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Molecular characterization of two *kiss* genes and their expression in rohu (*Labeo rohita*) during annual reproductive cycle

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ABSTRACT

Kisspeptin is an important regulator of reproduction in mammals and presumably non-mammalian species. In the Indian subcontinent, rohu (*Labeo rohita*) is a commercially important seasonal breeder freshwater fish species, but till date, the expression of kisspeptin gene during different phases of annual reproductive cycle has not been investigated. To address this, we cloned and characterized *kiss1* and *kiss2* full-length cDNA by RACE (rapid amplification of cDNA ends), and analyzed their expressions in brain–pituitary–gonad (BPG) axis by quantitative real time PCR (qRT-PCR) assay at various gonadal developmental stages of the annual reproductive cycle. Full-length rohu *kiss1* and *kiss2* cDNA encodes 116 and 125 amino acids respectively, and in the adult fish, they were widely expressed in brain, pituitary, gonad, liver, muscle, kidney, intestine and eye. In male, *kiss1* mRNA in brain and testis showed the highest level of expression during meiosis division of the gonad. The *kiss2* mRNA revealed the highest expression during recrudescence stages in the brain, spermiation stages in pituitary and post-spawning stages in testes. In females, significantly ($p < 0.05$) a higher level of *kiss1* transcript was expressed in brain and ovary, in the full grown oocyte stages, whereas during pre-vitellogenic and vitellogenic stages in pituitary. The *kiss2* gene expression was almost similar at various gonadal developmental stages in the brain and ovary, but, highest expression was detected in full grown oocyte stages in the pituitary. These results together may suggest the involvement of two *kiss* genes in the control of seasonal gonadal development in rohu.

1. Introduction

The importance of the kisspeptin system in the control of reproduction has been established in many vertebrates excluding avian species that lack kisspeptin (Um et al., 2010). Kisspeptins, the peptide products of the *Kiss1* gene, belonging to the RFamide family are ligands for the G protein-coupled receptor 54 (GPR54), now named as Kiss1r (Kotani et al., 2001; Muir et al., 2001; Roseweir and Millar, 2009). *Kiss1* was originally identified as tumor suppressor gene in breast cancer (Lee et al., 1996). The mutations in Kiss1r were associated with the idiopathic hypothalamic hypogonadism (IHH) syndrome in humans, which impaired puberty (de Roux et al., 2003; Seminara et al., 2003). Thus, it was demonstrated that kisspeptin and its receptor regulate the secretion of the gonadotropin-releasing hormone (GnRH), and in consequence this system was considered as a regulator of the brain–pituitary–gonadal (BPG) axis (de Roux et al., 2003; Seminara et al., 2003). The involvement of kisspeptin in this pathway has been demonstrated in different mammals through exogenous kisspeptin treatment, which elicited rapid increase in plasma levels of FSH and LH (Gottsch et al., 2004; Irwig et al., 2004; Dhillo et al., 2005; Navarro et al., 2005). Recent reports in different fishes also showed similar results (Nocillado et al., 2007; Felip et al., 2009; Kitahashi et al., 2009; Li et al., 2009; Beck et al., 2012; Zmora et al., 2012; Kim et al., 2014). In order to discuss the physiological role of kisspeptin systems identified by studies on different animals and fish species, the nomenclature referred to in Tena-sempre et al., 2012 has been adopted throughout this manuscript.

The existence of kisspeptin system in fish was demonstrated by the molecular cloning of *kiss1r* first in Nile tilapia, *Oreochromis niloticus* (Parhar et al., 2004), and subsequently in grey mullet, *Mugil cephalus* (Nocillado et al., 2007), cobia, *Rachycentron canadum* (Mohamed et al., 2007) and fathead minnow, *Pimephales promelas* (Filby et al., 2008). In most mammals, it was reported only one kisspeptin gene (*Kiss1*), with the exception of the platypus, a mammalian monotreme, in which two kisspeptin genes (*kiss1* and *kiss2*) were reported (Lee et al., 2009). Recent studies in non-mammalian vertebrates had revealed the presence of *kiss1* and *kiss2* in an Agnathan species, the sea lamprey,

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Table 1
Body length (BL), body weight (BW), gonadosomatic index (GSI) and germinal cell content of gonad at different phases of annual reproductive cycle in rohu.

	Preparatory (January)	Pre-spawning (April)	Spawning (June)	Post-spawning (September)
Male				
BW (gm)	825.00 ± 35.94	928.33 ± 93.64	1010.00 ± 76.59	1008.33 ± 81.05
BL (cm)	39.50 ± 1.14	40.33 ± 1.92	41.33 ± 1.49	42.08 ± 1.97
GSI (%)	0.22 ± 0.02	1.08 ± 0.08	2.04 ± 0.09	0.64 ± 0.05
Germinal cell content of gonad	Characterized by the presence of mostly primary and secondary spermatogonia and some spermatocytes	Testes in the spermatogenesis stage showing spermatocytes, spermatids and spermatozoa, with spermatocyte and spermatids being the dominant germ cell	Testes were filled with spermatozoa and were in spermiation stage and oozing of milt was observed when gentle abdominal pressure was given.	Testes showed mainly residual spermatozoa in seminiferous tubules long with some primary spermatogonia.
Female				
BW (gm)	775.00 ± 38.19	916.67 ± 62.80	1046.67 ± 76.5	826.67 ± 31.27
BL (cm)	39.17 ± 0.70	40.33 ± 1.00	41.83 ± 1.47	41.17 ± 0.60
GSI (%)	1.46 ± 0.12	7.92 ± 0.18	17.52 ± 0.43	5.16 ± 0.27
Germinal cell content of gonad	Pre-vitellogenic stage showed oogonia and primary oocytes.	Oocytes in the stage of vitellogenesis showing yolk granules in the yolk vesicles	Matured stage ovary characterized by full grown oocytes	A large number of empty follicles and some oogonia, primary oocytes and un-spawned atretic follicles

Table 2
Primers used for cDNA cloning and quantitative real time PCR analysis of *kiss1* and *kiss2* in rohu (*Labeo rohita*).

Primer	Nucleotide sequence (5′-3′)	Purpose
KP1F	CAGGGGAACTGATACTCATCCCAC	
KP1R	ACTCTGCAAGCATGTTCTGCTC	Partial cloning
KP2F	GGCGCTGATTCTCTTCATGT	
KP2R	TAAAGCATCATTGGCAGCAG	
Kiss1 RACE 3′	ACCTGCACCCATGGAGAAGTGCATCGA	
Kiss1 RACE 5′	CATGGGTGCAGGTCCTCAGCGAAACAC	RACE-PCR
Kiss 2 RACE 3′	ACCCAAACACAAGCACCTGCTGCCAA	
Kiss 2 RACE 5′	CGTCGTCTGAAGCACTGGGCTCGTCAA	
KP1QPCRF	CTTCTCCCAAGCTCTCGATG	
KP1QPCRR	ATAGCGGAGGCCAAAGGAAT	
KP2FQPCR	AGCCCAGTGCTTCAGACGAC	Real-time PCR
KP2RQPCR	AGCCCAAACGGGTTGTAGTT	
QELF1F	GAACTGTACCTGTGGGTCGTG	
QELF1R	CAACGTTGTCACCAGGAGTG	

Petromyzon marinus, and in amphibians (*Silurana tropicalis*, *Xenopus laevis*) (Felip et al., 2009). The presence of two kisspeptin gene, *kiss1* and *kiss2*, was reported in zebrafish, *Danio rerio* (Biran et al., 2008; Van aerle et al., 2008; Kitahashi et al., 2009), medaka, *Oryzias latipes*, (Kanda et al., 2008; Kitahashi et al., 2009), goldfish, *Carassius auratus* (Li et al., 2009; Yang et al., 2010), European seabass, *Dicentrarchus labrax* (Felip et al., 2009), yellowtail kingfish, *Seriola lalandi* (Nocillado et al., 2013), chub mackerel, *Scomber japonicas* (Selvaraj et al., 2010) and black rockfish, *Sebastes schlegelii* (Song et al., 2014). However, only one kisspeptin gene, *kiss2* was reported in the orange-spotted grouper, *Epinephelus coioides* (Shi et al., 2010), grass puffer, *Takifugu niphobles* (Shahjahan et al., 2010), Senegalese sole, *Solea senegalensis* (Mechaly et al., 2009) and Atlantic halibut, *Hippoglossus hippoglossus* (Mechaly et al., 2010).

Rohu (*Labeo rohita*), a seasonal breeder carp species, breeds once in a year during monsoon (rainy season). Different phases of annual reproductive cycle of rohu had previously been reported both in male (Gadekar and Baile, 2014) and female (Gadekar, 2014). But, there was no direct evidence of kisspeptins' involvement in the seasonal control of reproduction in rohu. As a first step to ascertain the involvement of kisspeptins in the seasonal gonadal development in rohu, we cloned and characterized *kiss1* and *kiss2* and investigated their basal expression in different tissues. Further, expression profiles of *kiss1* and *kiss2* transcripts in the BPG axis of adult fish during different phases of the annual reproductive cycle were carried out.

2. Material and methods

2.1. Ethical procedures

All animal handling procedures were approved by the Ethics and Animal Care Committee of the ICAR-CIFA established norms.

2.2. Fish and tissue sampling

Rohu broods were maintained in the brood-rearing pond of the Fish Nutrition and Physiology Division at the ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA), Bhubaneswar, India. For tissue distribution study, different tissues, namely brain, pituitary, ovary, testis, liver, kidney, muscle and intestine from adult spermiating male and late vitellogenic female rohu were quickly dissected and snap-frozen in liquid nitrogen. To examine the *kiss* mRNA level during different phases of the annual reproductive cycle, brain, pituitary and gonad tissues were collected during preparatory (January), pre-spawning (April), spawning (June) and post-spawning (September) phases. For sample collection, fish were captured and anesthetized in tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO) and their body length and body weight were measured. Gonads were removed and weighed to calculate the gonadosomatic index (GSI, gonad weight/ body weight × 100) and the sections of each gonad from individual fish were fixed in Bouin's solution for gonadal histology. Changes in body length (BL), body weight (BW), GSI and germinal cell content of gonad during different stages of the reproductive cycle are shown in Table 1.

2.3. Histological processing

After fixation, gonad samples were dehydrated in a series of ethanol solutions up to 100%, embedded in paraffin and sectioned at 5–7 μm using a WESWOX OPTIK rotary microtome (Model MT-1090A). Sections were stained with hematoxylin and counterstained with eosin. The stained tissues were subsequently observed under a light microscope to confirm the gonadal stages.

2.4. RNA extraction and cDNA preparation

Total cellular RNA from the collected tissues was extracted with Trizol (Invitrogen, USA) following the manufacturer's protocol and was treated with RNase free DNase I (Fermentas) to remove contaminating genomic DNA. The integrity of total RNA was checked by observing the band

Fig. 1. Nucleotide and deduced amino acid sequence of rohu *kiss1* and *kiss2* cDNAs. The putative signal peptide and kisspeptin-10 regions are underlined. The stop codon (TGA) is indicated by asterisks (*). The polyadenylation signal (AATAAA) is boxed.