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Identification, expression and sequence analysis of the gene *cyp19a1a* in the ovary of Indian major carp *Catla catla* throughout a reproductive cycle and in the Extragonadal tissues

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ABSTRACT

Aromatase is an enzyme responsible for a key step in the biosynthesis of estrogens, a product of a unique gene called *cyp19*. *Catlacatla* like other teleosts, expresses *cyp19a1a* gene mainly in the ovary, responsible for the production of aromatase enzyme. The abundance of the mRNA encoding *cyp19a1a* was determined by rtqRT-PCR in the tissues of the ovary of catla fish throughout the annual reproductive cycle. The expression of *cyp19a1a* has seen its peak level during the previtellogenic and vitellogenic period and the expression was very law during the time of resting period or postvitellogenic period. The expression level was prominent in the preparatory period, but it is lesser than previtellogenic and vitellogenic period. The expression of *cyp19a1a* is also seen in other tissues of brain, kidney, intestine, liver and gill. There was no expression of *cyp19a1a* in the male gonad. It confirms the significant role of this gene in the ovarian differentiation and in the different phases of reproductory cycle of *C. catla*. Phylogenetic results indicate that the gene *cyp19a1a* is evolutionally conserved throughout the vertebrate phyla.

Key words *: Aromatase, Cyp19a1a, Vitellogenic, HPF (hour post fertilization), Phylogeny.*

Introduction

In vertebrates, the growth and maturation of the ovarian follicle is dependent on the appropriate dynamics of sex steroid secretion, which is dictated by gene expression of the steroidogenic enzymes (Kumar *et al*., 2000). Steroid hormones produced by the gonads play a critical role in gonadal differentiation and sexual maturation and behavior in vertebrates in general (Fostier *et al*., 1983). Cyp19 is an essential enzyme catalysing the conversion of androgen into estrogens, a key biosynthetic step associated with sex differentiation and gonadal development (Conley and Hinshelwood, 2001) in vertebrates. It is the terminal enzyme in the steroidogenic pathway, the product of the cyp19a1a gene.The biosynthesis of estrogens occurs throughout the entire vertebrate phylum where *P450 arom* is mainly detected in the gonads (Simpson *et al.,* 2002).

Estrogens have long been regarded as important hormones for ovarian differentiation in non-eutherian vertebrates. For instance, administration of estrogens can reverse phenotypic males to females in birds (Scheib, 1983), reptiles (Larios *et al*., 1997) and teleosts (Kobayashi and Iwamatsu, 2005). Treatments with aromatase inhibitor (AI), that block aromatase activity, also resulted in the production of phenotypic males from females in birds (Hudson

et al., 2005), reptiles (Belaid *et al*., 2001) and fishes (Komatsu *et al*., 2006; Rashid *et al*., 2007). Endogenous estrogens are now proved to be key steroids and aromatase a key enzyme for ovarian differentiation in reptiles (Pieau and Dorizzi, 2004), birds (Villalpando *et al*., 2000) and fish (Guiguen *et al*., 2010). The specific over-expression of *cyp19a1a* during ovarian differentiation has been observed in many fish species including Nile tilapia (Ijiri *et al*., 2008), rainbow trout (Vizziano *et al*., 2007) and European seabass (Blázquez *et al*., 2008).

Cyp19a1a expression was preferentially expressed in carp ovaries, but also was present at much lower levels in testes and brains of both males and females(Tang *et al*., 2010). This expression pattern was also found in other species including zebrafish (Trant *et al*., 2001), sea bass (Blazquez and Piferrer, 2004) and wrasse (Choi *et al*., 2005). These studies support the idea that high *cyp19a1a* levels are essential for estradiol biosynthesis and ovary development in fish (Masaru *et al*., 1998). The analysis of real-time quantitative RT-PCR revealed that the clownfish *cyp19a1a* transcript was mainly expressed in the ovary of female (Kobayashi *et al*., 2010).Gonadal *cyp19a1a* transcript levels and enzymatic activity in ovarian follicles are also significantly increased during vitellogenesis in teleosts (Gen *et al*., 2001; Nunez *et al*., 2006). In European seabass, ovarian aromatase activity was found to be significantly higher after morphological gonadal sex differentiation (Blázquez *et al*., 2008). In the *Oncorhynchusrhodurus*, E2 has been detected in female serum soon after differentiation (Nakamura and Nagahama, 1993). In the Japanese medaka, expression of *cyp19a1a* has not been detected in the differentiating ovary but only later on at the onset of ovarian gametogenesis (Suzuki *et al*., 2004).

Physiological and molecular studies on the role of estrogens in fish have initially been developed very later mainly in relation to vitellogenesis (Ho, 1991). Studies have shown that transcript levels of gonadal aromatase (Cyp19a1a) are increased in association with aromatase enzyme activity during vitellogenesis (Gen *et al*., 2001). Immunohistochemical analysis revealed that cyp19a1a in miiuy croaker was localized exclusively in the cytoplasmic of thecal and granulosa cells surrounding the oocytes. Both the protein and mRNA levels of cyp19a1a were increased significantly at the stage III follicles (mid-vitellogenic) and then decreased along with vitellogenesis (Wei *et al*., 2017). Xiaowu *et al.,* 2018 expose that *cyp19a1a* is detected mainly in stromal cells around the oocytes of stage I ovary. Yufeng *et al*., 2016 reveal that *cyp19a1a* and *Foxl2* play a vital role in the gonad development of female Japanese flounder.

As we have seen above, estrogens are involved in various aspects of sexual differentiation, vitellogenesis, gonadal development, reproduction and reproductivebehaviors (Lange *et al*., 2003). So we can conclude that Cytochrome P450 aromatase (P450arom), the endoplasmic reticular enzyme catalyzing the production of estrogens from androgens, via production of estradiol-17b (E2) plays a potent role in preserving the continuity of life in diverse vertebrates including fishes by sustaining important physiological process, reproduction. But studies showing the significance of this gene throughout the reproductive cycle are very rare. In the present swot up we are trying to analyse the gene expression of *cyp19a1a* in the Indian major carp *Catlacatla* and its alteration in the annual reproductive cycle. So the outcome of the research is discussed in terms of its direct and indirect applications to aquaculture with a view to manipulate its breeding at a desired time of the year to meet market demand. Though many studies were carried out on the function of the *cyp19a1a* gene in many fish species including carp family, until now no attempt has been made with regard to Indian major carp in this direction.

Materials and Methods

Sample collection

The fish samples like gonads and other tissues of the Indian major carp, *C. catla* were collected from the Tamil Nadu Fisheries Department Corporation (TNFDC), Sathanoor Dam, situated about 200 km south of Chennai. Monthly samples were collected from the matured live fishes and the tissues were pooled in eppendorf tubes with RNA later and stored in –20 °C until RNA extraction.

Total RNA extraction

Total RNA was isolated from the 100 mg. of tissue using TRIzol method. The quantity and quality of RNA were determined by UV absorbance at 260 and 280 nm wavelength. Agarose gel electrophoresis has done and the clear 18S and 28S RNA bands has visualised by UV illuminator and gel documentation has done with the computer.

cDNA synthesis

One microgram of total RNA from the sample tissue was reverse transcribed using M- MuLV RT- PCR Kit according to manufacturer's instruction. The RT-PCR products were quantified and qualified by using spectrophotometer and gel electrophoresis.

Real-Time Quantitative RT-PCR

Suitable primers were constructed by appropriate software for the gene *cyp19a1a* and the housekeeping gene *beta actin*. Here *beta actin* will be used as control for the whole work.

*cyp19a1a*F: 52-TGGTGAGGARACTCYCATC-32

*cyp19a1a*R: 52-ACTBTCTTCTGNCAGGTGT-32

Beta Actin F: 52-CGGTTATCGTTG TAGGC ACG-32

Beta Actin R : 52-CACTGCCTGCACAAAGA ACT-32

Transcript abundance of *cyp19a1a* was quantified by rtqRT-PCR of total RNA isolated from the ovaries of catla fish throughout the year to find out the variation of the gene expression in the annual breeding cycle. rtqRT-PCR cycle conditions were 40 cycles with 95 °C for 10 minutes, 95 for 15 seconds and 60 for 1 minutes. rtqRT-PCR was carried out with SYBR Green fluorescent label using beta actin as an endogenous control.

Sequencing and Phylogenetic analysis

The PCR product was send for sequencing and sequence data is submitted to Genbank.. According to the sequence data multiple sequence alignment and phylogenetic analysis has done by using the software Mega 5.

Results

The onset of gonadal recrudescence was evident by the month of January and it is the following months which include the preparatory period. Even a slight increase in the gene expression can see throughout the preparatory period. Then a steady increase can see in the month of April which is the beginning period of previtellogenic phase and this is the time during which a maximum intensity of aromatase expression is seen. Then in the following months upto the month of August *aromatase* expression is almost in high level eventhough a slight decrease can see in the following months including both previtellogenic and vitellogenic phases. Then a steady decrease can see from the month of September to December which is the postvitellogenic period and during this time *aromatase* expression in a very low level. Yet again the preparatory phase starts from the month of January and expression level increasing suddenly during this period from the postspawning phase (Fig. 1).

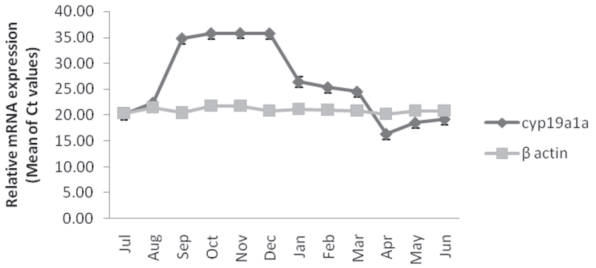

Fig. 1. The expression of *cyp19a1a* and the housekeeping gene *beta actin* in an annual reproductive cycle of *C. Catla*

The abundance of the mRNA encoding *cyp19a1a* was determined by qRT-PCR in the tissues of the ovary of catla fish throughout the annual reproductive cycle. As mentioned in previous findings the expression of *cyp19a1a* has seen its peak level during the previtellogenic and vitellogenic period and the expression was low down during the time of resting period or postvitellogenic period. The expression level was prominent in the preparatory period but it is slighter than previtellogenic and vitellogenic period. From this expression data we can understand that this gene has a significant role in the reproductary function. Because it is well expressed in all the important reproductory phases except postspawning period (Fig. 2).

The samples of testes from matured male fishes were also subjected to total RNA extraction and PCR amplification and the result shows no expres-

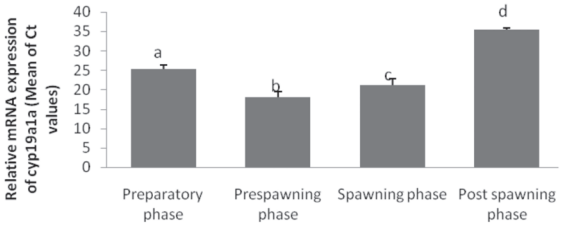

Fig. 2. The expression of *cyp19a1a* of *C. catla* in different reproductory phases of ovary (a differ from b and d: $p<0.01$; b differ from a and d: $p<0.01$; c differ from a and d: $p<0.01$)

sion of *cyp19a1a* in the male gonads. The expression of *cyp19a1a* was expressed in other tissues. Samples of brain, kidney, heart, muscle, intestine, liver, testes and gill were processed to check the expression of *cyp19a1a*. There was no expression of this gene in the samples of heart, muscle and testes. But *cyp19a1a* is clearly expressed in the tissues of brain, kidney, intestine, liver and gill. This results shows that *cyp19a1a* is not only present in the ovary alone but also in many other different tissues(Fig.3).

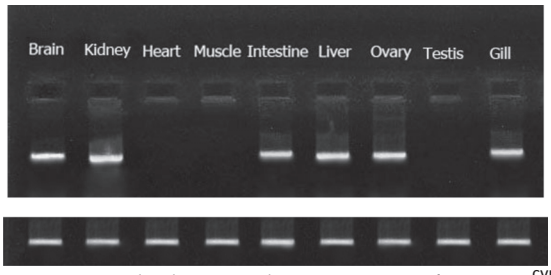

Fig. 3. Bands showing the expression of *cyp19a1a* and β *actin* in different tissues of *C. catla*

Partial sequencing of *cyp19a1a* in *C. catla* was successful and obtained 550bp sequence data. Blast result shows 99% similarity for the catla*cyp19a1a* sequence with other carp species. The sequence data is submitted to Genbank and theaccession number is KJ699355. Multiple Sequence Alignment was done with the sequence of *cyp19a1a* in *C.catla* with those of other vertebrate species. The 33 sequences used for analysis were downloaded from NCBIGenBank, and have the following accession numbers : *Carassiusauratus* (AB009336), *Carassiusauratus* x *Cyprinuscarpio* x *Carassiuscuvieri* (KC147010), *Cyprinuscarpio* (EU375455), *Carassiuscarassius* red var x *Cyprinuscarpio* (KC147011), *Gobiocyprisrarus* (GU220394), *Ovisaries* (Sheep) (NM001123000), *Capra hircus* (Goat)(NM001285747), *Monopterus albus* (EU841366), *Oreochromis niloticus* (AF472621), *Homo sapiens (Human)* (AC012169), *Danio rerio* (NM131154), *Anguilla japonica* (AY540622), *Oncorhynchus mykiss* (AM259379), *Oryziaslatipes* (NM001278879), *Hippoglossus hippoglossus* (AJ410171), *Acipens erschrenckii* (KC417317.1), *Mugilcephalus* (AY859426), *Dasyatissabina* (AF097513), *Mus musculus* (NM007810), *Xenopus laevis* (AB272088), *Sus scrofa* (Pig) (U92245), *Xenotilapia melanogenys* (KC684559), *Simochromis diagramma* (KC684578), *Ctenochromis horei* (KC684586), *Lamprologus lemairii* (KC684566), *Perissodus microlepis* (KC684563), *Pseudo simochromis curvifrons* (KC684583), *Callochromis macrops* (KC684560), *Simochromis babaulti* (KC684585), *Melanotaenia fluviatilis* (GU723457), *Perca flavescens* (DQ984126), *Anoplopoma fimbria* (KC112916) and *Amphiprion clarkia* (AB525197). Consenses phylogenetic tree was constructed based on the aminoacid sequences of *cyp19a1a* of *C. catla* using the UPGMA method (Fig. 4). These results indicate that the gene *cyp19a1a* is evolutionally conserved throughout the vertebrate phyla.

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 5.07623823 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 377 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

Discussion

P450arom plays a potent role in preserving the continuity of life in diverse vertebrates including fishes by sustaining important physiological process, reproduction. Its role in oogenesis, sexual behavior, sex change in hermaphrodites and temperature sensitive fishes are well characterized (Chang *et al.*, 2005). Many of the previous studies confirm that *cyp19a1a* is involved in gametogenesis and in regulation of vitellogenesis during reproductive cycle (Nakamura *et al.*, 2005). These kinds of molecular work on Indian major carps are very uncommon. So the main intention of this study was to determine systematically, for the first time, the expression profiles of the aromatase gene *cyp19a1a* in the ovary of a lower vertebrate *C. catla*, throughout an annual reproductive cycle.

Cyp19a1a expression was preferentially expressed in catla ovaries. This expression pattern was also found in other species including wrasse (Choi *et al.*, 2005) and Common carp (Tang *et al.*,2010). These studies support the idea that high *cyp19a1a* levels are essential for estradiol biosynthesis and ovary development in fish (Masaru *et al.*, 1998).

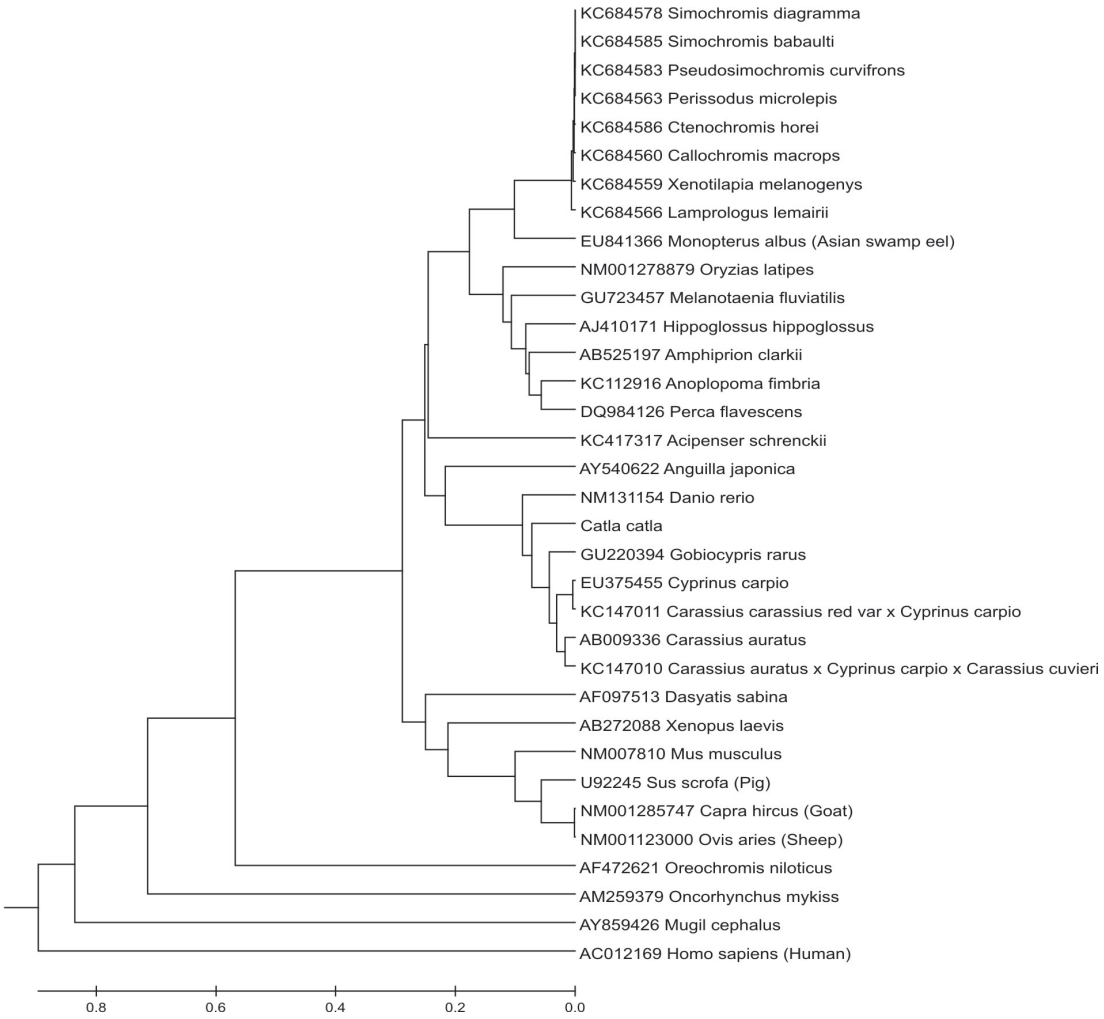

Fig. 4. Evolutionary relationships of 34 taxa of *cyp19a1a* with *C. catla* (UPGMA method)

Various approaches have been attempted to validate the significance of E2 and c*yp19a1* during ovarian differentiation and oogenesis across vertebrate phyla including teleosts. The expression of *cyp19a1a* incatlahas seen its peak level during the previtellogenic and vitellogenic period and the expression was very low during the time of resting period or postvitellogenic period. This gene was well expressed in all the important time of reproductory phases except postspawning period (Fig. 2). This result correlate with the findings in non-mammalian vertebrates that estrogens are thought to be essential for ovarian development (Wallace, 1985). Studies have shown that transcript levels of gonadal aromatase are increased in association with aromatase enzyme activity during vitellogenesis (Gen *et al.*, 2001).

From this study it is obvious that there is a comprehensible variation in the expression of *aromatase* gene in different reproductive phases in an annual cycle. It is also evident from the previous findings that each steroidogenic enzyme is expressed throughout the year and the expression is changed seasonally in the channel catfish ovary throughout a reproductive cycle (Sampath *et al.*, 2000). Current study proved that *aromatase* activity is much high during the time of previtellogenic and vitellogenic phases. Researchers establish that *cyp19a1a* gene expression in the developing ovary was relatively much higher than in testis, which was consistent with the high levels of *aromatase* activity and estradiol production during vitellogenesis (Sampath *et al.*, 2000; Blazquez *et al.*, 2008). These findings indicated that *cyp19a1a* gene has a key role in ovary

growth in teleosts.

Expression of *ovarian aromatase* correlated well with the activity of this enzyme in the different reproductory phases. The *cyp19a1* expression in channel catfish also correlated with plasma E2 levels (Kumar *et al.*, 2000). The transcript levels and enzyme activity precipitously increased during ovarian recrudescence, i.e., from preparatory phase to pre-spawning phase, a phase when the ovaries were filled mostly with vitellogenic and early post-vitellogenicoocytes, followed by a steep decline in the transcripts and activity of aromatase as the oocyte ensued maturation. A similar result of seasonal variation in *cyp19a1* transcript peaking just prior to oocyte maturation was observed in the follicular layer of zebrafish vitellogenic oocytes (Goto-Kazeto *et al.*, 2004). Yan *et al*., 2019 could prove this once again through their findings thatcyp19a1a transcript levels peaked in the mid-vitellogenicstage ovary and may play an important role in oocyte vitellogenesis in *Schizothorax prenanti*.

In this study it is found out that the aromatase gene expression is also seen in many other tissues. This *aromatase* activity or its transcript expression was detected in other tissues in addition to the ovary, including brain, pituitary, retina, kidney, and testis (Chiang *et al.*, 2001). Wong *et al.*(2006) also detected that in addition to the ovary, transcripts of cyp19a1a were also expressed at different levels in the brain, pituitary, gill, thyroid, retina, heart, head-kidney, trunk-kidney, spleen, intestine, testis, and ambisexual gonad, but not in liver or muscle. Some other studies also show that in addition to gonadal tissues, transcripts of *cyp19a1a* were expressed at different levels in the brain, eye, gill, heart and muscle, but not the intestine or liver (Choi *et al.*, 2005; Kobayashi *et al.*, 2010).

In summary, here identified the aromatase gene *cyp19a1a* in the Indian major carp *C. catla*, mainly ovary-derived. As in other species, the expression of *cyp19a1a* was predominately expressed in the ovary; however *cyp19a1a* expression were found in all major tissues tested. But there was no expression of *cyp19a1a* in the male gonad. Present study demonstrated the phase-dependent expression and activity of *cyp19a1a* in the ovarian tissues during ovarian cycle. Based on these results, we report specific role for *cyp19a1a* during ovarian differentiation and recrudescence. So Cytochrome P450 aromatase, the endoplasmic reticular enzyme catalyzing the production of estrogens from androgens, plays a potent role in preserving the continuity of life in diverse vertebrates including fishes by sustaining the important physiological process, reproduction. In short, it is now evident that the *cyp19a1a* gene, Cyp19a1a enzyme and estrogens are pivotal for the regulation of gonadal differentiation and in the different phases of reproductory cycle of *C. catla*.

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