10 individuals (six male and four female), as described previously. For RT-PCR detection, 460 ng of the total RNA from OB were reverse-transcribed using superscript III Reverse Transcriptase (Invitrogen) and random hexamers (Takara Bio Inc., Japan). The first-strand cDNAs were used as templates for PCR, using gene specific primers designed from open reading frame regions of Japanese anchovy GnRH1 and GnRH3 cDNAs (Table 1). All precautionary measures were taken to avoid false-positive results (Guilgur et al., 2007). The PCR amplification comprised activation of Ampli*Taq*Gold at 95°C for 9 min and then 35 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. RT-PCR analysis of β-actin mRNA was also performed for each sample and served as an internal control. Distilled water was used as a negative control to exclude any contamination from buffers and tubes. All PCR products were analysed using 3% agarose gels stained with ethidium bromide.

RESULTS

GnRH cDNAs sequences

The nucleotide sequence of cDNAs encoding the Japanese anchovy GnRH1 (hrGnRH), GnRH2 (cGnRH-II), and GnRH3 (sGnRH) were cloned and sequenced (Fig. 1). The full-length Japanese anchovy GnRH1 cDNA (GenBank accession no. JX406273) was 495 bp, excluding the poly-A tail, and contained an ORF of 267 bp encoding a precursor protein of 88 deduced aa with a predicted signal peptide of 22 aa. Two putative polyadenylation signal (AATAAA) were observed in the GnRH1 cDNA sequence; 1st and 2nd polyadenylation signal was recognized at 47 and 23 bp upstream of the poly-A tail region, respectively (Fig. 1A). The full-length Japanese anchovy GnRH2 cDNA (GenBank accession no. JX406274) consists of 637 bp, excluding the poly-A tail, and contained an ORF of 261 bp encoding a precursor protein of 86 deduced aa with a predicted signal peptide of 24 aa. A putative polyadenylation signal (ATAAAA) was recognized at 17 bp upstream of the poly-A tail (Fig. 1B). The full-length Japanese anchovy GnRH3 cDNA (GenBank accession no. JX406275) contained 366 bp, excluding the poly-A tail, having an ORF of 270 bp encoding a precursor protein of 89 deduced aa with a predicted signal peptide of 21 aa. A putative polyadenylation signal (AATAAA) was recognized at 11 bp upstream of the poly-A tail (Fig. 1C).

The three GnRH cDNAs found in the Japanese anchovy consisted of the characteristic signal peptides [GnRH1, 1–22 residues; GnRH2, 1–24 residues and GnRH3, 1–21 residues], GnRH decapeptides [GnRH1, 23–32 residues; GnRH2, 25–34 residues and GnRH3, 22–31 residues], cleavage site [Gly-Lys-Arg (G-K-R); GnRH1, 33–35 residues; GnRH2, 35–37 residues and GnRH3, 32–34 residues], and GnRH-associated peptides (GAP) [GnRH1, 36–88 residues; GnRH2, 38–86 residues and GnRH3, 35–89 residues].

Comparison of GnRH amino acid sequences of Japanese anchovy with other teleosts

The theoretical translation of three isoforms of Japanese anchovy GnRH cDNAs aligned with the GnRH amino acid sequences of representative species as shown in Fig. 2. The identities among the deduced amino acid sequences of GnRH precursors of the Japanese anchovy and other representative teleost species are presented in Table 2. The amino acid identity of Japanese anchovy GnRH1 ranges from 23.3–56.8% when compared with other teleost GnRH1 precursors. The American shad (a clupeiform species) GnRH1 (herring type) showed the highest sequence identity of 56.8%, whereas Japanese medaka (beloniformes) GnRH1 (medaka type) showed the lowest identity of 23.3%. The Japanese anchovy GnRH2 isoform showed 62.7–66.2% identity with the homologs of other representative species, with the highest amino acid identity to lake whitefish (66.2%) and the lowest to African catfish, Pejerrey, and Japanese medaka (62.7%). The Japanese anchovy GnRH3 amino acid sequence showed lower homology (35.0–40.2%) with other representative species than that of other two GnRH isoforms. The highest identity was found with Gilthead sea bream and African cichlid (40.2%) and the lowest to lake whitefish (35.0%).

Phylogenetic analysis

An unrooted phylogenetic tree based on the amino acid sequences of GnRH precursors revealed three major groupings of vertebrate GnRHs (GnRH1, GnRH2, and GnRH3;

Table 2. Amino acid sequence identity (%) of GnRH isoforms of the Japanese anchovy with those of other teleosts.

GnRH forms	Species	Percentage of identity (%) Japanese anchovy GnRH1	GnRH2	GnRH3	References
GnRH1 (hr)	American shad	56.8	21.5	21.0	Abraham (2004)
GnRH1 (sb)	Gilthead seabream	25.7	20.2	22.3	Gothilf et al. (1996)
GnRH1 (cf)	African catfish	37.5	21.5	17.8	Bogerd et al. (1994)
GnRH1 (m)	Japanese eel	27.9	22.1	20.0	Okubo et al. (1999)
GnRH1 (sb)	African cichlid	25.5	19.5	20.7	White et al. (1995)
GnRH1 (wf)	Lake whitefish	26.8	21.0	21.7	Vickers et al. (2004)
GnRH1 (pj/md)	Pejerrey	26.5	21.6	19.8	Guilgur et al. (2007)
GnRH1 (md/pj)	Japanese medaka	23.3	20.8	18.8	Okubo et al. (2000)
GnRH2	Gilthead seabream	26.9	63.9	24.7	Gothilf et al. (1996)
	African catfish	23.3	62.7	21.5	Bogerd et al. (1994)
	Japanese eel	25.5	65.5	24.5	Okubo et al. (1999)
	African cichlid	25.8	65.1	24.7	White et al. (1995)
	Lake whitefish	26.6	66.2	22.5	Vickers et al. (2004)
	Pejerrey	26.9	62.7	21.7	Guilgur et al. (2007)
	Japanese medaka	23.5	62.7	20.0	Okubo et al. (2000)
GnRH3	Gilthead seabream	20.6	25.0	40.2	Gothilf et al. (1996)
	African cichlid	20.6	25.0	40.2	White et al. (1995)
	Lake whitefish	15.4	20.8	35.0	Vickers et al. (2004)
	Pejerrey	20.8	23.7	39.1	Guilgur et al. (2007)
	Japanese medaka	21.6	26.0	38.1	Okubo et al. (2000)

Fig. 3). The Japanese anchovy hrGnRH precursor fits with GnRH1 group; while Japanese anchovy cGnRH-II and sGnRH precursors, each fitted with GnRH2 and GnRH3 group of the same GnRH isoforms from other teleost species.

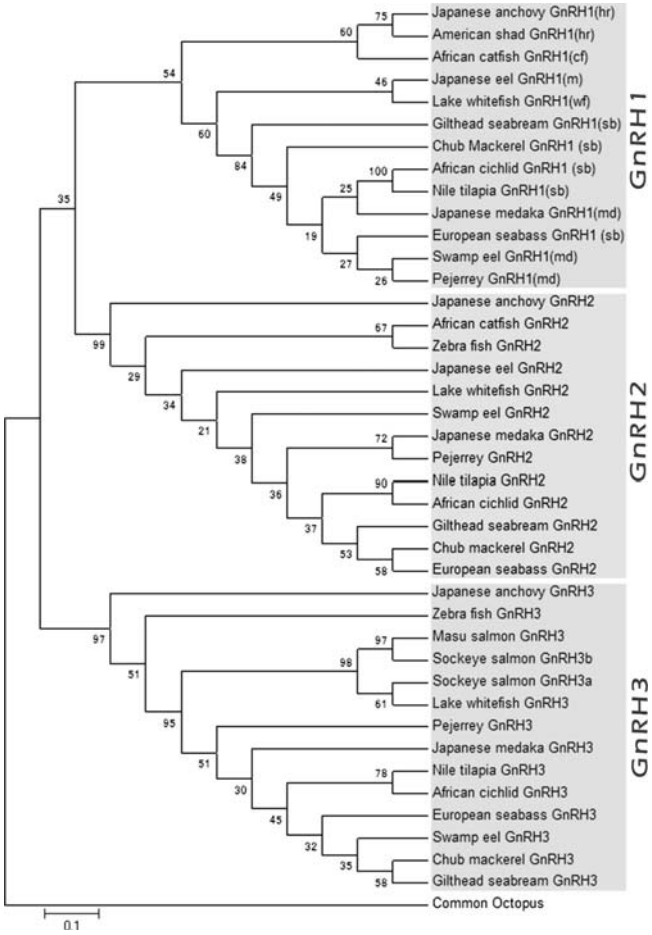

Fig. 3. Phylogenetic analyses were conducted in MEGA4 software based on amino acid alignment of the sequences of GnRH1, GnRH2, and GnRH3 in teleost fish. The evolutionary history was inferred using the neighbour-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap tests (10,000 replicates) are shown next to the branches. Octopus GnRH (*Octopus vulgaries*) was used as an outgroup. The GenBank accession number of the sequences: Japanese anchovy (GnRH1 JX406273, GnRH2 JX406274, and GnRH3 JX406275); American shad (GnRH1 AF536381); African catfish (GnRH1 X78049 and GnRH2 X78047); Japanese eel (GnRH1 AB026989 and GnRH2 AB026990); lake whitefish (GnRH, AY245104, GnRH2 AY245102, and GnRH3, AY245103); gilthead sea bream (GnRH1 SAU30320, GnRH2, U30325, GnRH3 U30311); African cichlid (GnRH1 AF076961, GnRH2, AF076962, and GnRH3, AF076963); Nile tilapia (GnRH1 AB101665, GnRH2, AB101666, and GnRH3, AF101667); Japanese medaka (GnRH1 AB041333, GnRH2 AB041330, and GnRH3 AB041332); chub mackerel (GnRH1 HQ108193, GnRH2 HQ108194, GnRH3 HQ108195); Swamp eel (GnRH1 AY858056, GnRH2, AY858054, and GnRH3, AY858055); European sea bass (GnRH1 AF224279, GnRH2 AF224282, and GnRH3 AF224280); Pejerrey (GnRH1 AY744689, GnRH2 AY744687, and GnRH3 AY744688); Zebrafish (GnRH2 AY094357 and GnRH3 AJ304429); sockeye salmon (GnRH3a D31868 and GnRH3b D31869); masu salmon (GnRH3 S44614) and Octopus (AB037165).

In-situ hybridization (ISH)

The three different riboprobe of GnRHs recognized their respective GnRH isoforms. All the ISH analyses were performed in both sexes, and their overall distribution pattern resembled in both sexes. The distribution of three GnRH mRNAs in the Japanese anchovy brain is summarized schematically in Fig. 4. For identifying brain regions, where GnRH mRNAs were localized, we referred brain atlas of Medaka (Ishikawa et al., 1999).

In the sagittal brain sections, GnRH1 mRNA-expressing neurons were detected in the ventral telencephalon (VT), nucleus preopticus (NPO) of the preoptic area (POA), and the anterior olfactory bulb (OB) regions. No hybridization signal was detected when sections were hybridized with the sense riboprobes (data not shown). GnRH1 neurons were distributed continuously in a horizontal layer in the VT of anterior POA (Fig. 5A, B); less number of GnRH1 neurons was localized in the anterior OB in comparison to the POA region (Fig. 5C). GnRH1 neurons were also localized in the NPO of POA (Fig. 5D). Fig. 5E showed GnRH1 neurons in the VT that were counterstained with Nissl stain. Nissl counterstained GnRH1 neurons in the OB are shown in Fig. 5F.

GnRH2 mRNA synthesizing neurons were localized in the nucleus of the medial longitudinal fasciculus (nMLF) of the midbrain tegmentum (MT) region (Fig. 6). Two or three GnRH2 neurons were observed in the nMLF (Fig. 6A). GnRH2 neurons were tightly arranged in the nMLF of MT (Fig. 6B); however, a cluster of GnRH2 neurons were observed in the nMLF (Fig. 6C) of different individual brains. Fig. 6D is the counterstained section of Fig. 6A, showing GnRH2 cells in the nMLF with Nissl counterstained. There was no hybridization signal when sections were hybridized only with the GnRH2 mRNA sense riboprobe (Fig. 6E). Figure 6F shows Nissl staining in GnRH2 cells in the nMLF of MT.

GnRH3 neurons were localized in the OB, olfactory nerve (ON), and transitional area between OB and ON (Fig. 7). High GnRH3 mRNA expression, in terms of both number of cells and signal intensity, was observed in the ventral OB region (Fig. 7A, B), whereas GnRH3 cells observed in the ON area were few in number (Fig. 7C). Figure 7D shows the GnRH3 neurons in the OB and ON that were counterstained

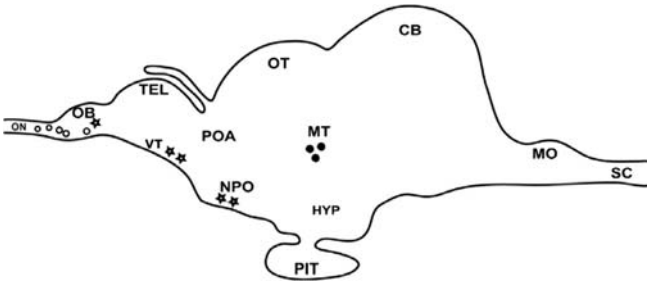

Fig. 4. Schematic illustration of the distribution of three GnRHs neurons in the brain of Japanese anchovy. GnRH1, GnRH2, and GnRH3 neurons are indicated with stars, closed circles, and open circles, respectively. CB, cerebellum; HYP, hypothalamus; MO, medulla oblongata; MT, midbrain tegmentum; NPO, nucleus preopticus; OB, olfactory bulb; ON, olfactory nerve; OT, optic tectum; PIT, pituitary; POA, preoptic area; SC, spinal cord; TEL, telencephalon; VT, ventral telencephalon.

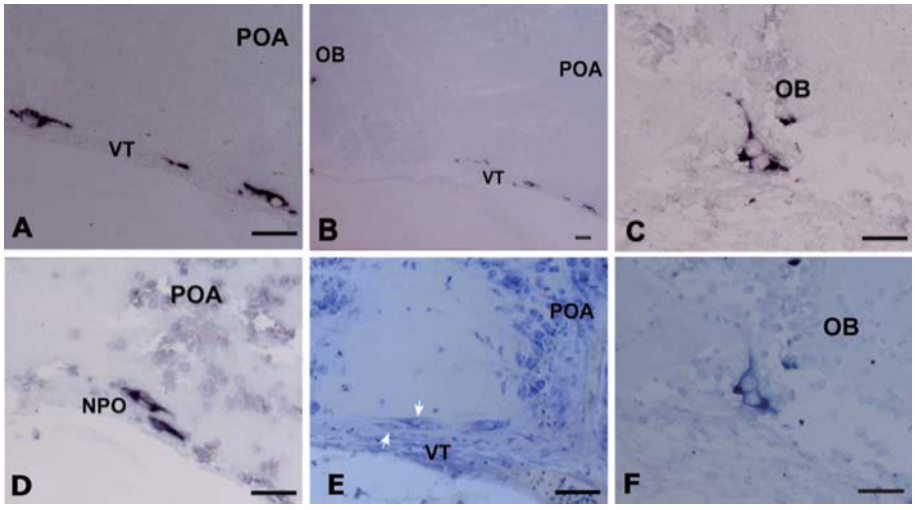

Fig. 5. Distribution of GnRH1 mRNA hybridization signals in the sagittal brain section of Japanese anchovy. **(A)** GnRH1 neurons in the ventral telencephalon (VT) of the anterior preoptic area (POA) hybridized with GnRH1 antisense riboprobe. **(B)** Low magnification picture of A. **(C)** GnRH1 mRNA hybridization signals in the anterior olfactory bulb (OB). **(D)** GnRH1 hybridization signals in nucleus preopticus (NPO) of POA. **(E)** Sagittal brain section showing GnRH1 neurons (arrows) in the VT stained with Nissl stain. **(F)** Nissl counter stained adjacent section (hybridized with sense probe) of **(C)**, showing staining in GnRH1 mRNA positive cells in the anterior OB. Scale bars = 100 μm.

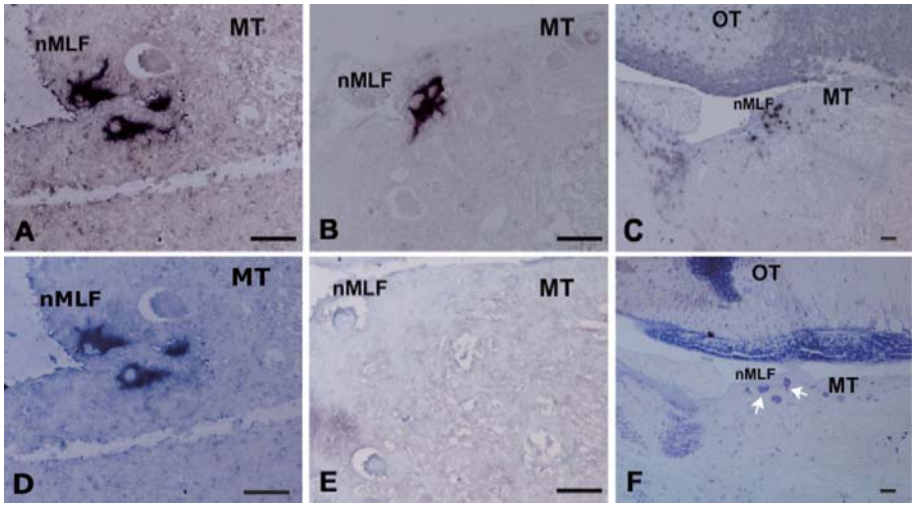

Fig. 6. Distribution of GnRH2 mRNA hybridization signals in sagittal section of the midbrain tegmentum (MT) of the Japanese anchovy brain. **(A–C)** GnRH2 neurons in the nucleus of the medial longitudinal fasciculus (nMLF) that hybridized with GnRH2 antisense riboprobe. **(D)** Nissl counterstained adjacent section (hybridized with sense probe) of **(A)** showing staining in GnRH2 cells in the nMLF. **(E)** Adjacent sagittal section of **(B)** hybridized with the GnRH2 sense riboprobe, showing no hybridization signal. **(F)** Sagittal brain section showing GnRH2 neurons (arrows) in the nMLF of MT that stained with Nissl stain. Scale bars = 100 m.

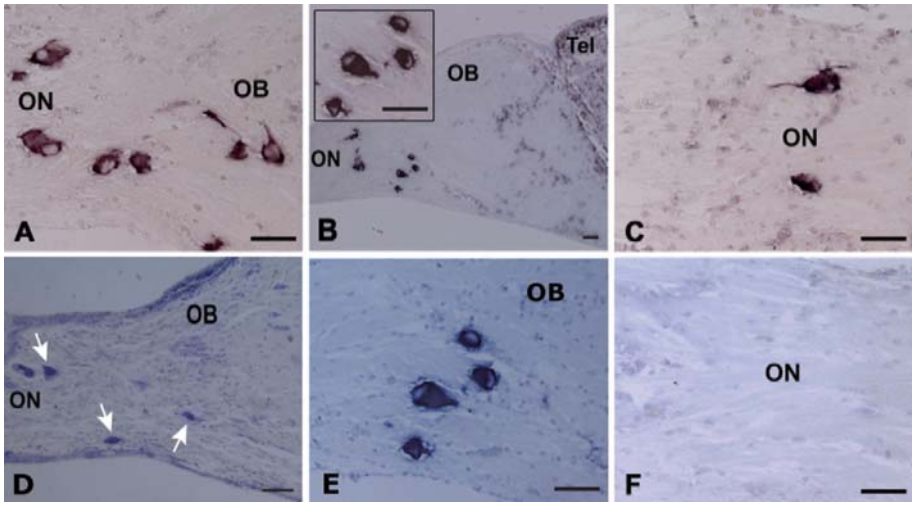

Fig. 7. Distribution of GnRH3 mRNA hybridization signals in sagittal section of the olfactory region of the Japanese anchovy brain. Sagittal brain section showing GnRH3 neurons in the olfactory region that hybridized with GnRH3 antisense riboprobe; **(A)** ventral olfactory bulb (OB), olfactory nerve (ON), and transitional region of OB and ON, **(B)** the OB and ON regions; the inset figure is at higher magnification, **(C)** the ON. **(D)** Sagittal brain section showing GnRH3 mRNA-positive cells (arrows) stained with Nissl staining. **(E)** Nissl counterstained adjacent section (hybridized with sense probe) of **(B)** showing staining in GnRH3 mRNA-positive cells in the OB. **(F)** Adjacent sagittal section of **(C)** hybridized with the GnRH3 sense riboprobe, showing no hybridization signal. Scale bars = 100 μm.

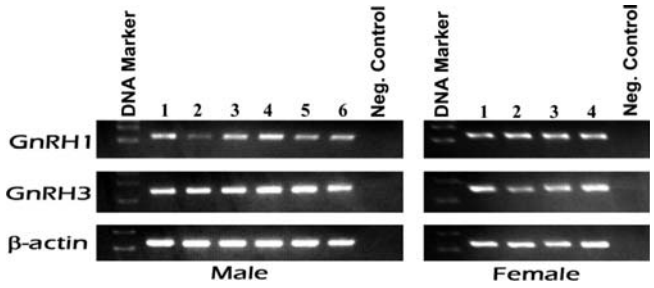

Fig. 8. RT-PCR analysis for the distribution of GnRH1 and GnRH3 transcripts in the olfactory bulb (OB) tissue of the brain of adult spermiating male and late vitellogenic female Japanese anchovy. The quality of the mRNA was confirmed by parallel amplification of β-actin. The numbers in the figure represent different individual male and female fish, respectively.

with Nissl. Fig. 7E is the Nissl counterstained section of Fig. 7B, showing GnRH3 neurons in the OB. Hybridization with only GnRH3 sense riboprobe showed no hybridization signal in the OB and ON (Fig. 7F). In the OB, GnRH3 and GnRH1 neurons were detected in the ventral OB and anterior OB, respectively.

Distribution of GnRH1 and GnRH3 mRNAs in the Olfactory bulb (OB)

RT-PCR analysis revealed that both the GnRH1 and GnRH3 transcripts were distributed in the OB region of Japanese anchovy brain (Fig. 8). The intensity of GnRH1 transcript was lower than the intensity of GnRH3 transcript.

DISCUSSION

In the present study, three GnRH cDNAs were cloned and sequenced from the brain of Japanese anchovy, and their distribution was analyzed using ISH. A previous report by Abraham (2004) reported cloning and sequencing of full-length cDNAs of all three GnRH isoforms from another clupeiform fish, but was unsuccessful except for the isolation of hrGnRH isoform. To our knowledge, this is the first report on cloning and sequencing of all three GnRH isoforms in a clupeiform fish.

The Japanese anchovy GnRH isoforms exhibited same sequence features as that of other known GnRH isoforms: a signal peptide at the N-terminus, the GnRH decapeptide, a conserved processing tri-peptide and the GnRH-associated peptide regions at the C-terminus. These conserved sequence features suggest that all these GnRH isoforms have a common ancestral origin. Alignment analysis indicated that the amino acid sequences of Japanese anchovy hrGnRH, cGnRH-II, and sGnRH isoforms grouped with other teleost GnRH1, GnRH2, and GnRH3 isoforms, respectively (Fig. 2). Organization of all three GnRH isoform clades in the present phylogenetic tree supports an expected relationship among fishes based on teleost evolution (Nelson, 1994). Based on the analysis of phylogenetic tree, it is found that there are three main lineages in the GnRH tree; GnRH1, GnRH2, and GnRH3. This observation agrees with the hypothesis of Dubois (2002) regarding GnRH evolution. In addition, phylogenetic analysis showed that the Japanese anchovy hrGnRH clusters with other known teleost GnRH1 isoforms (mGnRH, cfGnRH, wfGnRH, sbGnRH, and mdGnRH), suggesting that the Japanese anchovy hrGnRH isoform is a homolog of GnRH1. Further, it is found that hrGnRH has close phylogenetic relationship with cfGnRH. As expected, the Japanese anchovy GnRH2 and GnRH3 clustered with other teleost GnRH2 and GnRH3 isoforms, respectively (Fig. 3). To better understand the functional and physiological relevance of multiple GnRH isoforms, it is essential to know the localization and distribution pattern of GnRH isoforms in the brain and pituitary (Mousa and Mousa, 2003). In this study, differential distribution of three GnRH isoforms was demonstrated at mRNA levels by ISH using specific RNA probes. It was found that the three GnRH isoforms of Japanese anchovy were differentially expressed in different brain regions, with the exception of both GnRH1 and GnRH3 in the OB. GnRH1 neurons were predominant in the POA and VT regions. On the other hand, GnRH3 neurons were abundant in the OB and ON regions, while GnRH2 neurons were restricted to the nMLF of the MT region. Similar expression patterns were also reported in other perciform fishes such as European sea bass, *Dicentrarchus labrax* (González-Martínez et al., 2001) and Atlantic croaker (Mohamed et al., 2005).

The most interesting finding of the present study is the localization of GnRH1 and GnRH3 neurons in the olfactory bulb region. The distribution of GnRH1 neurons in the anterior OB, POA, and VT regions of the Japanese anchovy brain were similar as that of American cichlid, *Cichlasoma dimerus* (Pandolfi et al., 2005), European sea bass, (González-Martínez et al., 2001, 2002), and Atlantic croaker (Mohamed et al., 2005). RT-PCR analysis also indicated presence of GnRH1 and GnRH3 transcripts in OB of the Japanese anchovy brain, suggesting no possible cross-reaction of GnRH1 and GnRH3 probes. However, it is unclear whether GnRH1 neurons localized in the OB of Japanese anchovy innervate anterior pituitary regions. Recently, we have developed an antibody against hrGnRH decapeptide and future studies will help to clarify this observation. Previous studies in American cichlid, European seabass and Atlantic croaker detected GnRH1 producing neurons in the OB regions; however, it is still unclear whether these neurons innervate anterior pituitary as that POA GnRH1 neuronal population in these teleost fish. Further, these two GnRH populations were shown to co-exist in both OB and POA regions in the Atlantic croaker (Mohamed et al., 2005), South American cichlid (Pandolfi et al., 2005), and European sea bass (González-Martínez et al., 2001).

GnRH3 neurons of Japanese anchovy were localized in the ON, ventral OB, and transitional area between the OB and ON. Similar pattern of GnRH3 neuronal localization was also observed in the Atlantic croaker (Mohamed et al., 2005) and European sea bass (González-Martínez et al., 2001). GnRH2 neurons were exclusively detected in the nMLF of MT region. A similar localization of GnRH2 neurons were reported in several vertebrate species, including teleosts (Zandbergen et al., 1995; González-Martínez et al., 2001; Mohamed et al., 2005; Pandolfi et al., 2005). The presence of GnRH2 neurons in the MT was first reported in the platyfish, *Xiphophorus* sp. (Münz et al., 1981) and subsequently confirmed in the goldfish (Kah et al., 1986), masu salmon (Amano et al., 1991) and other teleost fishes. Presence of GnRH2 has now been reported in all vertebrate classes,

with the exception of the sea lamprey, *Petromyzon marinus* (Kavanaugh et al., 2008).

Although the developmental origins of GnRH1 and GnRH3 neurons are still controversial, GnRH3 neurons are thought to originate in the olfactory placode (Parhar et al., 1998; Wong et al., 2004). Pandolfi et al. (2005) showed that both forebrain GnRHs, i.e. GnRH1 and GnRH3, first arise in rostral olfactory regions in the South American cichlid. On the other hand, based on the expression pattern and ontogenic studies, several authors proposed that GnRH1 neurons originate from the POA, while GnRH3 neurons derive from the olfactory placode (Gothlif et al., 1996; Parthar et al., 1998; White and Fernald, 1998; Senthilkumaran et al., 1999; Pandolfi et al., 2005). In the Atlantic croaker and European sea bass, it has been shown that GnRH1 (sbGnRH) neurons first originate in the OB and VT regions and later migrate to their final position in the POA and HYP (Mohamed et al., 2005; González-Martínez et al., 2001). Recent studies indicate that, in mammals, GnRH1 neurons are derived from the neural crest (Forni et al., 2011).

The role of GnRH3 is not well established especially in multiple-spawning fishes with three different GnRH isoforms (Guilgur et al., 2007). The anatomical distribution of GnRH3 system in the Japanese anchovy brain suggests serving neuromodulatory role, as proposed in other teleost fishes, such as the modulation of sexual behaviour (Yamamoto et al., 1997; Ogawa et al., 2006). As GnRH2 neurons do not innervate into the pituitary of most teleosts (Senthilkumaran et al., 1999; Amano et al., 2002), GnRH2 seems not to be directly involved in gonadotropin secretion, especially in fishes expressing three GnRH isoforms; rather, it may have neuromodulatory function in fish, such as the electrical activity of vasotocin (VT) and isotocin (IT) neurons in the brain (Saito et al., 2003), conventional synaptic transmission (Kinoshita et al., 2007), the modulation of pineal function or melatonin release (Servili et al., 2010), and sensory processing of sexual or communicative stimuli (Maruska and Tricas, 2011). In a mammalian species (musk shrew, *Suncus murinus*), it has been demonstrated that GnRH2 coordinates energy and reproductive behaviour, including regulation of food intake (Temple et al., 2003; Kauffman and Rissman, 2004). GnRH1 (sbGnRH) neurons in the NPO are the main source of innervation into the pituitary (Senthilkumaran et al., 1999; Selvaraj et al., 2009) and GnRH1 neurons in the teleost preoptic nucleus innervate the pituitary (Prasada Rao, 1999). GnRH-expressing neurons in the POA project mainly to the adenohypophysis and are known to regulate gonadotropin secretion in fishes (Kim et al., 1995). The present result of the localization of GnRH1 (hrGnRH) in the POA suggests that hrGnRH neuronal axons might innervate into the pituitary and regulates gonadal development and maturation. However, this possibility will be examined in this species by immunohistochemistry using specific antibody to hrGnRH decapeptide.

In conclusion, the result of the present study demonstrates existence of three GnRH isoforms in the brain of clupeiform fish, Japanese anchovy. Distribution analysis indicated conservation of brain regions expressing three GnRH isoforms as that of other teleosts. However, both GnRH1 and GnRH3 neurons were localized in the OB region of Japanese anchovy. In addition, the localization of hrGnRH (GnRH1) in the POA suggests that GnRH1 may play a functional role in the gonadotropin regulation. The results of the present study provide important evidence for the presence of three GnRH isoforms in primitive teleosts.

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