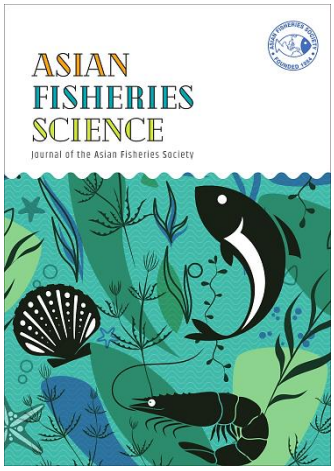

Molecular Characterisation and Reporter Gene Assay of the Three GnRH Isoforms and Two GnRH Receptors of a Clupeiform Fish, Japanese Sardine, *Sardinops sagax melanostictus* (Temminck & Schlegel, 1846)

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Abstract

The gonadotropin-releasing hormone (GnRH) is an important gene involved in fish reproduction and its action is mediated by GnRH receptor (GnRHR). Numerous GnRH-GnRHR studies have already been published in many teleost orders but information on reproduction-related genes for Clupeiformes is limited. To acquire a better understanding on the reproduction of Clupeiform fish species, GnRH isoforms and GnRHR paralogues of Japanese sardine, *Sardinops sagax melanostictus* (Temminck & Schlegel, 1846) were sequenced. Three GnRH isoforms (named as jsGnRH1, jsGnRH2, and jsGnRH3) and two GnRHR paralogues (named as jsGnRHR1 and jsGnRHR2) were isolated in the brain and pituitary of Japanese sardine. Based on phylogenetic analysis, jsGnRH1, jsGnRH2, and jsGnRH3 grouped into GnRH1, GnRH2, and GnRH3 forms, respectively, while jsGnRHR1 and jsGnRHR2 clustered into Type 2b and Type 1c receptors, correspondingly. The reporter gene assay showed high binding affinity of jsGnRHR1 to all three GnRH synthetic decapeptides, whereas jsGnRHR2 responded best to jsGnRH2 only. Clustering of jsGnRH1and jsGnRHR1 to other teleost GnRH1 and GnRHR2b, respectively, and activating the calcineurin/NFAT signalling pathway, suggests that these two genes are likely involved in fish reproduction.

Keywords: targeted sequencing, brain and pituitary hormones, phylogenetic analysis, sequence identity analysis, ligand binding affinity

Introduction

The brain-pituitary-gonadal (BPG) axis is a classical neuroendocrine hormonal pathway involved in fish reproduction (Weltzien et al., 2004). Gonadotropin-releasing hormone (GnRH) controls the hormone cascade via the BPG axis through the synthesis and release of gonadotropins (GtHs) in the pituitary (Lethimonier et al., 2004). Follicle-stimulating hormone (FSH) and luteinising hormone (LH) are the main GtHs that enhance sex steroid synthesis, which are necessary for gonadal development and maturation (Chang et al., 2009).

GnRHs are found in various vertebrate and invertebrate taxa, possessing multiple GnRH forms categorised as GnRH1, 2, and 3 (Fernald and White, 1999; Roch et al., 2011; Matsuyama et al., 2013; Muñoz-Cueto et al., 2020). In teleost fishes, two or three distinct isoforms can be found in each species (Carolsfeld et al., 2000; Adams et al., 2002; Roch et al., 2011). GnRH1 is mainly considered as the hypophysiotropic form, localised in the preoptic area (POA) region and hypothalamus (Yamamoto et al., 1998; Selvaraj et al., 2009). There are different GnRH1 forms found in fish which include catfish (cfGnRH), herring (hrGnRH), mammalian (mGnRH), medaka (mdGnRH), seabream (sbGnRH), and whitefish (wfGnRH). GnRH2, also known as chicken GnRH-2 (cGnRH-2) isoform, is localised mainly in the midbrain tegmentum area in vertebrates, with a possible role in neuromodulation (Kah et al., 2007). On the other hand,

GnRH3, also known as salmon GnRH (sGnRH) isoform, is expressed in the olfactory bulb, POA, and terminal nerve ganglion region, suggesting a role in sexual behaviour in teleosts (Kah et al., 2007). Actions of various GnRH isoforms are mediated by binding with different types of GnRH receptors (GnRHRs) in fish (Hapgood et al., 2005).

GnRHRs are G-protein coupled receptors (GPCRs) that belong to the rhodopsin β subfamily with seven transmembrane domains (TMDs) (Filipek et al., 2003). Only one or two GnRHRs have been found in mammals (Hapgood et al., 2005). However, some teleost species possess five to six GnRHR paralogues (Moncaut et al., 2005; Ciani et al., 2020). Although several paralogues have been discovered, there is presently no agreed-upon nomenclature for these GnRH receptors. Several investigators proposed various classifications dividing GnRHRs into two (Hildahl et al., 2011; Sefideh et al., 2014), three (Millar et al., 2004), or four (Ikemoto and Park, 2005) types separated into different subtypes.

GnRHR can interact with Gq/11 and Gs, two kinds of G proteins. Protein kinase C (PKC) pathway and Calcineurin/nuclear factor of activator T cell (NFAT) signalling pathway can be activated when Gq/11 is coupled (Naor, 1990; Fan et al, 2005), while the protein kinase A (PKA) pathway is activated when Gs is bound (Arora et al., 1998; Liu et al., 2002). The presence of numerous receptor paralogues in teleost pituitary cells makes the system more complex (Parhar et al., 2005).

Numerous GnRH-GnRHR studies on various teleost orders are well-documented, but information on reproduction-related genes for Clupeiformes is limited. More than ten Clupeiform fish species are used as food resources in Japan alone, and several of these species have already been listed as vulnerable species by the Japanese Ministry of the Environment (Ohkubo et al., 2010). Thus, there is a need to understand the fundamental aspects of the reproductive endocrinology of these Clupeiform fish species. One commercially-important Clupeid fish in Japan with a great potential for aquaculture is Japanese sardine, *Sardinops sagax melanostictus* (Temminck & Schlegel, 1846), also known as Japanese pilchard.

Recently, Nyuji et al. (2020) performed RNA sequencing (RNA-seq) of various genes, including the complete nucleotide Open Reading Frames (ORF) of three GnRH isoforms and one GnRH receptor in Japanese sardine. In the present study, targeted sequencing was conducted isolating three GnRH isoforms and two GnRH receptors in the brain and pituitary of Japanese sardine. Phylogenetic and sequence identity analyses were then performed to check the evolutionary relationships to other species. Furthermore, a reporter gene assay was conducted to study the binding efficiencies of the two GnRHR paralogues to the three GnRH ligands.

Materials and Methods

Fish brain and pituitary collection

The Japanese sardine samples used for brain and pituitary extraction were caught commercially in the wild (0+ years old). The fish were transferred, maintained, and fed with commercial dry pellets to an indoor tank at Fishery Research Laboratory, Fukutsu, Fukuoka, Japan. Around 20 fish with maturing or matured gonads were used in the present study. Each brain and a pool of pituitaries (8–10 fish) were flash-frozen in liquid nitrogen and was stored in –80 °C freezer for total ribonucleic acid (RNA) extraction. All of the sacrificed fish had adhered to the criteria set out by the Japanese government in legislation (No. 105) and declarations (No. 6).

cDNA sequencing of the three GnRH isoforms in Japanese sardine

Total RNA from the brain of Japanese sardine was extracted using ISOGEN RNA Extraction Kit (Nippon Gene, Japan), following the manufacturer's protocol. RNA extracts were treated with DNase using the DNA-free Kit (Thermo Fisher Scientific, USA). The first-strand cDNA library was prepared for partial sequencing using Superscript III Reverse Transcriptase (Invitrogen, USA) and Oligo-dT primers (Sigma-Aldrich, USA), following the manufacturer's protocol for reverse transcription. Degenerate primers for amplifying 3′ ends of all three GnRH isoforms previously used by Sukhan et al. (2013) were also used in the present study. The list of primers used is presented in Table 1. For partial sequencing, PCRs were performed in a final volume of 10 µL containing 5 µL of 2× Amplitaq Gold PCR Master Mix (Applied Biosystems, USA), 0.5 µL of each forward and reverse primers, 3.5 µL of PCR-grade water, and 0.5 µL of synthesised cDNA. Thermal cycling consisted of initial denaturation at 95 °C for 9 min, followed by 35 cycles at 94 °C for 1 min, 56–60 °C for 1 min, and 72 °C for 1 min. For the open reading frame (ORF), KOD FX Kit (Toyobo Co., Ltd., Japan) was used. PCRs were performed in a final volume of 50 µL containing 25 µL of 2× PCR buffer for KOD FX, 10 µL of 2 mM dNTPs, 10 µL of each forward and reverse gene-specific primers (GSPs), 0.25–1 µL of template DNA, 10–10.75 µL ultra-pure water (UPW), and 1 µL of KOD FX. Thermal cycling consisted of initial denaturation at 94 °C for 2 min, followed by 30 cycles at 98 °C for 10 sec, 55–60 °C for 30 sec, and 68 °C for 1:30 min. PCR products were purified from 1.5 % agarose gel using Nucleotrap (Takara Bio Inc., USA) and were subcloned into pGEM-T Easy Vector (Promega, USA). Plasmid DNA with insert cDNA was extracted using Cica Geneus Plasmid Prep Kit (Kanto Chemical Co., Japan) and was sent to Eurofins Genomics Co., Ltd, Tokyo, Japan for sequencing.

Table 1. Degenerate, specific, and reporter gene assay primers for DNA sequencing of jsGnRH1, jsGnRH2, jsGnRH3, jsGnRHR1, and jsGnRHR2 from Japanese sardine *Sardinops sagax melanostictus*.

Primers	Nucleotide sequences	Purpose
jsGnRH1 DP1 Fw	CARCACTGGTCNCAYGGNYT	Partial sequencing
jsGnRH1 DP2 Fw	CACTGGTCNCAYGGNYTNAG	
jsGnRH1 GSP1 Fw	GCTTGATGTGTTTTCAGAATGGAAGGG	ORF sequencing
jsGnRH1 GSP2 Fw	GTGTTTTCAGAATGGAAGGGAAACGC	
jsGnRH GSP1 Rv	GTTGTTTAGTCATAAGCTACATTTTCTCCCTCCA	
jsGnRH GSP2 Rv	GTTTAGTCATAAGCTACATTTTCTCCCTCCATCA	
jsGnRH2 DP1 Fw	CARCACTGGTCYCAYGGBTGG	Partial sequencing
jsGnRH2 DP2 Fw	TCYCAYGGBTGGTAYCCBGGDGG	
jsGnRH2 GSP1 Fw	GGAACATAGCCCCAGTGATGGTGTG	ORF sequencing
jsGnRH2 GSP2 Fw	CATAGCCCCAGTGATGGTGTGTGTG	
jsGnRH2 GSP1 Rv	AAGCCATCAGTGATGTCACTTCCTCCTC	
jsGnRH2 GSP2 Rv	GATGTCACTTCCTCCTCTGGAATTCTCTTG	
jsGnRH3 DP1 Fw	CAGCAYTGGTCNTAYGGNTGG	Partial sequencing
jsGnRH3 DP2 Fw	TAYGGNTGGCTDCCNGGNGG	
jsGnRH3 GSP1 Fw	GTCCAGTCTGAGCTTTAGATGTGTGTATGTGTG	ORF sequencing
jsGnRH3 GSP2 Fw	GTGTGTATGTGTGTGTGAGGTGTG	
jsGnRH3 GSP1 Rv	GCACATGTGCATTAGTCCAGGAGTACTCTTC	
jsGnRH3 GSP2 Rv	CCAGGAGTACTCTTCTAAATGGTCC	
jsGnRHR DP1 Fw	CTNCAGGCSATGTACTCYTGC	Partial sequencing
jsGnRHR DP2 Fw	TCYTGGGCYTTYGTSACBGTG	
jsGnRHR DP1 Rv	CAYAGWCCCARYAGGTAG	
jsGnRHR DP2 Rv	CARYAGGTAYGGAGTCCAGC	
jsGnRHR1 GSP1 Fw	GTCATCGTCAGCTCCTTCATC	3' RACE
jsGnRHR1 GSP2 Fw	CATCGTCAGCTCCTTCATCG	
jsGnRHR1 GSP1 Rv	CCATGCCACCGTCAGCTGGATTG	5' RACE
jsGnRHR1 GSP2 Rv	CATGCCACCGTCAGCTGGATTGTG	
jsGnRHR1 ORF1 Fw	CCAGATAAGGATCTACTAGATGCCTATGCCTTTG	ORF sequencing
jsGnRHR1 ORF2 Fw	GGATCTACTAGATGCCTATGCCTTTGTTGTACC	
jsGnRHR1 ORF1 Rv	CGAAAGTCTCACAGCAATTCATCAGAGTTG	
jsGnRHR1 ORF2 Rv	TCTCACAGCAATTCATCAGAGTTGGACAG	
jsGnRHR1 RGA Fw	CTGGCTAGCAACTCCTAAAAAACCGCCACCATGCCTATGCCTTTGTTGTACC	Reporter Gene Assay
jsGnRHR1 RGA Rv	TAGACTCGAGTCACAGCAATTCATCAGAGTTG	
jsGnRHR2 GSP1 Fw	CGTTCCACTTCGTCACACTC	3' RACE
jsGnRHR2 GSP2 Fw	CACTCTATGTCATACCCCTCTTGG	
jsGnRHR2 GSP1 Rv	CCTGCATGGCGAAGAGCTTGAGG	5' RACE
jsGnRHR2 GSP2 Rv	TGCATGGCGAAGAGCTTGAGGC	
jsGnRHR2 ORF1 Fw	AGCACACGCTACAGATGTCTGGCAG	ORF sequencing
jsGnRHR2 ORF2 Fw	CTACAGATGTCTGGCAGCGGCTCAC	
jsGnRHR2 ORF1 Rv	GGGAAAATCTACTCCCGGCATTTCAC	
jsGnRHR2 ORF2 Rv	TCTACTCCCGGCATTTCACACTGGG	
jsGnRHR2 RGA Fw	CTGGCTAGCAACTCCTAAAAAACCGCCACCATGTCTGGCAGCGGCTCACCCC	Reporter Gene Assay
jsGnRHR2 RGA Rv	TAGACTCGAGCTACTCCCGGCATTTCACACTG	

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

cDNA sequencing of the two GnRH receptors in Japanese sardine

Total RNA from the brain and pituitaries of Japanese sardine was extracted using ISOGEN (Nippon Gene), following the manufacturer's protocol. The first-strand cDNA library was prepared for partial sequencing using Superscript III Reverse Transcriptase (Invitrogen) and Oligo-dT primers (Sigma-Aldrich), following the manufacturer's protocol for reverse transcription. Degenerate primers for GnRHRs were designed from the conserved regions of various teleost fish GnRHRs previously used by Lumayno et al. (2017). The list of primers used is presented in Table 1. PCRs were performed in a final volume of 10 µL containing 5 µL 2× Amplitaq Gold PCR master mix (Applied Biosystems), 0.5 µL of each forward and reverse primers, 3.5 µL PCR-grade water, and 0.5 µL of synthesised cDNA. Thermal cycling consisted of initial denaturation at 95 °C for 9 min, followed by 35 cycles at 94 °C for 1 min, 60– 63.3 °C for 1 min, and 72 °C for 1 min. For 3′ end, 5′ end, and ORF sequencing, KOD FX Kit (Toyobo Co., Ltd., Japan) was used. PCRs were performed in a final volume of 50 µL containing 25 µL of 2× PCR buffer for KOD FX, 10 µL of 2 mM dNTPs, 10 µL of each forward and reverse GSPs, 0.25–1 µL of template DNA, 10–10.75 µL UPW, and 1 µL of KOD FX. Thermal cycling consisted of initial denaturation at 94 °C for 2 min, followed by 30 cycles at 98 °C for 10 sec, 55–65 °C for 30 sec, and 68 °C for 1:30 min. PCR products were purified from 1.5 % agarose gel using Nucleotrap (Takara Bio Inc.) and were subcloned into pGEM-T Easy Vector (Promega). Plasmid DNA with insert cDNA was extracted using Cica Geneus Plasmid Prep Kit (Kanto Chemical Co.) and was submitted to Eurofins Genomics Co. for sequencing.

Phylogenetic and sequence identity analyses

For homology searches of the Japanese sardine GnRHs and GnRHRs, the basic local alignment search tool (BLAST) was utilised. GenBank was accessed to collect amino acid (aa) sequences of 44 distinct GnRH precursors and 44 GnRHRs (www.ncbi.nlm.nih.gov). The HMMTOP 2.0 server was used to predict the TMD regions of jsGnRHR1 and jsGnRHR2 (Tusnády and Simon, 1998; 2001). MEGA6 software was used to create the phylogenetic tree using the neighbour-joining technique (Tamura et al., 2013). The GnRH and GnRHR of common octopus (*Octopus vulgaris* Lamarck, 1798) were used as outgroups. BioEdit Sequence Alignment Editor version 7.0.9 (Hall, 2007) was used to compute the aa sequence identity of Japanese sardine GnRHs and GnRHRs to other precursors.

GnRH synthetic decapeptides

Three GnRH synthetic decapeptides corresponding to Japanese sardine (js) GnRH1 (hrGnRH1; herring form; Glp-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly-NH2), jsGnRH2 (chicken-II form; Glp-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2), and jsGnRH3 (salmon form; Glp-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH2) were purchased from Sigma Life Science (Hokkaido, Japan) with purities of 95.2 %, 84.4 %, and 95.2 % respectively. The peptides were analysed by analytical HPLC and mass spectrometry. Each stock peptide was initially dissolved to 10^{-3} M with 1 % of final concentration using dimethyl sulfoxide (DMSO) and further diluted with 99 % of final concentration using ultra-pure water. Stock peptides were then aliquoted and stored in -80 °C freezer until ready for assay.

Reporter gene assay

The ORF of Japanese sardine GnRHRs containing lobster L21 sequence (which enhances translation efficiency) at the N-terminal were subcloned into the *Nhe1* and *Xho1* sites of the expression vector pcDNA3.1 (Invitrogen). The cDNA constructs for both GnRHRs were verified by sequencing. Transient transfection and cell culture conditions were followed according to Nyuji et al. (2013). Chinese hamster ovary (CHO) cells were grown at 37 °C in Ham's F-12 medium (Life Technologies, USA) supplemented with 10 % fetal bovine serum (Gibco, USA), 1 % HT supplement (Gibco), 50 U mL^{-1} penicillin (Nacalai Tesque, Japan), and 50 µg mL^{-1} streptomycin (Nacalai Tesque). One day before transfection, the cells were seeded into 6-well plates. Co-transfection of pc-jsGnRHR1/pc-jsGnRHR2 (1µg $well^{-1}$), pNFAT (1 µg $well^{-1}$; Agilent, USA), and pRL-TK (8 ng $well^{-1}$; Promega) was carried out with X-tremeGENE HP DNA Transfection Reagent (Roche, Germany). After transfection for 24 h, cells were removed and plated in 96-well plates (3 × 10^4 cells $well^{-1}$). After 44 h, cells were incubated for 6 h with a vehicle of decreasing concentrations (concentrations from 10^{-8} M to 10^{-19} M for jsGnRHR1; 10^{-5} M to 10^{-12} M for jsGnRHR2) of each synthetic peptides. Luciferase activity in the cell extract was measured using a Dual-Luciferase reporter system (Promega) in Lumat LB95701 luminometer (Berthold Technologies, Germany). The EC_{50} values were calculated from concentration-response curves utilising computer-ised nonlinear curve fitting with Prism 4 (Graphpad Software, USA).

Results

Sequences of the three GnRH isoforms and phylogenetic analysis

All the three GnRH isoforms (named as jsGnRH1, jsGnRH2, and jsGnRH3) were successfully isolated in the brain of Japanese sardine. Figure 1a shows jsGnRH1 (GenBank accession no. MW252048) ORF cDNA encoding 261 base pairs (bp) with 86 aa. The ORF cDNA sequence of jsGnRH2 (GenBank accession no. MW252049) comprises 261 bp with 86 aa (Fig. 1b). In addition, jsGnRH3 cDNA (GenBank accession no. MW252050) has an ORF of 240 bp with 79 aa (Fig. 1c).

All the characteristic signal peptides, GnRH decapeptides, cleavage sites, and GnRH-associated peptides were identified in all three GnRH cDNAs discovered in Japanese sardine, as presented in Figure 1.

Phylogenetic analysis revealed three main groups of GnRH comprising GnRH1, GnRH2, and GnRH3 (Fig. 2). Based on phylogenetic tree, jsGnRH1 (hrGnRH precursor) grouped with GnRH1, whereas the jsGnRH2 (cGnRH-II precursor) and jsGnRH3 (sGnRH precursor) clustered with GnRH2 and GnRH3, respectively.

GnRH aa sequences of Japanese sardine in comparison with other teleosts GnRHs

The potential translation of jsGnRH1, jsGnRH2, and

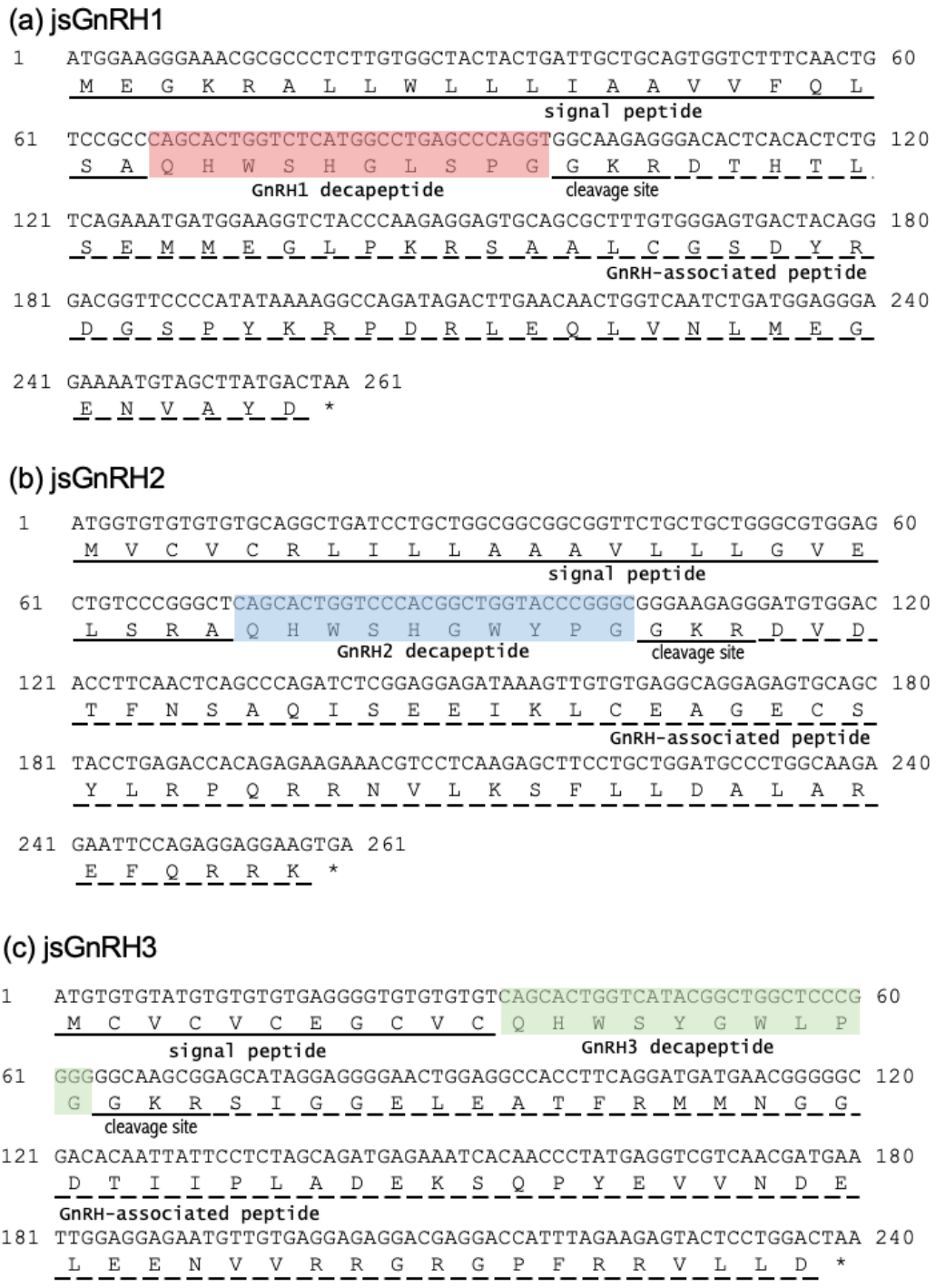

Fig. 1. Open reading frame sequences of (a) jsGnRH1, (b) jsGnRH2, and (c) jsGnRH3 from the brain of Japanese sardine *Sardinops sagax melanostictus*. The GnRH decapeptide region is highlighted. An asterisk (*) indicates the stop codon (TAA/TGA). GenBank accession numbers are MW252048 (jsGnRH1), MW252049 (jsGnRH2), and MW252050 (jsGnRH3).

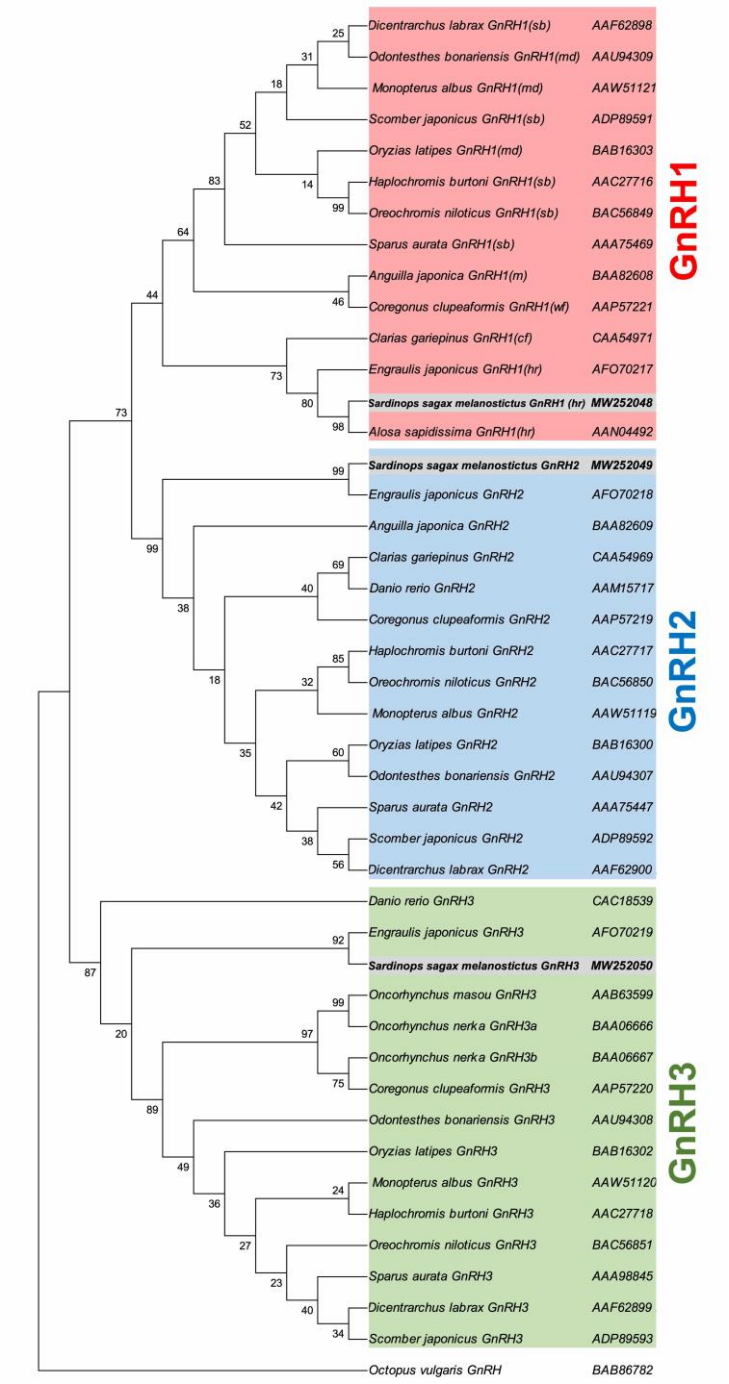

Fig. 2. Phylogenetic analysis of three GnRH forms in teleost fish. MEGA6 was used to conduct evolutionary studies using the neighbour-joining technique (Tamura et al., 2013). A total of 44 aa sequences were analysed. Each gene was tagged with its associated Genbank Accession number (protein ID) following the species' scientific name and GnRH categorisation. Gaps and incomplete data were removed from all spots. Common octopus GnRH was utilised as an outgroup.

jsGnRH3 aa sequences matched with related GnRH aa sequences of other fish species is depicted in Figure 3. Similarities between the deduced aa sequences from various GnRH precursors are presented in Table 2. Compared to other teleost GnRH1 precursors, the aa sequence identity of Japanese sardine GnRH1 varies from 24.7–90.6 %. American shad, *Alosa sapidissima* (Wilson, 1811); Clupeiformes GnRH1 (herring (hr) form) showed the highest similarity of 90.6 % and lowest to Japanese medaka, *Oryzias latipes* (Temminck & Schlegel, 1846); Beloniformes at 24.7 %. Homologues of other representative species exhibited 60.4–83.7 % similarity with the Japanese sardine GnRH2 isoform. The highest aa sequence identity was 83.7 % to Japanese anchovy, *Engraulis japonicus* Temminck & Schlegel, 1846; Clupeiformes and the lowest to Japanese medaka (60.4 %). The GnRH3 aa sequence from Japanese sardine had less homology (29.5–62.9 %) with other GnRH3 teleost species. Japanese anchovy had the highest similarity of 62.9 %, whereas sockeye salmon, *Oncorhynchus nerka* (Walbaum, 1792); Salmoniformeshad the lowest at 29.5 %.

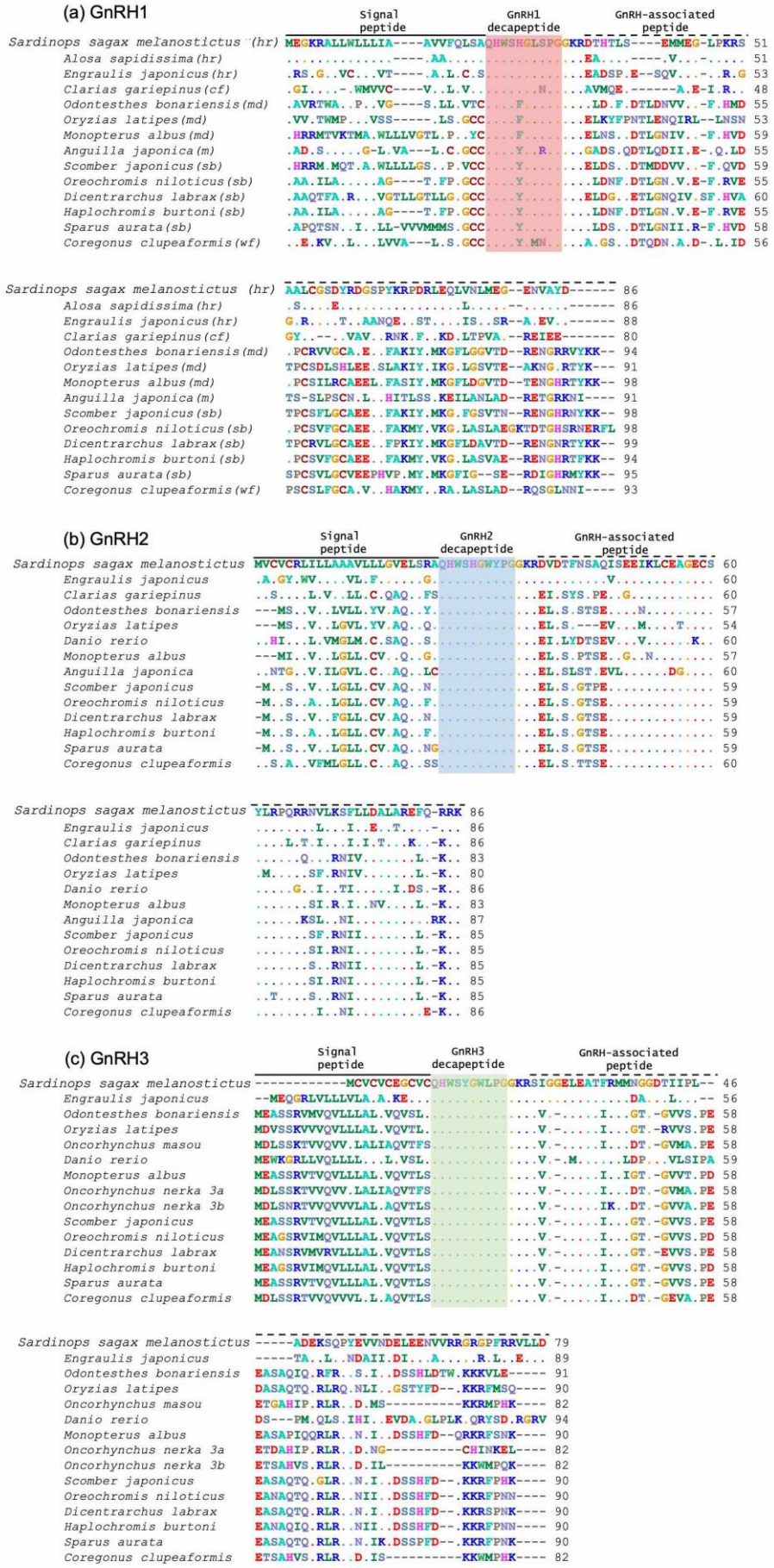

Fig. 3. Alignment of (a) GnRH1, (b) GnRH2, and (c) GnRH3. Dots show matching amino acid sequences. GnRH decapeptide is highlighted, whereas solid black lines show the aa corresponding to signal peptide, and black dashed lines indicate GnRH-associated peptide. GenBank Accession numbers of GnRH cDNA sequences are shown in Figure 2.

Table 2. The percentage of amino acid sequence identity between the Japanese sardine jsGnRH1, jsGnRH2, and jsGnRH3 with those of other teleosts species.

GnRH forms	Species	Percentage of identity (%)		
		Sardinops sagax melanostictus		
		GnRH1	GnRH2	GnRH3
GnRH1(hr)	*Sardinops sagax melanostictus*	100.0	24.1	15.6
GnRH1(hr)	*Alosa sapidissima*	90.6	24.1	13.7
GnRH1(hr)	*Engraulis japonicus*	55.6	21.7	14.7
GnRH1(cf)	*Clarias gariepinus*	45.3	24.1	15.0
GnRH1(md)	*Odontesthes bonariensis*	31.9	23.9	17.6
GnRH1(md)	*Oryzias latipes*	24.7	18.0	17.0
GnRH1(md)	*Monopterus albus*	30.6	20.4	16.0
GnRH1(m)	*Anguilla japonica*	30.8	26.0	18.4
GnRH1(sb)	*Scomber japonicus*	30.6	20.4	17.9
GnRH1(sb)	*Oreochromis niloticus*	31.6	23.0	20.5
GnRH1(sb)	*Dicentrarchus labrax*	25.2	18.8	18.8
GnRH1(sb)	*Haplochromis burtoni*	32.9	23.9	18.6
GnRH1(sb)	*Sparus aurata*	25.7	21.2	15.2
GnRH1(wf)	*Coregonus clupeaformis*	35.1	22.9	19.4
GnRH2	*Sardinops sagax melanostictus*	24.1	100.0	15.0
	Engraulis japonicus	25.2	83.7	14.0
	Clarias gariepinus	19.7	68.6	16.0
	Odontesthes bonariensis	23.5	65.1	14.4
	Oryzias latipes	22.9	60.4	15.4
	Danio rerio	21.9	62.7	15.0
	Monopterus albus	25.8	63.9	15.4
	Anguilla japonica	24.1	63.2	18.0
	Scomber japonicus	23.3	67.4	15.1
	Oreochromis niloticus	25.5	68.6	15.1
	Dicentrarchus labrax	23.3	68.6	15.1
	Haplochromis burtoni	25.5	68.6	15.1
	Sparus aurata	23.3	67.4	15.1
	Coregonus clupeaformis	21.9	69.7	14.0
GnRH3	*Sardinops sagax melanostictus*	15.6	15.0	100.0
	Engraulis japonicus	19.6	16.0	62.9
	Odontesthes bonariensis	19.5	23.6	34.6
	Oryzias latipes	20.4	21.2	32.6
	Danio rerio	17.4	21.2	38.9
	Monopterus albus	19.3	23.4	30.6
	Oncorhynchus nerka 3a	17.7	17.3	30.6
	Oncorhynchus nerka 3b	16.6	19.5	29.5
	Scomber japonicus	18.3	21.2	33.6
	Oreochromis niloticus	20.4	22.3	32.6
	Dicentrarchus labrax	19.3	22.3	33.6
	Haplochromis burtoni	20.4	22.3	32.6
	Sparus aurata	19.3	22.3	32.6
	Coregonus clupeaformis	16.6	18.4	31.6

Genbank Accession number (protein ID) for each species is presented in Figure 2.
The abbreviations used for GnRH1 forms in teleost fish are defined as: herring (hr); catfish (cf); medaka (md); mammalian (m); seabream (sb); and whitefish (wf).

Sequences of the two GnRHR paralogues and phylogenetic analysis

Two GnRHRs in the brain and pituitary of Japanese sardine named jsGnRHR1 and jsGnRHR2 were successfully isolated. The sequences were assigned the accession codes MW252051 and MW252052, respectively, in GenBank. The ORF for jsGnRHR1 encodes 1188 bp in length with 395 aa and contains seven TMD regions (Fig. 4a). Furthermore, the ORF for jsGnRHR2 encodes 1146 bp in length with 381 aa containing seven TMD regions shown in Figure 4b. Two distinct types with three subtypes each (Type 1: 1a, 1b, 1c; Type 2: 2a, 2b, 2c) of GnRHRs were revealed in phylogenetic analysis. The classification was adopted from Ciani et al. (2020) based on Sefideh et al. (2014). Octopus GnRHR was utilised as an outgroup. The jsGnRHR2 grouped to GnRHR1c, whereas jsGnRHR1 fitted to other teleost 2b GnRHRs (Fig. 5).

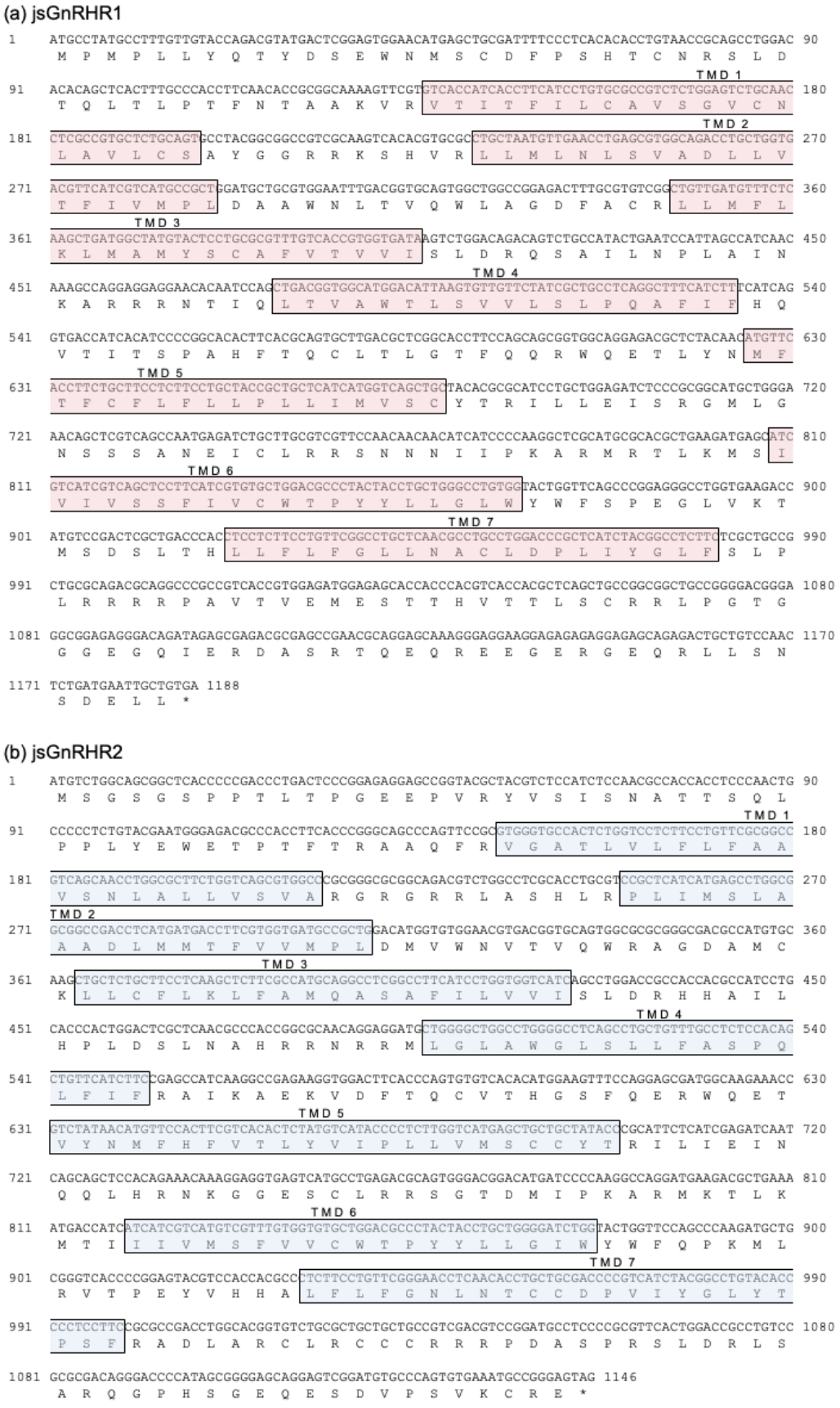

Fig. 4. Open reading frame of (a) jsGnRHR1 and (b) jsGnRHR2 from the brain and pituitary of Japanese sardine *Sardinops sagax melanostictus*. The HMMTOP 2.0 server was used to predict transmembrane domains (TMDs) (Tusnády and Simon, 1998, 2001). Seven TMDs are divided into boxes. An asterisk (*) denotes the stop codon (TAG/TGA).

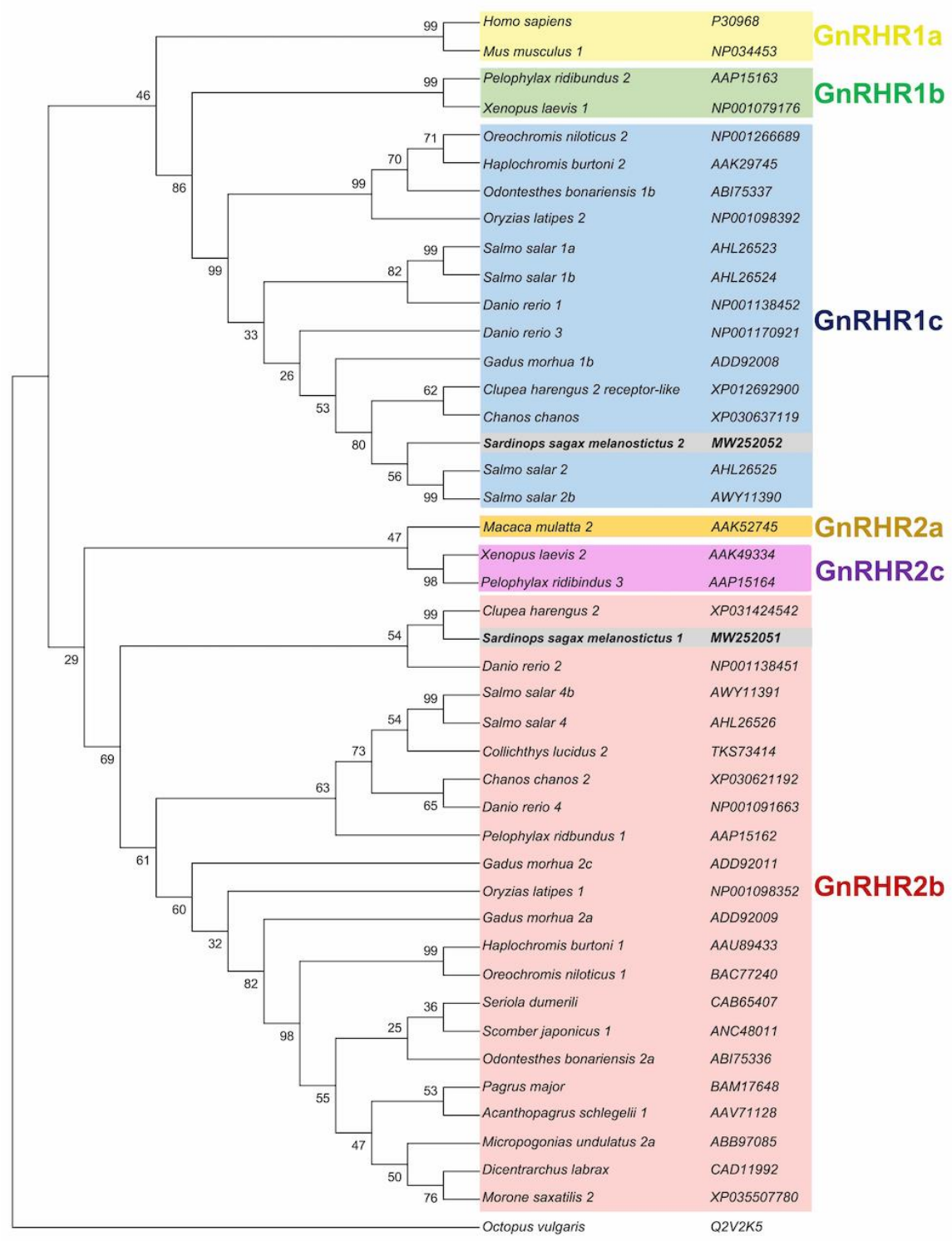

Fig. 5. Phylogenetic analysis of GnRH receptors in teleost fish. MEGA6 was used to conduct evolutionary studies using the neighbour-joining technique (Tamura et al., 2013). A total of 44 aa sequences were analysed. Each gene was labeled with its corresponding Genbank Accession number (protein ID), its scientific name, and the original designated code. Gaps and incomplete data were removed from all spots.

GnRHR aa sequences of Japanese sardine in comparison with other teleost GnRHRs

Putative translation of jsGnRHR1 and jsGnRHR2 matched with GnRHR aa sequences of GnRHR1c and GnRHR2b from representative species is depicted in Figure 6. Similarities between the deduced aa sequences from various GnRHR precursors are presented in Table 3. The aa sequence identity of jsGnRHR2 ranges from 30.8–88.4 % compared with other Type 1c GnRHRs. The putative Pacific herring 2 receptor-like (*Clupea harengus* Linnaeus, 1758; Clupeiformes) had the highest aa sequence identity (88.4 %), whereas the Nile tilapia 2, *Oreochromis niloticus* (Linnaeus, 1758); Cichliformes had the lowest

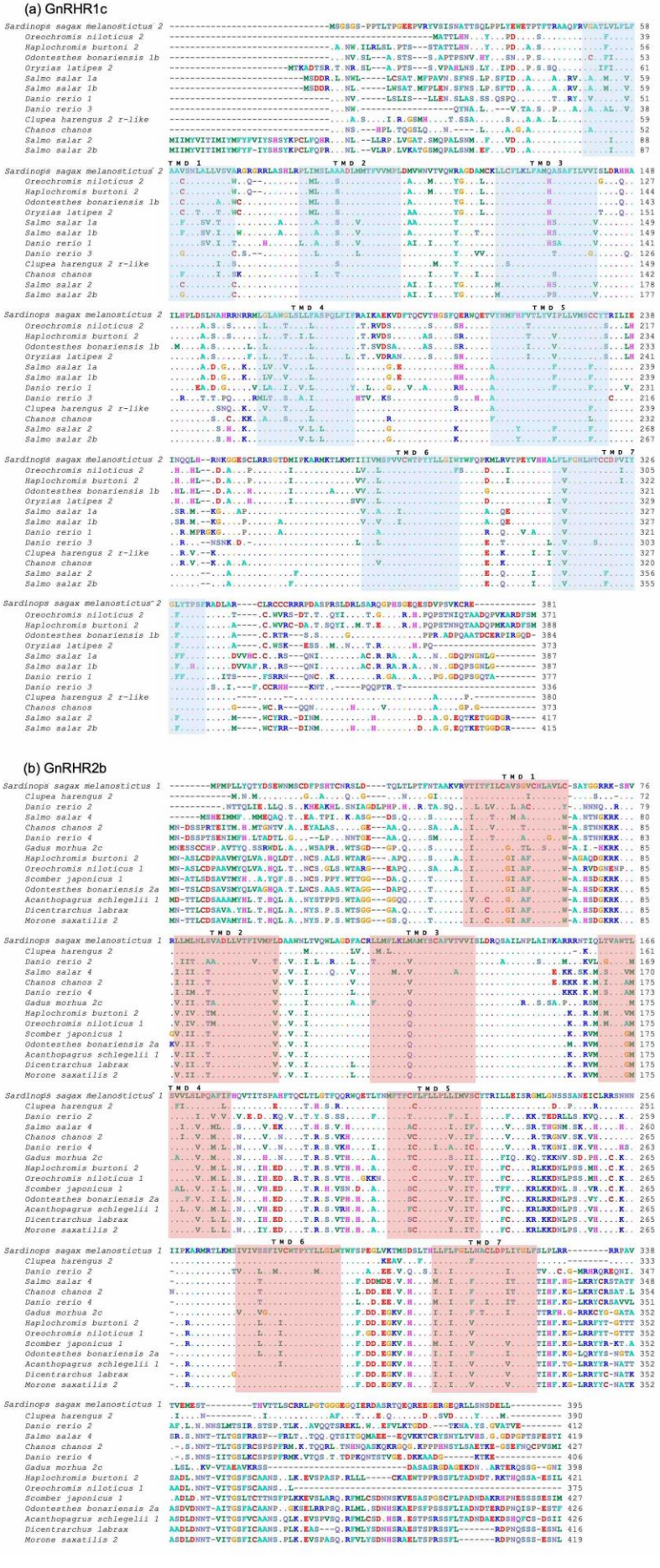

Fig. 6. Alignment of Japanese sardine GnRH receptors with other teleost GnRH receptor sequences. Dots show identical aa sequences. The seven transmembrane domain regions are divided into boxes. GenBank Accession numbers of GnRH receptor cDNA sequences are shown in Figure 5.

Table 3. The percentage of sequence identity between the Japanese sardine's aa GnRHR forms and those of other teleost species.

GnRHR types	Species	Percentage of identity (%)	
		Sardinops sagax melanostictus	
		jsGnRHR2	jsGnRHR1
GnRHR1a	*Homo sapiens*	35.5	33.6
	Mus musculus 1	35.2	33.6
GnRHR1b	*Pelophylax ridibundus 2*	48.7	38.7
	Xenopus laevis 1	49.7	40.2
GnRHR1c	*Sardinops sagax melanostictus 2*	100.0	40.0
	Oreochromis niloticus 2	30.8	26.0
	Haplochromis burtoni 2	69.2	39.8
	Odontesthes bonariensis 1b	71.7	40.4
	Oryzias latipes 2	68.9	40.1
	Salmo salar 1a	70.5	39.6
	Salmo salar 1b	70.5	39.8
	Danio rerio 1	69.5	41.2
	Danio rerio 3	67.0	38.7
	Gadus morhua 1b	34.1	21.7
	Clupea harengus receptor-like 2	88.4	39.2
	Chanos chanos	81.9	40.4
	Salmo salar 2	73.0	37.4
	Salmo salar 2b	72.2	37.5
GnRHR2a	*Macaca mulatta 2*	38.0	42.1
GnRHR2b	*Sardinops sagax melanostictus 1*	40.0	100.0
	Clupea harengus 2	38.4	86.0
	Danio rerio 2	38.2	55.5
	Salmo salar 4b	36.1	54.4
	Salmo salar 4	36.2	54.7
	Collichthys lucidus 2	37.2	56.9
	Chanos chanos 2	37.0	55.7
	Danio rerio 4	38.6	55.8
	Pelophylax ridibundus 1	36.9	48.2
	Gadus morhua 2c	22.8	30.1
	Oryzias latipes 1	36.8	52.5
	Gadus morhua 2a	33.5	46.0
	Haplochromis burtoni 1	37.9	52.3
	Oreochromis niloticus 1	39.3	51.7
	Seriola dumerili	37.7	52.3
	Scomber japonicus	37.4	51.9
	Odontesthes bonariensis 2a	38.8	51.6
	Pagrus major	38.4	52.8
	Acanthopagrus schlegelii 1	38.4	52.5
	Micropogonias undulatus 2a	38.4	52.1
	Dicentrarchus labrax	39.0	53.0
	Morone saxatilis	39.5	52.8
GnRHR2c	*Xenopus laevis 2*	40.6	46.8
	Pelophylax ridibundus 3	40.2	46.0

Genbank accession number (protein ID) for each species is shown in Figure 5.

(31.8 %). Compared to Type 2b receptors, however, the aa sequence identity of jsGnRHR2 ranges from 22.8–40 %, with the highest identity to jsGnRHR1. Human (*Homo sapiens* Linnaeus, 1758) and house mouse 1 (*Mus musculus* Linnaeus, 1758) belong to Type 1a GnRHRs, have 35.5 % similarities to jsGnRHR1. Rhesus monkey 2, *Macaca mulatta* (Zimmermann, 1780); Type 2a GnRHR representative and African clawed frog, *Xenopus laevis* (Daudin 1802); Type 2c GnRHR representative, have 38 % and 40.6 % aa

sequence identity to jsGnRHR1, respectively.

The aa sequence identity of jsGnRHR1 varies from 30.1–86 % compared to other Type 2b GnRHRs. The highest aa sequence identity was 86 % for putative Pacific herring 2 (Clupeiformes), while the lowest was 30.1 % for Atlantic cod 2c, *Gadus morhua* Linnaeus, 1758; Cichliformes. Compared with Type 1c receptors, jsGnRHR1 aa sequence identity ranges only from 21.7–40.4 %, with the highest identities to milkfish, *Chanos chanos* (Forsskål, 1775); Gonorynchiformes and pejerrey 1b, *Odontesthes bonariensis* (Valenciennes, 1835); Atheriniformes. Human and house mouse 1 both have 33.6 % aa sequence identity to jsGnRHR1. Rhesus monkey 2 and African clawed frog have 42.1 % and 46.8 % aa sequence identity to jsGnRHR2, respectively.

Comparison of ORF nucleotide sequences from targeted sequencing and RNA-seq

The Japanese sardine jsGnRH1 and jsGnRH2 ORF sequences obtained from targeted sequencing showed 100 % similarity to GnRH1 and GnRH2 from RNA-seq. On the other hand, GnRH3 and GnRHR2 ORF sequences from RNA-seq exhibited 97.92 % and 91.04 % similarities to jsGnRH3 and jsGnRHR2 ORF sequences from targeted sequencing, respectively. No complete ORF derived from RNA-seq is available for GnRHR1. Alignment analysis of the ORF nucleotide sequences from targeted sequencing and RNA-seq are presented in Supplementary Figures 1 and 2.

Ligand selectivity of Japanese sardine GnRH receptors

To investigate the ligand selectivity for jsGnRHR1 and jsGnRHR2, NFAT-luciferase (NFAT-Luc) was utilised as a reporter gene for calcineurin/NFAT signalling pathway. Japanese sardine GnRHR1 showed high potency to all three GnRH synthetic peptides (jsGnRH1, jsGnRH2, jsGnRH3) presented in Figure 7a. Whereas, jsGnRHR2 elicited high response to jsGnRH2 but low to jsGnRH1 and jsGnRH3 (Fig. 7b).

Discussion

In Clupeiform fish species, studies on the isolation of all three GnRH isoforms and GnRH receptors are minimal. Abraham (2004) attempted to clone all three forms of GnRH in American shad brain, but was only successful in isolating GnRH1. The first successful targeted sequencing study in a Clupeiform species of all three GnRHs was reported by Sukhan et al. (2013) in the brain of Japanese anchovy. Lavoué et al. (2007) reported a putative GnRH receptor of Pacific herring inferred from mitogenome sequences. Recently, Nyuji et al. (2020) reported the first deep RNA-seq analysis of Japanese sardine. A total of 115,173 non-redundant ORFs (partial or complete) were annotated in Japanese sardine, including GnRHs and GnRHRs (Nyuji et al., 2020). Targeted sequencing was performed in the present study to get more specific nucleotide sequences of the three GnRH isoforms and two GnRHR paralogues in the brain and pituitary of Japanese sardine.

In phylogenetic analysis, Japanese sardine jsGnRH1 clustered with other teleost GnRH1 suggesting an involvement in fish reproduction. On the other hand, jsGnRH2 grouped with other GnRH2 implying a possible role in neuromodulation, whereas jsGnRH3 clustered with other GnRH3 suggesting a role in sexual behaviour in fish. Future localisation studies in the brain and pituitary of Japanese sardine will help in understanding the functional role of these GnRH forms.

GnRH receptors, unlike its ligand, lack a well-defined categorisation system. The current work uses Sefideh

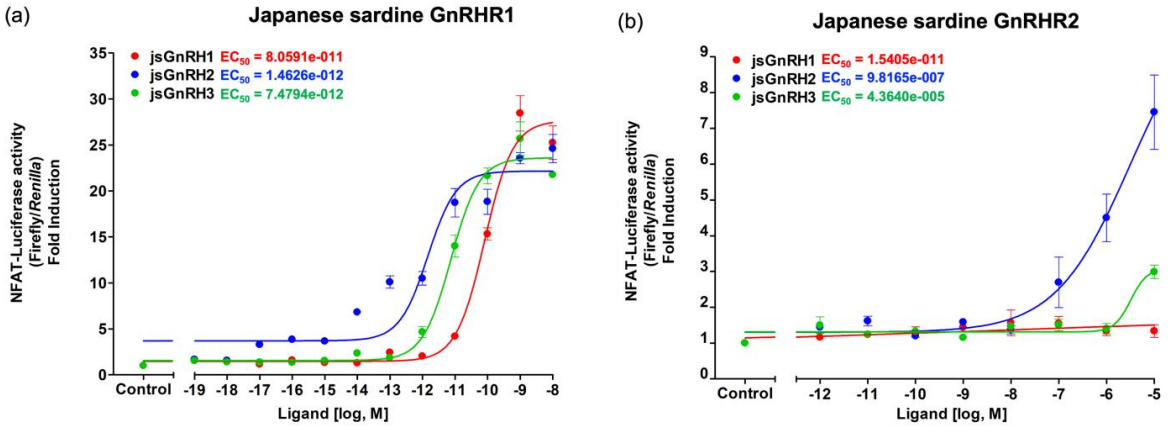

Fig. 7. Ligand binding affinity of the Japanese sardine GnRH receptors, jsGnRHR1 (a) and jsGnRHR2 (b) with NFAT-Luc. GnRH1, 2, and 3 from Japanese sardine were used to treat transfected cells. The results are given as a ratio of changes in firefly luciferase activity over *Renilla* luciferase activity. The jsGnRHR1 was performed in quadruplicate, whereas jsGnRHR2 was executed in triplicate. The data is presented as a mean with a standard deviation.

et al. (2014)'s nomenclature, dividing GnRH receptors into two major types, each with three subtypes. Using ORF aa sequences, phylogenetic analysis revealed that jsGnRHR2 grouped with Type 1c receptors, whereas jsGnRHR1 clustered with Type 2b receptors. Type 1c receptors have been implicated in the modulation of sensory, metabolic, and motor systems in *Astatotilapia burtoni* (Günther, 1894) and the Nile tilapia (Soga et al., 2005; Chen and Fernald, 2006). On the contrary, various studies have suggested the involvement of GnRHR2b receptor type in fish reproduction and gonadotropic function control, as exhibited in Nile tilapia (Soga et al., 2005), *A. burtoni* (Flanagan et al., 2007), Atlantic cod (Hildahl et al., 2011), chub mackerel (Lumayno et al., 2017), and Atlantic salmon (Ciani et al., 2020). In the present study, Japanese sardine jsGnRHR1 (clustered into GnRHR2b) may be involved in fish reproduction as well.

To check the ligand selectivity of the two isolated Japanese sardine GnRH receptors, NFAT-Luc was performed on jsGnRH1, jsGnRH2, and jsGnRH3. In the present study, NFAT-Luc was used as a reporter gene which can activate phospholipase C, elevating intracellular Ca^{2+}. Japanese sardine jsGnRHR2 responded best to jsGnRH2 but poorly to jsGnRH3 and jsGnRH1. However, all three GnRH synthetic peptides showed high potency to jsGnRHR1. According to transfected cell analyses in most fish species, GnRHR paralogues can be activated by various GnRHs, with GnRH2 being the most preferred, preceded by GnRH3, then GnRH1 (Lethimonier et al., 2004). According to Pfleger (2002), GnRH2 has a greater binding affinity in most non-mammalian vertebrate receptors, regardless of classification, due to the preset β-II' turn conformation.

In chub mackerel studies, only the GnRH1 (considered as hypophysiotropic form) neurons innervated anterior pituitary regions where FSH and LH are localised (Selvaraj et al., 2009). It is worth noting that GnRH2 neurons project broadly across the brain but do not innervate pituitary regions, implying a function in neuromodulation (Selvaraj et al., 2009). Unsurprisingly, reporter gene assay results showed that the chub mackerel GnRH receptor (clustered to GnRHR2b) involved in fish reproduction exhibited highest affinity to GnRH2, followed by GnRH3, then GnRH1. Previous assay studies in striped bass (*Morone saxatilis* (Walbaum, 1792)) (Alok et al., 2000) and *A. burtoni* (Flanagan et al., 2007) also showed similar order of affinity.

In the present study, the two isolated Japanese sardine GnRH receptors follow the affinity order GnRH2 > GnRH3 > GnRH1. It is very evident, however that the affinity of jsGnRH1 for jsGnRHR1 (up to 28-fold induction) is much higher in comparison for jsGnRHR2 (up to 1.5-fold induction). NFAT activation of jsGnRH1 to jsGnRHR1 suggests an importance on the synthesis and release of gonadotropin in the pituitary (Perrett and McArdle, 2013). In addition, activation of jsGnRH2 to jsGnRHR2 suggests that NFAT may mediate GnRH2 action (Armstrong et al., 2009). Based on the results, we can infer that jsGnRH1 and jsGnRHR1 may be involved in fish reproduction. Further studies are needed in understanding the physiological functions of GnRH-GnRHR system. Overall, these findings can serve as basis for further studies in the reproduction of Japanese sardine.

Conclusion

The findings show that the three GnRH isoforms and two GnRHR paralogues exist in the brain and pituitary of a Clupeiform fish, the Japanese sardine *Sardinops sagax melanostictus*. Clustering of jsGnRH1 to other teleost GnRH1 and jsGnRHR1 to other teleost GnRHR2b suggests that these two genes are likely involved in fish reproduction. Moreover, the calcineurin/NFAT signaling pathway activation of jsGnRHR1 to jsGnRH1 may play a role on the synthesis and release of GTHs in the pituitary. Further studies on gene expression at various reproductive stages and brain localisation of these genes will help in understanding the role of GnRH-GnRHR system in the reproduction of Japanese sardine.

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Conflict of interest: The authors declare that they have no conflict of interest.

Author contributions: Sanny David Pacheco Lumayno: Acquisition of data, analysis, and interpretation of data, drafting the manuscript, revising the manuscript. Kohei Ohta: Conception and design of the study, interpretation of data, revising the manuscript. Akihiko Yamaguchi: Conception and design of the study, interpretation of data, revising the manuscript. Michiya Matsuyama: Conception and design of the study, interpretation of data, revising the manuscript.

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(a) jsGnRH1

```
jsGnRH1_Targeted_Sequencing     ATGGAAGGGAAACGCGCCCTCTTGTGGCTACTACTGATTGCTGCAGTGGT 50
GnRH1_RNA_Seq                  ATGGAAGGGAAACGCGCCCTCTTGTGGCTACTACTGATTGCTGCAGTGGT 50
                               **************************************************

jsGnRH1_Targeted_Sequencing     CTTTCAACTGTCCGCCCAGCACTGGTCTCATGGCCTGAGCCCAGGTGGCA 100
GnRH1_RNA_Seq                  CTTTCAACTGTCCGCCCAGCACTGGTCTCATGGCCTGAGCCCAGGTGGCA 100
                               **************************************************

jsGnRH1_Targeted_Sequencing     AGAGGGACACTCACACTCTGTCAGAAATGATGGAAGGTCTACCCAAGAGG 150
GnRH1_RNA_Seq                  AGAGGGACACTCACACTCTGTCAGAAATGATGGAAGGTCTACCCAAGAGG 150
                               **************************************************

jsGnRH1_Targeted_Sequencing     AGTGCAGCGCTTTGTGGGAGTGACTACAGGGACGGTTCCCCATATAAAAG 200
GnRH1_RNA_Seq                  AGTGCAGCGCTTTGTGGGAGTGACTACAGGGACGGTTCCCCATATAAAAG 200
                               **************************************************

jsGnRH1_Targeted_Sequencing     GCCAGATAGACTTGAACAACTGGTCAATCTGATGGAGGGAGAAAATGTAG 250
GnRH1_RNA_Seq                  GCCAGATAGACTTGAACAACTGGTCAATCTGATGGAGGGAGAAAATGTAG 250
                               **************************************************

jsGnRH1_Targeted_Sequencing     CTTATGACTAA 261
GnRH1_RNA_Seq                  CTTATGACTAA 261
                               ***********
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(b) jsGnRH2

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jsGnRH2_Targeted_Sequencing     ATGGTGTGTGTGTGCAGGCTGATCCTGCTGGCGGCGGCGGTTCTGCTGCT 50
GnRH2_RNA_Seq                  ATGGTGTGTGTGTGCAGGCTGATCCTGCTGGCGGCGGCGGTTCTGCTGCT 50
                               **************************************************

jsGnRH2_Targeted_Sequencing     GGGCGTGGAGCTGTCCCGGGCTCAGCACTGGTCCCACGGCTGGTACCCGG 100
GnRH2_RNA_Seq                  GGGCGTGGAGCTGTCCCGGGCTCAGCACTGGTCCCACGGCTGGTACCCGG 100
                               **************************************************

jsGnRH2_Targeted_Sequencing     GCGGGAAGAGGGATGTGGACACCTTCAACTCAGCCCAGATCTCGGAGGAG 150
GnRH2_RNA_Seq                  GCGGGAAGAGGGATGTGGACACCTTCAACTCAGCCCAGATCTCGGAGGAG 150
                               **************************************************

jsGnRH2_Targeted_Sequencing     ATAAAGTTGTGTGAGGCAGGAGAGTGCAGCTACCTGAGACCACAGAGAAG 200
GnRH2_RNA_Seq                  ATAAAGTTGTGTGAGGCAGGAGAGTGCAGCTACCTGAGACCACAGAGAAG 200
                               **************************************************

jsGnRH2_Targeted_Sequencing     AAACGTCCTCAAGAGCTTCCTGCTGGATGCCCTGGCAAGAGAATTCCAGA 250
GnRH2_RNA_Seq                  AAACGTCCTCAAGAGCTTCCTGCTGGATGCCCTGGCAAGAGAATTCCAGA 250
                               **************************************************

jsGnRH2_Targeted_Sequencing     GGAGGAAGTGA 261
GnRH2_RNA_Seq                  GGAGGAAGTGA 261
                               ***********
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(c) jsGnRH3

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jsGnRH3_Targeted_Sequencing     ATGTGTGTATGTGTGTGTGAGGGGTGTGTGTGTCAGCACTGGTCATACGG 50
GnRH3_RNA_Seq                  ATGTGTGTATGTGTGTGTGAGGTGTGTGTGTGTCAGCACTGGTCATACGG 50
                               ********************* **************************

jsGnRH3_Targeted_Sequencing     CTGGCTCCCGGGGGGCAAGCGGAGCATAGGAGGGGAACTGGAGGCCACCT 100
GnRH3_RNA_Seq                  CTGGCTCCCGGGAGGCAAGCGGAGCATAGGAGGGGAACTGGAGGCCACTT 100
                               ************ ********************************** *

jsGnRH3_Targeted_Sequencing     TCAGGATGATGAACGGGGGCGACACAATTATTCCTCTAGCAGATGAGAAA 150
GnRH3_RNA_Seq                  TCAGGATGATGAACGGGGGCGACACAATTATTCCTCTAGCAGATGAGAAA 150
                               **************************************************

jsGnRH3_Targeted_Sequencing     TCACAACCCTATGAGGTCGTCAACGATGAATTGGAGGAGAATGTTGTGAG 200
GnRH3_RNA_Seq                  TCACAACCCTATGAGGTCGTCAACGATGAATTGGAGGACAATGTTGTGAG 200
                               ************************************* ************

jsGnRH3_Targeted_Sequencing     GAGAGGACGAGGACCATTTAGAAGAGTACTCCTGGACTAA 240
GnRH3_RNA_Seq                  GAGAGGTCGAGGACCATTTAGAAGAGTACTCCTGGACTAA 240
                               ****** **********************************
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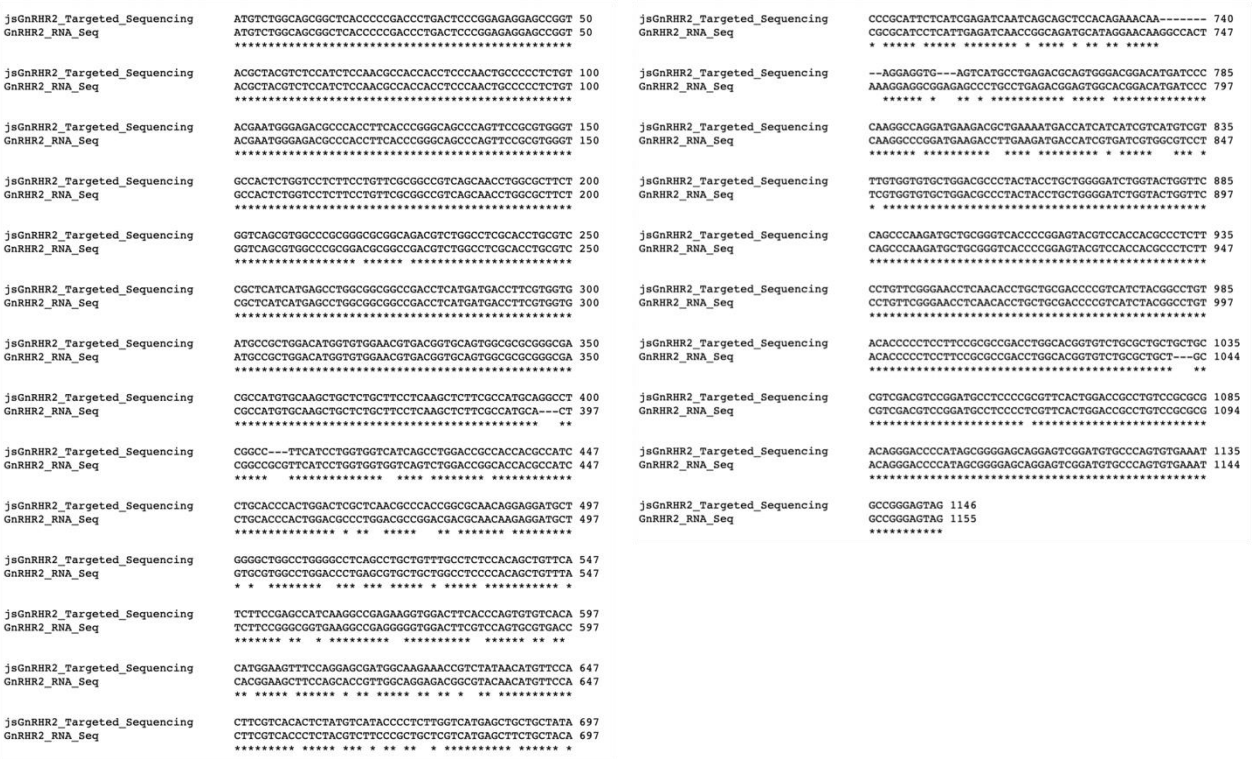
Supplementary Fig. 1. Alignment of GnRH isoforms from targeted sequencing and RNA sequencing (RNA-seq). The Genbank Accession numbers for RNA-seq derived GnRH1, GnRH2, and GnRH3 are ICPT01067198, ICPT01132793, and ICPT01060538, respectively (Nyuji et al. 2020).



Supplementary Fig. 2. Alignment of GnRHR2 from targeted sequencing and RNA sequencing (RNA-seq). The Genbank Accession number for RNA-seq derived GnRHR2 is ICPT01148960 (Nyuji et al. 2020).