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Analysis of Gonadotropin Releasing Hormone (GnRH), Leutinizing Hormone (LHb) and Follicle Stimulating Hormone (FSHb) Genes in Indian Spiny Loach (*Lepidocephalus thermalis*)

R. Hamsavalli^{1*}, J. Jaculine Pereira¹, K. Karal Marx³ and R. Jeya Shakila²

¹Department of Fisheries Biotechnology, ²Department of Fish Quality Assurance and Management, Fisheries College and Research Institute, Thoothukudi, India

³Institute of post graduate study, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, OMR campus, Chennai, India

*Corresponding author

ABSTRACT

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Lepidocephalus thermalis forms a potential candidate species for aquaculture, being a native species it also has high market demand with easy domestication procedure. RNA was extracted from the ovarian tissues of *Lepidocephalus thermalis* by following the TRIZOL reagent method and cDNA was synthesized. The purity of RNA, cDNA and PCR products were checked. The PCR reactions were carried out with gene specific primers of gonadotropin releasing hormone II, gonadotropin releasing hormone III, leutinizing hormone and follicle stimulating hormone genes and the PCR products size of the four genes were 270-bp for gonadotropin releasing hormone II, 250-bp for gonadotropin releasing hormone III, 290-bp for leutinizing hormone and 260-bp for follicle stimulating hormone. The results from present study will serve as a primary data for further research and development in the reproductive genomic aspects of Indian spiny loach (*Lepidocephalus thermalis*).

Introduction

India recites more than 10% of global fish biodiversity and it also ranks second in the world in total fish production. The capture fisheries were considered as the major contributor of marine sector whereas in inland fisheries, aquaculture constitutes nearly 77% of total production. India has a rich natural heritage and nurtures a unique biodiversity

placing it among the 12 most biodiverse countries. Out of 31,100 extant fish species 2438 are known from Indian Sub-Continent (Froese and Pauly, 2009). The index of biodiversity utilized for aquaculture in India was of the order of 0.13 (~85% from Indian major carps; ~5% air-breathing fishes; ~10% rest all species together). Hence, more species need to be brought into culture practice, for the sustainability of aquaculture (Ayyappan *et*

al., 2010). On that note *Lepidocephalus thermalis* forms a potential candidate species for aquaculture, being a native species it also has high market demand with easy domestication procedure. Although loaches have an ornamental value and native or endemic status in India, very few studies have been carried out so far on their reproductive biology.

Gonadotropin-releasing hormone (GnRH) was a neurodecapeptide, which plays an essential role in regulation of gonad development and final sexual maturation in vertebrate (Gharaei *et al.*, 2011). The secretion of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and other GnRH-associated peptide (GAP) were stimulated by GnRH, which was synthesized in the hypothalamus and in pituitary glands (Yaron, 1995). In teleost fishes, the presence of either two or three forms of GnRH has been well documented (Kah *et al.*, 2007). The gonadotropin (GTH) includes follicle stimulating hormone (FSH) and luteinizing hormone (LH), which plays significant role in the regulation of fish reproduction. Both FSH and LH are glycoproteins synthesized in the pituitary, consisting in a common α subunit (GP α) and a hormone specific β subunit (FSH β and LH β), which provides biological specificity (Yoshiura *et al.*, 1999). The role of FSH predominates during early gonadal recrudescence, including vitellogenesis and spermatogenesis, while function of LH was more related to final gonadal maturation, ovulation and spermiation.

The genomics of gonadotropin releasing hormone (GnRH), leutinizing hormone (LH), follicle stimulating hormone (FSH) genes will be useful for getting in depth knowledge about the genetic makeup of the species and also helps to understand the reproductive biology of Indian Spiny Loach (*Lepidocephalus thermalis*). By processing

this genomic information we would be able to develop a scientific breeding protocol for *Lepidocephalus thermalis* in the future. This will pave the way to produce the alternative species for freshwater fish farming community.

Materials and Methods

The loaches for the experiments were collected from Chola fish farm, Vaduvor, Thiruvavur District of Tamil Nadu. Stocking density was 40 fishes per 1000 liter tank and sufficient aeration was provided. The bottom of the tanks was filled with sand and small gravels to provide suitable substratum to the fishes. The water temperature was recorded as $26\pm 0.5^{\circ}\text{C}$ and the pH 8.3. The fishes were feed twice with pellet feed *ad libitum*. They were also fed with artemia biomass once in a week. Water exchange was done once in a fortnight. Water was also sprayed at the surface of the tank for 10-15 min at a time for twice or thrice a day to mimic natural monsoon condition, to induce maturity of the fishes.

RNA extraction and preparation of cDNA

Total RNA was extracted from ovarian tissues of *Lepidocephalus thermalis* using Trizol™ reagent (Favougen Biotech Corp., Taiwan). And cDNA was synthesized from total RNA (5 μg) using Revertaid Reverse Transcriptase Enzyme (Thermo Fisher Scientific India Pvt. Ltd., Mumbai) as per the manufacturer's instructions.

PCR amplification of cDNA of GnRH II, GnRH III, LH β and FSH β genes

PCR (Eppendorf AG, Germany) was performed to amplify the desired cDNA fragments from the template. PCR amplification was performed in a total volume of 25 μl volume including 21 μl of *Taq* 2X

PCR master mix red (1.5 U *Taq* DNA polymerase) with 1.5mM MgCl₂ (Ampliqon, Denmark), 1 µl forward primer, 1 µl of reverse primer and 2 µl of template cDNA. The PCR amplification conditions (Table 2) included an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 52°C (GnRH II and GnRH III) / 51°C (FSHb) / 53°C (LHb) for 40 s, 72°C for 1 min with a final extension of 72°C for 10 min followed by 4°C forever. Primer used for amplifying GnRH II, GnRH III, LHb and FSHb genes were in table 1. The PCR product sizes were determined by 2% agarose gel electrophoresis run along with DNA marker i.e., 100bp DNA ladder.

Results and Discussion

The total RNA was extracted from the ovarian tissues of loach and from the RNA (Fig. 1) isolates cDNAs of GnRH II, GnRH III, FSHb, LHb were synthesized using the specific reverse primers. The RNA was electrophoresed through 1% agarose gel containing ethidium bromide (Fig. 1). By using the synthesized cDNA of GnRH II, GnRH III, LHb and FSHb amplification was carried out by PCR to amplify the GnRH II, GnRH III, LHb and FSHb genes. PCR product sizes were 270 for GnRH II, 250 for GnRH III, 290 LHb and 260 for FSHb (Fig. 2 and 3).

Gonadotropin-releasing hormone (GnRH) is well known for its role in moderating gonadotropin release from the pituitary. GnRH is a member of family of neuropeptides that play a key role in the development and maintenance of reproductive function in vertebrates. The midbrain neuron population in fish is believed to be exclusively GnRH-II-producing neurons and these cells are suggested to play a role in reproductive behavior (Sherwood and Adams, 2005). The PCR amplified product size of

GnRH II gene examined in this study was 270 bp from ovarian cells of *Lepidocephalus thermalis* for the primers forward 5'-ATGGTGCTGGTCTGCAGGTG-3' and reverse 5'-GTAGGAAGTCTGCAAATGGT-3' whereas in hard-lipped barb (*Osteochilus hasselti*) Prayogo *et al.*, (2011) amplified a product of 253 bp from brain cells using the primers forward 5'-CCTAAGATGGTGCACATCTGCAGGCT-3' and reverse 5'-GGGCTCGAGTCTTTGGAAATCCCGT-3' and in catla (*Catla catla*) Rather *et al.*, (2015) amplified a product of 202 bp from brain cells using forward 5'-ATGGATCCGTGAGATTGCA CCA-3' and reverse 5'-GTTTCTTCCCTGGGTCTCAGGTAGC-3'. The brain cells were not used in this study because the size of the brain of *L.thermalis* is too small (insufficient in quantity) to carry out the genomic analysis, therefore the analysis was conducted on the ovarian tissue only.

GnRH III gene is expressed mainly in the olfactory system. GnRHIII constitutes the main hypophysiotrophic factor governing the release of gonadotropins from the pituitary gonadotropes and these neurons innervate the pituitary (Kah *et al.*, 1986). The highest content of pituitary GnRH is the form that is found in the preoptic neurons (Carolsfeld *et al.*, 2000; Collins *et al.*, 2001; Amano *et al.*, 2002). The amplified PCR product of GnRH III in the present study was 250 bp from ovarian cells of *Lepidocephalus thermalis* using primers forward 5'-CACAGCAGTTTTAGCATGGAGTG-3' and reverse 5'-ACACTCTTCCCC GTC TGTCGG-3'. Prayogo *et al.*, (2011) reported that in hard-lipped barb (*Osteochilus hasselti*), a product of 285 bp for GnRH-III from brain cells using the primers forward 5'-GGACCTAAG AGCATGGAGTGGAAAG GAAG-3' and reverse 5'-GGGCTCGAG CACTCTTCCCTCGTCTGTT GG-3'.

Gonadotropins (GTHs), follicle stimulating hormone (FSH) and luteinizing hormone (LH), are critical hormones in the regulation of reproduction in vertebrates, including fish (Yaron *et al.*, 2003). FSH is involved in the initiation of gametogenesis and regulation of gonadal growth, whereas LH mainly regulates gonadal maturation, spermiation and ovulation. FSH and LH are complex heterodimeric glycoproteins, consisting of a common α subunit and a hormone-specific β subunit, encoded by different genes (Pierce and Parson, 1981; Yoshiura *et al.*, 1999). Both subunits bind non-covalently into the gonadotropic cell, to form the biologically active dimeric hormone (Pierce, 1988). The first teleost LHb subunit gene to be isolated and sequenced was that of the Chinook

salmon, *Onchorhynchus tshawytscha* (csGtHIIb or csLHb) by Xiong and Hew (1991). The product size of LHb reported by Rather *et al.*,(2016) was 629 bp in catla (*Catla catla*) from brain cells using forward 5'-GTCCTACTAGCTGTTGCTCAAAGC TC-3' and reverse 5'-CATAGTGCACAGG CTGCAGTCGC-3' primers. In the present study, the PCR product size of LHb was 290 bp from ovarian cells of *Lepidocephalus thermalis* using the primers forward 5'-CAAGAGCCCATTTCAC-3' and reverse 5'-AGGCTGCAGTCGACAGCT-3'. The LHb product size in Zebra fish was 958 bp from brain cells (So *et al.*, 2005) using the primer forward 5'-CAAGAGCCCATTTCAC-3' and reverse 5'-AGGCTGCAGTCGACAGCT-3'.

Table.1 Primers selected for amplification of GnRH II, GnRH III, LHb and FSHbDNA

S.NO	GENE	PRIMERS	REFERENCE	
1	GnRH II	Forward	ATGGTGCTGGTCTGCAGGCTG	Kuo <i>et al.</i> , 2005
		Reverse	GTAGGAAGTCTGCAAATGGGT	
2	GnRH III	Forward	CACAGCAGTTTTAGCATGGAGTG	Kuo <i>et al.</i> , 2005
		Reverse	ACACTCTTCCCCGTCTGTCGG	
3	LHb	Forward	CAAGAGCCCATTTCAC	So <i>et al.</i> , 2005
		Reverse	AGGCTGCAGTCGACAGCT	
4	FSHb	Forward	CAGCTGTCGGCTCACCAATA	NCBI/Primer-BLAST(AB003583.1)
		Reverse	GCAAAGCAGTGTGGTTTCC	

Table.2 PCR protocol used for amplifying GnRH II, GnRH III, LHb and FSHbDNA

Step	Process	Temperature	Duration	
1	Initial denaturation	94°C	10 min	
2	Denaturation	94°C	30 sec	35 cycles
3	Annealing	52°C(GnRH II and GnRH III)	40 sec	
		53°C (LHb) 51°C (FSHb)		
4	Extension	72°C	1 min	
5	Final extension	72°C	10 min	

Fig.1 RNA isolated from *L.thermalis*; Lane 1: 100bp ladder; Lane 7- 8: Total RNA; Lane 6-5: Negative control

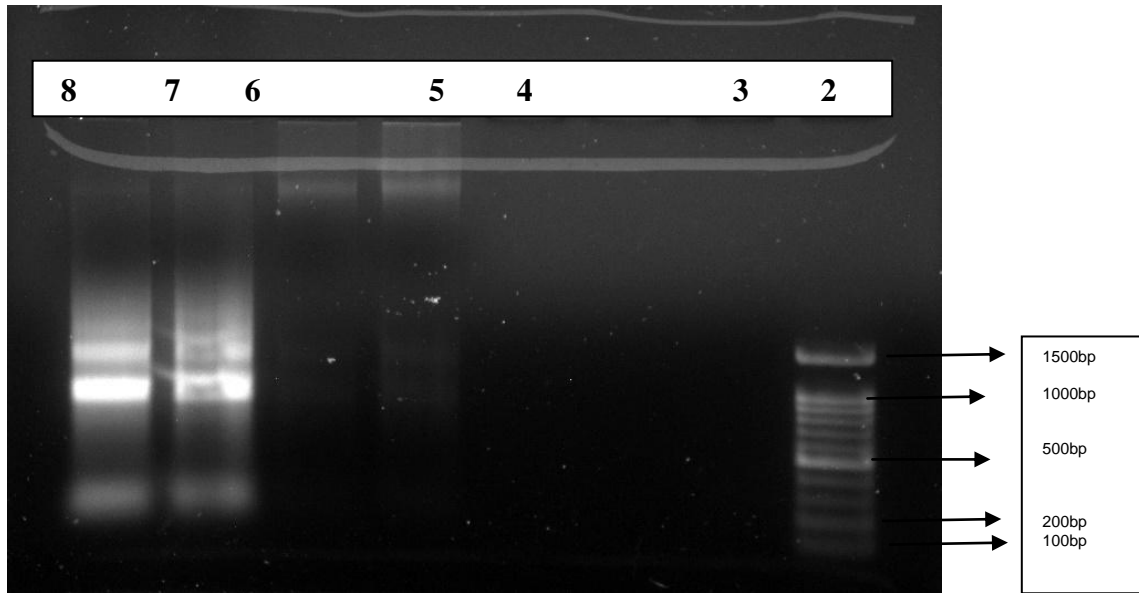


Fig.2 PCR amplified genes (FSHb, GnRH II, GnRH III) of *L. thermalis*; Lane 1: 100bp ladder ; Lane 3: GnRH II; Lane 5: GnRH III; Lane 6: LHb

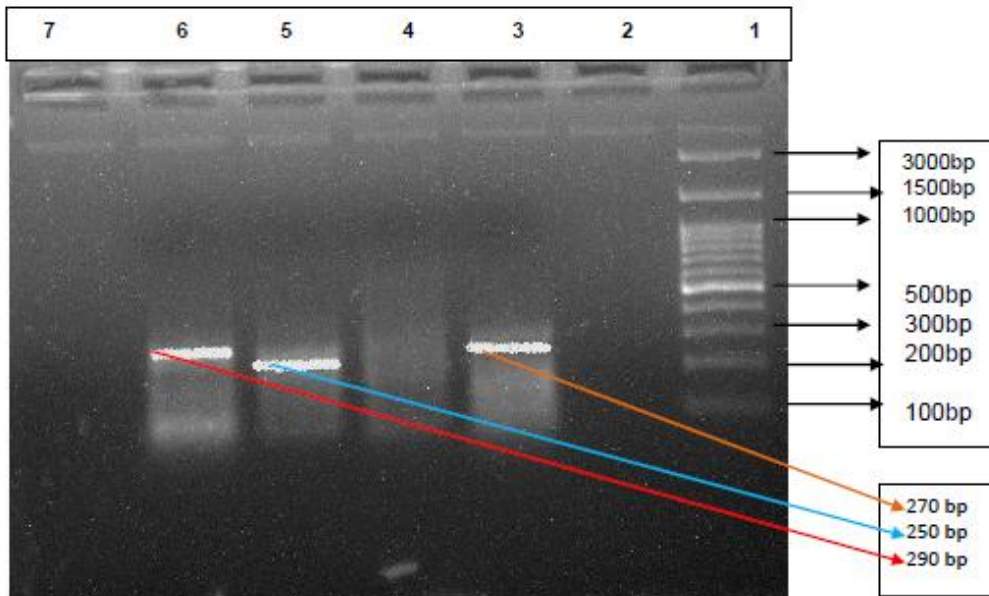
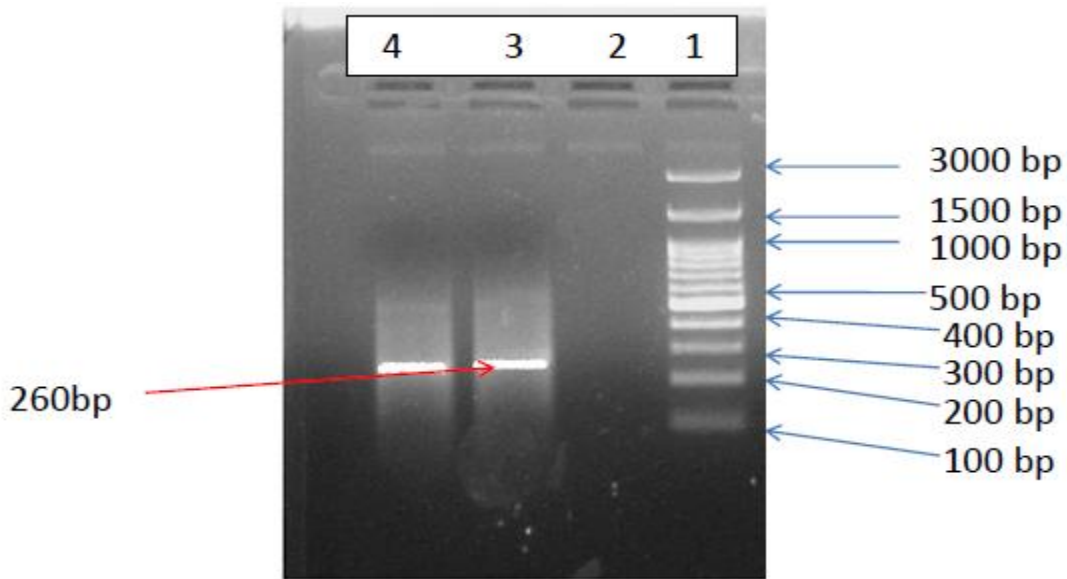


Fig.3 PCR amplified genes (FSHb) of *L. thermalis*; Lane 1: 100bp ladder; Lane 3-4: FSHb



The PCR product size of FSHb gene examined in this study was 260 bp from ovarian cells of *Lepidocephalus thermalis* using the primers, forward 5'-CAGCTGTCGGCTCACCAATA-3' and reverse 5'-GCAAAGCAGTGTTTGGT TTCC-3'. In zebra fish, So *et al.*, (2005) recorded 1038 bp size of FSH from brain cells using primers forward 5'-CATTGATTCCCA GATGAGGA-3' and reverse 5'-TTGCATGA CATACTCAGCAGCT-3'. This implies that the expression of GnRH, FSHb and LHb genes are not confined to brain and pituitary, but also express in the ovarian tissues. The identification of GnRH, FSHb and LHb genes synthesised in gonads gives a relatively new direction about their function. The first evidence for expression of GnRH in gonads was in rat ovary (Oikawa *et al.*, 1990). The first report of GnRH gene expression in the gonads (ovary and testis) of adult midshipman (*Porichthysnotatus*) was elucidated by Northern blotting (Grober *et al.*, 1995). A number of the GnRH forms are found in fish gonads of several fishes. For instance sGnRH, cGnRHIII and sbGnRH, are reported in testis of the cichlid, *Haplochromis burtoni* (White

and Fernald, 1998) by PCR; sGnRH (mRNA-1 and mRNA-2) and cGnRH-II are found in ovary and testis of rainbow trout by sequencing (Gray *et al.*, 2002);s GnRH (mRNA-2) was detected in ovary and testis of sockeye salmon by sequencing; GnRH-I was found in adult sea lamprey testis but not in the ovary as examined by Northern blotting (Suzuki *et al.*, 2002). Sherwood and Adams (2005) stated that GnRH was best known in vertebrates for its expression in neurons and play a role in stimulating the release of gonadotropins from the pituitary. However, expression of GnRH, FSHb and LHb along with their receptors was not confined to the brain and pituitary but is widespread in peripheral tissues. Two sites of interest were the ovary and testis because they express both the genes (GnRH,FSHb and LHb) and their receptors. Therefore the result of the present study was in agreement with earlier studies.

In conclusion, the results from present study will serve as a primary data for further research and development in the reproductive genomic aspects of Indian spiny loach (*Lepidocephalus thermalis*). It provides the

idea about maintenance of Indian spiny loach (*Lepidocephalus thermalis*) and the way of preparing the samples for maturation that helps in further analysis in the aspect of reproductive genomic study. The PCR products can be further analyzed and sequenced to get better knowledge about the gonadotropin-releasing hormone II (GnRH II), gonadotropin-releasing hormone III (GnRH III), luteinizing hormone (LH), follicle-stimulating hormone (FSH).

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