Identification, characterization and expression profiling of kisspeptin gene in *Catla catla* (Hamilton, 1822)

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by

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CERTIFICATE

Certified that the thesis entitled **"Identification, characterization and expression profiling of kisspeptin gene in *Catla catla* (Hamilton, 1822) "**is a record of independent bonafide research work carried out by **Mr. Mohd Ashraf Rather** during the period of study from October 2012 to October 2014 under our supervision and guidance for the degree of **Doctor of Philosophy (Fish Biotechnology)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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सारांश

किस पेपटीन जननागों के अत्यावश्यक नियंत्रक के रूप में आधुनिक रूप से पहचाना गया है, जो कि मछलियों में जननमन्यि के परिपक्वन एवं तरूणयता के अनुक्रमता के लिए उत्तरदायी है । वर्तमान कार्य का उद्देश्य किस पेपटीन जीन को कतला कतला में पहचानना तथा चित्रण करना था, ताकि इसके हस्तक्षेप से मछली के परिपत्रक अवधि को कम किया जा सके । इस संदर्भ में किस पेपटीनकिा-1, किस-2, किस1आर. तथा किस 2- आर का पूर्ण cDNA लम्ब अनुक्रम क्रमश: 754, 583, 1082 तथा 1376 बी.पी. पाया गया । किस-1, किस-2, किस1आर. तथा किस 2-आर के कोडींग क्रम ने क्रमश: 116, 125, 366 तथा 368 अमीनों एसीड के पेपटाइडस का संश्लेषण किया । किस-1, किस-2 के फाइलों जेबेकि अध्ययन ने जी. रारस *(G.raras)* तथा अनुक्रमित रूपर्स गोल्डफिस *(C.aratus)* के साथ अत्याधिक समानता दिखाया, जबकि किस पेपटीन रिसेप्क्स ने किस-1 आर तथा किस-2 आर ने *C.Carpio* तथा क्रमागत (C.aratus) के साथ अत्याधिक समानता दिखाई । इसी तरह PCR के परिणामों ने दिखाया कि mRNA मुख्य रूप से मस्तिष्क, अण्डकोष तथा जननग्रन्थि में अभिव्यक्त होता है । भ्रूणाध्ययन विश्लेषण से प्रतीत हुआ कि यह अभिव्यक्ति फ्राई स्टेज से शुरू होकर परिपक्वन के समय सर्वाधिक होता है ।काइटोजेन, नेनो कन्जुगेकेड किस पेपटीन - 10(CK-10) जिसका कण आकार 125 mm, पालीडिस्परसिटी इन्डेक्स 0.335 से 0.65 तथा जीटा पोटेनशीयल 34.95 mv का संश्लेषण किया गया और इसका मूल्यांकन नग्न किस पेपटीन - 10 के परिपेक्ष्य जनन हारमोनों के नियंत्रण के लिए किया गया । काइटोजेन नेनोपाटीकल्स ने k-10 कि 64 एनट्रॅपमेंट क्षमता दिखाई । ck-10 का मछलीयां से प्रशोधन दो नियंत्रित तथा निरंतर जनन हारमोनों के स्राव में 12 घंटे पर बड़े शिखर तथा नग्न किस पेपटीन - 10 ने छह घंटो के बाद शिखर में ह्रास दिखाया । उत्तकोंध्ययन प्रेक्षण ने जननग्रन्थि में उगोनीया को स्टेज -1 उसाइट दिखाया तथा नग्न किस पेपटीन प्रशोधन में ज्यादतर उसाइट स्टैज - 2 में दिखे । जबकि ck-10 समूह में ज्यादातर उगोनीया अध्योतर स्टेज में दिखे तथा बाकी उसाइट ने जनन ग्रन्थि का परिपक्वन दिखाया । इस प्रकार ck-10 समूह में परिपक्वन आयु को कम करने तथा मछली में अण्डजनन तथा निस्लेषण के संकालन में मदद करता है । वर्तमान अध्ययन अपने आप में किस पेपटीन जीन और काइटोजेन नेनो कन्जुगेटेड किस पेपटीन - 10(ck-10) पर कार्प मछलियों में प्रजनन के संदर्भ में अनूठा है ।

Abstract

Kisspeptin has been recently recognized as an essential regulator of the reproductive axis, which is obligatory for its timely activation for onset of puberty and maturation of gonads in fishes. The present work was carried out to identify and characterise kisspeptin gene in *C. catla* so that the age at maturity can be decreased by intervention of kisspeptin protein. Kiss1, kiss2, kiss1r and kiss2r have been cloned and characterized from the brain of *C. catla*. The full-length of cDNA sequence of kiss1, kiss2, kiss1r and kiss2r are 754, 583, 1082 and 1376 bp, respectively. The coding region of kiss1, kiss2, kiss1r and kiss2r encoded a peptide of 116, 125, 366 and 368 amino acids, respectively. Phylogenetic analysis of kiss1 and kiss2 genes showed high similarity with *G. rarus* followed by goldfish, *C. auratus.* However, kissppetin receptor *i.e.* kiss1r and kiss2r showed high similarity with *C. carpio* followed by *C. auratus.* The qPCR result showed that kiss1mRNA is mainly expressed in brain, testis and ovary. Ontogeny analysis showed that kiss1mRNA expression start from fry stage with peak in matured stage. Chitosan-nanoconjugated kisspeptin-10 (CK-10) with particle size of 125nm, polydispersity index of 0.335 to 0.65 and zeta potential of 34.95 mV were synthesized and evaluated against naked kisspeptin-10 for their role in regulation of reproductive hormones. Chitosan nanoparticles showed 64% entrapment efficiency for K-10. Treatment of fish with CK-10 showed controlled and sustained surge of the reproductive hormones with peak at 12h, while naked kisspeptin-10 treated fish showed decline in hormone levels after 6h. Histological observation of ovary in control group contained oogonia with stage-I oocytes. In naked kisspeptin-10 treatment, most of oocytes are at stage II. However, in case of CK-10 group, it contained oogonia with advance stage having yolky oocytes which showed maturation of ovary under captive condition. The sustained release of this CK-10 will help in reducing age at maturity, synchronization of ovulation and spawning in fish. This is the first report on molecular cloning of kisspeptin gene and use of chitosan-nanoconjugated kisspeptin-10 (CK-10) for reproduction in Indian Major Carps.

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1.INTRODUCTION

Neuroendocrinology is the field of science which links neurobiology and endocrinology. It is the study of control exerted by brain onto the endocrine system. These two tightly-linked mechanisms contribute to the adaptability of an organism to change in environment (Zohar *et al.,* 2010). Fish neuroendocrinology is a recent area of research. The hypothalamic-pituitary-gonadal (HPG) axis regulates puberty in fish, primarily through the hypothalamic secretion of gonadotropin-releasing hormone (gnrh). Gnrh is a decapeptide hormone which stimulates the release of the following gonadotropins from the anterior pituitary: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Ankley and Johnson 2004; Weltzien *et al.,* 2004) for gamete growth. FSH and LH, secreted by the pituitary gland, play vital roles in the gametogenesis and production of steroid hormone in gonads (Levavi *et al*., 2010)

Knowledge about the mechanism of regulatory gnrh neurons and their roles in the control of gonadotropic axis has boosted considerably in recent years (Sempere *et al*., 2012). A major breakthrough in the field took place in late 2003, when kisspeptins, the product of Kiss1 gene, were first suggested to be important players in the central control of reproduction. This hypothesis comes from human and rodent data showing that inactivating mutations of G-protein-coupled receptor (GPR54) induced a state of hypogonadotropic hypogonadism (DeRoux *et al*., 2003; Seminara *et al*., 2003). In relatively short period of time, a massive number of experimental evidence has accumulated that clearly support an essential role of kisspeptins in the stimulation of gnrh secretion (Roa *et al*., 2008; Oakley *et al*., 2009; Pineda *et al*., 2010).

In case of mammals, broad neuroanatomical studies have recognized the existence of discrete populations of Kiss1 neurons in key hypothalamic areas, such as arcuate nucleus (ARC) and in rodents, the anteroventral periventricular region (AVPV) (Roa *et al*., 2008; Oakley *et al*., 2009; Pineda *et al*., 2010). Functional analyses have confirmed the involvement and assumed relevant roles of kisspeptin in virtually all aspects of reproductive physiology.These roles are likely to include: (a) the process of sexual differentiation; (b) the proper timing of puberty; (c) the dynamic control of gonadotropin secretion,

via stimulation of gnrh neurons; (d) the transmission of the negative feedback effects of sex steroids; (e) the mediation of the positive feedback effects of estrogen and, hence, generation of the pre-ovulatory surge of gonadotropins; (f) the metabolic regulation of fertility; and (g) the control of reproductive function by environmental (photoperiodic) cues (Roa *et al.,* 2008; Oakley *et al*., 2009; Pineda *et al*., 2010; Anglemont *et al*., 2010; Sempere *et al*., 2012). Most of the initial information in the field of kisspeptin physiology came from studies in different mammalian species, including laboratory rodents and primates (Oakley *et al*., 2009; Anglemont *et al*., 2010; Pineda *et al*., 2010). However, in the last few years there was significant increase in studies aiming to define the genomic organization and functional characterization of the kisspeptin system in different non-mammalian species, including fish.

Similar to mammals, Kiss1 has been cloned in fish models such as medaka and zebrafish (Biran *et al.,* 2008) and its involvement in gonadotropin release (Felip *et al.,* 2009) and signaling of sexual maturation/puberty (Felip *et al.,* 2009) have been confirmed. Besides Kiss1, a second Kiss gene, namely Kiss2, was also identified in lower vertebrates including the bony fish. Many teleost species have two paralogous genes of kisspeptin, kiss1 and kiss2. Medaka, (Oryzias latipes) in which kiss1 and kiss2 are expressed in distinctive hypothalamic neuron populations, is a good model system for the study of central regulation of reproduction (Akazome *et al.,* 2010). Two Kiss genes arose early in vertebrate evolution by gene duplication and Kiss2 gene might have been lost recently in the mammalian lineage (Felip *et al.,* 2009). Apart from the core sequence of kisspeptin-10, sequence homology between Kiss1 and Kiss2 is rather low (20–25%). When comparing kisspeptin-10 sequences encoded by the two genes, a distinct pattern of amino acid substitutions at position 1 and 10 were noted, with the ‘‘Y–Y form” (YNL/WNSFGLRY) for Kiss1 and ‘‘F–F form” (FNY/FNPFGLRF) for Kiss2 (Kitahashi *et al.,* 2009). In zebrafish, Kiss1 and Kiss2 are expressed in different locations within the hypothalamus (Kitahashi *et al.,* 2009) and may exert differential actions on LH and FSH secretion/expression (Felip *et al.,* 2008; Kitahashi *et al.,* 2009). Genome database searches showed that both genes are present in non-placental vertebrate genomes. Indeed, phylogenetic and genome mapping analyses suggest that kiss1and kiss2 are paralogous genes originated by duplication of an ancestral gene while kiss2 is lost in placental mammals. Kiss1 and

kiss2 mRNAs are present in brain and gonads of sea-bass, medaka and zebrafish (Kitahashi *et al.,* 2009; Akazome *et al.,* 2010). Most teleost species possess kiss2 gene, however, there is also reports that both kiss1 and kiss2 genes are present in the genomes of zebrafish, medaka, sea bass, goldfish and chub mackerel (Kitahashi *et al.,* 2009; Akazome *et al.,* 2010).

Year round breeding of IMC particularly rohu (*Labeo rohita*) and *Catla catla* is being practiced in eastern India. However, the mechanism of reproduction during non-spawning seasons is not clear yet. Kisspeptin gene can play a role in such induced breeding. But till date, there is no report on molecular characterization of kisspeptin genes, which are now considered as new gatekeepers for puberty and reproduction in IMC.

The mammalian Kissr1 (kisspeptin receptor) gene includes five exons and four introns, and its structure is well conserved among vertebrates (Sempere *et al.,* 2012). However, it has been reported that the medaka kissr3 gene is made of six exons and five introns (Sempere *et al.,* 2012). The existence of kissr (kisspeptin receptor) mRNA splicing isoforms has been reported in several teleost species including the senegalese sole (two kissr2 isoforms; Mechaly *et al.,* 2009); yellowtail kingfish (five kissr2 isoforms) and Southern bluefin tuna (two kissr2 isoforms; Nocillado *et al.,* 2012); zebrafish (five kissr3 isoforms; Onuma & Duan 2012); goldfish (two kissr2 isoforms; Li *et al.,* 2009); and European eel (three kissr3 isoforms; Pasquier *et al.,* 2011). However, there is no report so far on the molecular characterization of kisspeptin receptors in Indian Major Carp.

Biological activity of kisspeptin protein was tested *in-vivo* by administration of the core decapeptides in many fish. In goldfish, kisspeptin injection resulted in the increase of luteinizing hormone (LH) plasma levels (Li *et al.,* 2009). In European sea bass, systemic administration of kisspeptin peptide increased LH and FSH plasma levels (Felip *et al.,* 2009). Both KISS1- 15 and KISS2-12 peptides were potent in inducing the release of reproductive hormones in the *Morone* species (Zmora *et al.,* 2012). Kisspeptin-10 stimulated the release of luteinizing hormone-releasing hormone (LHRH) from in vitro hypothalamic explants and peripheral administration of Kisspeptin-10 increased plasma LH, FSH and total testosterone (Thompson *et al.,* 2004). Kisspeptin-10 plays an important role in the HPG axis

activity and affected sexual maturation and final maturation of the gonads of clownfish (Kim *et al.,* 2014). Saadeldin *et al* (2011) reported the role of kisspeptin in enhancing oocyte maturation in vitro but has an adverse impact on hatched blastocysts during in vitro culture. In striped bass females, Kiss1 stimulated fshβ expression and gonadal development while, hypophysiotropic gnrh1 and gnrh receptor expression remained unchanged (Zmora *et al*., 2014). The above studies collectively point out that kisspeptins are likely to be the most potent elicitors of gnrh/gonadotropin secretion.

Nanodelivery method is gaining popularity day by day owing to its advantage over the conventional method. Chitosan acts as a penetration enhancer by opening the tight epithelial junction and hence is particularly exploited in protein and vaccine delivery (Van *et al.,* 2001). Nanoparticles of chitosan have been extensively examined for their potential in the development of controlled delivery and release of proteins, antigens, oligonucleotides and genes (Kavshima *et al.,* 1985). This helps to overcome the problem of short life of the kisspeptin-10 in blood and avoids use of multiple injections to enhance reproductive efficacy. Yet, there is no information on the administration of kisspeptin-10 through nanoparticles and its effect on IMC.

Three IMCs *viz*., Catla (*Catla catla)*, Rohu (*Labeo rohita*) and Mrigal (*Cirrhinus mrigal*) contribute to the bulk of aquaculture production in Indian and neighboring countries like Bangladesh, Nepal and Pakistan. *Catla catla* is one of the major freshwater carps native to Indian sub-continent and introduced in many other countries as exotic species. Among the three IMCs, *Catla* is the fast growing fish and requires precise environmental conditions for spawning. It attains sexual maturity late both in captivity and natural conditions. Thus, understanding molecular and structural information of kisspeptin gene and their receptors in *Catla catla* will provide valuable information on the molecular control of reproduction in this particular fish. With this background, the present study has been proposed with the following objectives –

1. To identify and characterize the kisspeptin genes in *Catla catla.*
2. To study ontogeny of kiss1 gene expression in *Catla catla.*
3. To determine the effects of commercially available kisspeptin on hormonal profile of *Catla catla* using nanodelivery system.

2. REVIEW OF LITERATURE

2.1. The kisspeptin system

Kisspeptins (formerly known as Metastin), are peptide products of Kiss-1 gene, which was first discovered by Lee *et al.* (1996) as a metastasis suppressing gene in malignant melanoma cells. As it was discovered in Hershey, it was decided to name the gene after Hershey's famous chocolate kisses and ever since it has been exploited by researchers. Since mutations in the gene encoding the kisspeptin receptor *Kiss1r* were found to cause hypogonadotropic hypogonadism (de Roux *et al.,* 2003; Seminara *et al.,* 2003), the main focus of kisspeptin research has revolved around its role in reproduction. In humans, the gene product of *Kiss1* is translated into a pre-pro-peptide which is cleaved into a 54 amino acids long peptide, and amidated at the carboxy-terminal which is called kisspeptin-54 (Muir *et al*., 2001; Ohtaki *et al.,* 2001). Compared to human kisspeptin-54 (Terao *et al.,* 2004) in rodents, the same cleavage sites give rise to a 52 amino acids long amidated peptide with some sequence differences.

All amidated kisspeptins containing at least 10 amino acids of the carboxy-terminal have been shown to effectively activate Kiss1R (Kotani *et al*., 2001; Mikkelsen *et al.,* 2009). Kiss1r activation gives rise to gonadotropin-releasing hormone (gnrh)-dependent release of luteinising hormone (LH) and sex steroids (Gottsch *et al*., 2004; Mikkelsen *et al*., 2009), and kisspeptin is as such a potent activator of the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis comprises hypothalamic gnrh neurones, the gonadotropins LH and follicle-stimulating hormone (FSH) at the pituitary level and the sex steroids such as testosterone and oestradiol from the gonads. The vital control of sex steroids are maintained by the pulsatile release of gnrh to the pituitary portal system, which intermediates pulsatile release of LH and FSH from the anterior pituitary. These LH and FSH in turn stimulate sex steroid release from the gonads. Sex steroids have negative feedback on gnrh secretion, except during the preovulatory gnrh surge, where a positive feedback loop, essential for proper ovulation, is created. Although gnrh is regulated by sex steroids, gnrh neurones do not express sex steroid receptors and sex steroids thus

regulate gnrh release indirectly. Kisspeptin neurones on the other hand, express sex steroid receptors (Lehman *et al*., 2010), and several lines of evidence suggest that kisspeptin regulates gnrh release, as described in the following section and depicted in Fig. 1.

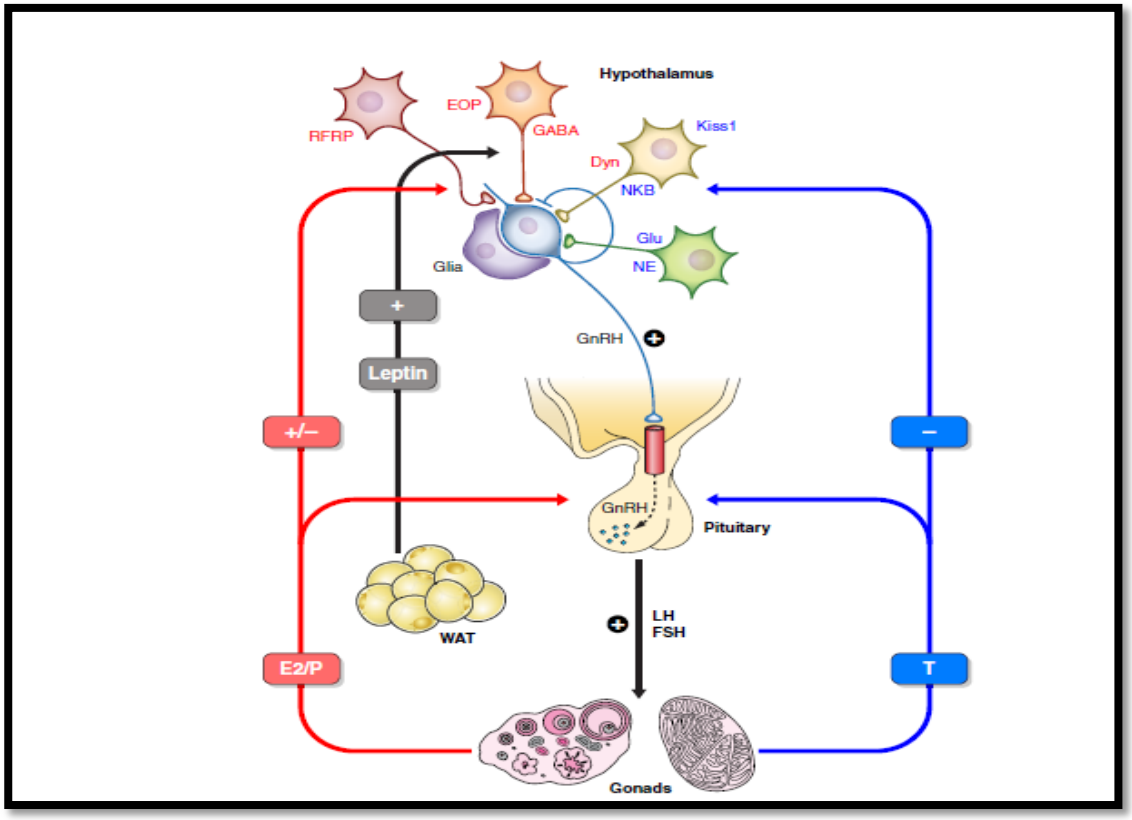


Fig.1.The HPG axis and the role of kisspeptin in gnrh secretion (Source: Leonor *et al.,* 2012)

Kisspeptin was found to be vital for gnrh secretion. It has already been discovered that an inactivating mutation in Kiss1r suffered from hypogonadotropic hypogonadism in humans, and that Kiss1R knockout mice displayed the same infertile phenotype (de Roux *et al.,* 2003; Seminara *et al*., 2003). These findings prompted scientists to investigate the nature of the hypothalamic kisspeptin system, due to which major progression in the understanding of hypothalamic regulation of gnrh release has been made. Findings that support the central role of kisspeptin in regulation of Gnrh secretion include the discovery that gnrh neurone soma (Clarkson and Herbison, 2006) as well as gnrh fibres at the level of the median distinction (Ramaswamy *et al.,* 2008) are innervated by kisspeptin-immunoreactive fibres and gnrh neurones express *Kiss1r* mRNA (Han *et al*., 2005; Irwig et *al.,* 2004),

suggesting that kisspeptin regulates gnrh neurones directly. This is further supported by the finding that kisspeptin depolarises isolated gnrh neurones (Han *et al*., 2005), and the finding of synchrony between kisspeptin pulses and gnrh pulses in monkeys (Keen *et al.,* 2008). Kisspeptin neurones are activated during the preovulatory LH surge and infusion of anti-kisspeptin antibody into the preoptic area prevents the preovulatory LH surge in rats (Kinoshita *et al*., 2005). It is well-documented that kisspeptin administration elicits release of LH to the circulation, which is blocked by gnrh antagonist pre-treatment (Gottsch *et al.,* 2004; Irwig *et al*., 2004; Mikkelsen *et al*., 2009). Concurrently, these findings strongly suggest a direct role for kisspeptin in the regulation of gnrh neurones.

In the rodent, kisspeptin is expressed in two hypothalamic areas; one is the rostral periventricular area of the third ventricle including the antero-ventral periventricular nucleus (AVPV) and the other is the arcuate nucleus (ARC)(Clarkson *et al.,* 2009;Mikkelsen and Simonneaux, 2009; Smith *et al*., 2005a). However, species differences exist and the kisspeptin neurone population in the AVPV is only found in rodents, whereas in humans the magnocellular paraventricular nucleus (Hrabovszky *et al.,* 2010) and in sheep the preoptic area (Smith, 2009) is a possible equivalent to the rodent AVPV. Even in rodents, agreement in distribution is obtained mainly at the mRNA level, where rats and mice have similar *Kiss1* mRNA distribution (Kauffman *et al.,* 2007; Smith *et al*., 2005b), but at the peptide level, differences exist (Overgaard *et al*., 2013).

2.2. Nomenclature of kisspeptin system

There have been some differences regarding the nomenclature of the elements of the kisspeptin system. Efforts have been made to sum up and integrate different terminologies used in the field during the last years. Whereas the general guidelines of the Human Genome Organization Gene Nomenclature Committee (HGNC) and the International Union of Pharmacology (IUPHAR) have been considered (Gottsch *et al*., 2009), the conventional terminology among the scientific community has been also taken into account as a means to maintain unity with the authoritative literature in this field. Some considerations for the nomination of genes, mRNA transcripts and proteins will be briefly made below.

Gottsch *et al.* (2009) which follows to the general indications of the HGNC, *KISS1* and *Kiss1* are used to name the human and non-human (primate and non-primate) genes, respectively. A similar terminology is adopted for the corresponding mRNA transcripts. This gene/mRNA was initially termed Kiss-1, as a way to recognize the origin of the research team that identified this factor, from Pennsylvania, (homeland of the famous Hershey *Kiss* chocolates), and its metastasis suppressor (sequence) activity; hence, the use of "SS." Yet, the use of the hyphenation and mixture of upper and lowercase letters has been progressively eliminated from the literature. In addition, the peptide products of *KISS1/Kiss1* are globally termed kisspeptins, following the original proposal of Kotani *et al*. (2001), with the abbreviation Kp followed by a numeric extension to indicate the amino acid length (e.g., Kp-10). About the receptor, suggestions have been made recently to use the terms KISS1R and Kiss1r to refer to G protein-coupled receptor GPR54 in human and non-human species, respectively, following again the indications of HGNC (Gottsch *et al.,* 2009).

2.3. Elements of kisspeptin

Kisspeptin are a family of basically related peptides, encoded by the *KISS1/Kiss1*gene, that act through binding and subsequent activation of the G protein-coupled receptor GPR54. The elements (genes and peptides) of this ligand-receptor system were sequentially identified between 1996 and 2001, but their close association with reproductive physiology was not recognized at that time (Sempere *et al.,* 2006; Roa *et al*., 2008). In fact, *KISS1* and its products, kisspeptins, were originally catalogued as metastasis suppressors. By the use of subtractive hybridization in melanoma cell lines with different metastatic capacity, *KISS1* mRNA was identified in 1996 as a selectively overexpressed transcript in tumour cells with low invasiveness (Kokay *et al.,* 2011). This initial finding was followed by further characterization of the anti-metastatic potential of the *KISS1* transcript (Lee *et al.,* 1997) and cloning and chromosomal localization of human *KISS1* gene (496). However, it was not until 2001 when three independent studies provided full characterization of the peptide products of the *KISS1* gene (Kotani *et al*., 2001; Muir *et al*., 2001; Ohtaki *et al*., 2001). Based on structural similarities and its common origin as KISS1- derived peptides, the term *kisspeptins* was coined to globally define

this family (Kotani *et al.,* 2001). This name became popular and displaced the initial terminology of metastin.

Kisspeptins are derived from the differential proteolytic processing of a single precursor. In the human, the kisspeptin precursor comprises 145 amino acids, with a putative 19-amino acid signal sequence, two potential dibasic cleavage sites (at amino acids 57 and 67), and one site for terminal cleavage and amidation (at amino acids 121–124) (Kotani *et al*., 2001; Ohtaki *et al.,* 2001) which generates the biologically active kisspeptins (Figure 2). Certainly, proteolysis of prepro-kisspeptin gives rise to a 54-amino acid peptide (kisspeptin-54), initially termed metastin because of its capacity to inhibit tumour metastasis, which has been considered the major product of the *KISS1* gene (Ohtaki *et al.,* 2001). Other peptide fragments of the kisspeptin precursor have been identified, such as kisspeptin-14, kisspeptin-13 and kisspeptin-10 (Kotani *et al.,* 2001) that share the COOH-terminal region of the kisspeptin-54 molecule, where they harbour an Arg-Phe-NH_2 motif characteristic of the RF-amide peptide family.

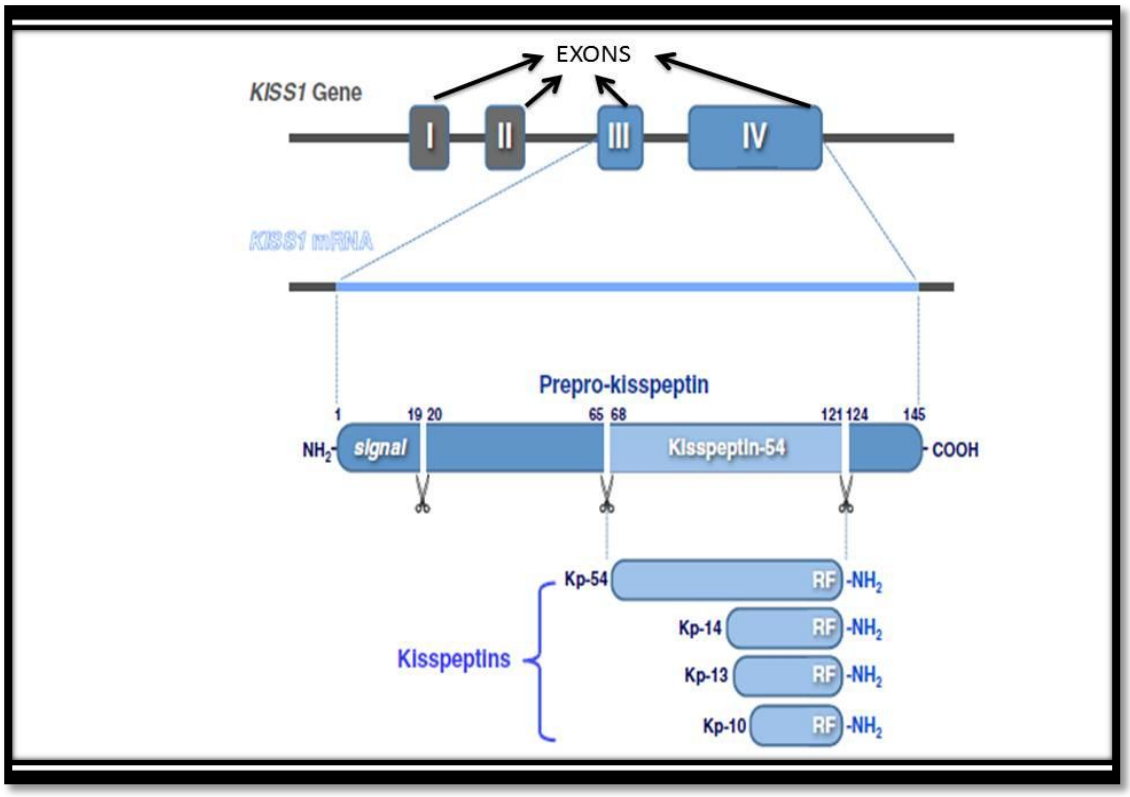
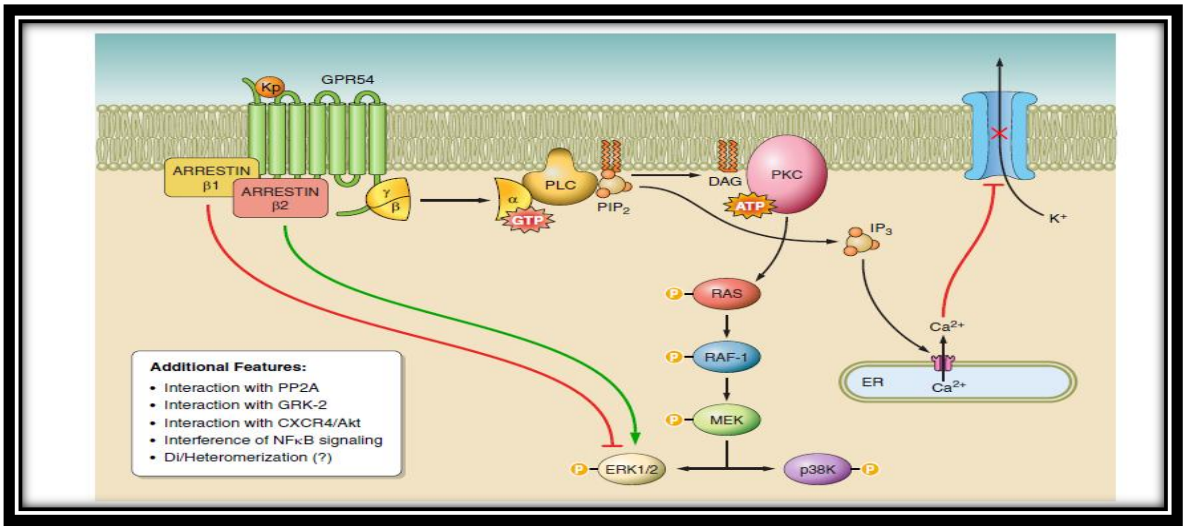


Fig.2. Major structural features of kisspeptins, the products of the Kiss1 gene (Leonor *et al.,* 2012)

Different kisspeptins are generated by proteolytic cleavage form a common precursor, prepro-kisspeptin, encoded by the *Kiss1* gene. In human, the *KISS1* gene is composed by four exons, the first two being noncoding exons (West *et al.,* 1998). However, an alternative genomic organization, with three exons (the first one being noncoding) has also been proposed (Luan *et al*., 2007). The human kisspeptin precursor (prepro-kisspeptin) contains 145 amino acids, with a 19-amino acid signal peptide and a central 54-amino acid region, kisspeptin-54 (Kp-54; formerly termed metastin). Lower molecular weight forms of kisspeptins include Kp-14, Kp-13, and Kp-10; the latter corresponds to the common COOH-terminal 10-amino acid stretch containing the RF-amide motif that is sufficient to fully activate GPR54.

2.4. Kisspeptin signalling via GPR54

The mapping of the signalling pathways employed by GPR54 was initiated already in 2001 by the use of different cell systems (Kotani *et al*., 2001; Ohtaki *et al.,* 2001). GPR54 signalling and its potential regulation is summarized in Figure 3.



(Source: Leonor et al., 2012)

Fig.3. Schematic presentation of the major signaling pathways recruited upon GPR54 activation by kisspeptin

2.5. Detection of the reproductive roles of kisspeptins

Even though the elements of the Kiss1 system had been already identified in 2001, their reproductive dimension remained unsuspected until the end of 2003. In that year, two separate reports (De Roux *et al.,* 2003; Seminara *et al*., 2003) documented the presence of deletions and inactivating mutations of the *GPR54* gene in patients suffering familial or sporadic forms of idiopathic hypogonadotropic hypogonadism, a rare condition of impuberism, defective gonadotropin secretion, and infertility of central origin. These explanations were the first to focus the crucial roles of GPR54 and its ligands in the control of key aspects of reproductive function. Such essential findings in humans were reinforced by the simultaneous report that mice engineered to lack functional GPR54 were a complete phenocopy of affected patients (Seminara *et al*., 2003), a condition that was later confirmed in Kiss1 null mice (Lapatto *et al*., 2007), even though Kiss1 KO (*knockout mouse)* animals appear to have a slighter reproductive impact than Gpr54-deficient mice (Colledge *et al*., 2009). This mixture of genetic (human) and functional genomic (mouse) studies concrete the way for the analysis of the physiological relevance and underlying mechanism of the reproductive actions of kisspeptins.

2.6. Molecular characterization of kiss/kissr system in non-mammalian vertebrates

Identification of the Kiss/kissr as a new regulatory system of Gnrh, and accordingly gonadotropin secretion, has revolutionized the understanding of the mechanisms for the neuroendocrine control of reproduction in mammals, and has also initiated related research in other vertebrate species (Sempere *et al.,* 2010; Zohar *et al.,* 2010). From 2004 onwards, a number of studies reported the isolation and (partial) characterization of kiss/kissr genes in non-mammalian vertebrates (Lee *et al.,* 2009; Akazome *et al*., 2010; Um *et al*., 2010). Divergent to mammals where, with the exception of the platypus (monotreme), only one gene coding for the ligand and one for the receptor are present. But compelling evidence has now demonstrated the presence of two distinct genes encoding kisspeptins (kiss1 and kiss2), and up to four different genes encoding their cognate receptors, gpr54s, in non-mammalian vertebrates, such as amphibians, reptiles and preferentially, bony fish (Um *et al.,* 2010; Zohar *et al.,* 2010).

2.7. Kiss/kissr system in fish

In fish, the first report of cloning of kiss genes in fish were released only in 2008 in zebrafish (*Danio rerio*) (Aerle *et al*., 2008). Following this, kisspeptin genes have been characterized from zebrafish (Kitahashi *et al*., 2009), medaka (*Oryzias latipes*) (Kanda *et al.,* 2008; Kitahashi *et al*., 2009), sea bass (*Dicentrarchus labrax*) (Felip *et al*., 2009), goldfish (*Carassius auratus)* (Li *et al*., 2009), grass puffer (*Takifugu niphobles)* (Shahjahan *et al*., 2010), orange-spotted grouper (*Epinephelus coioides)* (Shi *et al*., 2010), chub mackerel (*Scomber japonicas*) (Selvaraj *et al*., 2010) and striped bass (*Morones axatilis*) (Zmora *et al*., 2011). All of these teleosts possess the kiss2 gene, while the kiss1 gene is present only in the genomes of zebrafish, medaka, sea bass, goldfish and chub mackerel. All of the above studies have uniformly agreed that kisspeptins are involved in the process of sexual differentiation; the proper timing of puberty and the dynamic control of gonadotropin secretion, via stimulation of gnrh neurons etc. in fishes too.

2.8. Tissue distribution and expression of kisspeptin gene and Kissr system in fish

In fish, expression of the genes encoding kisspeptins and their receptors have been found in different parts of the brain namely, hypothalamus, telencephalon, thalamus, mid-brain tegumentum, olfactory bulbs and tracts, optic tectum, optic nerves, medulla oblongata, cerebellum and pituitary (Sempere *et al.,* 2012). The expression of these genes has also been reported in different tissues including testis, ovary, heart, muscle, stomach, intestine, spleen, liver, kidney, adipose tissue, pancreas, gills, eye and skin, to a variable extent depending on the fish species and gene (Oakley *et al*., 2009). The report on the expression of a non-mammalian gpr54 in gnrh neurons in the brain of tilapia (*Oreochromis niloticus*) provided the first evidence of a potential association of gpr54 with the gnrh system (Parhar *et al*., 2004). Two distinct kisspeptin receptor transcripts (gpr54-1b and gpr54-2b) have been isolated in a variety of fish species and its expression was examined by quantitative PCR (Sempere *et al*., 2012). The detection of gpr54-1b and gpr54-2b mRNAs in brain and gonads at different reproductive stages indicates their potential role in reproduction, although their functional importance may vary among gender and species (Oakley *et al*., 2009; Zohar *et al*., 2010).

Through in situ hybridization (ISH), fish neuronal kiss1 cell bodies in two hypothalamic nuclei, the nucleus posterioris periventricularis (NPPv) and the nucleus ventralis tuberis (NVT), was identified in medaka for the first time (Kanda *et al*., 2008). The number of neurons of NVT was larger in breeding than in non-breeding fish, and more numerous in males than in females. From these effects, it was concluded that kiss1 system is pivotal for the regulation of reproduction in that species. Later, Kitahashi *et al*. (2009) cloned a novel kisspeptin gene (kiss2) in zebrafish and medaka. Using ISH and laser capture micro-dissection coupled with real-time PCR, kiss1 mRNA expression in the ventromedial habenula and the periventricular hypothalamic nucleus, and kiss2 expressing neurons in the posteriortuberal nucleus and the periventricular hypothalamic nucleus was identified. Simultaneously, kiss1 and kiss2 genes were cloned in sea bass (Felip *et al*., 2009). Neuroanatomical distribution of their expression has been characterized by ISH assays, which point out that the mediobasal hypothalamus and the nucleus of the lateral recess are important area of expression of both kiss1 and kiss2 genes in european sea bass. Besides, kiss1 expressing cells were also found at the level of the habenular region in this fish species (Escobar *et al*., 2010). Two kisspeptin receptors genes have been characterized in the sea bass (Carrillo *et al*., 2009) and fascinatingly gpr54-1b and gpr54-2b-expressing cells have been shown to be mostly located in the same regions as their cognate ligands (Escobar *et al*., 2010). Furthermore, these authors observed that gnrh1-expressing neurons co-express gp54-2b indicating that they are target for kisspeptins via gpr54, in line with the functional observations of effective LH-releasing effects of kiss2 in this species.

2.9. Physiological actions of kisspeptins: major regulators of gonadotropin secretion and puberty in fish

The biological effects of kiss-10 forms after complete administration have been studied in some fish species. As significant end-points, the expression of gnrh genes in the brain and of gonadotropin subunits in the pituitary has been measured, and variations in gonadotropin levels in blood monitored. In early-mid pubertal fathead minnow, kiss1–10 injection provoked an increase in the expression of gpr54-2b and gnrh3 in the brain, but not of gnrh2 (Filby *et al*., 2008). However, systemic injections of kiss1–10 and kiss2–10 in sexually mature female zebrafish did

not stimulate any expression variation in gnrh3 (hypophysiotropic form) or gnrh2 (Kitahashi *et al*., 2009). In orange spotted grouper, whose genome contains only kiss2, the administration of the kiss2–10 peptide in sexually mature females evoked an increase in hypothalamic expression of gnrh1 (Shi *et al*., 2010). Lastly, in sea bass, no variation of Gnrh1 content was detected in brain or pituitary after injection of kiss1–10 or kiss2–10 in prepubertal and pubertal fish (Felip *et al*., 2009)

At the pituitary level, total administration of kisspeptins induced detectable responses in all cases, either in gene expression or hormone secretion. In female zebrafish, kiss2–10 was significantly more potent than kiss1–10 inducing expression of the FSHβ and LHβ subunits (Kitahashi *et al*., 2009). In female orange spotted grouper, kiss2–10injection also activate an increase in Fshβ expression, but had no effect on expression of the LHβ gene(Shi *et al*., 2010). In the European sea bass and goldfish, the effects of kiss-10 administration on gonadotropin release from the pituitary were analyzed by measuring gonadotropin levels in blood. In sea bass, kiss2–10 was more potent thankiss1–10 in inducing LH and FSH release in pre-pubertal fish, and LH secretion in pubertal males (Felip *et al*., 2009). On the other hand, kiss1–10 administration in goldfish significantly increased blood LH levels in a dose dependent manner, while kiss2–10 showed no effect (Li *et al*., 2009). Selvaraj *et al.* (2010) suggest the possible involvement of two Kiss genes in the brain and Kiss1 in the gonads of chub mackerel during seasonal gonadal development. Kiss2/Kiss1r system plays an important role in the regulation of reproductive function in the spawning period of grass puffer, possibly through the stimulation of gnrh1 secretion (Shahjahan *et al*., 2010). Kissr1 and kissr2 possible contribute in the seasonal ovarian development of female and males of chub mackerel (Ohga *et al*., 2013). kisspeptin-10 stimulated the release of luteinizing hormone-releasing hormone (LHRH) from in vitro hypothalamic explants and peripheral administration of kisspeptin-10 increased plasma LH, FSH and total testosterone (Thompson *et al*., 2004). Kisspeptin-10 plays an important role in the HPG axis activity and affected sexual maturation and final maturation of the gonads of clownfish (Kim *et al*., 2014). Saadeldin *et al*. (2011) shows kisspeptin enhances oocyte *in vitro* maturation but has an adverse impact on hatched blastocysts during *in vitro* culture. In striped bass females, Kiss1 stimulated *Fsh*β expression, although

hypophysiotropic *gnrh1* and *gnrh* receptor expression remained unchanged (Zmora *et al*., 2014).

It is now recognized that kisspeptins play an important role in the timing of puberty in animals. While characterization of the roles of kisspeptins in the control of puberty in fish is still at its infancy, recent studies performed in the very diversified and evolutionary ancient group of teleosts, such as zebra fish, gray mullet (*Mugil cephalus*), fathead minnow (*Pimephales promelas*), tilapia and cobia (*Rachycentron canadum*), have suggested that kisspeptin pathways are also involved in timing the onset of puberty in these non-mammalian species (Mohamed *et al*., 2007; Nocillado *et al*., 2007; Biran *et al*., 2008; Filby *et al*., 2008; Martinez *et al*., 2008).

2.10. Nanoparticles for drug delivery

Nanotechnology is a highly promising technology that spans many areas of science and technological applications. Among the recent advancements in science, nanotechnology is fast emerging as the new science and technology platform for the next generation of development and transformation of agri-food systems (Kuzma and Verhage 2006). Rapid advancements in nanosciences and nanotechnologies in recent years have opened up new horizons for many industrial and consumer sectors that have been regarded as the hotbed of a new industrial revolution including agriculture and allied sectors.

Nanotechnology has provided the opportunity of delivering drugs to specific cells using nanoparticles. The overall drug consumption and side-effects may be lowered significantly by depositing the active agent in the specific region only in minimum dose. The major goals for research of nano-bio-technologies in drug delivery include:

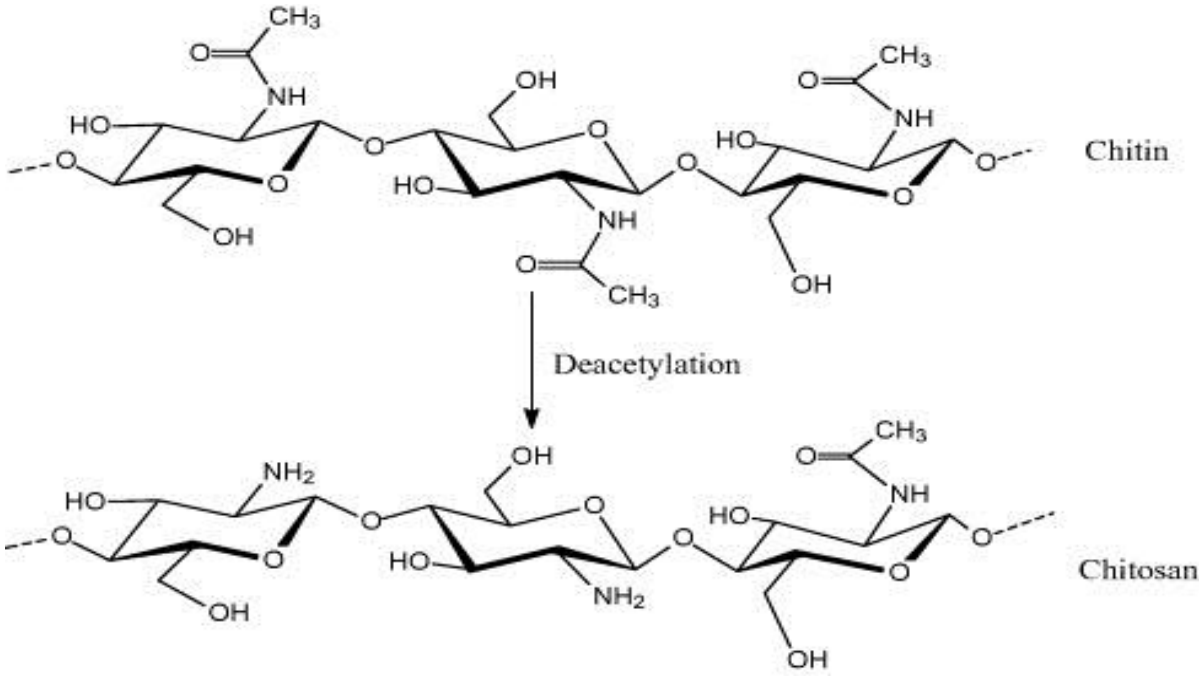
- More specific drug targeting and delivery,
- Reduction in toxicity while maintaining therapeutic effects,
- Greater safety and biocompatibility, and
- To improve drug bioavailability

There are several types of nanoparticles such as Polymeric nanoparticles, like chitosan, poly-lactide-coglycolide acid (PLGA), inorganic nanoparticles e.g silver, gold, carbon nanoparticles like fullerenes, carbon nanotube, quantum dots, liposome, etc have been used for drug delivery .

2.11.Use of chitosan for development of nanoparticles and delivery

Various concerns have been expressed over the biocompatibility of the nanoparticle that have been developed and its ill effect on the biological system. Hence focus is on for developing biodegradable nanoparticles. A number of different polymers, both synthetic and natural have been utilized in formulating biodegradable nanoparticles (Moghimi *et al.,* 2001). Of these polymers, chitosan and poly (lactic-*co*-glycolic acid) (PLGA) and polylactides (PLA) have been the most extensively investigated for drug delivery (Jain, 2000).

Chitosan is a natural cationic biopolymer formed by the hydrolysis of chitin. One visible improvement of this substance is that it can be obtained from ecologically sound natural sources, namely shrimp shell and crabs. Together with chitin, chitosan is the second most abundant polysaccharide subsequent to cellulose. However contrasting cellulose, the employ of chitosan as an excipient in pharmaceutical formula is a pretty new development. Chitosan has been widely premeditated in the biomedical field and has been found to be highly biocompatible. In addition to that, chitosan is a biodegradable polysaccharide derived by partial deacetylation of chitin, which is copolymer of glucosamine and N-acetyl-d-glucosamine linked together by ß(1,4) glycosidic bonds. The structural formula of chitin and chitosan is given below



Chitosan has been widely used in pharmaceutical and medical areas, due to its favourable biological properties such as biodegradability, biocompatibility, low toxicity, hemostatic, bacteriostatic, fungistatic, anti-cancerogen, properties (Hejazi *et al.,* 2003). Nanoparticles of chitosan has been extensively examined for its potential in the development of the controlled release drug delivery system like peptides, protein antigens, oligonucletiodes and genes (Kavshima *et al.,*1985; Rajeshkumar *et al*., 2009; Ramya *et al*., 2014). Insulin–chitosan nanoparticles were prepared by the ionotropic gelation of chitosan glutamate and tripolyphosphate penta sodium and simple complexation of insulin and chitosan (Dyer *et al.,* 2002). Polymeric nanoparticles have been extensively studied as particular carrier in the pharmaceutical and medical fields because of their controlled and sustained release properties, sub cellular size and biocompatibility with tissue and cells (Reis *et al.,* 2006). Chitosan acts as a penetration enhancer by opening the tight epithelial junction and hence is particularly exploited in protein and vaccine (Van *et al.,* 2001). Chitosan nanoparticles for delivery of polypeptides such as insulin, tetanus toxoid, and diphtheria toxoid are widely explored (Xu and Du 2006; Vander *et al*., 2003). Chitosan reduced gold nanoparticles as novel carriers for transomucosal delivery of insulin were developed (Devika *et al.,* 2007). High molecular weight chitosan solution has already been proposed as vehicles for protein delivery (Chun *et al*., 2007).

3. MATERIALS AND METHODS

3.1. Animals and tissue sampling

Adult *Catla catla* used in the experiment were collected from Powarkherda (Hosangabad, Madhya Pradesh) Centre ICAR-Central Institute of Fisheries Education (CIFE) Mumbai, India. The animals were brought to wet laboratory of CIFE, Mumbai and were acclimatized for 20 days. The fish were kept in FRP tank (500L) and were daily fed twice with commercially available fish feed pellets containing 30% of protein content. For cDNA cloning and tissue distribution analysis, brain, ovary, testis, intestine, kidney, gills and liver samples were collected in RNA *later*™ (Qiagen, Germany) and stored at -80^{o}C.

3.2. Chemicals, reagents and kits

Chemicals used in this study were generally of molecular biology grade. Agarose, ethidium bromide,TAE buffer, LB agar and LB broth were purchased from HiMedia, India. Ampicillin and ethanol were procured from Amresco, USA. 2x PCR master mixes, nuclease free water, 6x gel loading dye, X-gal and IPTG were purchased from Thermo Fisher scientific, USA. Trizol reagent was procured from Invitrogen (USA). Taq DNA polymerase and restriction endonucleases such as *Bam*HI, *Eco*RI and *Hind*III were acquired from Thermo Fisher scientific, USA. T4DNA Ligase was provided with InsTAclone PCR Cloning kit, Revert aid reverse Transcriptase (RT) was delivered with Revert Aid First Strand cDNA Synthesis kit **(**Thermo Fisher scientific, USA) and Maxima SYBER Green qPCR Master mix (Thermo Fisher scientific, USA). Kits used in this study were SMARTer™ RACE cDNA Amplification Kit (Clonetech, USA)**,** Gel Extraction Kit (Thermo Fisher scientific,USA) and Qaigen (USA).GeneJET plasmid miniprep Kit (Thermo Fisher scientific, USA) .

3.3. Equipments and Laboratory ware

ABI PCR system (Applied Biosystems, USA), TaKaRa PCR Thermal Cycler Dice (Takara bio, Japan) Nanodrop spectrophotometer (Thermo Fisher scientific, USA),horizontal gel electrophoresis apparatus (Bio-Rad, USA), shaking incubator (ORBITEK, India), gel documentation system(DNr Bio-Imaging Systems,Israel), biophotometer (Eppendorf, Germany), tissue homogenizer (Tissue

Tearor, USA) thermostatic water bath (ESCY, USA), electronic balance (OHAUS, USA), tabletop centrifuge 5430 (Eppendorf, Germany), incubator (Wadiegarfl India), microwave oven (Kenstar, India), autoclave (TOMY, Japan), Hot owen (Newtronic, India), micropipettes (Eppendorf, Germany) and UV transillummator (UVP, USA), Real time PCR (LightCycler® Roche, USA), 96-well low-profile semi-skirted PCR plate for the fast block of Life Technologies PCR and qPCR thermal cyclers (Thermo Fisher scientific, USA) were the equipments used. The glassware used in this study was from Borosil, India and plasticware from Tarsons, India.

3.4. Bacterial strains and vector

3.4.1. Bacterial strains

E. coil DH5α, a recombinase A deficient amber suppressing strain available at Molecular biology lab, Fish Genetics and Biotechnology Division, Central Institute of Fisheries Education was used as host strain. The genotype of DH5α is supE44 ΔlacU169 (ψ80 lac Z ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1.

3.4.2. Culture conditions

DH5α culture was maintained on LB agar plates and stored at 4°C for regular use. Sub-culturing was done every 20 days to maintain the cultures. For long term storage, 1.5ml aliquot of overnight (O/N) grown LB broth culture was transferred to a 2ml screw-cap tube and 0.5ml of autoclaved glycerol (60%) was added. The tube was vortexed briefly to ensure complete dispersal of glycerol, snap frozen in liquid nitrogen and stored at -80°C indefinitely. To recover the culture, frozen surface of the culture was scraped with a sterile pipette tip, streaked on a selective agar plate and incubated overnight at 37°C.

3.4.3. Vector/Plasmid

Plasmid pTZ57R/T having ampicillin resistance gene provided along with InsTAclone PCR cloning kit (Thermo Fisher scientific, USA) was used for T/A cloning of the PCR amplified products.

3.5. Nucleic acid isolation

3.5.1. Total RNA isolation

Total RNA was isolated from brain and gonads of *C. catla* following Sambrook *et al*., (2001) with slight modifications. Tissue sample (100mg) was taken directly in a 2.0 ml microfuge tube and homogenized in 1ml Trizol™ reagent (Invitrogen, USA) using sterile forceps and tissue homogenizer (Tissue Tearor, USA) for 20-30s at r.t. The homogenate was incubated for 5 min at r. t. to allow lysis and was centrifuged for 5 min at 10000 rpm. The aqueous phase was transferred to a fresh tube. To the lysate, 200µL of chloroform (0.2 vol/ml Trizol™ reagent) was added and mixed by vigorous vortexing and incubated at r. t. for 10 min. The sample was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected without disturbing the interface and transferred to a fresh 1.5 ml tube. The RNA was precipitated by adding 500µL of ultrapure isopropanol (0.5 vol/ ml Trizol™ reagent) for 10 min at r. t. and subsequent centrifugation at 12000 rpm for 15 min at 4°C. The precipitated RNA was washed twice with 1ml of 70% ethanol. The RNA pellet was vacuum dried for 10 min and dissolved in 30 µL of nuclease free water and stored at -80°C for further use.

3.5.2. Quantification of RNA

The concentration of the isolated RNA was measured using Nanodrop spectrophotometer (Thermo Fisher scientific, USA). Nucleic acid concentration was obtained directly in terms of ng/µL along with the 260: 280 ratios. The ratio of the absorbance at 260 nm and 280 nm provided the estimate of the purity of the isolated RNA.

3.5.3. Gel electrophoresis of RNA

The integrity of the isolated RNA was checked by electrophoresing on 1% agarose gel. Gel casting apparatus used for electrophoresis of RNA was soaked in 1% SDS in ethanol (2hrs, 37^{o}C) then rinse with DEPC treated water and heated to 100^{o}C for 15 minutes to inactivated RNase enzymes. Appropriate amount of agarose (0.4 g) was added to 40 ml of 0.5 X TAE buffer and melted in a microwave oven till the solution became completely clear. The solution was cooled and 2µL of ethidium bromide was added and mixed thoroughly. The solution was poured into the comb fixed gel tray and allowed to solidify. The comb was removed carefully and the gel tray was placed in the gel electrophoresis buffer unit containing 0.5 X TAE buffer.

Appropriate volume of RNA (2 µL) was mixed with 1 µL of 6X gel loading buffer and loaded into the gel slots. The unit was connected to power supply and the gel was run at 80 V for 1h. The gel was then taken out and documented in a gel documentation unit (Bio-Rad, USA).

3.5.4. DNase treatment

Total RNA isolated using Trizol™ reagent contains significant amounts of genomic DNA and RNA was treated with 'RNase free' DNaseI (MBI Fermentas, USA) before cDNA synthesis. A mixture of 1 µg of total RNA, 1 µL of Buffer (10x) and 1µL DNAse was taken in PCR tube made up to 10 µL by adding nuclease free water. The reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 2µL of 25mM EDTA followed by a 10 min exposure at 65°C.

3.5.5. Reverse Transcriptase PCR (RT - PCR)

The mRNA pool was converted to its complementary DNA using the Revert Aid reverse transcriptase First Strand cDNA Synthesis kit (Thermo Fisher scientific, USA) as per the manufacturer's instructions. Briefly, 1 µg of the RNA sample and 1 µL of oligo-dT was mixed with nuclease free water to make a final volume of 10 µL in a PCR tube. This mixture was then incubated at 60°C for 5 minutes and immediately chilled in ice for 5 min subsequently 4 µL of 5X reaction buffer, 2µL of 10Mm dNTP mix, 1 µL of RiboLock RNase inhibitor (20 u/µL) and 1 µL of RevertAid M-MuLV Reverse Transcriptase (200u/ µL) were added to above mixture and make the final volume made upto 20µL.The solution was mixed thoroughly, centrifuged in table top centrifuge and incubated at 4°C for 1h and the reaction was terminated by incubated at 70°C for 5 min. The synthesized first strand cDNA was directly used in PCR to amplify the gene using specific primers.

3.6. Primers

Specific primers were designed for PCR amplification of partial sequences of kisspeptin genes kiss1, kiss2, kiss receptor (kiss1r) and kiss2r transcripts based on the conserved regions of kisspeptin coding sequences from the closely related fish species using Gene Runner software, version 3.05. The factors such as primer length, %GC content, melting temperature, annealing temperature, secondary structures were taken into consideration while designing the primer sets.

Primers were synthesized commercially by Eurofin, company, Bangalore. The primers were received in lyophilized from the supplier. They were dissolved in sterile TE buffer (pH 7.0) as per the instructions received to get a stock solution of 100 pmol/ µL concentration. The stock solution was then diluted to get a working concentration of 10pmol/ µL with NFW. List of primers used in this study is given in Table 1.

Table1. List of primers used for PCR amplification

Primer name	Purpose	Primer sequence
Kiss1F	Normal PCR	ATCACTCCAGGTTCTCAGGGGAACTGA
Kiss1R	Normal PCR	CATAGCGGAGGCCGAAGGAATTGAG
GSP1- 3'	RACE	GGTGCAGGTCCTCAGCGAAACACATGGTG
GSP2- 3'	RACE	AATCTCAATTCCTTCGGCCTCCGCTATCATA
GSP1- 5'	RACE	GGAGACCACCACCATGTGTTTCGCTGAG
GSP2- 5'	RACE	GAGGACCTGCACCCATGGAGAAGTGCATTG
Kiss2F	Normal PCR	AGTGGAGCGGAGGCAGTTTGATGAGCC
Kiss2R	Normal PCR	AGGGCGTGCCTGAAATAATTACCCACA
GSP1- 3'	RACE	ACGGACTCTGACGGACCCAAACACAAGCA
GSP2- 3'	RACE	CAGATGGAAGGACGTGGTTTGCTTGGTTG
GSP1- 5'	RACE	AATCGATGTTTGCAGGAAATGTGACTCG
GSP2- 5'	RACE	GCTTGCGTCGTCTGAAGCGCTGGGCTCG
Kiss1rF	Normal PCR	GCTCCATTGGAAACTCCTCTATGGAGGA
Kiss1rR	Normal PCR	AATAGGGTTGATAGAGGAGTTGGCATAG
GSP1- 3'	RACE	AGGTCCACCTGCTCTCAGAGAGAACCATC
GSP2- 3'	RACE	GTGGTCATTGTTGTTCTCTTCACCATCTGC
GSP1- 5'	RACE	CATTGAGGAGTTTCCAATGGAGCTGTTCA
GSP2- 5'	RACE	AGACTTGTGAAAGACCTTGAAACAAGATGG
Kiss2rF	Normal PCR	CCACTGAGGTTGCAGAACTAATCTTGTG

Kiss2rR	Normal PCR	GCCTCTTCTGAATGAGAGATGGAAAGG
GSP1- 3'	RACE	CGGTCCACAGACGTACTGCACCGAGGCC
GSP2- 3'	RACE	GATCACCATCTGCATGTGTTACACCTTC
GSP1- 5'	RACE	ATTAGTTCTGCAACCTCAGTGGTGCTGTTG
GSP2- 5'	RACE	CACTGGCACCAGCCAGGCGTCTGTCAGTGG
UPM	RACE	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGC
NUM	RACE	AAGCAGTGGTATCAACGCAGAGT

3.7. PCR amplification

The composition of 20µL PCR reaction mixture was given as given below.

Reaction components	**Volume**
10X reaction buffer	2.5 µL
10mM dNTP	0.5 µL
Forward primer	1 µL
Reverse primer	1 µL
cDNA	1 µL
Taq polymerase	0.25 µL
$MgCl_2$	2.5 µL
Water	11.25 µL
Total	20 µL

Amplification was carried based on Tm of primer of that particular gene, using the following PCR conditions in thermal cycler (Takara, Japan). Amplification was performed in a 96-well Takara PCR System (Takara, Japan). PCR conditions included an initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 0.30 sec, 58°C and 60°C for 0.45 sec min and 72°C for 1 min and a final extension of 10 min at 72°C for kiss1 gene. PCR product were visualized on 1% agarose gel. The appropriate bands were eluted from agarose gel as given below.

3.8. Elution of PCR product from the gel

The selected DNA band was excised from the gel and eluted using Gel Extraction kit (Qiagen, USA). The excised gel was loaded in a microcentrifuge tube and weighed. Three volumes of Buffer QG were added to 1 volume of the gel and the tubes were incubated at 50°C for 10 min in a thermostatic waterbath. The tubes were vortexed every 2-3 min during the incubation. One volume of isopropanol was added to the dissolved gel-buffer mixture and was mixed properly. Then the samples were applied to the spin columns and centrifuged at 10,000 rpm for 1 min to bind the DNA to the column and the flow through was discarded. The column was washed with 0.75ml of wash buffer to remove the buffer and other impurities from DNA. The column was centrifuged again to remove the residual wash buffer. Following this, the spin column was placed in a new sterile 1.5 ml micro centrifuge tube and 30µL of elution buffer was added to the center of the column membrane. The spin column was allowed to stand for 1 min and was centrifuged at 10,000 rpm for 3 min. The resulting DNA fragment was checked on a 1.5% agarose gel.

3.9. T/A Cloning

PCR products amplified by Taq polymerase have an extra single A added to 3' end of the PCR product. It allows these amplicons to be cloned into vectors that have been specifically designed to carry a T overhang on the complementary strand (called T/A vectors). The desired DNA fragments were cloned into pTZ57R/T vector using InsTAclone™ PCR Cloning Kit (Thermo Fisher scientific, USA) following manufacture protocol. The resultant PCR product with ‘A’ overhang was mixed with the vector in 3:1 proportion of molar ends in 1X ligase buffer. Five units of T4 DNA ligase were added and the ligation mixture was incubated at 16°C

overnight. Finally 3µL of ligation mixture was transformed into *E. coli* DH5α competent cells.

3.10.1. Ligation

The insert was ligated into a pTZ57R/T vector provided with the InsTAclone PCR Cloning kit (Thermo Fisher scientific, USA). The ligation mixture was prepared out in 30µL total volume by adding the following components, 6 µL of 5X Ligation buffer, 3µL of pTZ57R/T vector, 4 µL of purified PCR fragment, 1µL of T4 DNA Ligase and make the final volume upto 30µL with nuclease free water. The reaction mixture was mixed by pipetting and incubated at 16°C overnight. The ligation mixture vector used for the transformation was DH5α strain of *E. coli.*

3.10.2. Transformation

Transformation was carried out using the InsTAclone PCR Cloning kit (Thermo Fisher scientific USA) as per the protocol supplied by the manufacturer. DH5α strain of *E. coli* was inoculated into 5 ml of LB broth taken in a sterilized test tube and the culture was incubated overnight at 37°C in a shaking incubator. An amount of 1.5 ml of fresh C-medium was taken in a micro-centrifuge tube which was pre-warmed at 37°C. To this solution, 150 µL of the overnight culture was added and incubated at 37°C for 20 min in a shaking incubator at 160 rpm. The bacterial culture thus obtained was used for transformation. T-solution was prepared by mixing equal volumes of T-solution (A) with T-solution (B) and was kept on ice. *E. coli* culture was centrifuged at 10,000 rpm for 1 min. Cell pellet was re-suspended in 300 µL of T-solution and the tube was incubated on ice for 5 min. Again the cells were pelleted down at 10000 rpm for 1 minute and the supernatant was discarded. The cells were resuspended in 120 µL of T-solution and incubated on ice for 5 min. For 2 minutes 2.5 µL of ligation mixture was held in ice, to which 50 µL of the re-suspended cells were added and incubated on ice for 5 min. Finally the cells were spread plated on the pre warmed X-gal-IPTG and Ampicillin added LB agar plates and incubated overnight at 37°C in an incubator until the transformed bacterial colonies were visible.

3.10.3. Analysis of recombinant clones

3.10.3.1. Blue white selection

The primary confirmation of the clones was done by a-complementation and white colonies were selected from IPTG/X-gal Ampicillin plates. The colonies that were fully or partially blue were discarded. The isolated white colonies were picked up to prepare a master plate. The confirmation of clone was done by colony PCR followed by RE digestion of the isolated plasmid.

3.10.3.2. Colony PCR

White colonies were screened for the presence of inserts using colony PCR. Colony PCR was performed with gene specific primers for rapid confirmation of recombinant clones. The white colonies were picked individually using sterile disposable inoculation loop and suspended in 10 µL of TE. The PCR reaction mixture comprised of 10 µL of 2X PCR master mix, 1 µL each of 10 pmoles gene specific forward and reverse primers, 1 µL colony suspended in TE solution and 7 µL nuclease free water. The PCR cycling conditions included an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30s, annealing at 55°C and extension at 72°C for 1 min. Final extension was done for 5 min. The insert size was determined by running the PCR product on an agarose gel against a molecular size standard.

3.11. Plasmid DNA isolation

Plasmid DNA from the recombinant clone was isolated using the Gene JET plasmid miniprep kit (Thermo Fisher scientific, USA) as per the manufacturer's protocol. A positive colony was picked from the master plate and grown overnight in 5 ml LB broth with 50 µL /ml ampicillin at 37°C in a shaking incubator. The bacterial culture was harvested by centrifugation at 12,000 rpm for 5 min to obtain the cell pellet. The bacterial cells were then re-suspended in 250 µL of resuspension solution (P1) by vortexing. Then 250 µL of lysis solution (P2) was added and mixed well by inverting the tube followed by incubation at room temperature for less than 5 min. To this mixture 350 µL of neutralization solution (P3) was added and mixed immediately by inverting the tube. This mixture was then centrifuged at 12,000 rpm for 10 min. The supernatant was pipetted out and transferred to the spin column with a collection tube. The spin column was centrifuged at 12,000 rpm for 1 min and the flow through was discarded. Then, 500 µL of wash buffer was added to the spin column and centrifuged at 12,000 rpm for 1 min. The flow through was discarded and 750 µL of

diluted wash solution was added to the spin column which was centrifuged at 12,000 rpm for 1 min. The flow through was discarded and the column was again centrifuged at 12,000 rpm for 1 min to remove the residual wash buffer. The spin column was placed in a new collection tube and 30 µL of nuclease free water was added to the center of the spin column and it was allowed to stand for 1 min. Then the spin column was centrifuged at 12,000 rpm for 1 min and the recombinant plasmid DNA was eluted. The integrity of the isolated plasmid was checked on 1% agarose gel.

3.12. Restriction enzyme digestion of recombinant plasmid

Restriction digestion was performed to confirm the presence of the insert cloned in pTZ57R/T vector. An aliquot of the purified plasmid DNA was digested with Eco *RI* and Hind*III* using conditions specified by the manufacturer. A total volume of 20 µL reaction mixture was prepared by adding 1 µL of purified plasmid DNA, 2 µL of 10x fast digestion buffer, 1 µL of Hind*III* and 13 µL of nuclease free water This reaction mixture was incubated at 37°C for 10 min. The insert sizes were confirmed against a molecular size standard. All the inserts were confirmed by sequencing.

3.13. Sequencing

The confirmed recombinant plasmid was sequenced using ABI Big DYE terminator method (Eurofins, Bangalore, India). The sequence from the sequencer was obtained in a chromatogram. The data obtained in chromatogram was analyzed using Chromas LITE 201 software. The obtained partial sequence was having vector sequences attached to its flanking ends which were removed and BLASTn was done to confirm its identity.

3.14. Rapid Amplification of cDNA Ends (RACE)

The full-length cDNA was obtained by amplification of *cDNA* ends using RACE (Rapid Amplification of cDNA Ends) technique. RACE is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and either the 3' or 5' end of the mRNA. Hence it is called 5' RACE or 3'RACE depending on which end of the mRNA is amplified. This methodology of amplification with single-sided specificity has been described by

others as “one-sided" PCR or "anchored’ PCR. In this study both 5' and 3' RACE were performed using SMARTer™ RACE cDNA Amplification Kit (Clonetech, USA) using manufacturer’s protocol.

3.14.1. RACE PCR cDNA synthesis

For 5' and 3' RACE, cDNA was synthesized according to manufacturer’s protocol as given below-

Step I:-

5X First-Strand Buffer	2.0 µl
DTT (20 mM)	1.0 µl
dNTP Mix (10 mM)	1.0 µl
Total Volume	4.0 µl

The contents were properly mixed in a PCR tube and kept at room temperature. The resulted mixture was called as Buffer Mix.

Step II:

Following reagents were added in separate PCR tubes, mixed properly and centrifuged briefly.

For preparation of 5’ -RACE-Ready Cdna	**For preparation of 3’-RACE-Ready cDNA**
1 µl RNA(1000ng/µl)	1 µl RNA
1.0 µl 5’ -CDS Primer A	1.0 µl 3’-CDS Primer A:
0.75 µl NFW and 1 µl of the SMARTer IIA oligo	2.75 µl NFW

After centrifugation, the tubes were incubated at 72°C for 3 min, then cooled at 42°C for 2 min. After cooling, tubes were centrifuged briefly for 10 seconds

at 14,000 rpm to collect the contents at the bottom. Put the buffer mix in a microcentrifuge tube and added, 0.25 µl RNase inhibitor (40U/µl), 1.0 µl SMARTScribe™ Reverse Transcriptase (100 U) and mixed thoroughly. Denatured RNA from Step II was added to the above master mix, for a total volume of 10 µl. The contents of the tubes were mixed gently by pipetting, and the tubes were centrifuged briefly to collect the contents at the bottom. Tubes were incubated at 42°C for 90 min in hot-lid thermal cycler and the reaction was terminated at 70°C for 10 min. The first-strand reaction products were diluted with Tricine-EDTA Buffer and stored at –20°C. Gene specific primers were designed from the partial sequences of kisspeptin cDNA for amplification of flanking regions. As per the 3' RACE and 5' RACE protocol guidelines two sets of gene specific primers; GSP1 and GSP2 (nested) were designed for amplification of both the ends using software Gene runner V 4.0.9.68.

3.14.2. RACE PCR amplification

3.14.2.1. 3' RACE

The first strand cDNA prepared using oligo dT- adapter primer was amplified using a gene specific primer (GSP1) and a universal primer mix (UPM) (Table 1). The PCR conditions were optimized to obtain the specific product. To minimize nonspecific amplification touchdown PCR was used. After the primary PCR, nested PCR was performed using kisspeptin genes 3’ GSP2 primers and Nested universal primer mix (UPM) (Table 1), with diluted primary PCR products.

3.14.2.2. 5' RACE

The 5’ RACE was performed by using gene specific primer kisspeptin genes 5’ (Gsp1) and universal primer mix supplied with Clonetech kit (Table1). The touchdown PCR was performed to minimize nonspecific amplification. Following the primary PCR, nested PCR was performed using kisspeptin genes 5’ GSP2 primers (Table1) and 5’ Nested universal primer mix with diluted primary PCR products. After amplification both 5' and 3' PCR products were run on gel electrophoresis and expected bands were excised from the gel and then elution, ligation, transformation, colony PCR, plasmid extraction and conformation of plasmids were done in same way as described above for partial PCR product. The confirmed recombinant plasmid was sequenced using ABI Big DYE terminator method (Eurofins, Bangalore,

India). The sequence from the sequencer was obtained in a chromatogram. The chromatogram was converted into FASTA format by using the software Chromas LITE v.2.1.1 software. The resulted sequences were analyzed and then confirmed by using BLASTn from NCBI.

3.15. *In silico* sequence analysis

The partial, 5' and 3' sequences were aligned to generate a full length cds of kisspeptin genes cDNA using Gene Runner .V 4.0.9.68 software. *In silco* analysis of kisspeptin genes was done using different bioinformatic tools.

3.15.1. Bioinformatics tools

The confirmed recombinant plasmid was sequenced using ABI Big DYE terminator method (Eurofins Pvt. Ltd., Bangalore, India). The sequence data, obtained as a chromatogram, was converted to FASTA format using Chromas Lite 2.01 software. Homology searches of kisspeptin genes CDS was done with the basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ORF analysis was performed using NCBI ORF finder. Multiple sequence alignment of the deduced amino acid sequence was performed using Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/).The conserved protein domains were predicted by the simple modular architecture research tool (SMART) based on the sequence homology (http://smart.embl-heidelberg.de/).

3.15.2. Phylogenetic analysis

C. catla kisspeptin gene sequences were analysed using BLASTn and BLASTp programs provided by the online BLAST (basic local alignment search tool) software at the NCBI (National Center for Biotechnology Information) database for finding homology with other sequences. Highly similar sequences of the kisspeptin gene from different fish and other animal species were downloaded from NCBI GenBank. A neighbour-joining phylogenetic tree was constructed based on the deduced amino acid sequences of other reported genes using MEGA 6.0 software (Tamura *et al*., 2011) applying the amino acid substitution type and Poisson model (Zuckerkandl & Pauling, 1965).

3.16. Tissue distribution and ontogeny expression profile

Real-time PCR amplifications were carried out in LightCycler® Real Time PCR detection system (Roache, Switzerland). For tissues distribution sample from brain, gill, intestine, muscle, liver, kidney, ovary and testis were taken from three fishes and pooled. Animals were anesthetized using clove oil before dissection. Brains were collected using sterile scissors and forceps, taking care to avoid contamination and used for RNA isolation. For ontogeny study all development stages including unfertilized eggs and advance stages was screening for gene expression study. Primer used in this are study are given in Table 2.

Table 2. List of primers used for tissue distribution and ontogeny study

Name	Purpose	Sequences
qKISS1F	Tissue distribution and ontogeny	CATGGTGGTGGTCTCCAGAAAG
qKISS1R	Tissue distribution and ontogeny	GTAGCCGACCCAGTGATCCAAG
qbeta actin F	Real time PCR	ATGGTGTCACCCACACTGTGC
qbeta actin R	Real time PCR	GAAGTCAAGAGCCACATAGCAGA

3.17. Preparation of Chitosan Nanoparticles

Chitosan nanoparticles were prepared based on ionic gelation method (Calvo *et al.* 1997) of chitosan and tri-polyphosphate (TPP) anion with little modification. Briefly, 2mg of chitosan was dissolved in 100 ml aqueous acidic solution to obtain the cation of chitosan. This aqueous solution was prepared with 80 ml of water, 15 ml of TPP and 5 ml of acetic acid. The solution was subjected to the constant magnetic stirring for 10 min and the pH of the solution was adjusted to 6.5. During the process involving chemical reaction, chitosan undergoes ionic gelation and precipitates to form spherical particles.

3.17.1.Nano-conjugation of the Kisspeptin-10 (K-10) with chitosan (CK-10)

For conjugation of the nanoparticles, a high pressure magnetic stir process was used. A stock solution of **kisspeptin-10 or KISS-1 (**metastin 45-54 amide; Sigma, USA) that has 80% similarity with fish species was prepared with 0.5 µg/ml of water (Kim *et al.,* 2014). From this stock solution, 500µl was added to chitosan nanoparticles. Then concentration of the protein, i.e., **Kisspeptin-10 (K-10)** in the solution was measured at 660 nm (Lowry *et al*., 1951). After that the solution was subjected to high pressure magnetic stirring for 60 min and kept for overnight in refrigerator at 4°C. Next day, solution was centrifuged at 20,000rpm, supernatant was collected and the concentration of protein was again determined (Lowry *et al.,* 1951). The entrapment efficiency (EE) of the K-10 in chitosan was calculated using the formula;

$$EE = \frac{\text{Total k-10- Free k-10 in supernatant}}{\text{Total k-10}}$$

3.17.2. Characterization of CK-10 Nanoparticles

Mean particle size and zeta potential of CK-10 were determined by using Horiba scientific Nanoparticle Analyzer SZ-100 series (Kyoto, Japan) at 25°C. Particle size analysis is performed by dynamic light scattering (DLS) method. For this, 10µl of the nanoconjugate suspension sample was mixed with 90 ml of deionized water and added to a cuvette. The cuvette was well-shaken by hand and placed immediately inside the sample holder of the particle size analyzer. Once the required intensity was reached, analysis was performed to obtain the mean particle size (n=3) and polydispersity index (PDI) of the sample. Measurement of the zeta potential gives an idea about the stability of nanoparticles and it was determined based on laser doppler electrophoresis technique in the same instrument.

3.18. *In vivo* experiment

Adult fishes (average body weight 150-200g) were used for the experiment study. The animal experiment in this study included three treatments with three replicates. Each replicate contained four male and four female fish in each tank. The first group was maintained as a control in which fishes were given

phosphate *buffer* saline (PBS). In second group, intra-muscular injection of K-10 @ 0.2 ml/kg body weight of fish and in last group chitosan conjugated K-10 (CK-10) was given. The injections were given using a 1.0 ml syringe fitted with 22 gauge needle. Blood was collected at 2^{nd}, 4^{th}, 6^{th} 8^{th} and 12^{th} hour post-injection in all the groups. At pituitary level, effect of kisspeptin-10 was realized by analysing LH and FSH hormones from serum of fish. Similar 11-keto testosterone and 17-β oestradiol was measured by using immunoassay (EIA) kit (Cayman Chemical, USA) as per manufacturer's instructions. The ELISA plates were read using Biotek Microplate Instrumentation (Winooski, VT).

3.18.1 Collection, Preparation of Tissue Samples and Histology

After two months of administration of kisspeptin, fishes were anaesthetized with clove oil (50 µl/l)) until they remained motionless. When there was cessation of heartbeat/respiration and the fish were unresponsive to external stimuli and had lost all reflexes, fish were removed from water. Ovary was dissected out and fixed in aqueous Bouin's fluid for 24 h. After fixing, tissues were washed in 70% alcohol in various changes till the yellow colour of picric acid was removed. Further, the ovaries were dehydrated in ascending grades of ethanol, cleaned in xylene and embedded in paraffin wax (58–60°C congealing point). From the paraffin block, section of 6–8 µm thickness were prepared using a rotary microtome and stretched on albumenised slide. The slides were fixed at 60°C overnight. The next morning, sections were deparaffinised in three changes of xylene and dehydrated in descending grades of alcohol to distilled water. The slides were stained in haematoxylin for 20 min, differentiated in 1% of alcohol and blued in ammonia water. After washing, sections were stained with eosin (working) for 10min. Dehydrated and cleaned sections were then mounted in DPX (Distrene, Plasticiser, Xylene) (Luna 1968) and observed under microscope to record gonadal changes through microphotography.

3.19. Ethics Statement

The research undertaken complies with the current animal welfare laws in India. The care and treatment of animals used in this study were in accordance with the guidelines of the CPCSEA [(Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment & Forests (Animal

Welfare Division), Govt of India] on care and use of animals in scientific research. The study was approved by the Board of Studies and authorities of the Central Institute of Fisheries Education (Deemed University), Mumbai-61. As the experimental fish *C. catla* is not an endangered fish, the provisions of the Govt. of India's Wildlife Protection Act of 1972 are not applicable for experiments on this fish.

3.20. Statistical Analysis

The differences in transcript levels between treatments were tested for statistical significance using one-way ANOVA. Data on serum hormone levels was subjected to analysis of variance (ANOVA), followed by Duncan's Multiple Range Test with the help of SPSS-16.0 version software. All the data analysis was expressed as mean ± standard error.

4. RESULTS

4.1. Isolation of total RNA

The total RNA isolated from the brain of *C. catla* was quantified using Nanodrop spectrophotometer (Thermo Fisher scientific, USA) and concentration was found to be 2000ng/µl. The ratio of absorbance at 260/ 280nm was 1.98, which indicated that the purity of the isolated RNA was good. The integrity of the extracted RNA was verified by running it on a 1% agarose gel, which showed clear separation of 28S and 18S rRNA bands.

4.2. PCR amplification of kisspeptin gene partial cDNA

A partial cDNA fragment of kiss1, kiss2 and kiss1r and kiss2r (kisspeptin gene) were amplified from the brain total RNA of *C. catla* using RT-PCR. The partial cDNA fragments of 200, 220, 900 and 982bp were amplified using the specific primer sets for kiss1, kiss2 and kiss1r and kiss2r, respectively (Fig. 4 - 7). For full-length characterisation of kiss1, kiss2, kiss1r and kiss2r, 5´ and 3´ flanking regions of the partial sequence were amplified using RACE-PCR.

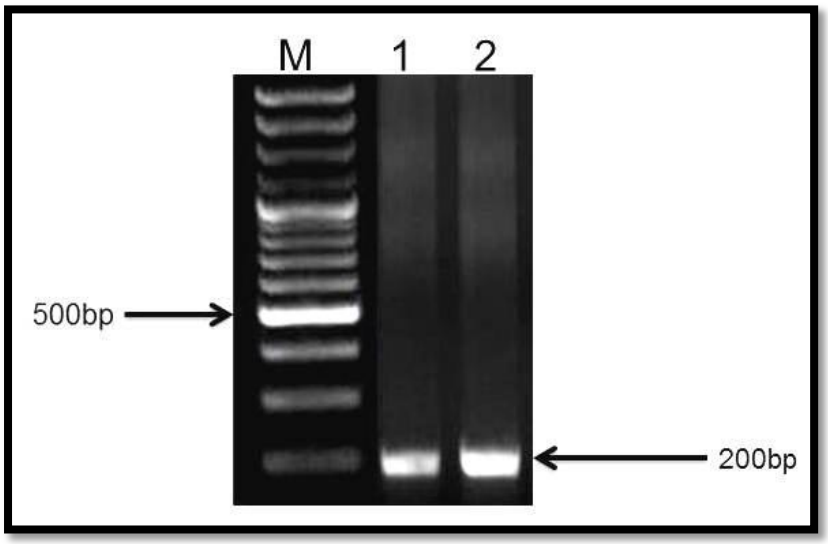


Fig. 4. PCR amplification of kiss1 cDNA fragment of amplicon size 200bp. Lane M; 100bp plus ladder (Thermo Fisher scientific USA), lane 1-2: Amplified products at 60°C and 58°C annealing temperature

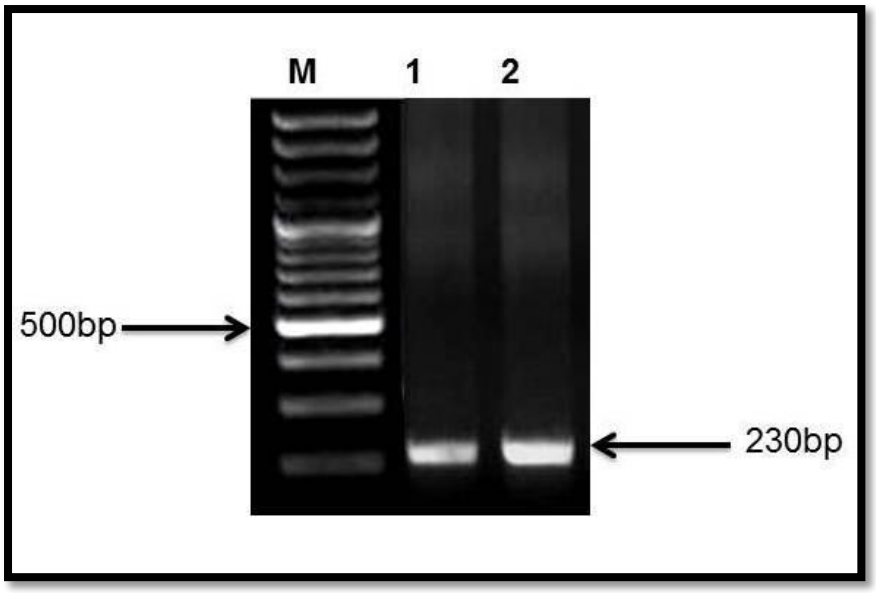


Fig. 5. PCR amplification of kiss2 cDNA fragment of amplicon size 230bp. Lane M; 100bp plus ladder (Thermo Fisher scientific, USA), lane 1-2: Amplified products at 62°C and 60°C annealing temperature

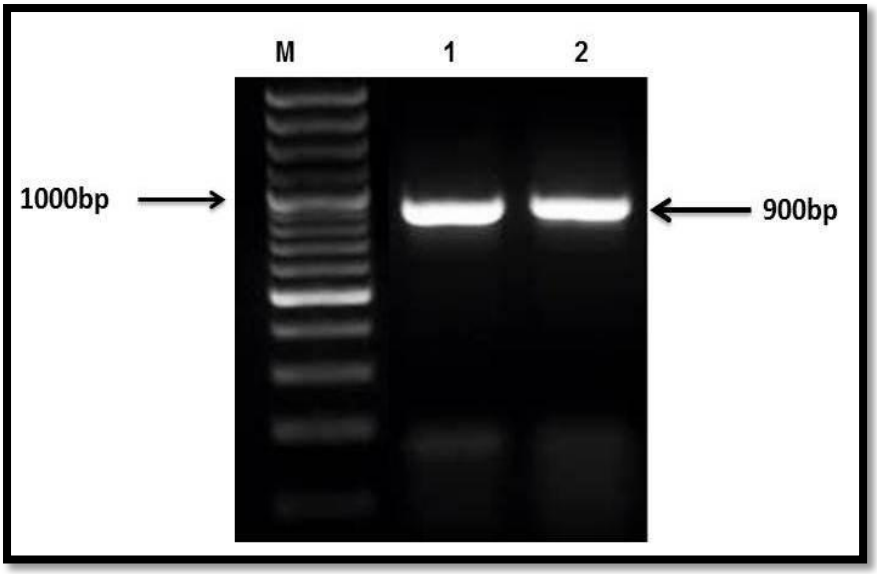


Fig. 6. PCR amplification of kiss1r cDNA fragment of amplicon size 900bp. Lane M: 100bp plus ladder (Thermo Fisher scientific, USA); lane 1-2: Amplified products at 64°C annealing temperature

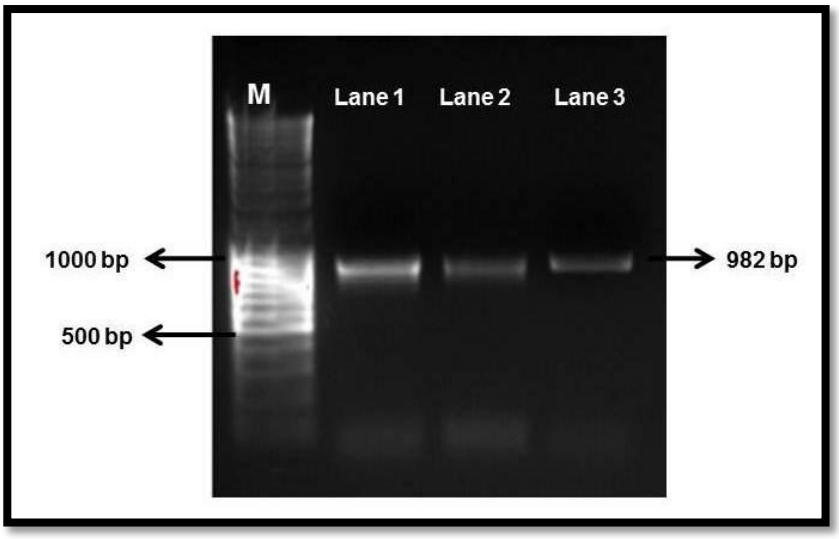


Fig. 7. PCR amplification of kiss2r cDNA fragment of amplicon size 982 bp. Lane M; 100bp plus ladder (Thermo Fisher scientific, USA); lane 1, 2 and 3 kiss2r amplified product at 62°C annealing temperature

.4.3. Cloning and screening of the recombinants

PCR product was extracted from the agarose gel and T/A cloned into pTZ57R/T vector. The DH5α strain of E. coli transformed with pTZ57R/T vector produced blue and white colonies in the X-gal/IPTG/ampicillin LB Agar. White colonies were selected as recombinant clones (Fig. 8). Transformation of DH5α cells with recombinant plasmid was confirmed by colony PCR using the gene specific primers. Plasmids purified from positive colonies were checked on 1% agarose gel.

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o cids,
respectively. The annotation of kisspeptin genes is given in figures 9-12

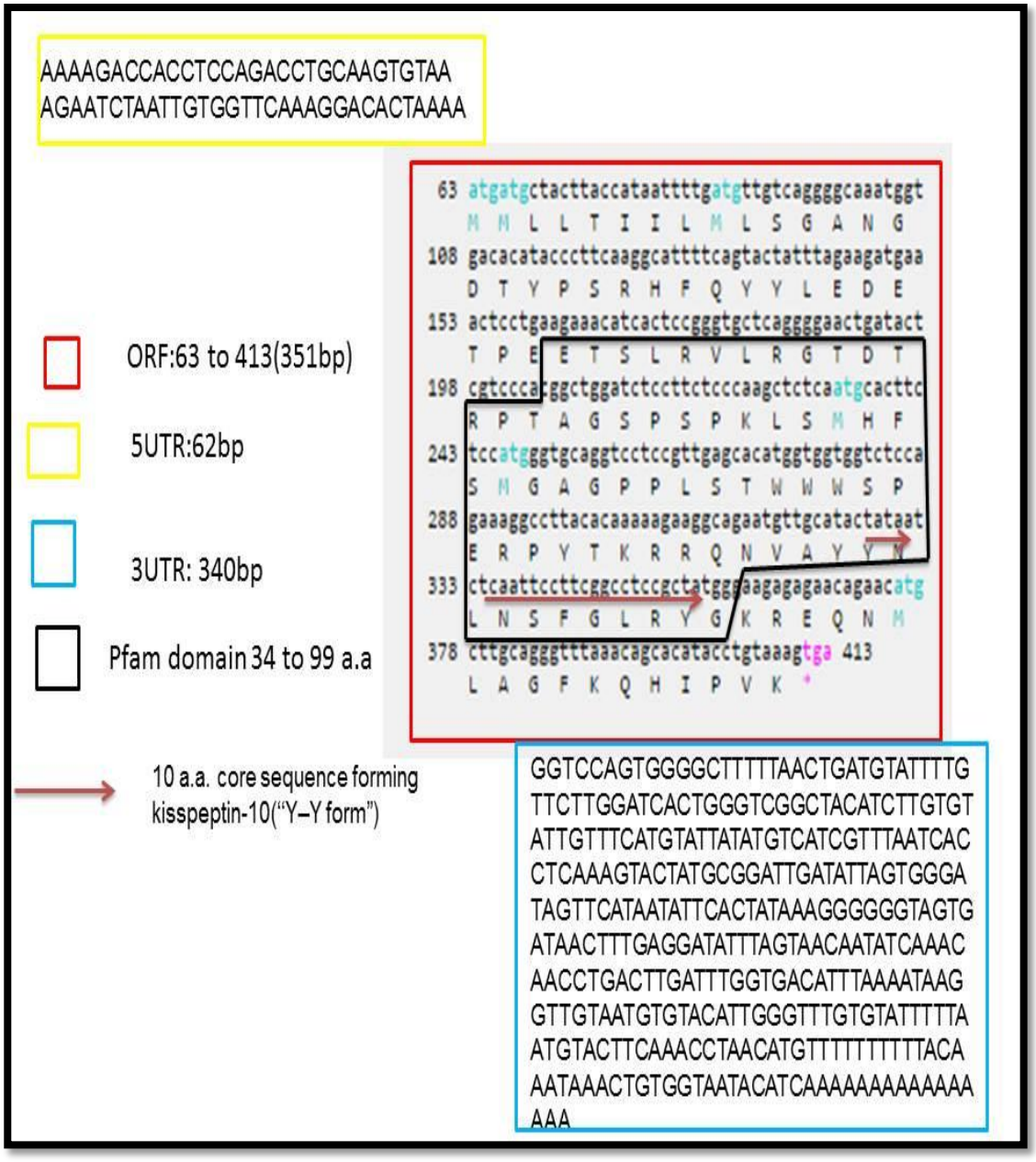


Fig. 9. Nucleotide and deduced amino acid sequence of *kiss1* cDNA

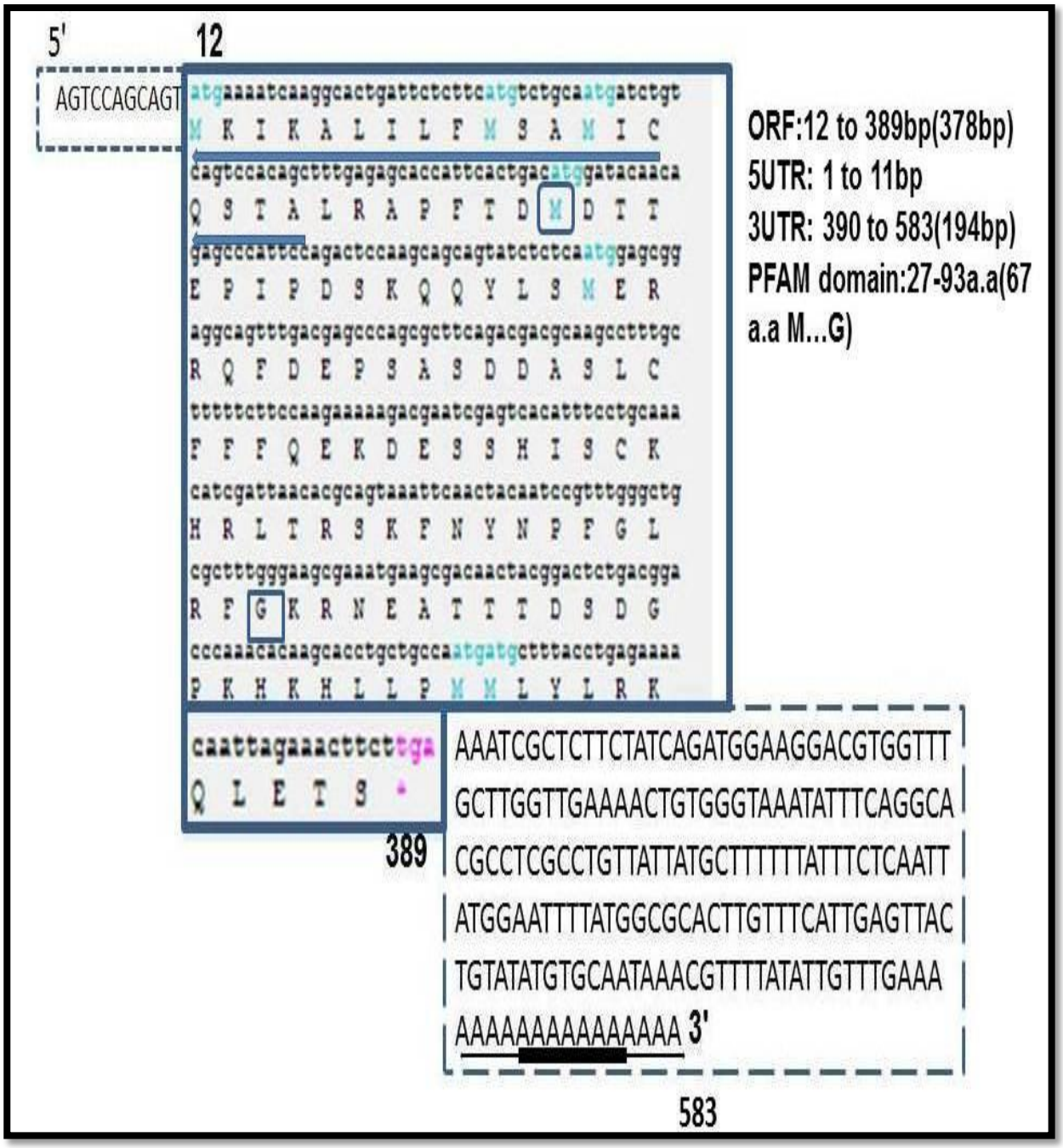


Fig.10. Nucleotide and deduced amino acid sequence of *kiss2 cDNA*

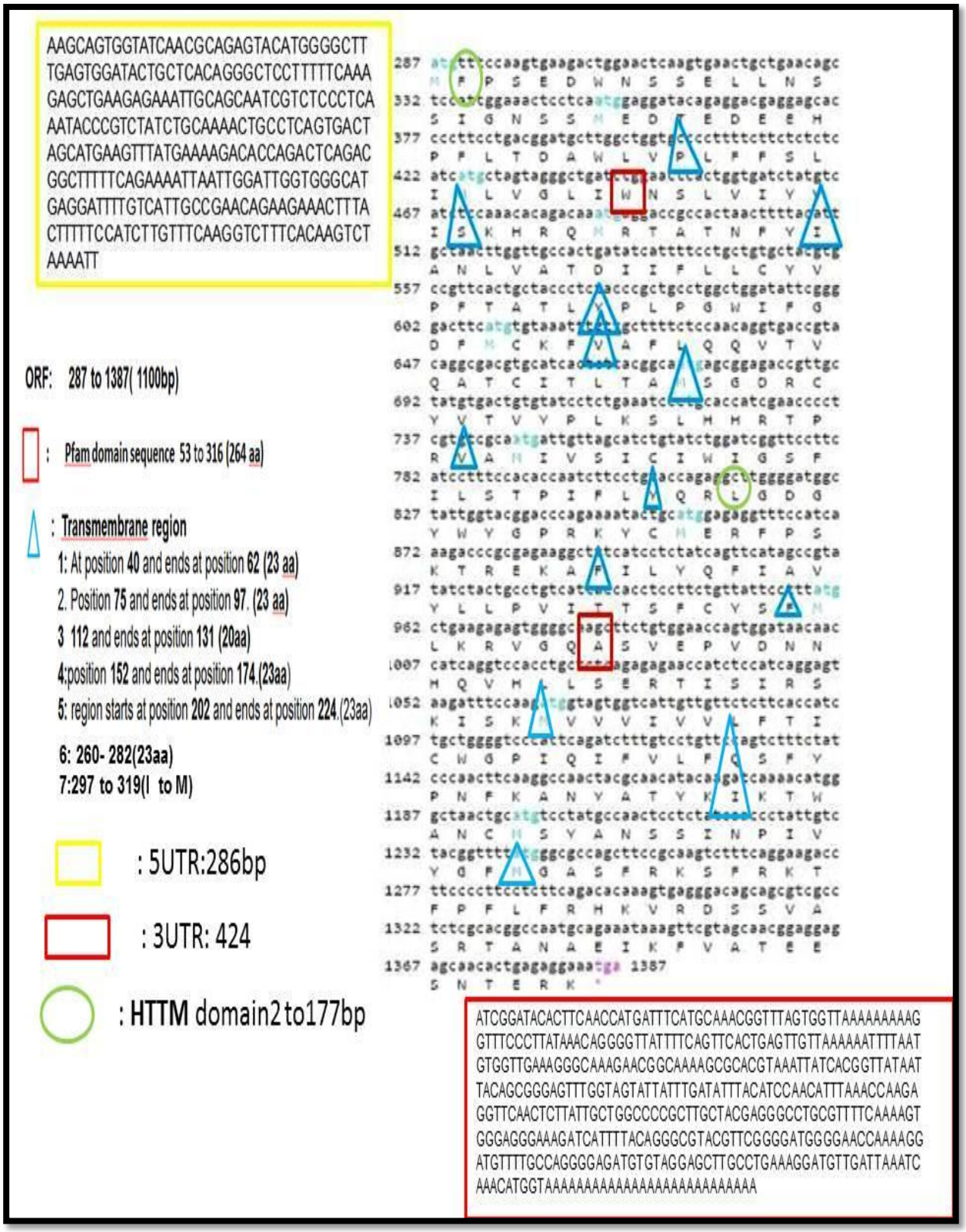


Fig.11**.** Nucleotide and deduced amino acid sequence of *kiss1r* cDNA

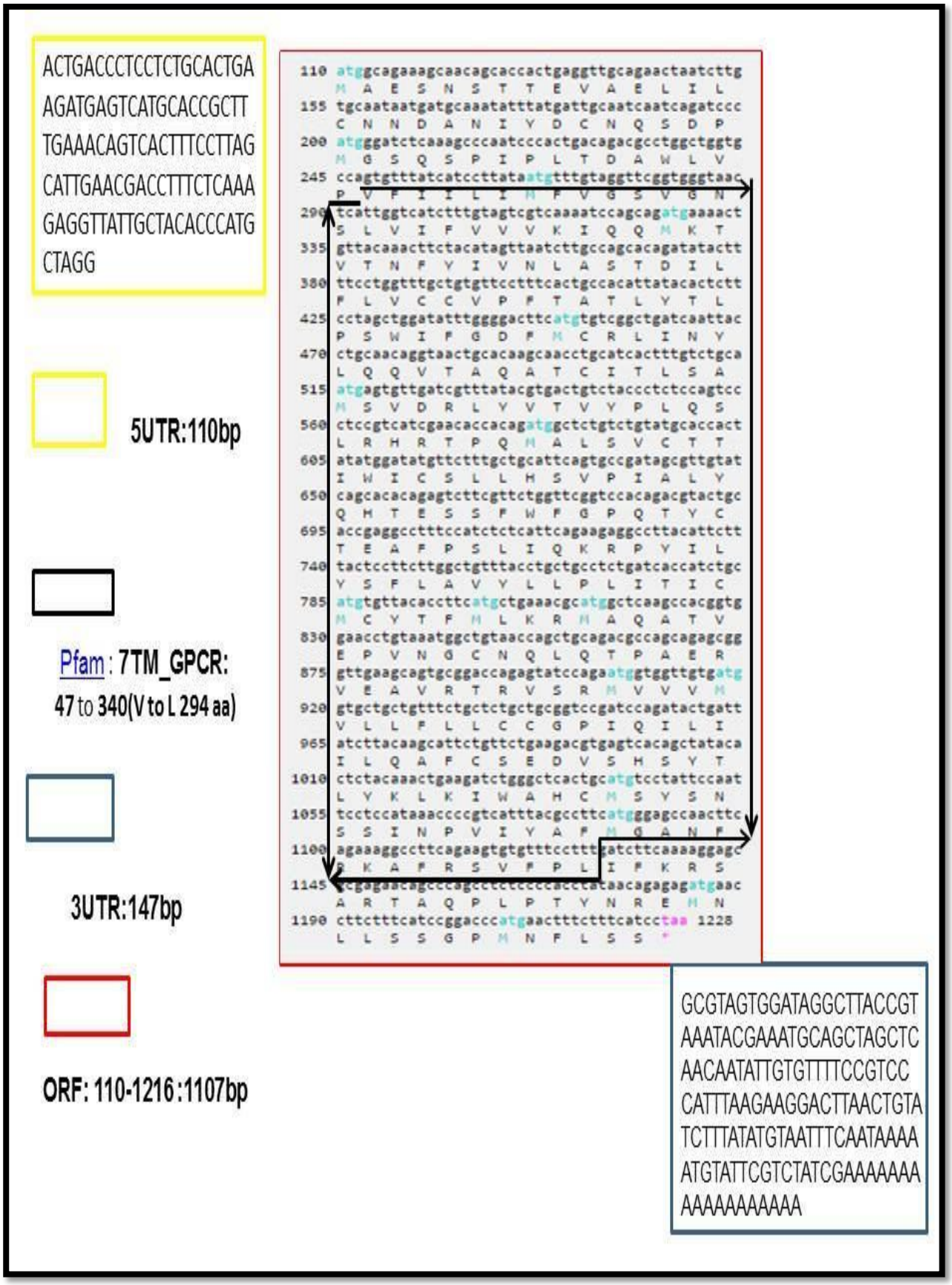


Fig.12. Nucleotide and deduced amino acid sequence of *kiss2r* cDNA

4.5. Proteomic analysis

The deduced amino acid sequence was subjected to various online sequence analysis tools. The amino acid sequences of different fish species and mammals were downloaded from NCBI and multiple alignments was done by using clustal omega. Comparisons of the 10 amino acid core sequences forming kisspeptin-10 in Kiss1 and Kiss2 ORFs in vertebrates are shown in figure 13 and 14. In this study, a.a. substitutions at position 1 and 10 can be noted in the ‘‘Y–Y form” and ‘‘F–F form” of kisspeptin-10 encoded by Kiss1 and Kiss2, respectively. Protein blast by uniprot (Table 3, 4) revealed that the Kiss1 protein domain of *C. catla* was highly similar to other fish species with highest identity with *Gobiocypris rarus* (93%), Danio rerio (86%) while kiss2 with *Megalobrama amblycephala* (92%) and *Gobiocypris rarus* (93%). Analysis of the predicted amino acid sequence of *C. catla* kiss1r and kiss2r revealed seven TMDs, a characteristic of membrane-bound G-protein coupled receptors (Fig. 15 and 16). Kiss1r protein domain of *C. catla* was highly similar with *Danio rerio* (91%) and *Gobiocypris rarus* (91%). However, in case of kiss2r it is more alike with *Cyprinus carpio* (91%) followed by *Carassius auratus* (89%, Table 5 and 6).

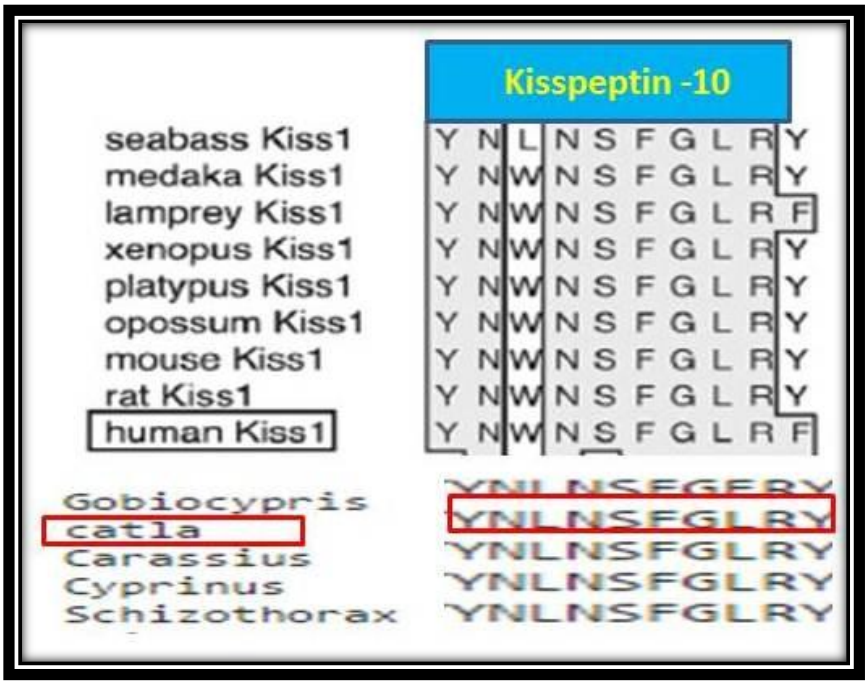


Fig.13. Core sequence coding for kisspeptin-10 of Kiss 1appears to be same at position 1 and 10 within ‘‘Y–Y form”.

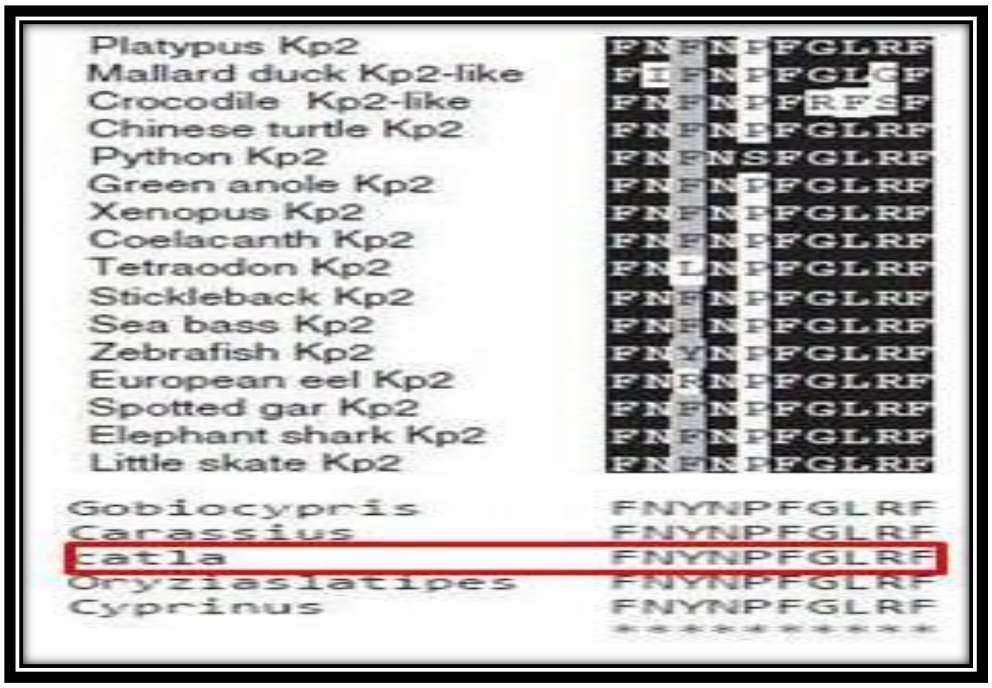


Fig.14. Core sequence coding for kisspeptin-10 of Kiss 2 appears to same at position 1and 10 within ‘‘F–F form”.

Table 3. Alignment score of *C. catla* kiss1 amino acid sequences predicted by Uniprot database

Sr.no	Accession no.	Species	Length	Identity
1	**TR:V5JDG7_MEGAM**	***Megalobrama amblycephala***	**125**	**92%**
2	**TR:W5VNM9_9TELE**	***Gobiocypris rarus***	**124**	**92%**
3	**TR:C6JW21_CARAU**	***Carassius auratus***	**122**	**87%**
4	**TR:B6ZIV2_DANRE**	***Danio rerio***	**125**	**83%**
5	**TR:I4AWS9_CYPCA**	***Cyprinus carpio***	**80**	**92%**

Table 4. Alignment score of *C. catla* kiss2 amino acid sequences predicted by Uniprot database

Sr.no	Accession no.	Species	Length	Identity
1	**TR:W5VPH4_9TELE**	***Gobiocypris rarus***	**116**	**93%**
2	**TR:A8C5J1_DANRE**	***Danio rerio***	**116**	**86%**
3	**TR:B8Y247_CARAU**	***Carassius auratus***	**115**	**81%**
4.	**TR:I4AWS8_CYPCA**	***Cyprinus carpio***	**82**	**86.0**

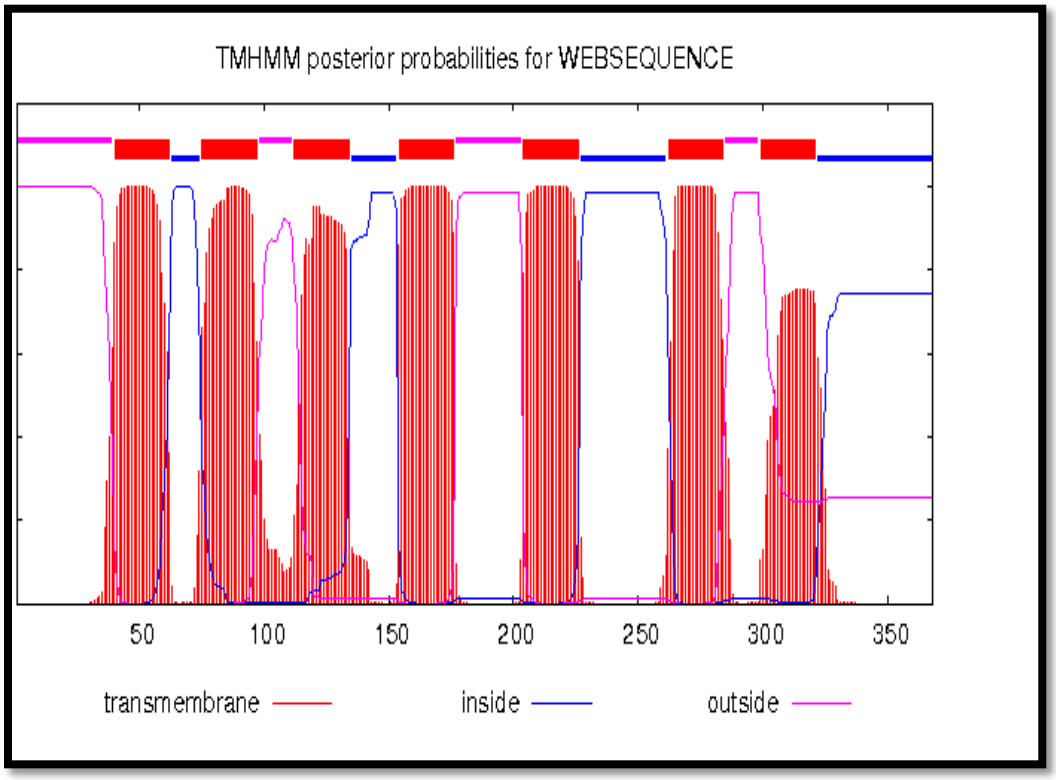
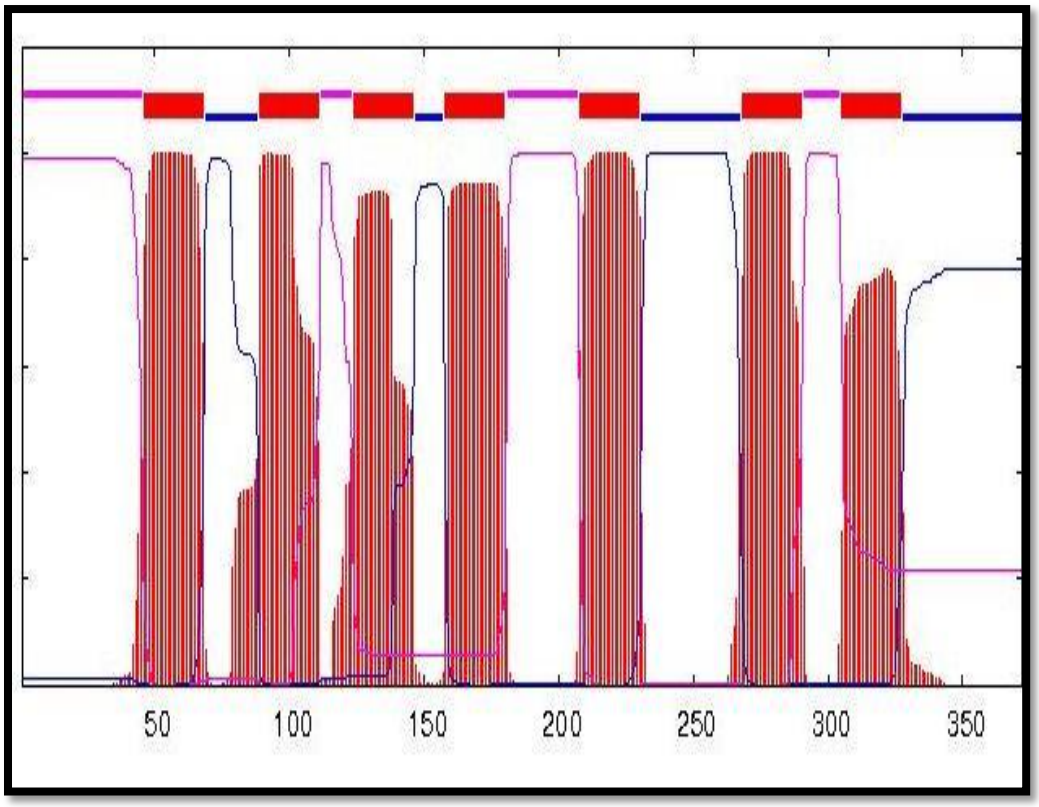


Fig.15. Seven Putative transmembrane domains (TMDs) identified in kiss1r cDNA



outside	1	45
TMhelix	46	68
inside	69	88
TMhelix	89	111
outside	112	123
TMhelix	124	146
inside	147	157
TMhelix	158	180
outside	181	207
TMhelix	208	230
inside	231	267
TMhelix	268	290
outside	291	304
TMhelix	305	327
inside	328	372

Fig.16. Seven putative transmembrane domains (TMDs) identified in kiss2r cDNA

Table 5. Alignment score of *C. catla* kiss1r amino acid sequences predicted by Uniprot database

Table 6. Alignment score of *C. catla* kiss2r amino acid sequences predicted by Uniprot database

Sr. no	Accession no.	Species	Length	Identity (%)
Sr.no	**Accession no.**	**Species**	**Length**	**Identity**
1	**TR:A8CAH3_DANRE**	***Danio rerio***	**355**	**91%**
2	**TR:W5VNI9_9TELE**	***Gobiocypris rarus***	**365**	**91%**
3	**TR:I4AWS6_CYPCA**	***Cyprinus carpio***	**366**	**91%**
4	**TR:B8Y249_CARAU**	***Carassius auratus***	**366**	**90%**
5	**TR:D2I9G4_THUMA**	***Thunnus maccoyii***	**377**	**83%**
1	**TR:I4AWS7_CYPCA**	***Cyprinus carpio***	**366**	**91%**
2	**TR:B8Y250_CARAU**	***Carassius auratus***	**366**	**89%**
3	**TR:W5VVI4_9TELE**	***Gobiocypris rarus***	**371**	**84%**
4	**TR:A8CAH4_DANRE**	***Danio rerio***	**364**	**81%**

4.6. Phylogenetic analysis of kisspeptin genes

In order to compare the sequence similarities, all the *kisspeptin* protein sequences known or predicted in Chordates in the GenBank were selected and aligned using Clustal omega program. A phylogenetic rooted tree was constructed by the neighbour-joining method to find out evolutionary relationship of *kisspeptin* gene using MEGA 6 software (Fig.17). The sequences were clustered into two main clades comprising mammals and fishes. In the phylogenetic tree, *C. catla* kiss1 showed the closest relationship with another cyprinid *Gobiocypris rarus* while kiss2 showed the closest relationship with *Carassius auratus,* which also belongs to Cyprinidae family. However, *C. catla* kiss1r and kiss2r showed highest similarity with *Cyprinus carpio* followed by *Carassius auratus* (Fig. 18).

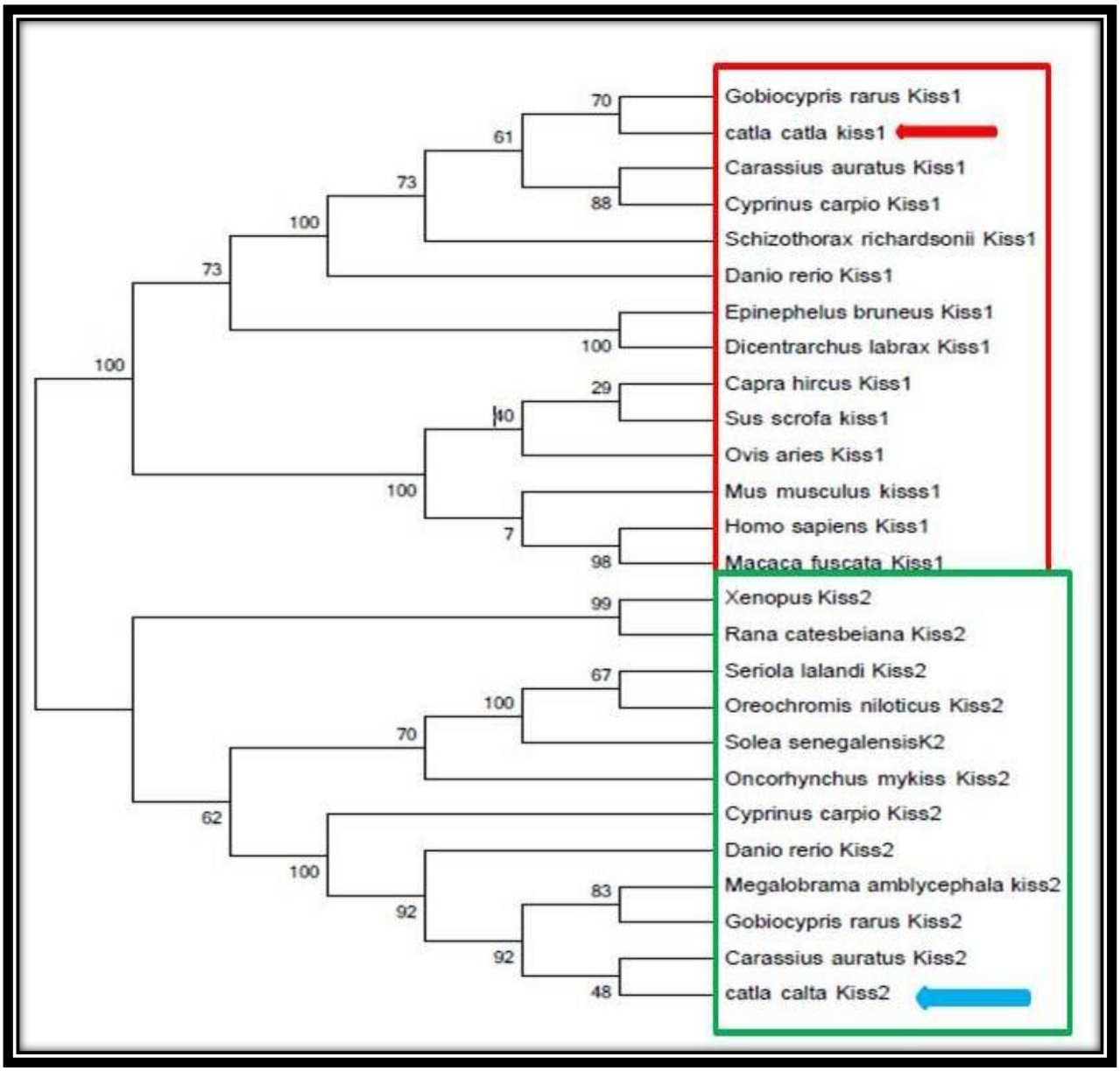


Fig. 17. Phylogenetic tree analysis of the deduced amino acid sequences of kiss1 and kiss2.

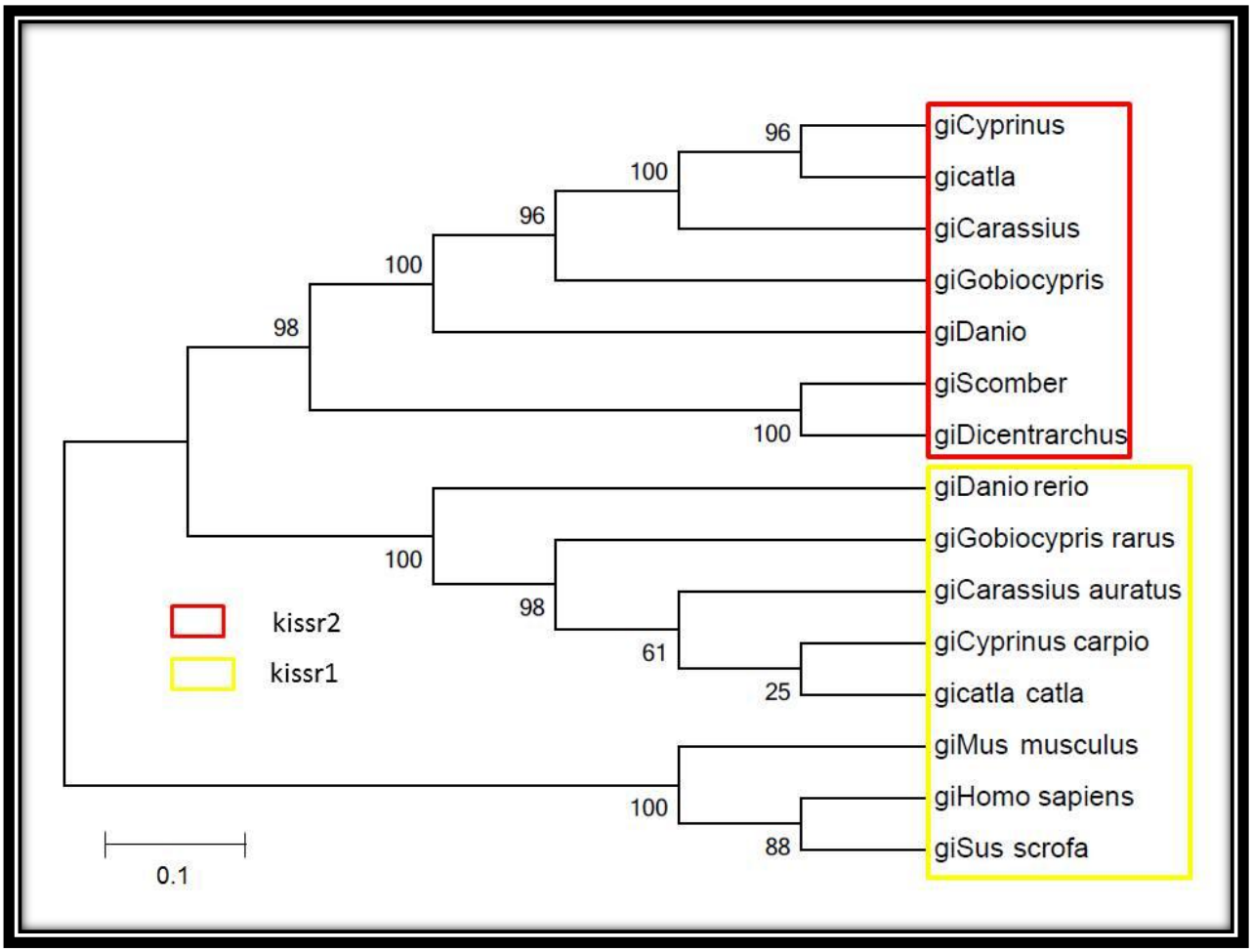


Fig.18. Phylogenetic tree analysis of the deduced amino acid sequences of kiss1r and kiss2r

4.7. Ontogeny and tissue distribution expression pattern of kiss1 gene

The ontogeny pattern of *kiss1* gene studied in different development stages of *C. catla* by semi-quantitative RT-PCR and qRT-PCR revealed that mRNA transcripts is highly expressed in mature phase followed post-spawning (Fig.19 and 20). *Kiss1* mRNA gene expression was not observed prior to hatching and then increased significantly after juvenile stage.

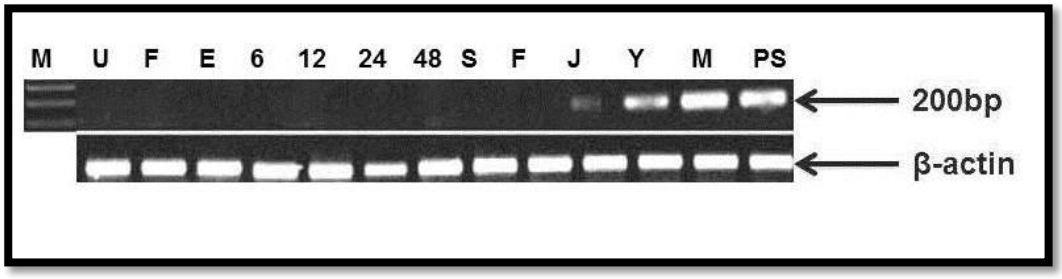


Fig.19. *mRNA* expression of kiss1 determined by *semi-quantitative* PCR in 13 different life stage of *C. catla*

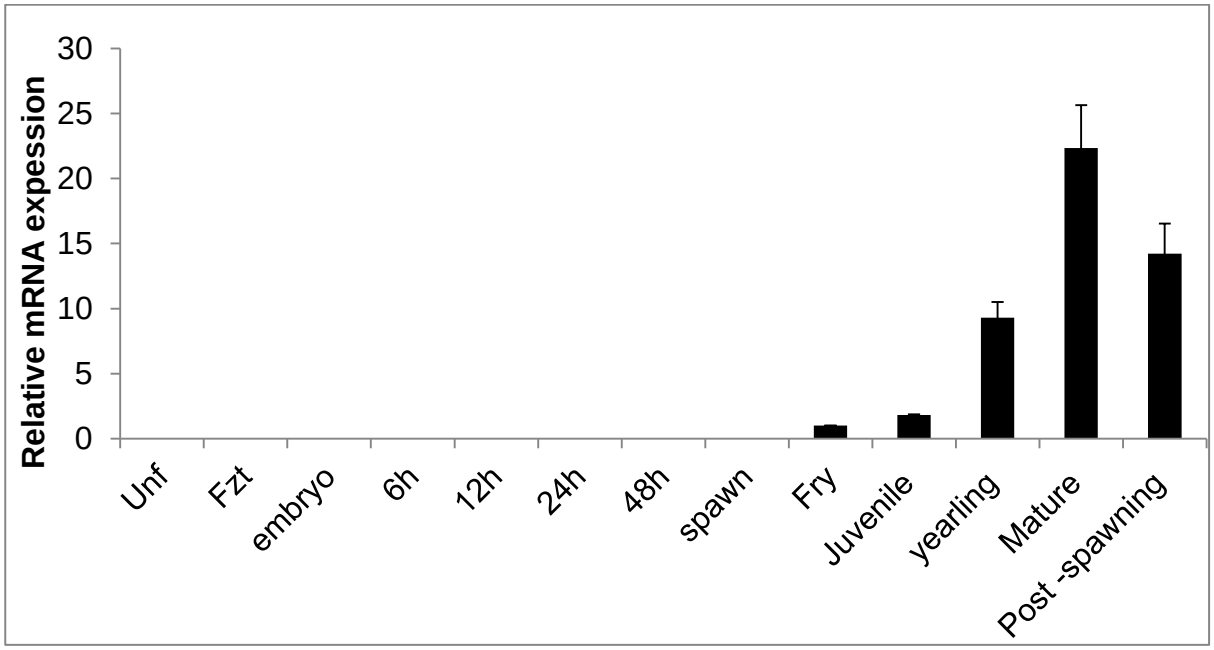


Fig. 20. mRNA expression of kiss1 determined by *Real- time* PCR in 13 different life stages of *C. catla*

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Kiss1 gene mRNA transcript was detected in most of fish tissues tested. The level of mRNA transcripts was found mainly expressed in brain, followed by testis, ovary and intestine. However, a low expression level was observed in tissues like kidney, liver, and gill in semi-quantitative RT-PCR which was further confirmed with qRT-PCR (Figs 21 and 22).

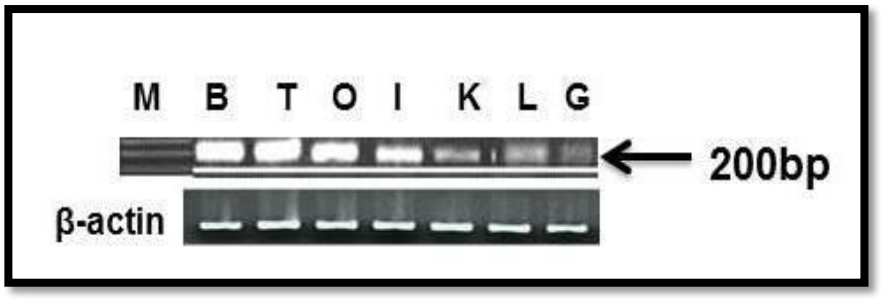


Fig. 21. Tissue distribution of kiss1 in 7 different tissues of *C. catla as* determined by *semi-quantitative* RT-PCR.

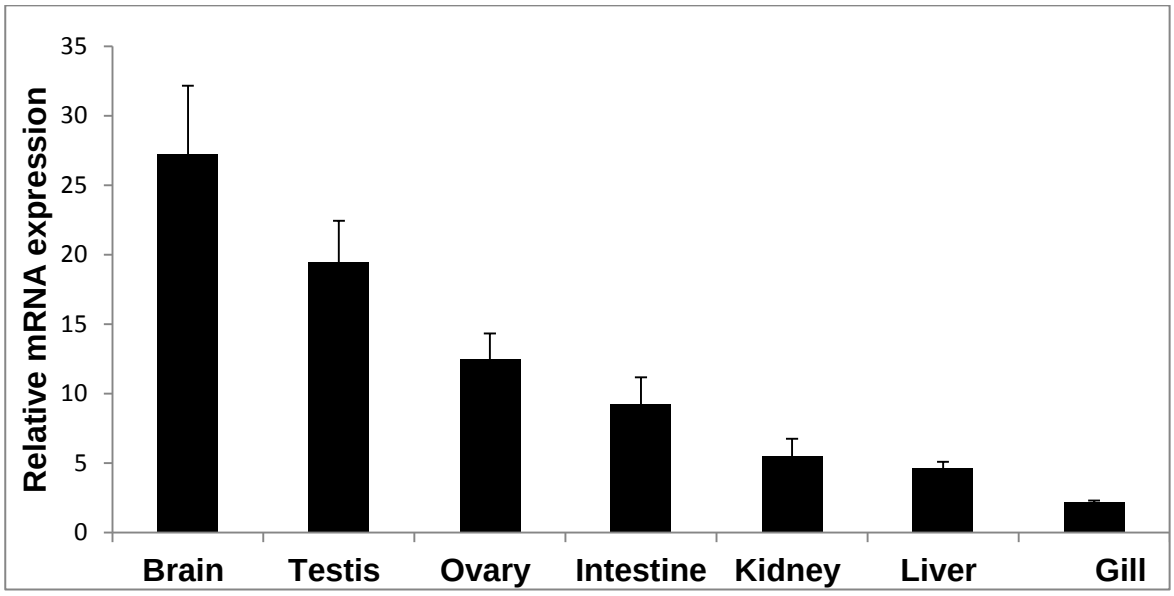
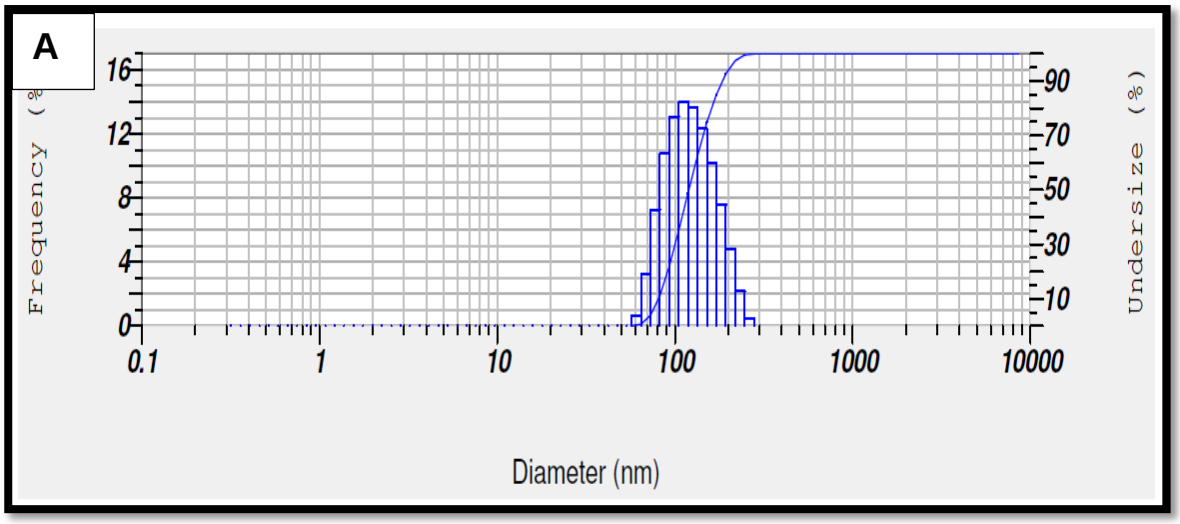


Fig. 22. qRT-PCR analysis of kiss1 expression in different tissues of adult *C. catla.*Data for real-time PCR were expressed as mean ± S.E.M (n=3) ($p<0.05$ is significant level)

4.8. Physicochemical characterization of Nanoparticles

The mean particle size of the CK-10 nanoparticles was found to be 125 nm. The distribution of particle size for CK-10 nanoparticles is given in Fig. 4**.** In general, nanoparticles were well dispersed and pretty stable as evinced by physico-chemical characteristics like polydispersity index (0.335 to 0.65) and zeta potential (34.95 mV). Chitosan nanoparticles showed 64% entrapment efficiency for K-10.



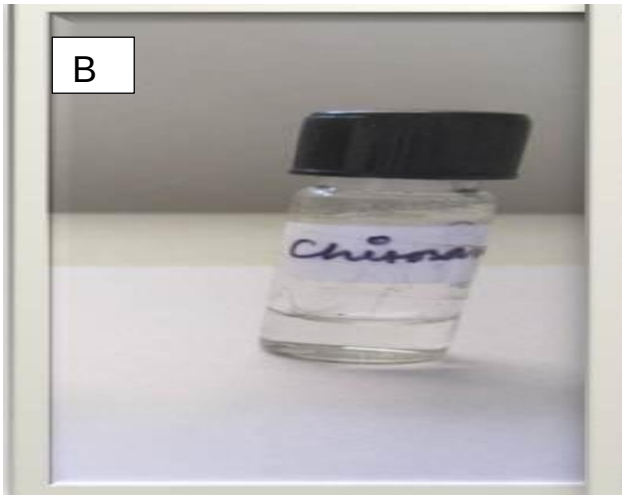


Fig. 23. A) Particle size distributions of chitosan kisspeptin -10 (CK-10).B) Nanoformulation of CK-10.

4.9. Serum hormone levels

The treatment of fish with K-10 and CK-10 showed significant effect on hormonal levels of LH, FSH, 11-KT and17-βE2 ($P<0.05$). Nano-conjugated kisspeptin-10 (CK-10) induced sustained and controlled release of hormones with highest ($P<0.05$) surge at 12 hrs. In case of K-10 treatment, hormone levels reached its peak at 5-6 hrs and then sharply declined (Figure 24 and 25)

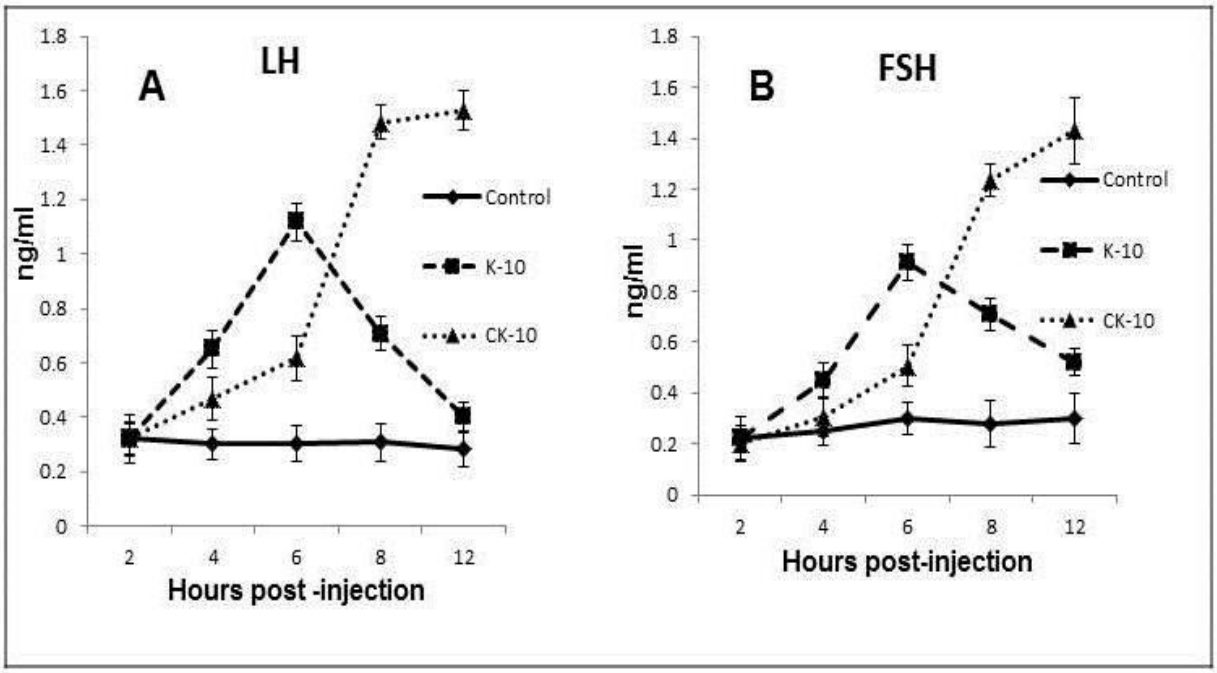


Fig. 24. Serum level of various reproductive hormones (FSH, LH) in *C. catla* following injection of different preparations of Kisspeptin-10 during 12 hr period. Means differ significantly (P<0.05) at that hour point. Each point is mean ± SE of three observations

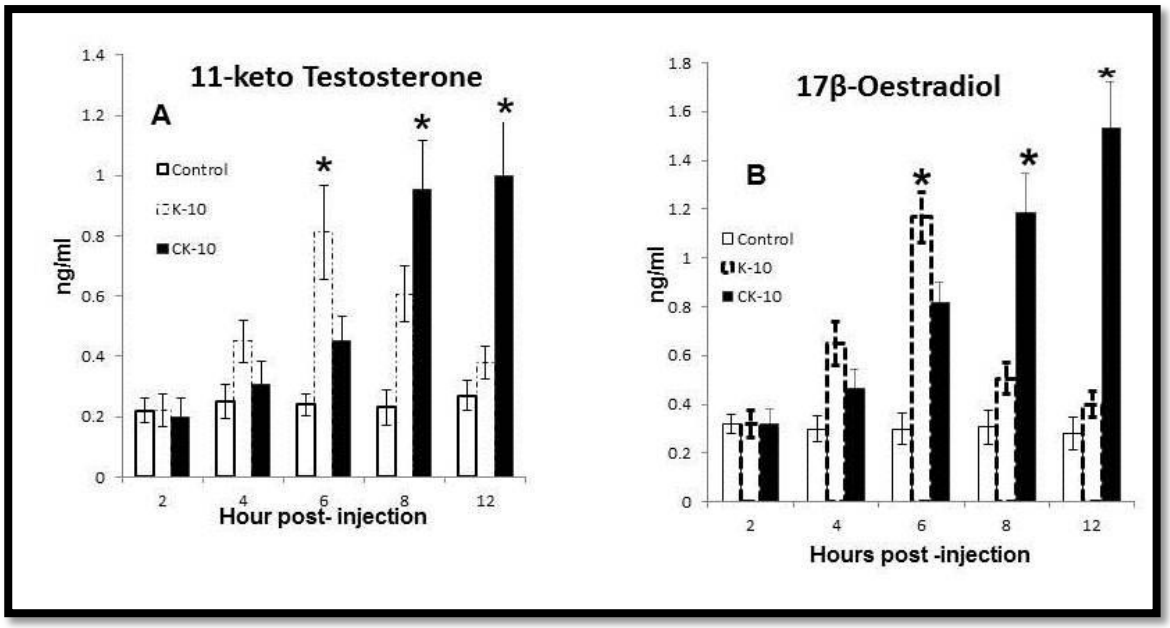


Fig. 25. Serum level of various reproductive hormones (11-KT and 17-β E2) in *C. catla* following injection of different preparations of Kisspeptin-10 during 12 h period. Significant difference ($p < 0.05$) between the treated and control group is indicated with asterisk at that hour point.

4.10. Histological study of gonads

The histological observation of ovaries from different groups are given in Figure 26.Histological studies of ovary in control fish contained oogonia, stage I oocytes. In naked kisspeptin-10 treatment most of oocytes are at stage II. However in case of CK-10 groups contained oogonia, with stage III yolky oocytes which show maturation of ovary under captivity conditions.

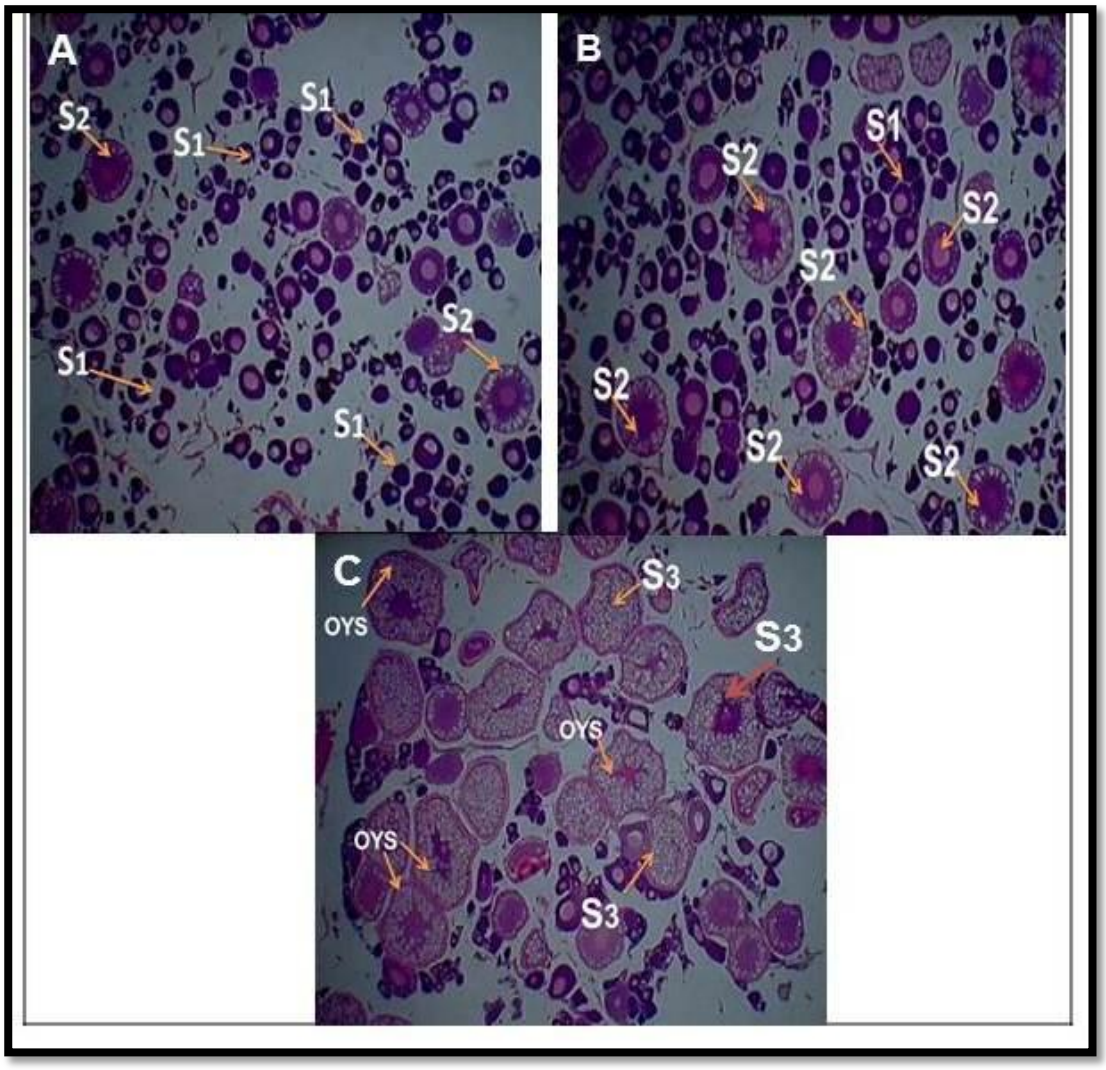


Fig. 26.Histology of ovary from *C. catla* injected with different preparations of kisspeptin -10. A) Control group with stage I oocytes (S1), B) K-10 group, oocytes are at stage I and II(S1and S2). C) CK-10 oocytes group III yolky oocytes(S3).

5. DISCUSSION

5.1. Cloning and characterization of kisspeptin genes

Kisspeptin has appeared as a key player in the regulation of many physiological functions, among which the most recognized is elicitation of gnrh secretion (Seminaria et al., 2003; Smith et al., 2006). Experimental evidence has strongly suggested the involvement of kisspeptin in many physiological processes of reproduction, such as generation of pre-ovulatory gnrh surge (Lehman et al., 2010), maturation and timing of puberty onset (Amstalden et al., 2010; Clarkson et al., 2010), positive and negative feedback of sex steroids on gonadotropin secretion (Terasawa et al., 2010), metabolic regulation of fertility (Roa et al., 2008) and photoperiodic control of reproduction in seasonal breeders (Chalivoix et al., 2010). Sex and species-specific differences of kisspeptin neurons in signalling hypothalamic gnrh cells has been observed (Homma et al., 2009; Kauffman et al., 2009). The effect of gonadal steroids on kisspeptin expression in the hypothalamus and gonadotropin secretion in adult females and males in early post natal life in rodents has been studied by Kauffman et al. (2009).

Based on molecular cloning and comparative synteny using data mining in genomic data bases, the presence of a Kisspeptin gene system has been confirmed in lower vertebrates, including fish species (Van *et al.,* 2008). In the present study, kiss1, kiss2, kiss1r and kiss2r sequences were cloned and functionally evaluated in *C. catla*. The coding region of kiss1 encoded a peptide of 116 amino acids, which is similar to other fishes such as *D. rerio* (Van *et al.,* 2008) *C. auratus* (Yang *et al.,* 2010) and *G. rarus* (Yang *et al.,* 2014).

ORF (open reading frame) of kiss1 cDNA is 351bp, which is same as *G. rarus* (Yang *et al.,* 2014). The full-length *C. catla* kiss2 cDNA is 583 nuclotides, encoding a polypeptide of 125 amino acids with an ORF of 378bp. In *D. rerio,* full-length kiss2 cDNA is 588bp, coding for a protein of 125 amino acids (Shahjahan, 2013). Our results are similar to that of Wen *et al.* (2013) who worked on *Megalobrama amblycephala*, and reported an ORF of 378bp for kiss2 encoding a putative protein of 125 amino acid residues. Yang *et al.* (2014) characterized *G. rarus* kiss2 mRNA having an ORF of 381bp encoding a putative protein of 126 amino

acid residues. However, in case of *C. auratus,* the kiss2 mRNA is 555bp (ORF 369) encoding 122 amino acids (Yang and Wong 2009).

The core sequence, namely kisspeptin-10, encoded by the two genes exhibits conserved substitutions at position 1 and 10 with Tyr in Kiss1 (‘‘Y–Y form”) and Phe in Kiss2 (‘‘F–F form”). The Core sequence of *C. catla* kiss1 showed 80% similarity with human kiss1 and 90-100% similarity with other carp species (Yang *et al.,* 2010). However, in case of kiss2 the core sequence is 100% conserved among carp fishes and 80-90% with other animals. Sequence alignment at the protein level has revealed that the *C. catla* kiss1 is highly homologous to that of *G. rarus* (93% a.a identity) and kiss2 with *M. amblycephala* and *G. rarus* (92% a.a identity).

In numerous fish species, expression of kiss2r was shown to increase during the onset of puberty and the spawning period (Mohamed *et al.,* 2007; Nocillado *et al.,* 2007; Biran *et al.,* 2008; Filby *et al.,* 2008; Martinez *et al.,* 2008; Mechaly *et al.,* 2009; Shahjahan *et al.,* 2010; Migaud *et al.,* 2012). These outcomes suggest that the kiss2r has a dominant role in the reproduction of many fish species while the function of kiss1r remains unknown. kiss1r having an ORF of 1101bp which codes putative protein of 366 amino acid residues in *C.auratus* (Li *et al.,* 2010) and kiss2r codes putative protein 366 amino acid. In case of *C. Carpio,* kiss2r also been found to be 1812 and 1375bp encoding a polypeptide of 366 and 368 amino acids, respectively. The kiss1r ORF region was identified from 287 to1387bp (1101bp) in the cDNA. In *C. carpio,* kiss1r full-length cDNA is 1809 bp with an ORF of 1101bp (Cao *et al*., 2012). In *C. auratus* too, the kiss1r has an ORF of 1101bp encoding a putative protein of 366 amino acid residues (Li *et al*., 2010), while the kiss2r also encoded a protein of 366 amino acids. In case of *C. carpio,* kiss2r also encoded a putative protein of 366 amino acids. However, in case of *G. rarus* and *D. rerio* kiss1r encoded a protein of 365 and 355 amino acids, respectively. Sequence alignment at the protein level has revealed that kiss1r is highly homologous *D. rerio*, *G. rarus* and *C. carpio* (91% a.a. identity) while kiss2r is homologous to *C. carpio* (91% a.a. identity) followed by *C. auratus* (89%).

Analysis of the predicted amino acid sequence of *C. catla* kiss1r and kiss2r revealed seven TMDs (Trans-membrane domain), a characteristic feature of membrane-bound G-protein coupled receptors (Muir *et al.,* 2001). The high degree of

structural similarity in the kiss1r has been demonstrated in various teleost species (Nocillado *et al.,* 2007; Van *et al.,* 2008; Lee *et al.,* 2009; Mechaly *et al.,* 2009, 2010). The teleost kiss1r contains three putative glycosylation sites at its N-terminus, similar to that observed in mammals (Colledge, 2004). The presence of a number of putative protein kinase C (PKC) phosphorylation sites suggests that the receptor potentially conveys its signal via the PKC pathway (Stafford *et al.,* 2002; Stathatos *et al.,* 2005). Activation of protein kinase A (PKA) pathway as well as the PKC pathway has been shown to be evoked by binding of kisspeptin to its receptors in zebrafish (Biran *et al.,* 2008; Lee *et al.,* 2009) and goldfish (Li *et al.,* 2009). In goldfish, kiss1 and kiss2 activate different signalling pathways depending on the receptor subtypes: kiss1 activates both PKA and PKC pathways through kiss1ra and PKA pathway through kiss1rb while kiss2 only activates the PKC pathway through kiss1ra but both pathways through kiss1rb (Li *et al*., 2009). In chub mackerel, *Scomber japonicas* kissr1 signal is transduced via the PKC pathway while kissr2 activity is transduced via both PKC and PKA pathways (Ohga *et al*., 2013).

5.2. Ontogeny and tissue distribution expression

The expression of kiss1 and kiss1r during pubertal development has been now studied in several fish species. In zebrafish, mRNA levels of kiss1 increased during development, starting at the intermediate phase, reaching maximum levels during the advanced phase and remained high in adulthood (Kitahashi *et al.,* 2009). In contrast, in chub mackerel, expression was highest in pre-pubertal fish (Selvaraj *et al.,* 2010 & 2012). In the red seabream *(Pagrus major),* kiss2 expression in neurons of the nucleus recessi lateralis increased during the first spawning season in both sexes (Shimizu *et al.,* 2012). In the present study also, maximum expression was measured during the mature stage. RT-PCR analysis of seabass revealed that the kiss1 expression starts in juvenile stage and reaches maximum in mature fish (Felip *et al.,* 2009). Our results corroborate well with the above study. A related situation was reported in the striped bass (*Morone saxatilis*), where an increase in kiss2 mRNA expression was observed in mature fish, particularly in females (Zmora *et al.,* 2012). These ontogeny studies including the present one on the kisspeptin system in fish support their involvement in regulating puberty in fishes.

In fish, expression of the genes encoding kisspeptins and their receptors has mainly been found in different regions of brain. The expression of these genes has also been reported in a range of other tissues including testes, ovary, stomach, intestine, spleen, liver, kidney, adipose tissue, pancreas, gills to a variable extent depending on the fish species and gene (Oakley *et al.,* 2009). In goldfish and chub mackerel, kiss1 gene is found to be highly expressed in brain and gonads (Selvaraj *et al.,* 2010; Li *et al.,* 2013). Similar observation was also made in the present study.

5.3. Evolutionary and Phylogenetic relationship

Regarding kisspeptin gene and kisspeptin receptor diversity, contrasting situations are found in different vertebrate phyla. In teleosts, two situations have been reported. One Kiss gene and one Kissr gene are present in some species such as fugu *(Takifugu niphobles),* tetraodon *(Tetraodon nigroviridis),* and stickleback *(Gasterosteus aculeatus).* However, a second Kiss as well as a second Kissr gene have been characterized in some species including zebrafish *(D. rerio*) (Biran *et al.,* 2008), goldfish (*C. auratus*) (Li *et al.,* 2009), medaka (Oryzias latipes) (*Lee et al.,* 2009). The present study revealed the presence of two Kiss genes in *C. catla*. Phylogenetic analysis revealed that Kiss1 and Kiss2 are clustered into two separate clades. Similar results were also reported by Li *et al.* (2010) and Selvaraj *et al.* (2010) in chub mackerel.

5.4. Characterization of Nanoparticles

Nanodelivery method is gaining popularity day by day owing to its advantage over the conventional method. Nanoparticles of chitosan have been extensively examined for their potential in the development of controlled release delivery of peptides, oligonucletiodes and genes (Kavshima *et al.,* 1985). Chitosan acts as a penetration enhancer by opening the tight epithelial junction and hence is particularly exploited in protein and vaccine (Van e*t al.,* 2001). Nanoconjugation of K-10 with chitosan increases the stability of the K-10, which otherwise has short lifetime in the blood (Bos *et al.,* 2012). Such nanodelivery has quite a pronounced advantage over multiple injections for enhancing maturation in fishes. The ionotropic gelation method used in the present study has already been successfully employed to prepare chitosan nanoparticles for the delivery of peptides and proteins (Chae *et*

al., 2005; Bahreini *et al*., 2014). Due to the reaction involving complex formation between oppositely charged species (negatively charged groups of the penta sodium tri-polyphosphate and the positively charged amino groups of chitosan), chitosan undergoes ionic gelation and precipitates to form spherical particles. Bovine serum albumin (BSA) protein-loaded nanoparticles prepared by using same technique were in the size range of 200–580 nm, and encapsulation was 61.1% (Gan *et al .,* 2007). Wang *et al.* (2008) developed estradiol loaded chitosan nanoparticles by the same technique with the particles mean size of 269 nm and zeta potential of +25.4mv. In another study, estradiol loaded Poly D, L-lactide-*co*-glycolide (PLGA) particle with loading efficiency of 51% and zeta potential value of 92.4±3 was developed (Mittal *et al.,* 2007). Using insulin as a model drug, positively charged chitosan coated PLGA nanoparticles were prepared with average size of <150 nm (Zhang *et al.,* 2012). Delivery of fish hormones and pheromones by using chitosan nanoparticle in fishes has also been reported (Rather *et al.,* 2013; Sharma *et al.,* 2014). Chitosan nano-encapsulated RNA with particle size of 250nm was developed and applied for increased growth performance, immunity and survival against bacterial challenge in fish (Ferosekhan *et al.,* 2013). It is clear from above discussion that the nanoparticles developed in our study were much smaller in size and more stable with higher entrapment efficiency than the above reports. Nanoparticles having smaller size gives better result as they have more surface area to volume ratio and *can easily pass through biological barriers.*

5.5. Exogenous administration of kisspeptin-10

Recently kisspeptin, a novel neuropeptide encoded by the metastasis suppressor gene has been identified as potent regulators of reproduction, particularly for the onset of puberty (de Roux *et al.,* 2003; Seminara *et al.,* 2003; Churcher *et al*., 2014). Kisspeptin plays an important role in regulating reproduction and stimulates FSH and LH secretion before reproduction and ovulation (Chang *et al.,* 2012) and also controls the gnrh secretion (Seminara *et al.,* 2005; Popa *et al.,* 2008). Biological activity of kisspeptin was tested *in-vivo* by administration of the core decapeptide (Li *et al.,* 2009). In goldfish, kisspeptin injection resulted in increase in luteinizing hormone (LH) plasma levels (Li *et al.,* 2009). In the European seabass, systemic administration of kisspeptin increased LH and FSH plasma levels (Felip *et al.,* 2009). Both Kiss1- 15 and Kiss2-12 peptides were potent in inducing the release of

reproductive hormones in the Morone species (Zmora *et al.,* 2012). The results of the present study also confirmed the regulation of the hypothalamo-pituitary hormone by kisspeptin in the experimental species. Kisspeptin-10 has been found to stimulate gonadotropin release including luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone in numerous vertebrates including humans, rodents and fishes (Gottsch *et al.,* 2004; Dhillo *et al.,* 2005; Cho *et al.,* 2009; Felip *et al.,* 2009; Li *et al.,* 2009; Beck and Welch, 2010; George *et al.,* 2011). However, there are no reports on the administration of chitosan-conjugated kisspeptin-10 (CK-10) for fish reproduction and present work is a novel approach on this aspect.

Various chitosan-conjugated nanoparticles of hormones, proteins and oligo-nucleotides have been developed for treating diseases such as steroid hormone disorders (Leuschner *et al.,* 2006) for aquatic and terrestrial animals. In goldfish, *C. auratus*, intra-peritoneal administration of kisspeptin resulted in an increase in serum LH (Li *et al.,* 2009). In grouper, *Epinephelus coioides*, intra-peritoneal injection of kiss-2 decapeptide significantly increased gnrh and FSH transcript levels at 6 and 12 h post-injection (Shi *et al.,* 2010). Similar observations have been made in the present study too. The hormonal profiling in the present study suggests that *C. catla* pituitary is receptive to naked as well as chitosan nano-conjugated K-10. In juvenile European seabass *(Dicentrarchus labrax)* both kiss1and kiss2 decapeptides induced FSH and LH secretion within 1–2 h post-injection after which it decreased (Felip *et al*., 2009). In our study, the hormonal peaks of naked K-10 administered group declined after 4-6 h post injection while a sustained and controlled release of the endogenous hormone showing enhanced peak at 12 h has been observed for chitosan nano-conjugated K-10. This can be attributed to the enhanced resistance of chitosan nano-conjugated K-10 against enzymatic degradation, which results in controlled and sustained release of kisspeptin-10 leading to oocyte maturation.

Physiological increase in the serum gonadotropin level in *C. catla* as observed in the current study is in agreement with that of Drori *et al*. (1994). The effect of nanoparticle size on endogenous hormone levels (LH and FSH) and duration of release has already been reported (Mittal *et al.,* 2007). Our results on sustained elevated levels of LH over a period of 12 h corroborate well with the study by Mananos *et al.* (2002), in which enhanced reproductive output (milt production)

has been observed in spermiating males of European seabass (*D. labrax*) in response to controlled-release of gnrh analogue. Sustained delivery systems of gonadotropin-releasing hormone analogue (Larsson *et al.,* 1997) have been successfully used to synchronize reproduction in fishes particularly those maturing late. So, the nano-formulation developed in the present study can be used to enhance maturation and reproductive output in fishes. This establishes the potential of low cost chitosan-conjugated kisspeptin-10 nanoparticles as a new means of manipulating reproduction in fish. This is the first report on the development of chitosan-conjugated kisspeptin-10 nano particles for application in fish reproduction.

5.6. Histological examination

The effect of kisspeptin-10 administration on onset of puberty at tissue level in *C. catla* was also studied in the present study. *C. catla* is a seasonal breeder that is known to mature at the age of 2.5 – 3 years. In the present study, we utilized both naked and nanoparticle-based delivery systems to administer core Kiss1 decapeptide in pre-pubertal catla. In naked kisspeptin-10 treatment most of oocytes are at stage II. However in case of CK-10 groups, it contained oogonia with stage III yolky oocytes which show maturation of ovary in captive conditions. Studies involving kisspeptin treatment in teleosts have shown its stimulatory effect at the hypothalamic-pituitary axis (Filby *et al.,* 2008; Felip *et al.,* 2009; Kitahashi *et al.,* 2009; Zmora *et al.,* 2012). Exogenous kisspeptin administration accelerates gonadal development in striped bass *i.e.* mature white bass treated with kisspeptins showed advancement in oocyte development (Beck *et al.,* 2012). About 75% of the Kiss1 female striped bass had a more advanced stage of oocyte development, including several larger oocytes (230 mm) with numerous lipid droplets. Similar observation was obtained in present study also. Nocillado *et al.* (2013) utilized slow-release implants to chronically administer the core kiss1 and kiss2 amidate decapeptides, result of which suggest that kisspeptins modulate the early gonadal development of male yellowtail kingfish**.** In another study, gonadal development and sexual maturation in cinnamon clownfish was observed after administration of same concentration of kisspeptin-10 (Kim *et al.,* 2014). In the present, sustained delivery systems of kisspeptin-10 will help in enhancing gonadal maturation and reproductive output in fishes due to long lasting surge of reproductive hormone in fish. The implant is similar to that designed for the decapeptide analog of GnRH that releases

the hormone over a period of about 2 weeks, with the highest release on the first week (Zohar and Mylonas, 2001; Mylonas *et al.,* 2007) which gonal development in fishes. Sustained delivery systems of gonadotropin-releasing hormone analogue (Larsson *et al.,*1997) have been successfully used to synchronize reproduction in fishes particularly which mature late. So, nano-formulation developed in the present study can be used to enhance maturation and reproductive output in fish

5.7. Conclusion

The aim of the study was to clone, sequence and characterise kisspeptin gene and their receptors in *C. catla*. The study successfully cloned and sequenced the full-length cDNA of kiss1, kiss2, kiss1r and kiss2r and this forms the first report on the identification and characterisation of these genes from Indian Major Carps. The kisspeptin gene and their receptors of *C. catla s*howed close sequence similarity with other cyprinid fishes. To understand the involvement of kisspeptin-10 in the process of controlling gonadotropin hormone secretion, LH, FSH, 11-KT and 17-β oestradiol were determined after administration of kisspeptin-10. The treatment of fish with K-10 and CK-10 showed significant effect on hormonal levels of LH, FSH, 11-KT and17-βE_2. These results highlight new means of manipulating reproduction in fish in terms of gonadal development. However, further research in this field needs to be carried out to elucidate the role of kisspeptin-10 in reproductive physiology of fish.

6. SUMMARY

Kisspeptins are peptide products of kiss1 gene, which was first discovered in 1996 as a metastasis suppressing gene in malignant melanoma cells. Mutations in gene encoding kisspeptin receptor Kiss1r were found to cause hypogonadotropic hypogonadism. The main focus of kisspeptin research has revolved around its role in reproduction since 2003. All amidated kisspeptin containing at least 10 amino acids of the carboxy-terminal have been shown to effectively activate Kiss1r. Kiss1r activation gives rise to gonadotropin-releasing hormone (gnrh) dependent release of LH and FSH. These LH and FSH in turn stimulate sex steroid release from the gonads. Sex steroids have negative feedback on gnrh secretion, except during the preovulatory gnrh surge, where a positive feedback loop, essential for proper ovulation, is created. Although gnrh is regulated by sex steroids, gnrh neurones do not express sex steroid receptors and sex steroids thus regulate gnrh release indirectly. Kisspeptin neurones on the other hand, express sex steroid receptors and several lines of evidence suggest that kisspeptin regulates gnrh release.

Catla catla is the most popular fish in aquaculture in India and in other south Asian countries. Among the Indian Major Carps (IMC), Catla attains sexual maturity late, both in captivity and natural conditions. Thus, understanding molecular and structural information regarding kisspeptin genes and their receptors in *C. catla* not only provides the basic information about the kisspetin gene system in IMC, but also provides valuable information on the molecular control of reproduction in IMC. To understand involvement of kisspeptin-10 in the process of controlling gonadotropin hormone secretion, hormonal profiles of LH FSH, 11-KT and 17-β oestradiol were determined after administration of different preparation of kisspeptin-10.

Adult *C. catla* used in the experiment were collected from Powarkherda centre of ICAR-Central Institute of Fisheries Education (CIFE), Mumbai, India and total RNA was isolated from the brain of *C. catla.* A partial cDNA fragment of kiss1, kiss2, kiss1r and kiss2r (kisspeptin gene) were amplified from the isolated total RNA using RT-PCR. A cDNA fragment of 200, 220, 900 and 982bp was amplified using the specific

primer set. For full-length characterisation of kisspeptin genes, 5´ and 3´ flanking regions of the partial sequence were amplified by using RACE PCR.

In present study, cloning and sequencing of full-length gene kiss1, kiss2, kiss1r and kiss2r has been successfully carried out and this forms the first report on the identification and characterisation of these genes from IMC. The full-length cDNA sequence of 754, 583, 1786 and1376 bp were obtained for C. catla kiss1, kiss2, kiss1r and kiss2r, respectively. The coding region of these four cDNAs encoded a peptide of 116, 125, 366 and 368 amino acids. Phylogenetic analysis of kiss1 and kiss2 genes showed high similarity with *G. rarus* followed by goldfish, *C. auratus*. However, kissppetin receptor i.e. kiss1r and kiss2r showed high similarity with *C. carpio* followed by *C. auratus*.

The core sequence, namely kisspeptin-10, encoded by the two genes exhibits conserved substitutions at position 1 and 10 with Tyr in Kiss1 (‘‘Y–Y form”) and Phe in Kiss2 (‘‘F–F form”), respectively. Core sequence of kiss1 have 80% similarity with human kiss1 and 90-100% similarity with other carp fishes determine biological activities of kisspeptin. However in case of kiss2 core sequence is 100% conserved among carp fishes and 80-90% with other animals.

The ontogeny pattern of kiss1 gene studied in different development stages of *C. catla* by semi-quantitative RT-PCR and qRT-PCR revealed that mRNA transcripts is highly expressed in mature stage followed by the post-spawning phase. Kiss1 gene mRNA transcript was detected in most of the fish tissues tested. The level of mRNA transcripts was found to express in brain, followed by testis, ovary and intestine. However, a low expression level was observed in tissues like kidney, liver, and gill in semi-quantitative RT-PCR which was further confirmed with qRT-PCR.

Nanodelivery method is gaining popularity day by day owing to its advantage over the conventional method. In present study, chitosan nanoparticles were prepared following the ionic gelation method. For conjugation of the chitosan nanoparticles with kisspeptin-10 (CK-10), a high pressure magnetic stir process was used. Chitosan nanoparticles showed 64% entrapment efficiency for kisspeptin-10. The mean particle size of the CK-10 nanoparticles was found to be 125 nm. The

nanoparticles were well dispersed and pretty stable as evinced by physico-chemical characteristics like polydispersity index (0.335 to 0.65) and zeta potential (34.95 mV). Chitosan nanoparticles (CK-10) showed 64% entrapment efficiency for K-10.

The treatment of fish with K-10 and CK-10 showed significant effect on hormonal level of LH, FSH, 11KT and 17-β-oestradiol. CK-10 induced sustained and controlled release of hormones with highest ($P<0.05$) surge at 12hrs. In case of K-10 treated fish, hormone levels reached its peak at 5-6hrs and then sharply declined. Histological observation of ovaries from different groups clearly showed influence of CK-10 in gonad and oocytes maturation in treated fish. This nanoformulation developed in the present study will help to overcome the problem associated with maturity and reproduction in fish by sustained and controlled release of the endogenous hormone. This is the first report on the development of chitosan-conjugated kisspeptin-10 nanoparticles for application in fish reproduction.

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