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EXPRESSION OF ENZYME P450C17 ENCODING GENE CYP17 IN THE BRAIN OF *Labeo rohita* (HAM.)

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ABSTRACT

Brain is a steroidogenic organ which produces steroids. Our study has been focused to analyze the gene expression in the brain of an Indian Major Carp (IMC), *Labeo rohita*. This is the first attempt to report the gene expression of CYP17 which codes for the steroidogenic enzyme P450c17 in *L. rohita*. The brain samples were collected and Total RNA was isolated. The isolated RNA was reverse transcribed into cDNA using RT-PCR. The synthesized cDNA was then added with the gene specific primer and subjected to PCR. Two bands at 402bp and 550bp were obtained which confirms the CYP17 gene expression. The PCR product was sequenced. The obtained sequences of *L. rohita* were multiple aligned with mammals and with fishes. Phylogram were also plotted to know the relationship, similarity and evolutionary relationship of *L. rohita* with other organisms which shows that *L. rohita* is far distantly placed in the evolutionary tree.

INTRODUCTION

Neurosteroids are steroids that are synthesized *de novo* in the brain from cholesterol (Baulieu, 1991; Corpechot *et al*., 1981) and include allopregnanolone, DHEA, PREG, their sulfate and fatty acid esters. The human cytochrome P450c17 has two distinct activities responsible for the synthesis of glucocorticoid and sex steroid precursors from PREG. This 56kDa steroidogenic CYP is located in the zona fasciculata and zona reticularis of the adrenal cortex (and the gonad tissues), where it catalyzes the pivotal step in the formation of glucocorticoids in the former and androgens in the latter; zona glomerulosa lacks CYP17 and consequently produces mineralocorticoids. Zwain and Yen (1999), proved the presence of gene responsible for steroidogenesis in neural cells. The key enzyme for androgen synthesis, cytochrome P450c17 hydroxylase was identified in astrocytes, oligodendrocytes and neurons. Wang and Ge (2004), reported the cloning and expression of ovarian P450c17 in gonadal and extra gonadal tissues of zebra fish. This is the first attempt to report the presence of P450c17 in the brain of an IMC, *L. rohita*. This study mainly deals with the analysis of the P450c17 gene identification, expression and sequencing. Although the two types of P450c17 reported earlier, the present study will give an idea about the existence of the two types of gene in the *L. rohita* also or not.

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MATERIALS AND METHODS

Sample collection

Brain samples of *Labeo rohita* were collected from the Lake Sevilimedu located at Kanchipuram. Fishes were captured in live condition and they were dissected out to get brain sample. The brain tissues were fixed in sterilized vials containing RNA later and kept in ice and they were stored at -70˚C until analysis.

Total RNA isolation

Total RNA was isolated by homogenizing the whole brain sample (200mg) with 500 µl of Tri Reagent (Sigma) and 200 µl of DEPC water. After homogenizing the sample, it was then incubated at -20˚C for 5 minutes. 0.2ml of chloroform was added and incubated at -20˚C for 5 minutes. It was then centrifuged at 12,000rpm (4˚C) for 15 minutes. Supernatant was collected in a fresh tube. Total RNA was precipitated by adding equal volume of isopropanol and it was stored at -20˚C

for 45 minutes. The sample was centrifuged again at 12,000 rpm (4˚C) for 15 minutes. Total RNA was obtained as a pellet and added 75% ethanol (7.5 ml of Ethanol was mixed with 2.5ml of DEPC water) it was centrifuged at 12,000 rpm for 5 minutes and air dried the RNA pellet, and dissolved by adding 40 µl of DEPC water. It was then freezed and stored at -20˚C for half-an-hour. It was then tested with 1.2% agarose gel for its purity.

Synthesis of first strand cDNA

Total RNA was isolated from the brain of *L. rohita*. The separated RNA has been reverse transcribed into cDNA using RT-PCR method. A clean PCR tube was taken to this 1 µl of the sample, 1 µl of Oligo (dT) $_{18}$ primer, 12 µl of de-ionized water was added and it was spinned gently for few seconds in a micro centrifuge. This mixture was incubated at 70˚C for 5 minutes. After incubation 4 µl of 5X reaction buffer, 0.5 µl of Ribonuclease inhibitor (40 µl/dl), 2 µl of 10mM dNTP mix were added. It was mixed gently and centrifuged. This mixture was incubated at 42˚C for 5 minutes. After the period of incubation it was added with M-MuLV reverse transcriptase (20U/µl) to make it around 20 µl volume. It was incubated finally at 42˚C for 60 min and 25˚C for 10 min. The reaction was stopped by heating at 70˚C for 10 min and chilled on ice. PCR products were then tested with agarose gel electrophoresis. cDNA was quantified to measure the concentration of DNA using the Smart spec plus spectrophotometer.

Polymerase Chain Reaction

2 µl of cDNA was taken in a sterilized PCR tube with 1 µl of CYP17 primers of sense and antisense. The following primers were used.

TYPE		SEQUENCE (5'-3')
P450c17-I	Forward	GACAGCCTGGTGGACATCTT
	Reverse	GATCTCTCTGCACGTGGTCA
P450c17-II	Forward	AAGGACTTTGCTGGACGACCGAG
	Reverse	TGTCCCAGTGATGGGGGTCGTGGTG
	Forward	CCAGAGAGGTTCTCCTGCTG
	Reverse	TGGACAACAGCTCCTCACAG

25 µl PCR master mix consists of all basic components: Taq DNA Polymerase, dNTPs and reaction buffer (1.5mM Magnesium chloride) were added. The PCR amplification was used to check the expression of the mRNAs of CYP17 enzyme in the brain sample. The temperature followed in the amplification is as follows: 94°C for 2 minutes in 1 cycle. 95°C for 30sec, 48°C for 30sec and 72°C for 1 minute in 35cycles finally holding temperature is 4°C. The PCR products of gene specific primer of CYP17 along with 100bp DNA Ladder were then subjected to 1.2% agarose gel electrophoresis. After running the gel, the images of specific bands were captured using UV trans illumination under Gel documentation system.

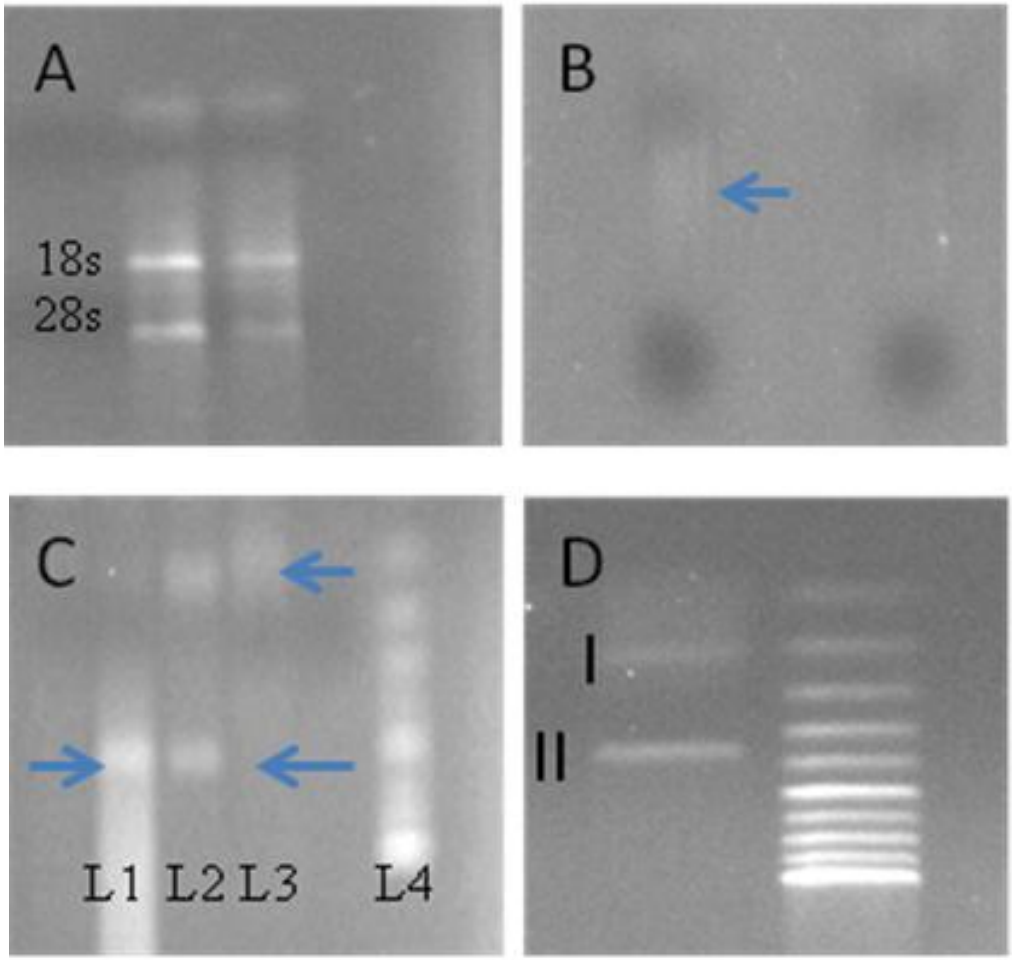

Fig.1. A. Shows the total RNA, B- shows the cDNA, C- L1 shows the P450c17 type II, L2 shows P450c17-I and II and L4 shows the100bp marker DNA, and D- shows the P450c17 type I, II and marker DNA

Sequencing

The PCR product was sequenced and the mRNA sequences of CYP17 obtained for *L. rohita* were shown in the result. The mRNA sequences of CYP17 of *L. rohita* were multiple aligned with vertebrate organisms to know the similarity, relationship and evolutionary significance of *L. rohita* with other organisms.

RESULTS

Total RNA from the whole male brain of *L. rohita* was isolated and checked the purity of the RNA separation (Fig. 1.A). The isolated RNA was quantified. The isolated RNA was reverse transcribed into cDNA using RT-PCR. The cDNA strand is shown in (Fig. 1.B) cDNA was used to catch the specific P450c17 gene with the gene specific primer designed for type-I and type-II genes by using PCR at specific annealing temperature to find out the gene expression. The gel was loaded with 100bp DNA Ladder and PCR product with the primer of both P450c17 type I and II. The PCR product showing the bands were represented in (Fig. 1.C) Lane 1 shows 100bp DNA ladder, L2 and L6 shows no band for P450c17 type I, L3 shows the band for P450c17 type I, L4 shows the band for P450c17 type I and II, and L5 shows the band for P450c17 type II. DNA was quantified. Two bands approximately 400bp and 600bp were obtained (Fig. 1.C and D). The PCR product was sequenced. The obtained sequence of *L. rohita* was multiple aligned with mammals and with fishes. Sequences was analyzed, phylogram showing the similarity of P450c17 type I of fishes with *L. rohita* were shown in Fig. 2 and Fig. 4.

It reveals that the tree was bifurcated into two branches with *G. aculeatus* at one end and with *L. rohita* at the other end. *T. rubripes* has originated from a separate point from the Phylogram tree. *D. rerio* and *L. rohita* came from the same branch reveals that they have originated from the same point and they share similar conserved region. The phylogaram analysis reveals that *L. rohita* is phylogenetically far from *G. aculeatus* which was at the top of the tree. Phylogram tree showing the similarity of P450c17-II of fishes along with *L. rohita* were shown Fig. 3. The tree reveals that *G. aculeatus* and *T. rubripes* originated from the same point. Whereas *M. albus* and *L. rohita* were originated from the separate branch. *G. aculeatus* is the distantly related species and *M. albus* is the closely related species to *L. rohita*. The phylogram states that *H. sapiens* has originated separately in the evolutionary tree at one end and with *L. rohita* at the other end. *R. norvegicus* and *M. musculus* have originated from the same branch revealed they share similar conserved region. *L. rohita* is far distantly placed in the evolutionary tree.

DISCUSSION

P450c17 enzyme expression has been reported in different tissues in vertebrates. P450c17 expression in the CNS and PNS has been proposed first by Compagnone *et al*. (1995). In the brain of adult mammals, the regional distribution of the mRNA encoding P450c17 has been studied by RT-PCR (Stromstedt and Waterman, 1995; Kohchi *et al*., 1998) but the localization of the mRNA at the cellular level remains unknown. Previous reports have shown the ability of cat brain and pituitary tissues (Ficher and Baker, 1978), rat neurons and astrocytes (Zwain and Yen, 1999), rat hippocampal tissues

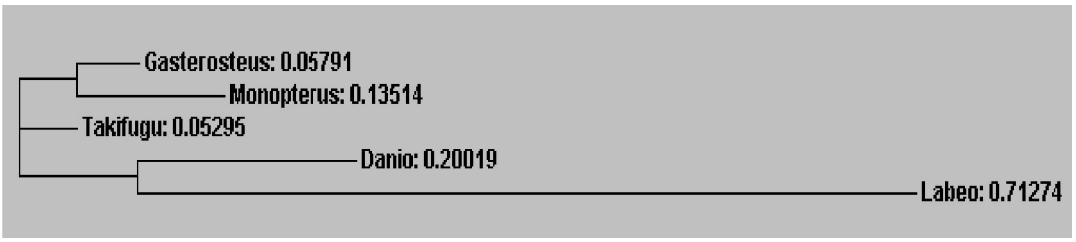

Fig. 2. Phylogram of P450c17 type I of fishes with the obtained sequence from *Labeo rohita* showing its evolutionary distance and relationship with other fishes

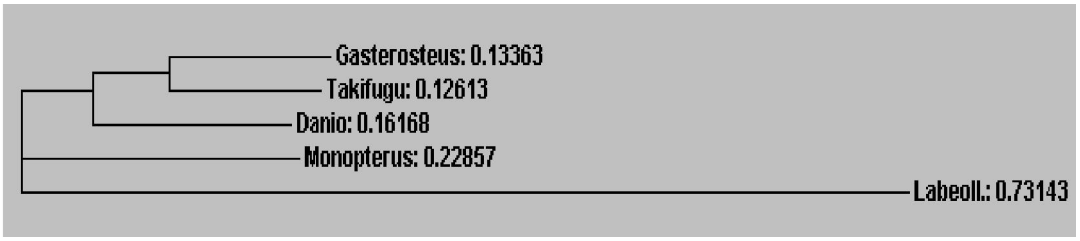

Fig. 3. Phylogram sequence of P450c17 type II of fishes with the obtained *Labeo rohita* type II showing its evolutionary distance and relationship with other fishes

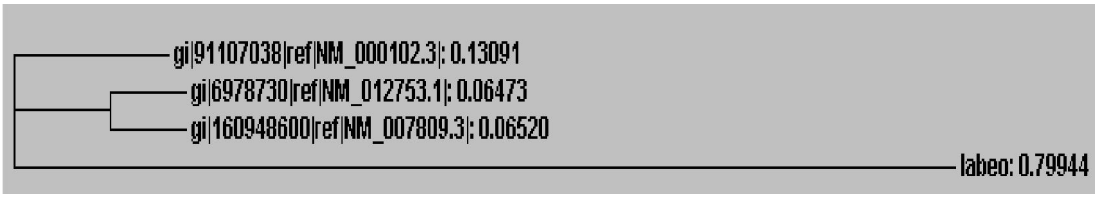

Fig. 4. Phylogram of P450c17 type I of vertebrates along with *Labeo rohita* showing its evolutionary distance and relationship with each other

(Hojo *et al*., 2004) and quail brain tissues (Matsunaga *et al*., 2002) to convert Δ5P and P into DHEA and Δ^4 through a P450c17-dependent mechanism. The zonal distribution of P450c17 has been studied in the adrenal cortex of the guinea pig (Shinzawa *et al*., 1988; Colby *et al*., 1993) Rhesus monkey (Mesiano *et al*., 1993), and the human fetus (Breault *et al*., 1996). Hamsters differ from other rodents in that they express adrenal P450c17 (Cloutier *et al*., 1997) and produce cortisol as their major corticosteroid (Le Houx *et al*., 1992). Le Goascogne *et al.* (1991), reported immunoreactive P450c17 in rat gonads but not in the adrenals, thus confirming the absence of expression of this cytochrome in this species. Immunohistochemical observations of $P450_{scc}$, 3β-HSD, P450c17 and P450 arom have been reported in the corpus luteum of numerous species, including humans (Suzuki *et al*., 1993), bears (Tsubota *et al*., 2001), horses (Albrecht *et al*., 2001) dogs (Nishiyana *et al*., 1999), pigs (Guthrie *et al*., 1994) seals and sea lions (Ishinazaka *et al*., 2001), sheep and cows (Conley *et al*., 1995). Immunological studies of Weng *et al* (2005), proposed that immunostaining for P450c17 and P450arom was observed in syncytiotrophoblast of the placenta in pregnancy (120 days). Do Rego *et al*., (2007) reported the first exhaustive mapping of P450c17-immunoreactive cells in the brain and pituitary of a vertebrate. The anatomical distribution and cellular localization of P450c17-immunoreaactive structures in the CNS and pituitary of the frog was reported by Tremblay *et al*., (1994).

Zhou *et al*., 2007 reported the two P450c17 types encoded by two different genes in Fugu, tetraodon, stickleback, tilapia (*Percomorpha*), medaka (*Atherinomorpha*) and zebrafish (*Ostaruiohysi*), which belong to three different clades in the phylogenetic tree (Nelson, 1994). They did an *in silico* search on the genomes of Xenopus, chicken, mouse, rat, and human but failed to obtain any P450c17-II-like sequences. They have reported from the sequences and structure of the medaka P450c17 type I and II genes that the two are completely different. Many report explaining the expression of the enzyme P450c17 in different organisms in different tissues. But till date, no report confirms the expression of the enzyme P450c17 particularly in the brain of an Indian carp, *L. rohita*. Our results confirm the presence of the gene CYP17 that codes for the enzyme P450c17 in the brain of *L. rohita* by analyzing the gene expression through PCR technique. In our experiment P450c17-I and II was observed and its mRNA sequence were partial, which suggests that both the types are expressed in fishes. Results of our experiment are the first attempt to report both the types of P450c17 particularier on the brain of *L. rohita*. The obtained mRNA sequence of both the types of P450c17 of *L. rohita* were multiple aligned with different classes of vertebrates and with fishes to know its similarity, evolutionary distance and relationship with other organisms. Results from our experiment concludes that brain is a steroidogenic organ by producing the steroidogenic enzymes.

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