

## Original Research Article

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## Molecular Cloning of Retinoic-Acid Metabolizing Cytochrome P450 26B1 (Cyp26B1) and Expression Analysis during Different Reproductive Phase in *Clarias magur* (Hamilton 1822)

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### ABSTRACT

Retinoid signalling is crucial to vertebrate embryonic development. The Cyp26B1 is one of the key catabolic enzymes, involved in the inactivation of all-trans retinoic acid and helps in the maintaining of retinoic acid availability for retinoid signalling. In the present study, for the first time, we cloned and characterized the full coding sequence of Cyp26B1 gene in *Clarias magur*. The full-length cDNA sequence of Cyp26B1 gene was 1782 bp (ORF 1533 bp) encoding 511 amino acids. Signal peptide analysis revealed that Cyp26B1 is a secretory protein as reported in other vertebrates. The hydropathy profile predicted that Cyp26B1 is hydrophilic in nature. The overall quality factors of Cyp26B1 protein by ERRAT analysis was 56.19%, indicating the instability of proteins. The expression profile generated for the preparatory, spawning, 6h and 16h post Ovotide™ injection stages, revealed the significantly higher expression (P<0.05) of Cyp26B1 mRNA in the brain of female fish compared to the ovary at all developmental stages and peaked at spawning stage. Whereas in male fish, the mRNA expression was significantly higher at 16h post Ovotide™ injection stages in the brain and minor expression obtained at all the stages of testis. Our findings suggest that maintenance and clearance of retinoic acid are essential at every developmental stage in *C. magur* and Cyp26B1 might play important role in germ cell development ultimately affect the reproductive physiology.

#### Keywords

Cyp26B1, Retinoic acid, Preparatory phase, Ovotide™, Retinoid signalling

#### Article Info

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### Introduction

It is well studied that retinoids were involved in many developmental processes. Through binding to its receptors RARs and RXRs,

retinoids participate in the retinoid signalling and ultimately control the sex-specific meiosis initiation timing and regulate spermatogenesis in vertebrates (Chung *et al.*, 2004; Feng *et al.*, 2015). However, vertebrates cannot synthesize

the retinoids *de novo* while metabolize it from its precursor vit. A (Zhao *et al.*, 2005). In all animal including vertebrates, vit. A which is essential in reproduction (Clagett-Dame and Knutson, 2011) is converted into trans tetraenoic acid (RA) through retinol dehydrogenase enzymes (Aldh1a 1/2/3; Ross *et al.*, 2000). Since any signalling is determined by the feedback processes, therefore excess RA is converted into its the inactive polar forms 4-hydroxy-retinoic acid and 4-oxo-retinoic acid through the Cyp26s enzymes (Ross *et al.*, 2000). In vertebrates, three Cyp26s (including Cyp26A1, Cyp26B1 and Cyp26C1) have been reported which all involved in the catabolism of RA. Meiosis is an indispensable process of sexual reproduction which has been reported to be initiated at the same time in female and males (Pradhan *et al.*, 2015).

The entry of germ cell into the meiotic stage is determined by the RA through star8 dependent pathway and the level of RA is finally regulated by the balance between its synthesis and degradation through Aldh1a 1/2/3 and Cyp26A1 and Cyp26B1 respectively (Abu-Abed *et al.*, 2001; Yashiro *et al.*, 2004).

Cyp26A1 and Cyp26B1 exhibit less than 30% amino acid identity with other cytochromes and therefore could not be classified into any of the previously defined subfamilies hence come under new P450 26 subfamily (Nelson *et al.*, 1993). However, with only 40% sequence similarity between Cyp26A1 and Cyp26B1, broad expression pattern, different catalytic activities with no biologically important differences were reported between Cyp26A1 and Cyp26B1 in the formation of active metabolites of RA (Topletz *et al.*, 2012). Recent studies in some fishes revealed that Cyp26A1, rather than Cyp26B1, was considered as the major RA degrading enzyme (Rodríguez-Marí *et al.*, 2013; Lau *et al.*, 2013; Feng *et al.*, 2015). However, low or no expression of cyp26B1 induced an excessive

initiation of meiosis, resulted in the apoptosis of pre-matured spermatogonia through an elevated level of RA (MacLean *et al.*, 2007). Only a few reports on the characterisation of this gene are available in fish making it difficult to understand its actual function.

*Clarias magur*, the Indian walking catfish is a seasonal breeder which breeds during the rainy season, July-August (Joy *et al.*, 2000). In captivity, the male brooder needs to be sacrificed to obtain milt while in the natural environment, this fish breeds easily without any complications. The physiology involved in the reproduction of this fish needs to be explored. Hence, the main aim of the present study was to study the structural and functional aspects of RA metabolic gene Cyp26B1 which govern the retinoid signaling. The mRNA expression levels were analysed in different reproductive phases. Furthermore, the mRNA expression was studied after the injection of synthetic GnRH analog (Ovatide<sup>TM</sup>).

## Materials and Methods

### Sample and tissue collection

The wet lab experiment was conducted at the Freshwater Fish Farm, ICAR-CIFE, Powarkeda, Madhya Pradesh, India. Nine fishes were sampled from each stage among the 4 reproductive stages of *C. magur* (i) Preparatory (ii) Mature; (iii) Post Ovatide<sup>TM</sup> injection 6h and (iv) Post Ovatide<sup>TM</sup> injection. For preparatory phase, fishes were sampled in the month of February while fishes for spawning phase were collected in August. Ovatide<sup>TM</sup> (Hemmo Pharmaceuticals Pvt. Ltd., Mumbai), was injected @ 0.5 ml kg<sup>-1</sup> body weight to male and 1.0 ml kg<sup>-1</sup> (Sharma *et al.*, 2010) to female fishes and sampling was done after 6h and 16h of injection. In all cases, brain and gonadal tissues were collected, kept in RNAlater<sup>TM</sup> (QIAGEN, USA) and stored at

-80 °C until further analysis. Three individuals pool tissue were used to extract total RNA.

### **RNA extraction and cDNA synthesis**

Total RNA was extracted using Trizol™ reagent (Invitrogen, USA) and treated with DNase I (Thermo Scientific, USA) to remove the genomic DNA contamination following manufacturer's guidelines. The quality and quantity of the purified total RNA were determined using agarose gel electrophoresis and NanoDrop spectrophotometer (Thermo Scientific, USA). 1 µg of DNase treated total RNA was reverse transcribed to cDNA using Oligo dT primer and RevertAid™ reverse transcriptase First Strand cDNA Synthesis kit (Thermo Scientific, USA) as per the manufacturer's instructions.

### **Molecular cloning of Cyp26B1 cDNA and RACE amplification**

Primers to amplify partial cDNA of Cyp26B1 were designed using gene runner primer designing tool and synthesized commercially by Eurofins Genomics India Pvt Ltd, India. PCR amplification was performed in a 96-well Takara PCR System (Takara, Japan) with a total volume of 25 µL containing 2.5µL of 10× Taq buffer, 0.5µL of 10mM each dNTP mix, 2.5µL of 25mM MgCl<sub>2</sub>, 1µL each of sense and antisense primers (10 pmol), 1µL of cDNA (500ng), 0.25µL of 5U/µL Taq DNA polymerase and 16.25µL of nuclease-free water. The annealing temperature of each primer set was optimized by touch-down as well as gradient PCR.

The partial cDNA fragments of 504 bp was obtained and eluted from the gel. The eluted PCR product was cloned into pTZ57R/T vector (Thermo Scientific, USA), sequenced and confirmed by BLASTn similarity search. After partial cDNA amplification, gene-specific primers (Table 1) for 3' and 5' RACE

(Rapid Amplification of Complementary DNA Ends) PCR were designed from the identified partial sequence as per the guidelines provided with the RACE-PCR kit (Clontech, Japan). The reaction was carried out for both 3' and 5' ends to obtain the full-length cDNA sequence of the genes comprising of the open reading frame (ORF), 3' and 5' untranslated (UTR) regions. The desired PCR products were purified, cloned and sequenced. The sequences were confirmed by BLASTn software (<http://www.ncbi.nlm.nih.gov/blast>).

### **Amino acid sequence analysis and ORF prediction**

The open reading frame (ORF) of the full-length cDNA sequence obtained through RACE-PCR was determined using NCBI ORFfinder tool (<https://www.ncbi.nlm.nih.gov/orffinder/>). The amino acid sequence of the coding region was deduced using Gene Runner and conserved domains were identified by NCBI Conserved Domain search tool (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>).

### **Phylogenetic analysis**

The deduced amino acid sequences of the Cyp26B1 from *C. magur* (Indian magur), *I. punctatus* (Channel catfish), *D. rerio* (Zebrafish), *C. carpio* (Common carp), *Tachysurus fulvidraco* (Yellowhead catfish), *Silurus meridionalis* (Chinese large-mouth catfish), *Homo sapiens* (human), *Mus musculus* (Mouse), *Ovis aries* (Sheep) and *Bos taurus* (Cattle) were subjected to multiple sequence alignment analysis using CLC Genomics Workbench 9 program software (QIAGEN, USA). The sequence alignment obtained was used to construct a Neighbour-joining tree with 1000 bootstrap replicates using MEGA 7.0 software (Kumar *et al.*, 2016).

### **In silico analysis of *C. magur* Cyp26B1 sequence**

Physiochemical parameters of the *C. magur* Cyp26B1 amino acid sequence were analysed using online ProtParam tool at ExPASy (Gasteiger *et al.*, 2005). Secondary structure of amino acid sequence was predicted through online tools like PSIPRED V.3 (McGuffin *et al.*, 2000) and SOPMA (Geourjon *et al.*, 1995), while ProtScale program (Gasteiger *et al.*, 2005) was used to analyze the protein hydropathy profile. SignalP 4.0 software was used to predict the signal peptides (Petersen *et al.*, 2011).

### **Homology modeling of Cyp26B1**

The 3D model of *C. magur* Cyp26B1 was generated using the amino acid sequences of closest homologs for which X-ray crystal structures were available. The solution of NMR structure of *Saccharomyces cerevisiae* Cyp26B1 (PDB code: c4lxjA) was used as a template. The homology model was generated using Modeller 9v12 software.

### **Model evaluation**

The stereochemical properties of *C. magur* Cyp26B1 3D model were analysed through Ramachandran plots from RAMPAGE server (Lovell *et al.*, 2002) and overall quality factor by ERRAT analysis (Colovos *et al.*, 1993). Ramachandran plot tells the percentage of amino acid residues in the favorable and disallowed regions.

The ERRAT program evaluates the statistics of non-bonded interactions between different atom types and plots the value of the error function versus position of a nine-residue sliding window, calculated by a comparison with statistics from highly refined structures. PyMOL 4.4.0 software was used to obtain the superimposition between model and template

and also to diagrammatically represent the structures. Ligand binding sites were predicted using I-TASSER program (Roy *et al.*, 2010) and located on predicted protein model using RasMol software (Version 2.7.5.2).

### **Protein-protein interactions of *C. magur* Cyp26B1**

The interactions of *C. magur* Cyp26B1 protein with other proteins involved in the pathway of steroidogenesis and spermatogenesis were determined using online software STRING (Szklarczyk *et al.*, 2014).

### **Real time mRNA expression of Cyp26B1 in brain and gonads**

The basal mRNA expression analysis of Cyp26B1 mRNA in tissues of *C. magur* was carried out in LightCycler450 Real-time PCR detection system (Roche, USA). The reactions were performed in 10  $\mu$ L reaction mix volume containing 5  $\mu$ L of 2 $\times$  Maxima<sup>TM</sup> SYBR Green qPCR master mix (Thermo Scientific, USA), 0.5  $\mu$ L of (0.3 pM) each gene-specific primer (Table 1) and 2  $\mu$ L (20 ng) of cDNA and 2  $\mu$ L of nuclease-free water. The default thermal profile was used for PCR amplification and it consisted of initial denaturation at 95 °C/10 min, followed by 40 cycles of denaturation at 95 °C/20 s, annealing at 64 °C/20 s and extension at 72 °C /30 s. All reactions were run in triplicate and repeated twice.

Relative expression of genes was determined using comparative Ct method ( $2^{-\Delta C_t}$ ) (Livak *et al.*, 2001). Two putative housekeeping genes (HKG), Elongation factor 1-alpha (EF1 $\alpha$ ) and  $\beta$ -actin were statistically analyzed by the comprehensive algorithm of NormFinder, Bestkeeper and Genorm to assess their transcriptional expression stability. Here,  $\beta$ -actin was selected as HKG for gene normalization.

### **Ethics statement**

The guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare Division), Government of India on care and use of animals in scientific research were followed to care and rear the animals used in the present study. The study was approved by the Board of studies and authorities of ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Mumbai-61.

### **Statistical analysis**

The one-way analysis of variance (ANOVA) of statistical package SPSS 17.0 (USA) was used to test the statistical significance of differences in mRNA transcript levels.  $P < 0.05$  was considered as statistically significant. The results were expressed as mean  $\pm$  SEM (bars).

## **Results and Discussion**

### **Molecular cloning of *C. magur* Cyp26B1 gene**

The partial cDNA fragment of Cyp26B1 (545 bp) was cloned from *C. magur* testis. The full-length cDNA fragment of 1782 bp (ORF 1533 bp) was obtained by 5'/3' RACE-PCR technique. The ORF of *C. magur* Cyp26B1 gene encoded a putative protein of 511 amino acids. The sequence obtained in the present study was submitted to NCBI GenBank (Accession number: LC365786).

### **Physicochemical properties and secondary structure analysis of Cyp26B1 protein**

The *C. magur* Cyp26B1 protein was shown to have apparent molecular weight (MW) of 57241.19 Da with pI of 8.78. The secondary structure of *C. magur* Cyp26B1 predicted by

PSIPRED server showed the presence of 17  $\alpha$ -helices, 27 coils and 10  $\beta$ -sheets in the protein as shown in Figure 1. The structural percentage of amino acids of Cyp26B1 predicted by SOPMA software revealed that 246 amino acids (48.14%) have an alpha helix, 70 (13.70%) have extended strand, 41 (8.02%) have a beta turn, and 154 amino acids (30.14%) have random coil structures. *C. magur* Cyp26B1 protein exhibited 31 serine (Ser), 10 threonine (Thr), and 5 tyrosine (Tyr) phosphorylation sites that were uniformly distributed throughout the polypeptide chain as predicted by NetPhos 3.1 Server. As the Cyp26B1 belongs to P450 superfamily which is a large family of haem-thiolate proteins involved in the oxidative degradation of various compounds. They are particularly well known for their role in the degradation of environmental toxins and mutagens. CDD search revealed the presence of four conserved motifs (CR1-CR4) and a conserved heme-binding domain in all members of the P450 superfamily including Cyp26B1 (Fig. 2).

### **Signal peptide analysis of Cyp26B1 protein**

The signal peptide of the *C. magur* Cyp26B1 protein as determined by SignalP 4.1 server by keeping D-cutoff value as sensitive (0.34) is shown in figure 3. The comparison was made among signal peptide graphs of *Clarias magur*, *Danio rerio* (a model fish) and *Ictalurus punctatus* (closely related). All the five indexes scores of *C. magur* proteins (max C, max Y, max S, mean S, and mean D) were more similar to the score of *I. punctatus* and less to the *D. rerio*. The D-score (Discrimination score) is the weighted average of the mean S and the max Y scores, this is the score mainly used to discriminate signal peptides from non-signal peptides (Petersen *et al.*, 2011). In case of Cyp26B1 protein the D value (Discrimination score) was more (0.453) than the corresponding cut-off value. Thus, Cyp26B1 showed the presence of signal

peptide from 1-11 residue position confirming its secretory nature.

### Sequence and phylogenetic analysis

Sequencing result of Cyp26B1 was analyzed using BLAST tool at NCBI for similarity search. The deduced amino acid sequence of *C. magur* Cyp26B1 deduced amino acid revealed 76%, 91% and 96% identity with human, zebrafish and channel catfish sequences, respectively. Multiple sequence alignment of *C. magur* deduced amino acid sequence of Cyp26B1 with sequences from other fish species showed well-conserved regions across the sequences (Fig. 2). The catalytic domains of Cyp26B1 protein were found to be highly conserved across all the species used for multiple alignment analysis. The identity level of *C. magur* Cyp26B1 amino acid sequence with other species is presented in figure 4. The Neighbour-joining tree for Cyp26B1 was prepared which differentiated the closely related species in separate clades. The catfishes formed one cluster while cyprinids formed a separate group (Fig. 5). Bovine species grouped in to a single cluster while human formed sister group. Cyp26B1 of *C. magur* was closely related to Chinese large-mouth catfish and channel catfish followed by cyprinids. This finding is consistent with the classification and evolutionary status of these species.

### Hydropathy analysis of the Cyp26B1 protein

The hydropathy profile of the Cyp26B1 protein was determined by ProtScale program which demonstrated that the aspartic acid (D) at residue position 423 of the polypeptide chain exhibited the highest hydrophilicity (hydrophobic parameter: -3.189) whereas valine (V) at residue position 22 showed the highest hydrophobicity (hydrophobic parameter: 2.822). In Cyp26B1 protein hydropathy graph, the hydrophilic area was

also greater than hydrophobic area (Fig. 6). Therefore, the whole Cyp26B1 peptide chain showed hydrophilic character.

### 3D structure prediction and analysis

Since no PDB file has been reported for Cyp26B1 in human so NMR structure of *Saccharomyces cerevisiae* Cyp26B1 protein (PDB code: c4lxjA) with maximum sequence identity was considered as best hits and used as template to generate 3D model. Pymol software was used to obtain the final 3D ribbon structure of Cyp26B1 PDB sequence obtained from the modeler software (Fig. 7). The ligand binding sites of Cyp26B1 protein were valine at positions 115, 367 and 478; leucine at positions 130, 293, 361, 441 and 445; alanine at positions 131, 193, 296, 297, 304 and 446; histidine at position 138; arginine at positions 142, 372 and 438; phenylalanine at positions 149 and 433; threonine at position 300; proline at positions 366, 432 and 477; glycine at positions 370, 434 and 442; isoleucine at position 395 and cysteine at position 440 (Fig. 8).

The overall structural similarity between the *C. magur* Cyp26B1 protein model with its ortholog (2VE3) from cyanobacteria is shown as backbone superposition with RMS value of 4.68 (Fig. 9). Ramachandran analysis of *C. magur* Cyp26B1 3D model revealed that 94.3% of the residues are in the most favored regions while 4.2% are in the additional allowed region (Fig. 10). The data figure indicated that the 3D model generated were highly stable. The overall quality factors of modelled *C. magur* Cyp26B1 protein in ERRAT analysis was 56.198% (Fig. 11).

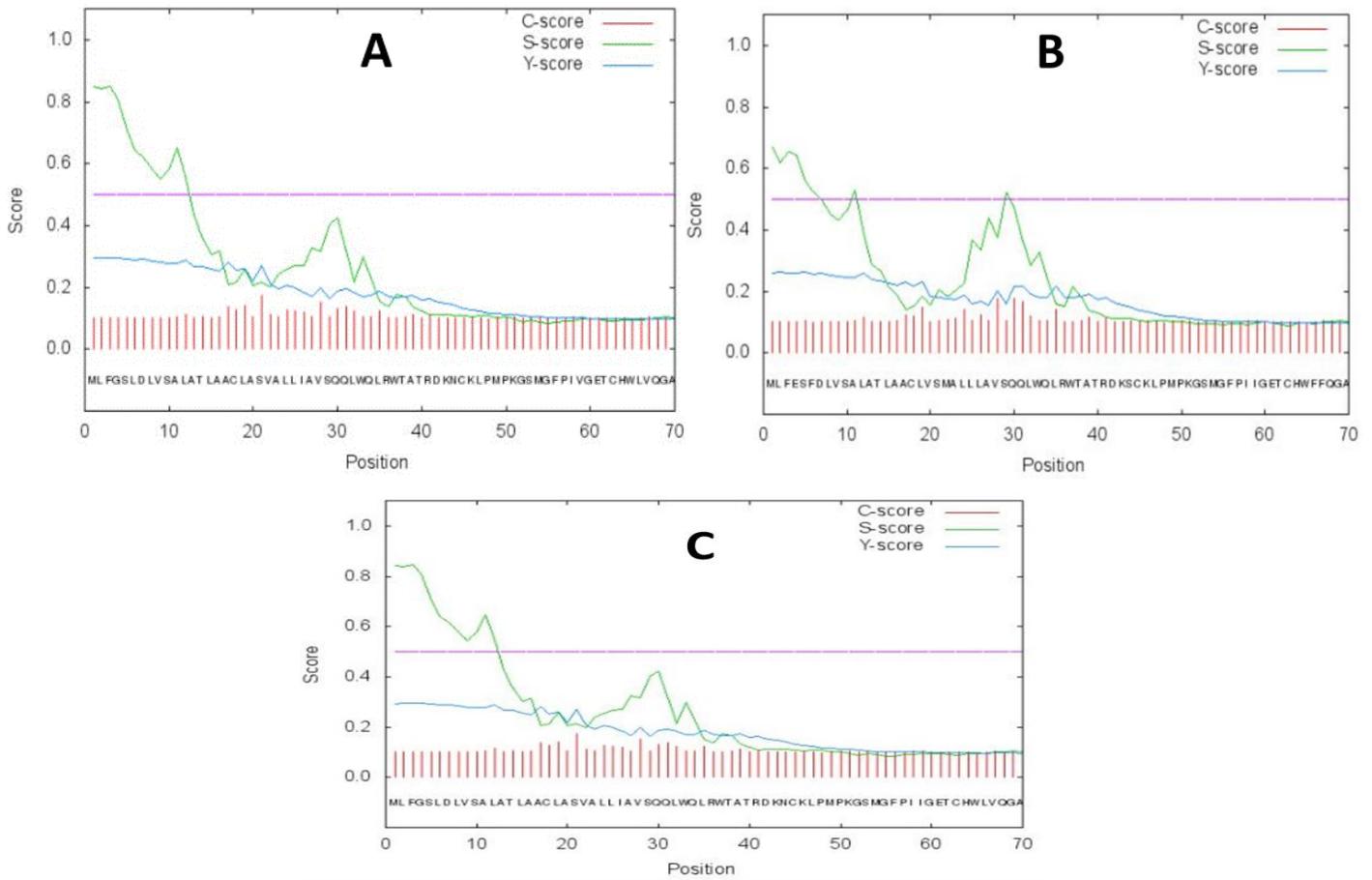
### Protein-protein interactions of Cyp26B1

The protein-protein interactions of Cyp26B1 protein with that of other proteins is presented in figure 12.





**Fig.3** The predicted signal peptide of *C. magur* (A) *D. rerio* (B) and *I. punctatus* (C) Cyp26B1 proteins. The signal peptide was predicted by the Signal P 4. 1 online tool



**Fig.4** Graph showing percentage similarity of *C. magur* Cyp26B1 amino acid sequence with other species including fishes and mammals

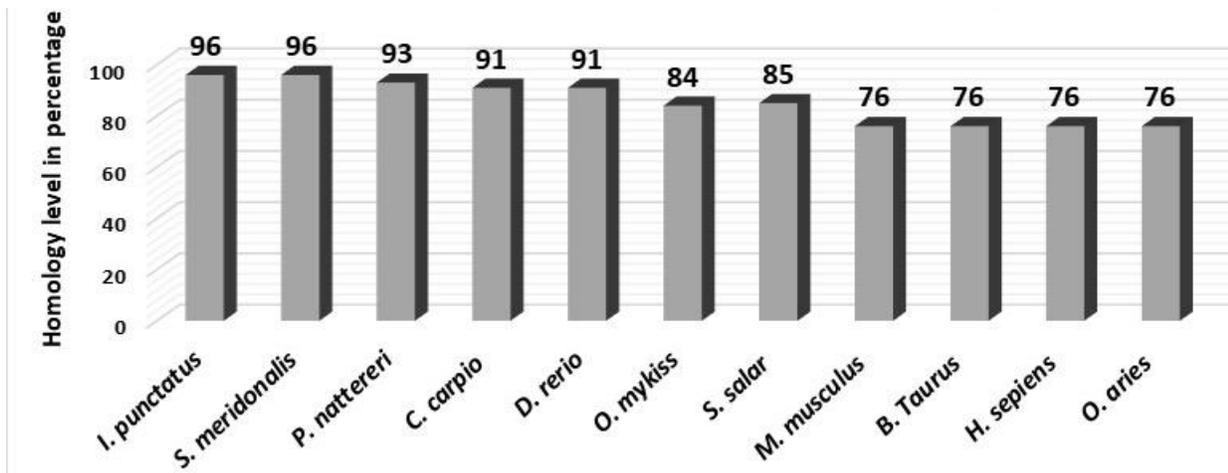


Fig.5 Phylogenetic analysis of *C. magur* Cyp26B1

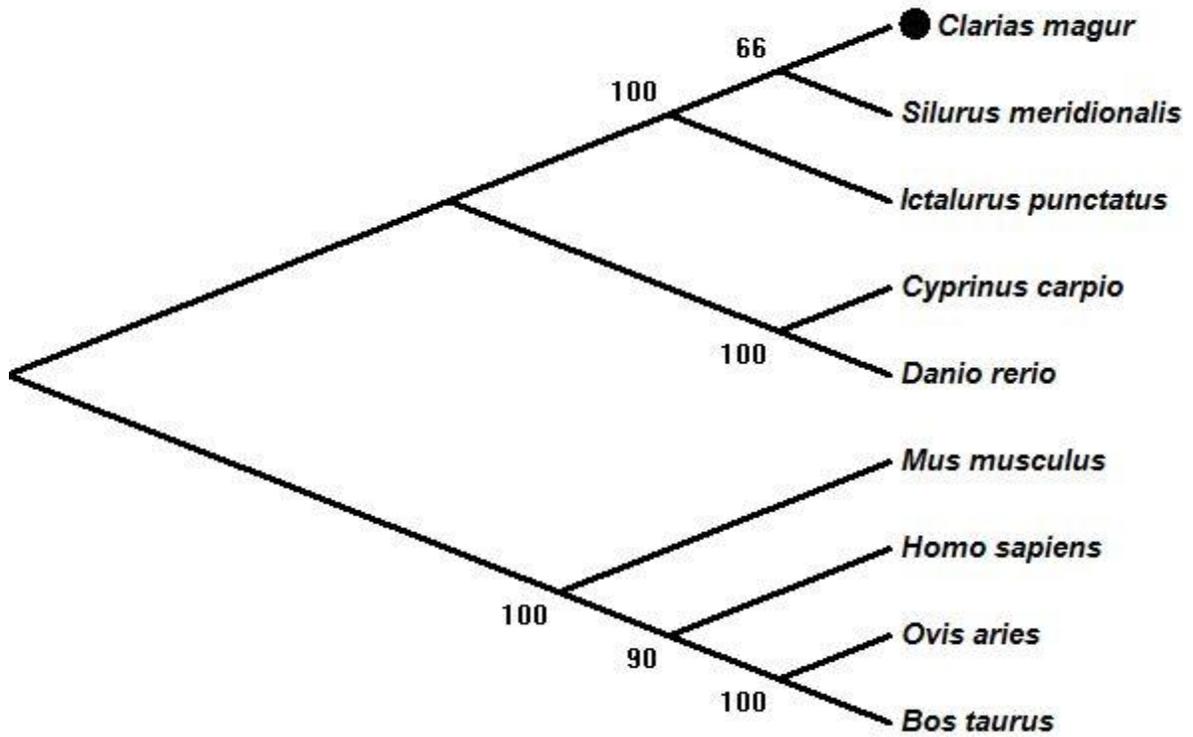
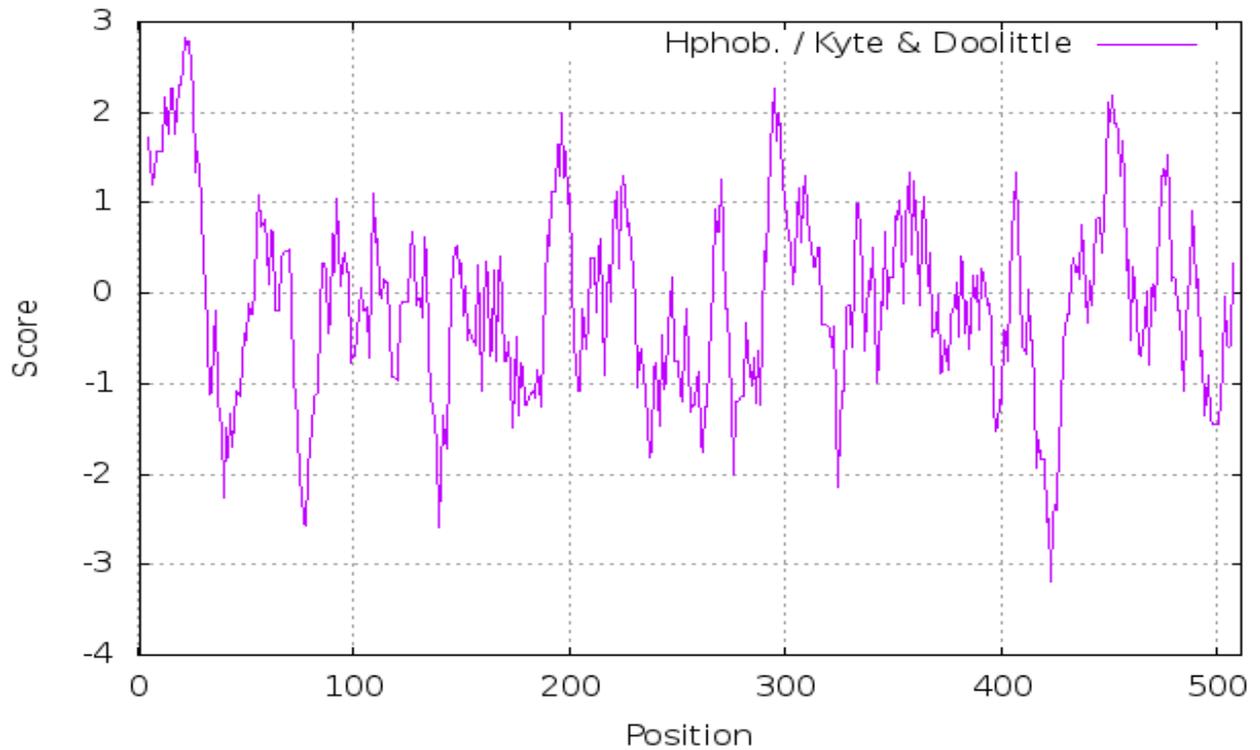


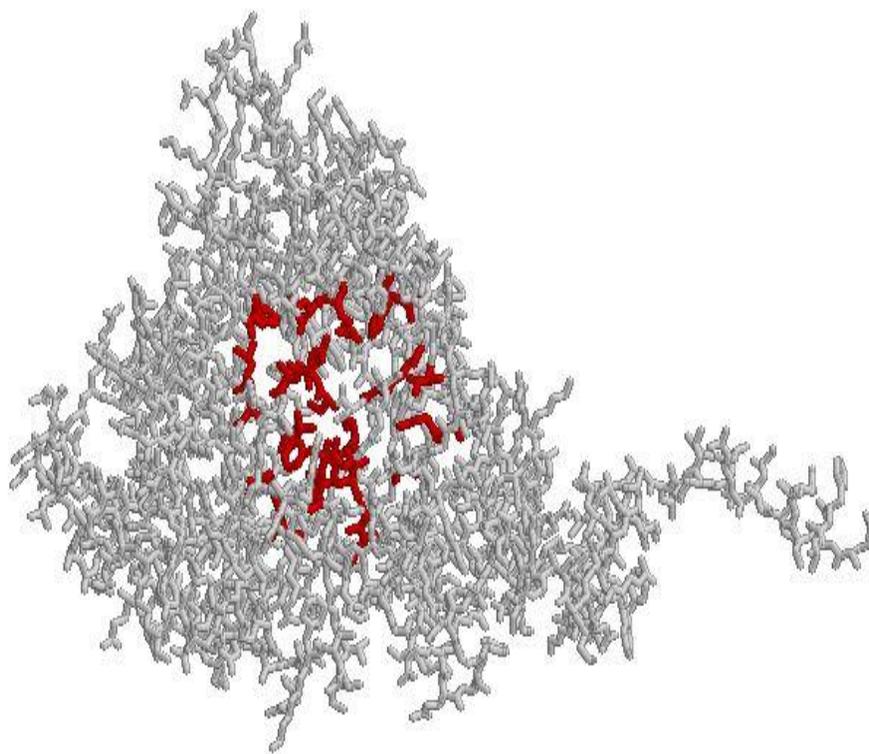
Fig.6 The hydropathy profile of *C. magur* Cyp26B1 protein



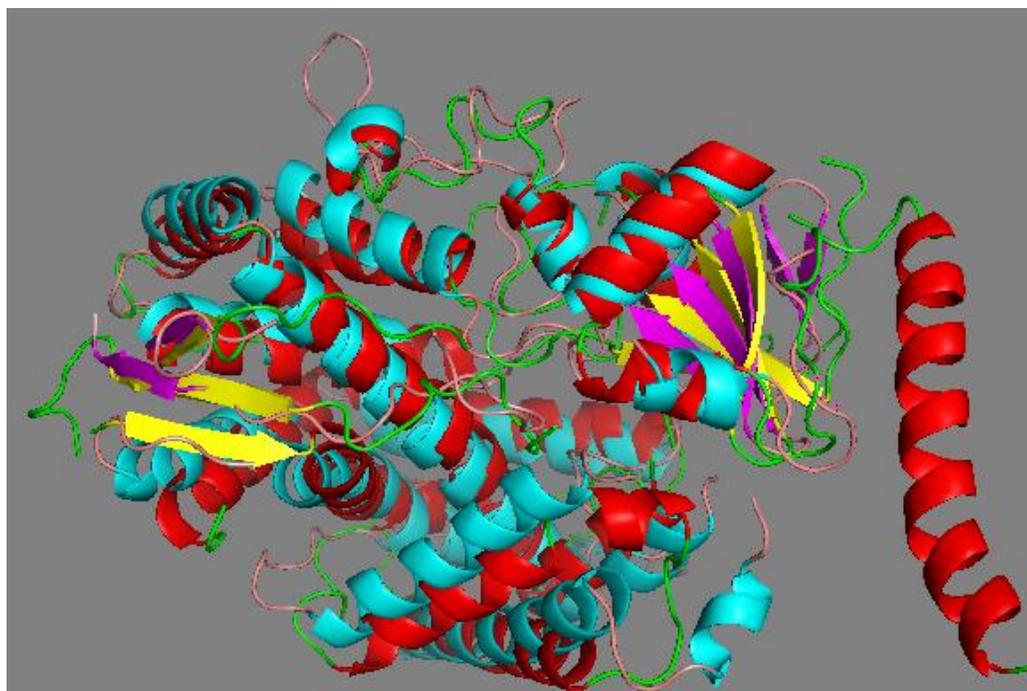
**Fig.7** Ribbon diagram showing the tertiary structure of *C. magur* Cyp26B1 (B) protein. Alpha helices are shown in red colour, beta sheets in yellow colour, and coils are shown in green colour



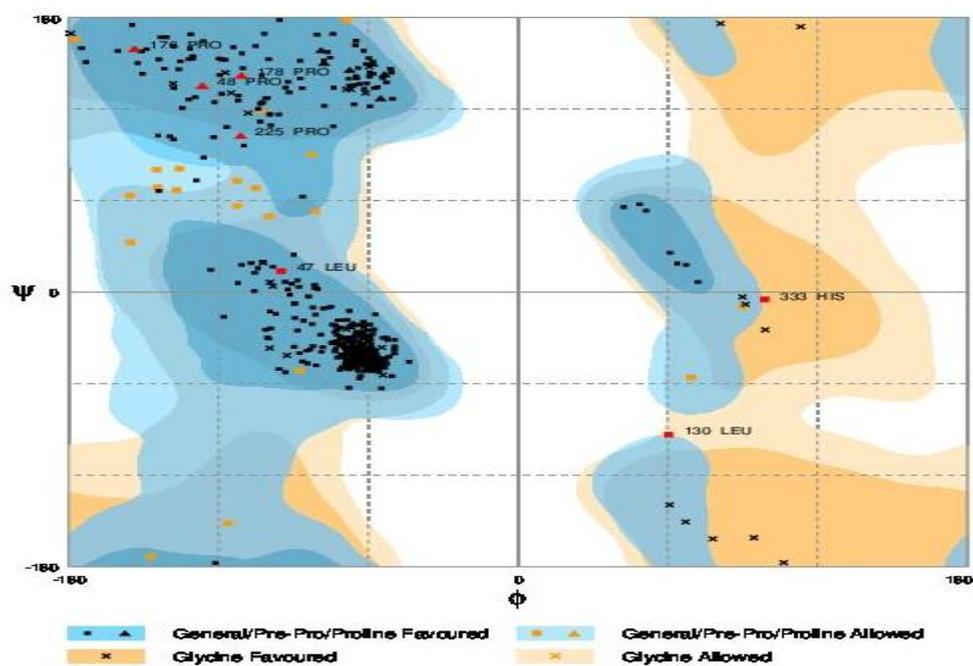
**Fig.8** Stick diagram of Cyp26B1 protein. The red colour sticks of the structure are showing the ligand binding sites



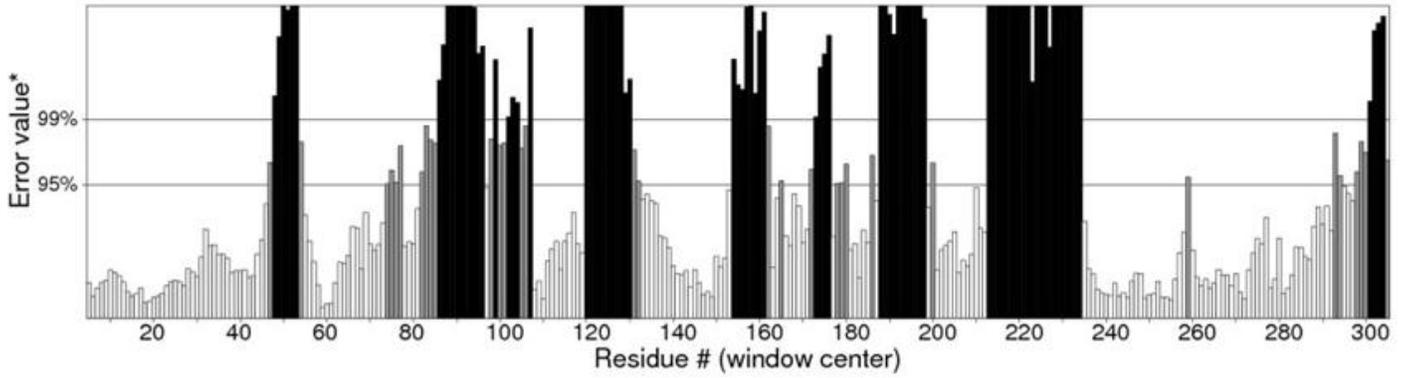
**Fig.9** Superimposition of modelled Cyp26B1 protein of *C. magur* (Red, yellow and green) with template 2VE3 (Cytochrome P450) (blue, violet and orange). Red and Blue indicate helices, yellow and violet indicate sheets and green and orange indicate coils



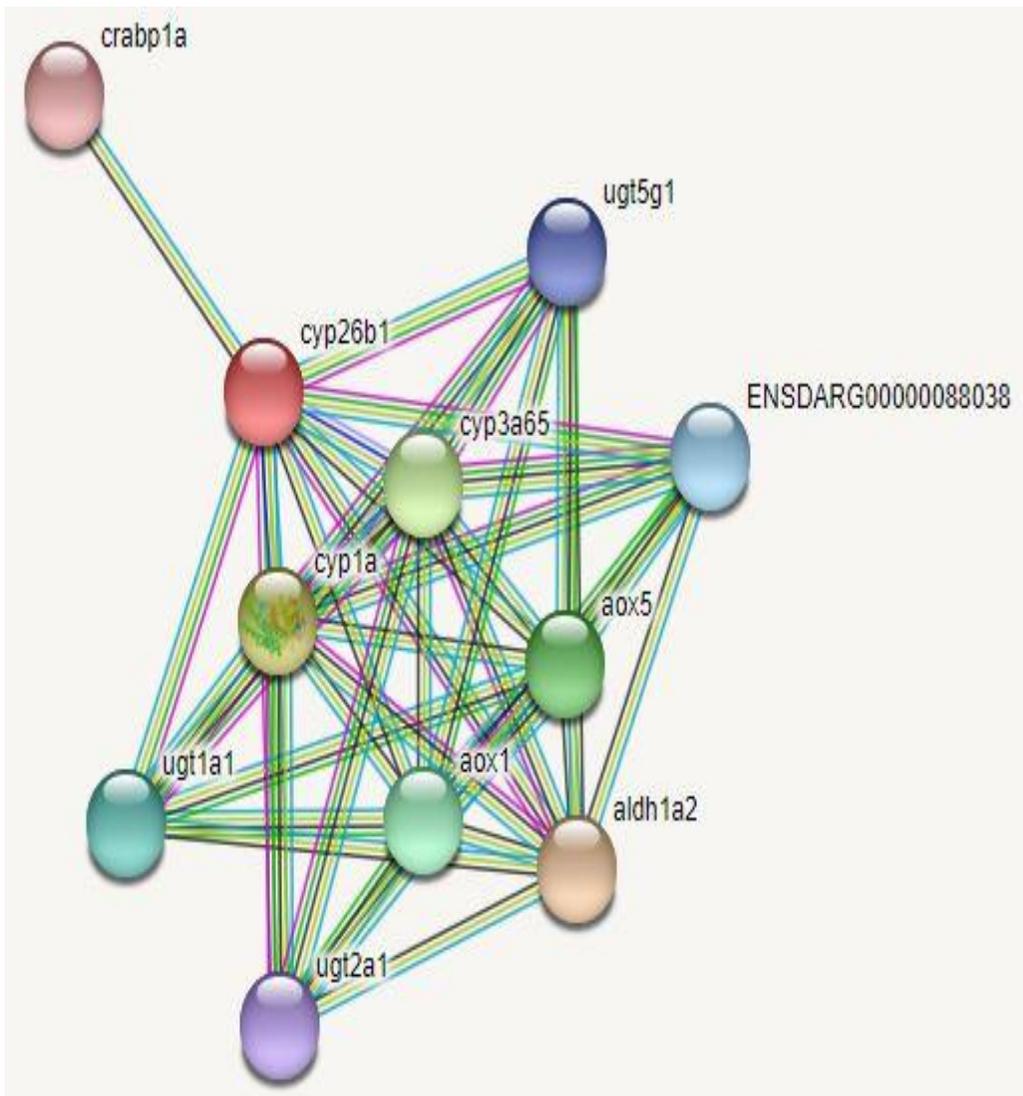
**Fig.10** Ramachandran plot (RAMPAGE) of *C. magur* Cyp26B1 showing the dihedral angles Psi and Phi of amino acid residues. The residues which lie in most favoured regions are shown in blue curves and the residues which lie in additional allowed regions are in yellow curves



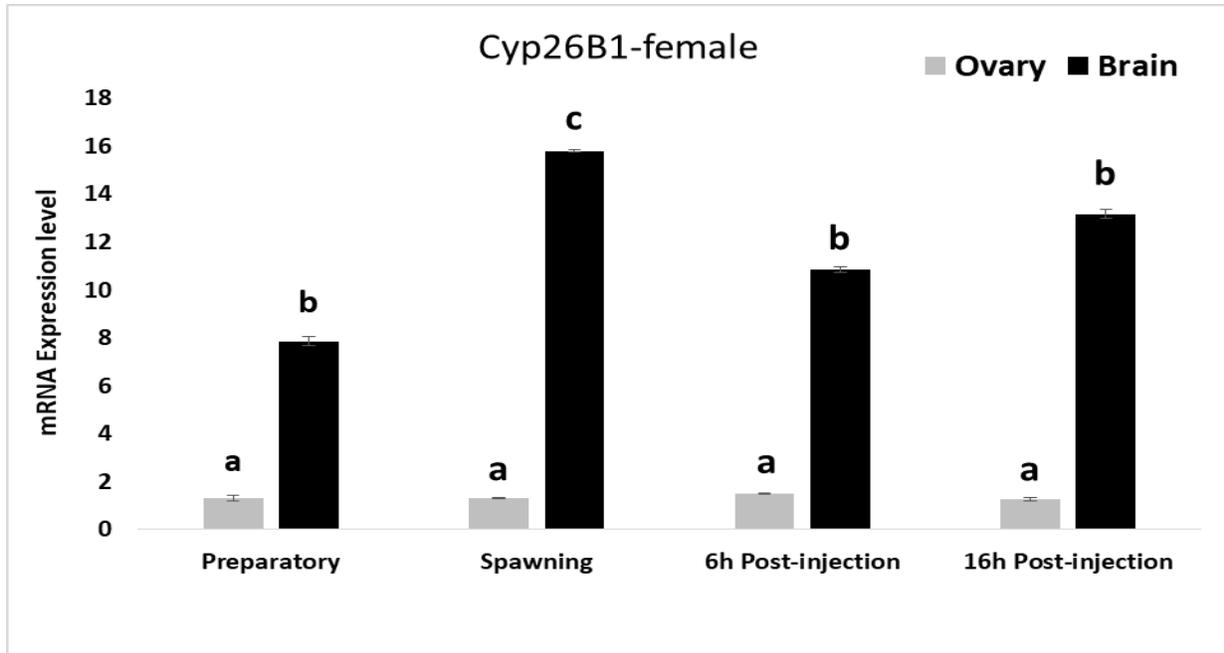
**Fig.11** ERRAT plot analysis of modelled *C. magur* Cyp26ACyp26B1 (B) proteins



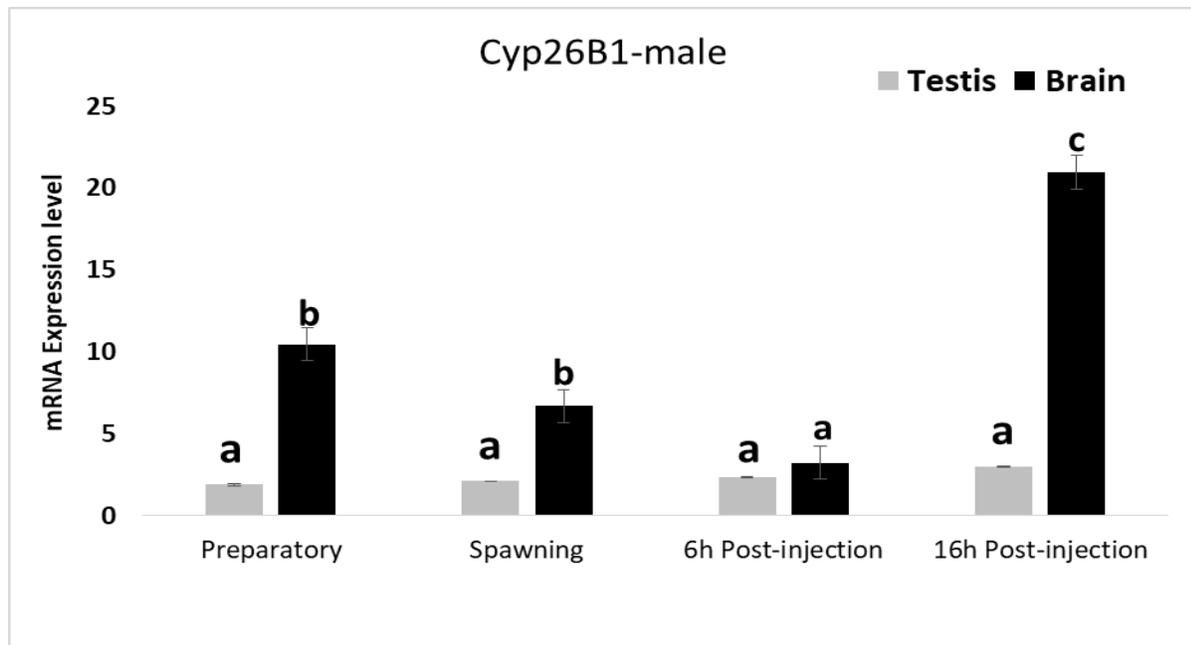
**Fig.12** Interaction networks of *C. magur* Cyp26B1 with other proteins as predicted by STRING



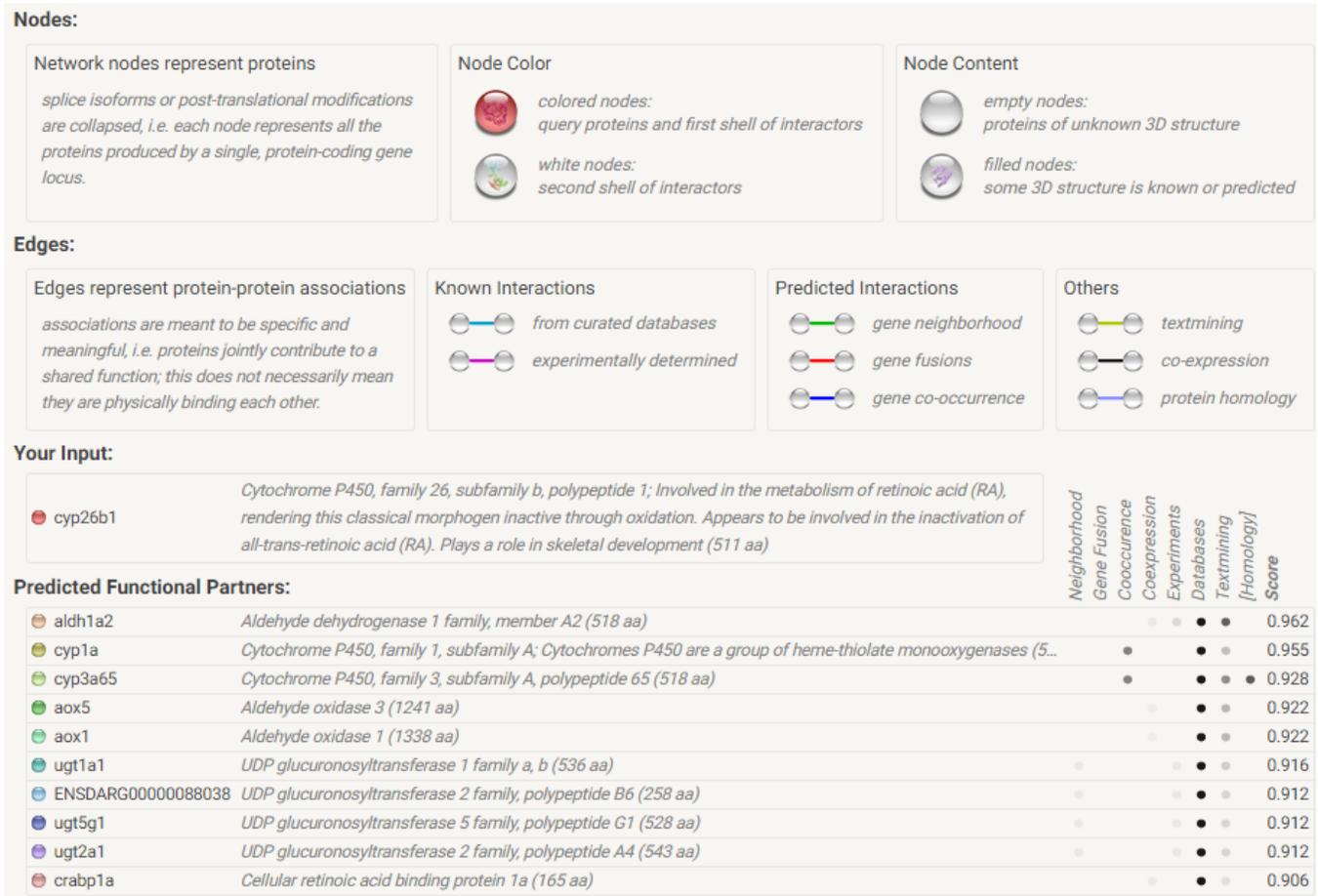
**Fig.13** qRT-PCR analysis of Cyp26B1 mRNA expression in ovary and brain of different maturation stages of *C. magur* female. The relative expression of Cyp26B1 normalized with  $\beta$ -actin was calculated using comparative Ct method. Data for real-time PCR were expressed as mean  $\pm$  S.E.M (n = 3)



**Fig.14** qRT-PCR analysis of Cyp26B1 mRNA expression in testis and brain of different maturation stages of *C. magur* male. The relative expression of Cyp26B1 normalized with  $\beta$ -actin was calculated using comparative Ct method. Data for real-time PCR were expressed as mean  $\pm$  S.E.M (n = 3)



Supp. Fig.1 Supplementary file of string Cyp26B1



The interaction revealed that Cyp26B1 protein has a strong association with aldehyde dehydrogenase 1 family, member A2 (aldh1a2) followed cytochrome P450 gene family (cyp1a and cyp3a65) with a confidence score of 0.962, 0.955 and 0.928 respectively (see supplementary file).

**Expression analysis of C. magur Cyp26B1 gene**

The mRNA expression pattern of Cyp26B1 gene in C. magur adult male and female fishes was determined through qRT-PCR in brain and gonads at four different maturation stages namely preparatory, spawning (before injection), 6h post-Ovatide™ injection and

16h post-Ovatide™ injection (post-spawning). The Cyp26B1 gene in female fish was expressed in ovary and brain of all selected stages with significantly higher mRNA expression in the brain. However, no statistically significant differences in mRNA expression were observed in the ovary at various maturation stages (Fig. 13). In the female brain, highest mRNA expression of Cyp26B1 was observed at spawning stage followed by hormone stimulated stages and least at the preparatory stage. Similarly, in case of male fish Cyp26B1 gene showed higher mRNA expression in the brain compared to testis with significantly peak expression at 16h post-Ovatide™ injection stage in brain (Fig. 14). The expression of

Cyp26B1 observed in testis was very less. We cloned the full-length cDNA sequence of Cyp26B1 (ORF 1533 bp) gene from *C. magur* using 5'/3' RACE-PCR strategy. The full-length cDNA sequence of Cyp26B1 has also been reported in zebrafish (Zhao *et al.*, 2005). The predicted ORF and deduced amino acid sequence was similar to other fish species like channel catfish (XP\_017327166.1), and zebrafish (NP\_997831.1). The catalytic active site motifs including heme-binding domain of Cyp26B1 gene and similarity analysis between different vertebrates revealed that these motifs are highly conserved (Fig. 2). Cyp26B1 has a PFGGGVVRXCLGKXLAXLF LKLLAXELA heme-binding domain (highlighted in Fig. 2) located at the C-terminal end. The cysteine residue of the active site motif serving as a critical catalytic residue, directly involved in heme-binding and surrounded by several highly conserved amino acids. The present results of Cyp26B1 gene sequence were in agreement with other reports confirming that these motifs are highly conserved in most of the Cyp26 subfamily members (White *et al.*, 1996; White *et al.*, 1997; MacLean *et al.*, 2001; Li *et al.*, 2016).

The phylogenetic analysis of *C. magur* Cyp26B1, with other species, revealed high similarity with *I. punctatus*, *D. rerio* and other catfishes. The *C. magur* Cyp26B1 showed 76% similarity with its orthologs from human which is similar to the homology obtained in zebrafish (Xi *et al.*, 2015).

The prediction of ligand binding sites in a protein helps to understand its putative function and molecular interaction with other proteins or molecules (Rausell *et al.*, 2010). Many ligand binding sites have been predicted in *C. magur* Cyp26B1 amino acid sequence out of which few have been revealed through homology modeling in human (Shimshoni *et al.*, 2012). This is the

first report on the putative ligand binding sites in any catfish species and will greatly help in understanding the molecular mechanism of action of these important proteins in the oxidative metabolism of all-trans-retinoic acid.

The hydropathicity analyzed using the Kyte-Doolittle algorithm predicted that *C. magur* Cyp26B1 protein is more hydrophobic at N-terminus region due to the presence of a highly hydrophobic region of nonpolar amino acids and is believed to function as a transmembrane signal anchor. Similar kind of hydropathy profile has been observed in its human ortholog by White *et al.*, (1997). Overall the Cyp26B1 protein of *C. magur* is hydrophilic in nature like its orthologs from zebrafish and human (White *et al.*, 1996; White *et al.*, 1997; Zhao *et al.*, 2005). From the RAMPAGE server, the percentage of residues in favored regions of Cyp26B1 obtained from Ramachandran plot were 94.3% which is in contrast to the earlier report on human (Karlsson *et al.*, 2008).

From the above mentioned background it is clear that RA plays crucial role in the regulation of genes involved in cell proliferation, differentiation and tissue function, hence essential for germ cell development. Though over and under expression of genes involved in the synthesis and degradation of RA resulted into the disturbed embryonic development therefore it is essential to maintain the homeostasis of RA. For mRNA expression analysis of Cyp26B1 in *C. magur*, adult male and female of two maturation stages preparatory and spawning were taken. To see the changes in gene expression after ovotide induction, 6h and 16h post induction stages were also taken. Study of mRNA expression was done by qRT-PCR. The mRNA expression results of Cyp26B1 in fish ovary were not in contrast to the results of other reports in which they

found a good expression of Cyp26B1 in the ovary (Zhao *et al.*, 2005). We found very minor expression of Cyp26B1 in ovary of *C. magur*. As the role of RA described in earlier studies, it should be required at the preparatory stage of fish in agreement to this, the present study showed significantly higher mRNA expression of Cyp26B1 in the brain at spawning and 16h post-Ovatide™ injection stages compared to the preparatory stage in female fish. Lower expression of Cyp26B1 in brain and ovary at preparatory stage precluded degradation of RA which suggested the need of RA at the preparatory stage for the regulation of meiotic initiation. As suggested by Lau *et al.*, 2013, both higher and lower expression of RA resulting from disrupted *aldh1a2* and *cyp26s* expressions are involved in disturbed meiotic entry in gonad. Significantly higher expression of Cyp26B1 at spawning stage might be responsible for the depletion of RA. Similarly, the mRNA expression of Cyp26B1 was also very less in testis compared to the brain of male fish. An earlier study on mouse suggested the role of Cyp26 genes in early development (Ray *et al.*, 1997) while in case of *C. magur*, the Cyp26B1 was found to be least expressed at the preparatory stage in testis. The brain of male fish showed the Cyp26B1 expression but decreased gradually from preparatory stage and peak at 16h of Ovatide™ injection stage. This significant observation if studied in detail might give a clue regarding the reproductive dysfunction of males during induced breeding in captivity. The mRNA expression of Cyp26B1 in *C. magur* needs further study on the controlled expression of Cyp26B1 gene under controlled conditions.

In summary, the present study reports full-length coding sequence of Cyp26B1 gene from *C. magur*. The sequence was analysed using various bioinformatics tools to predict the structural and functional features. The ligand binding sites predicted in the protein

can be used for drug designing in future. The differential mRNA expression analysis of the gene at various maturation stages revealed lowest expression of Cyp26B1 in the ovary and testis compared to brain of both the sexes. Preparatory stage of female brain showed very less expression while spawning stage showed peak expression. To the best of our knowledge, this is the first report of cloning and structural analysis of Cyp26B1 full-length cDNA in Indian catfish.

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### Conflict of interest

Authors declare that they have no actual or potential conflict of any interests.

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