Gene Reports 10 (2018) 54–65

Contents lists available at ScienceDirect

Gene Reports

journal homepage: www.elsevier.com/locate/genrep

Identification, cloning and expression profile of *sycp3* during gonadal cycle and after siRNA silencing in catfish

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ARTICLE INFO

Keywords:
Germ cells
Catfish
Testis development
PEI transfection
Proteomics
Sycp3

ABSTRACT

Molecular mechanisms underlying germ cell development, proliferation and maturation in teleosts revealed the importance of transcription factors through functional genomic approaches which is often comparable to mammals. In the present study, a proteomic approach was employed to find novel molecules with reference to germ cell development in teleosts. Based on this, current work utilized two-dimensional (2D) gel electrophoresis combined with matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry in an attempt to identify and compare the proteins which are expressed differentially in the male gonads of catfish during juvenile and adult stages. The 2D gel analysis identified the Sycp3 protein which was found to be up regulated in juvenile testis when compared to adult stages, and then the full length cDNA was cloned using the peptide sequence data from catfish testis. The spatio-temporal expression pattern of *sycp3* was explored during the critical time period of gonadal development as well as at different phases of recrudescence in catfish using real-time PCR, followed by localization analysis by *in situ* hybridization and immunohistochemistry. In addition, *in vivo* transient knockdown using PEI mediated *sycp3*-esiRNA in adult catfish during recrudescence showed a decrease in *sycp3* expression, which also affected the expression level of various testis-related genes. Present findings suggest that Sycp3 might have a potential role in the development and maintenance of testicular function in catfish.

1. Introduction

The processes of germ cell and gonadal development are the basis of sexual reproduction in vertebrates. Particularly in teleosts, these processes are under the influence of a cascade of molecular, genetic and physiological mechanisms. A crucial step in attaining sexual maturity is the entry of germ cells into meiosis, followed by their progression into viable gametes to produce either eggs or sperm. Although various transcription factors and genes which are implicated in gonadal development have been identified, there is still inadequate information to decipher underlying mechanisms and specific mode of actions. Earlier reports in teleost species in this context are mainly from daily or fortnight breeders, and there is a lack of similar data in annual breeders. To gain more insight, present study aimed to identify genes/factors regulating meiosis in order to understand germ cell development. Taking this into account, a proteomic approach was employed involving a two dimensional (2D) electrophoretic separation of proteins from adult and juvenile testis of the catfish *Clarias gariepinus*, excision of differentially expressed spots, in-gel digestion and identification of the resulting peptides matrix assisted laser desorption/ionization time-of-flight (MALDI TOF/TOF) mass spectrometry. Upon rigorous analysis the synaptonemal complex protein-3 (Sycp3) was identified which was found to be up regulated in juvenile testis when compared to adult stages.

SYCP3 is a meiosis-specific component of the synaptonemal complex, essential for the synapsis of homologous chromosomes, and is a marker of meiosis in mammals (Page et al., 2006). The *Sycp3* gene encodes the SYCP3 protein which is a major component of the synaptonemal complex (SC), a meiosis-specific structure which comprises of two parallel lateral regions and a central element (Botelho et al., 2001). Many studies, mostly in mammalian gametogenesis, have shown its crucial role in chromosome pairing, recombination, synapsis, and also in relation with their expression during sex differentiation of gonads and the stages of meiotic prophase in mouse oocytes (Di Carlo et al., 2000). Its importance in gametogenesis and fertility has been studied in mammals as well as in a few teleost species such as medaka and zebrafish (Iwai et al., 2006; Ozaki et al., 2011). Male mice that

Abbreviations: 2D-electrophoresis, 2 dimensional gel electrophoresis; Dph, days post-hatch; esiRNA, endonuclease prepared small interfering RNAs; hCG, human chorionic gonadotropin hormone; IF, immunofluorescence; IHC, immunohistochemistry; ISH, *in situ* hybridisation; MALDI-TOF/TOF, matrix assisted laser desorption/ionization time-of-flight; PEI, polyethylenimine; SC, synaptonemal complex; Sycp3, synaptonemal complex protein 3

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https://doi.org/10.1016/j.genrep.2017.10.009
Received 1 February 2017; Received in revised form 8 October 2017; Accepted 23 October 2017
Available online 26 October 2017

carry a null-mutation in the *Sycp3* gene have been found to be sterile with complete loss of spermatocytes (Yuan et al., 2000). Male specific genes such *Dmrt1* knockdowns have also been observed to have an effect on expression and localization patterns of SYCP3 (Agbor et al., 2013). Expression profile analysis of SYCP3 in rats revealed its detection from day 15 of postnatal period and its increased expression thereon might possibly corroborate with similar findings in fish during critical stages of gonadal development (Liu et al., 2012) where its expression was found to be significantly elevated during early spermatogenesis. In black porgy, *Sycp3* along with *dazl* and *dmc1* have been found to be essential for meiotic competency, and was downregulated with estrogen treatment which affects meiotic arrest in the germ cells (Lau et al., 2013). Although prior studies have established its role in meiosis and gametogenesis mostly in mammals, further information about the involvement of this testis-critical gene would be essential to elucidate its role in the process of gonadal germ cell development during critical stages, in teleost species where very limited reports are available in this context. Such studies, hence, may contribute to the pool of numerous genes that are responsible for the complex process of germ cell development, proliferation and maturation in teleosts. This assumes more importance where certain annually breeding teleosts undergo recrudescence to complete gonadal cycle repeatedly.

To enable this view, at first, based on the identified Sycp3 protein sequence using MALDI-TOF, degenerate primers were designed and cloned the full length cDNA of *sycp3* from catfish testis. The spatio-temporal expression pattern of *sycp3* was then analysed during the critical time period of gonadal development and recrudescence in catfish using real-time PCR. Later, polyclonal antibodies were raised in rabbit based on deduced amino acid sequences to localize the protein using immunohistochemistry (IHC) or immunofluorescence (IF) while transcripts in testis were identified using *in situ* hybridization (ISH). In addition, endonuclease prepared small interfering RNAs (esiRNA) targeted against *sycp3* gene was transfected in catfish testis *in vivo* and analysed its transient knockdown effects as well as the impact of the silencing on other genes crucial for germ cell and testicular development.

2. Materials and methods

2.1. Animal breeding and sample collections

Catfish at different age groups were reared in fresh water tanks (25 ± 2 °C) under ambient conditions. Mature male and gravid female fishes were used for *in vitro* fertilization to obtain catfish larva as per the method described by Raghuveer et al. (2011). Maturity in catfish is usually attained approximately in a year. The *in vitro* fertilization was carried out in accordance with the seasonal reproductive cycle culminating in spawning phase. Catfish *C. gariepinus* exhibits an annual seasonal pattern of reproductive cycle which can be divided in four different phases, preparatory (February–April), pre-spawning (May–-June), spawning (July–November) and regressing or post-spawning phase (December–January). This pattern of gonadal attenuation and recrudescence which is characteristic of annual breeders like catfish compared to continuously mature species is crucial in highlighting the roles of genes during development as well as recrudescence.

Gravid females were selected and injected with 2500 IU of human chorionic gonadotropin (hCG) intramuscularly and intraperitoneally. After ~14 h, mature eggs were stripped from the female by applying gentle pressure on the abdomen. Milt was collected from the testes by dissecting it out from the male followed by thorough mincing on ice. Both were mixed carefully in a sterile bowl with gentle shaking to allow fertilization. The embryos were then transferred to tanks connected with circulating filtered tap water. Hatching generally takes 30–36 h after fertilization. After hatching, the fries were kept in the incubator with filtered water. Once the yolk sac of the fries gets completely absorbed, live tubeworms were fed *ad libitum* daily. The water tanks were aerated and filled with filtered tap water, and maintained under normal photoperiod and ambient temperature (23 ± 2 °C). Gonads of male and female catfish (adult, 400 dph and juvenile, 100 dph) were dissected out and snap frozen in liquid nitrogen, stored at − 80 °C for proteomic analysis/total RNA isolation. Juvenile fish gonads (n = 20, for a single biological sample) were pooled individually for males and females to obtain three biological samples. Animals were briefly anesthetized with 100 mg/L of ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) in mild ice-cold water and samples were dissected out for further analysis. Sampling for fish was performed by following the general guidelines and with the approval of the Institutional Animal Ethics Committee, University of Hyderabad.

2.2. Two-dimensional gel electrophoresis and MALDI-TOF-MS analysis

2.2.1. Sample preparation and tissue extraction

Adult and juvenile catfish gonads were collected and homogenized for protein extraction in a buffer containing 6 M Urea, 4% CHAPS, 50 mM DTT and 100 mM PMSF on ice. Centrifugation was done at 12,000 × *g* at 4 °C for 30 min. The supernatant was then carefully extracted, precipitated, and used for 2D gel electrophoresis. Protein concentration was measured using the method of Bradford (1976).

2.2.2. 2D electrophoresis

Clean-up was done for the supernatant from the gonadal tissue homogenate with 2D Clean-up kit (GE Healthcare Life Sciences, USA) as per the manufacturer's instructions. This process was followed by dissolving the separated protein precipitate in rehydration buffer constituting 7 M Urea, 2 M Thiourea, 4% CHAPS, 2% Ampholytes of pH 3–10, 70 mM DTT and 3.5% Bromophenol blue. Immobiline Drystrips (3–10 pH 18 cm, GE Healthcare, USA) were rehydrated in 350 µl of the protein solution for ~20 h under low viscosity mineral oil (Genei, Bangalore, India). The rehydrated IPG strips containing 350 µg protein for each sample were then subjected to Isoelectric Focusing in Ettan IPGphor3 gel apparatus (GE Healthcare) at 20 °C for 70,000 Volt hours (Vh)-50 V-1 h, 500 V-5 h, 500 V-5 h, 10,000 V-8 h, 500 V-10 h (a total of 70,000 V hours). The strips were equilibrated in equilibration buffer after IEF (75 mM Tris-HCl pH 8.8, 30% glycerol, 2% SDS and a trace of bromophenol blue) containing 10 mg/ml of DTT, followed by 20 min in equilibration buffer containing 25 mg/ml of iodoacetamide (an alkylating agent to reduce cysteine residues preventing disulfide bond formation). SDS-PAGE was carried out as illustrated by Laemmli (1970) and the gels were then stained using coomassie brilliant blue R-250.

2.2.3. Image analysis

The gels were stained with coomassie brilliant blue R-250, and computer analyses were carried out using Image Master 2-D Platinum (GE Healthcare) system. Three separate gels were analysed for both juvenile and adult samples. The spots that were found to display significant changes in their expression were chosen for further analysis.

2.2.4. In-gel digestion, protein identification and database search

In-gel digestion and MALDI-TOF-MS analysis was performed with a MALDI-TOF/TOF mass spectrometer (BrukerAutoflex III smartbeam, BrukerDaltonics, Bremen, Germany) according to the method described by Shevchenko et al. (1996) with slight modifications. Coomassie brilliant blue R-250 stained protein spots were carefully excised from the gels. Destaining was done with 100 µl of 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate (NH_4HCO_3) for five times. Then, the gel pieces were treated with 10 mM DTT in 25 mM NH_4HCO_3 and incubated at 56 °C for 1 h. This was followed by treatment with 55 mM iodoacetamide in 25 mM NH_4HCO_3 for 45 min at room temperature (25 ± 2 °C), washed with 25 mM NH_4HCO_3 and ACN, dried in speed vac (Labconco, San Diego) and rehydrated in 20 µl of 25 mM NH_4HCO_3 solution containing 12.5 ng/µl trypsin (sequencing grade, Promega,

Wisconsin, USA). This mixture was incubated on ice for 10 min and kept overnight for digestion at 37 °C. After digestion, it was centrifuged for 10 min and the supernatant was collected in a fresh eppendorf tube. The gel pieces were re-extracted with 50 µl of 1% trifluoroacetic acid (TFA) and ACN (1:1) for 15 min with frequent vortexing. The supernatants were pooled together and dried using speed vac and reconstituted in 5 µl of 1:1 ACN and 1% TFA. From the above sample, 2 µl of it was mixed with 2 µl of freshly prepared α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50% ACN and 1% TFA (1:1) and 1 µl was spotted on target plate. Protein identification was performed by database searches (PMF and MS/MS) using MASCOT program (http://www.matrixscience.com) employing Biotools software (BrukerDaltonics). The similarity search for mass values was done with existing digests and sequence information from NCBInr and Swiss Prot database. The search parameters were: fixed modification of carbamidomethyl (C), variable modification of oxidation (M), enzyme trypsin, peptide charge of 1 + and monoisotropic. According to the MASCOT program, the hits with the probability of $P < 0.05$ were considered statistically significant which were only accepted for protein identification. The peptide obtained from MALDI-TOF from the protein sample was highly homologous to the peptide KVMMDTQQQEMATVR identified in *Oncorhynchus mykiss*, and the translated nucleotide sequence from this peptide was used to design the degenerate reverse primer for cDNA cloning.

2.3. Molecular cloning of sycp3 from catfish testis

Total RNA was isolated from adult and juvenile catfish testis using TRI reagent (Sigma). The concentration was measured by NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). First strand cDNA synthesis was carried out using the isolated total RNA by Primescript RT-PCR (TaKaRa) Reverse transcriptase with 1 µl oligo d(T) primer, 1 µl dNTPs, 4 µl reaction buffer, 0.25 µl RNase inhibitor and 5 µg of total RNA in a 20 µl reaction at 42 °C. Different sets of degenerate primers for *sycp3* were designed (Table 2) by aligning the existing sequences of siluriforms in NCBI data base using Lasergene software (release 3.05; DNASTAR, Madison, WI, USA). The amplicon obtained was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), sequenced, analysed and confirmed as catfish *sycp3* partial cDNA. The full length sequence of *sycp3* cDNA was obtained by RACE strategy. Gene specific primers (GSP) were designed using sequence information from the partial cDNA sequence of *sycp3*. The 5′ and 3′ cDNA templates for RACE were prepared using SMARTer™ RACE cDNA amplification kit (Clontech, Mountainview, CA, USA) as per the manufacturer's protocol. Then, touchdown PCR reactions were carried out using 5P, 5N, 3P and 3N primers (Table 2) along with universal primer A mix, nested universal primer and Advantage® 2 PCR kit (Clontech) to obtain the 5′ and 3′ ends. All thermal cycling conditions were done as per the manufacturer's protocol. The RACE products were gel purified, cloned into pGEM®-T easy vector (Promega), subsequently sequenced and analysed by NCBI-BLAST.

2.4. Quantitative RT-PCR (qPCR) analysis of sycp3 for tissue distribution, reproductive cycle and ontogeny studies

Adult catfish ($n = 5$), both male and female from late preparatory phase were sacrificed to get different tissues (brain, heart, muscle, liver, kidney, testis, ovary). Testis samples were collected at different time intervals of the reproductive cycle for phase expression analysis. Samples of developing gonads from both male and female were also collected. In catfish, gonadal differentiation occurs from 35 to 50 days post-hatch (dph), developing into either a testis or ovary. Hence, gonad samples from different stages of development were collected (0, 10, 20, 30, 40, 50, 100, and 200 dph). For 0 dph, whole body without head and tail regions was used while for 10 to 40 dph mesonephric gondal complex has been dissected out for experiments. Wherever necessary, sample pooling was done to obtain 5 biological samples. Total RNA was extracted from these tissues using TRI-reagent® (Sigma) as per the manufacturer's protocol. Reverse transcription was carried out with Verso® reverse transcriptase enzyme (Thermo Fisher Scientific Inc., Waltham, MA, USA), random hexamers and 1 µg of total RNA as per the manufacturer's instructions. β-actin (internal control) amplification was done to confirm the quality of the cDNA templates. qPCR primers for *sycp3* were designed for an amplicon length of ~150-250 bp. The expression pattern of *sycp3* was analysed by qPCR in various tissues, and also during gonadal development and reproductive cycle in the testis. Real-time PCRs were carried out using SYBR® Green Master mix (Clontech) in a ABI Prism® 7500 fast thermal cycler (Applied Biosystems, Foster City, CA, USA) at 95 °C (15 s), 60 °C (1 min) for 40 cycles. The resulting amplicons were then sequenced and verified to confirm the specificity of the PCR amplification followed by dissociation curve analysis with sequence detection software (Applied Biosystems). *18S rRNA* was used as a reference gene due to its stable expression based on our previous studies. Each sample was run in triplicate for the qPCR assays using the *sycp3* specific primers and no template controls were also done which yielded no amplification. Cycle threshold (C_t) values were obtained from exponential phase of PCR amplification and *sycp3* expression was normalized against the expression the internal control gene, *18S rRNA* to generate a ΔCt value (C_t of target gene − C_t of control). Differential gene expressions were then calculated using $2^{-\Delta Ct}$ method. All primers used are listed in Table 1.

2.5. hCG induction in vivo and in vitro

In vivo and *in vitro* hCG induction on adult catfish at early pre-spawning phase was done as per the protocols described by Murugananthkumar et al. (2016). In brief, for *in vivo* induction, 5000 IU of hCG (Trade name: Pubergen; Sanzyme Ltd., Shameerpet TS, India) dissolved in 100 µl of saline was loaded into an osmotic pump (ALZET® osmotic pumps, Cupertino, 116 CA, USA) and saline was used for control groups. Fishes were anesthetized with 100 mg/L of MS-222 (Sigma). A small incision of about 8 mm was cut in the intraperitoneal and saline or hCG loaded osmotic pump were then implanted into the peritoneal cavity close to the gonads. The incision was sutured using sterile 30 mm catgut and the fishes were carefully monitored for 3 days and then maintained in glass tanks. After the experimental period of 21 days, the fishes were sacrificed and samples were taken for analysis.

Table 1
List of identified protein spots by 2D-gel electrophoresis followed by MALDI-TOF/TOF from adult and juvenile testis of male catfish.

Name of spot	Expression pattern	Molecular weight in kDa	Protein name	Accession number	Species identified	Score
UR1	Upregulated	27.9	Synaptonemal complex protein 3	gi|217416450	*Onchorynchus mykiss*	60
UR2	Upregulated	47.5	P2X purinoceptor 1-like protein	gi|348541227	*Oreochromis niloticus*	55
DR1	Downregulated	35	Annexin A	gi|225705972	*Osmerus mordax*	45
DR2	Downregulated	15.3	Hemoglobin cathodic subunit alpha	gi|115502083	*Tetradon nigroviridis*	57

For *in vitro*, an adult male catfish was sacrificed during preparatory phase and the testis was dissected out under sterile conditions and kept it in ice-cold Leibovitz (L-15) culture medium (Sigma). Tissue slices of about 50 μm thickness were then cut from the testis using a McIIwain tissue chopper (Vibratome, Ted Pella Inc., Redding, CA, USA). The testicular slices were transferred to tissue culture plates containing 2 ml of L-15 medium supplemented with 10 mM HEPES and antibiotics (penicillin, 100 IU/ml; streptomycin, 0.1 mg/ml). The slices were then kept for 24 h at 20–22 °C in the presence 100 IU/ml of hCG. Physiological saline was used for control. Testis samples were collected at intervals of 2, 4, 6, 12, 20, and 24 h from both hCG treated and control. The collected tissues were briefly washed with ice-cold 1 × PBS, pH 7.4. Total RNA extraction and cDNA synthesis were done, and relative expression was calculated as previously explained.

2.6. Polyclonal antibody generation for Sycp3

Based on the deduced amino acid sequence of Sycp3, an antigenic peptide, CLQQWETDVKKSEDQ was synthesized and conjugated to keyhole limpet hemocyanin carrier protein commercially (Biotech Desk Pvt. Ltd., Secunderabad, India). The peptide dissolved in phosphate buffer saline (PBS; pH 7.4) was then injected into three-month old New Zealand white rabbits. The rabbits were housed and handled with the specific approval and as per the guidelines of Institutional Animal Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA approval No.151/1999 dt. 22.07.1999). Pre-immune serum was collected from the blood drawn from the lateral ear vein before the peptide was injected. Injection was done subcutaneously with 500 μg of antigenic peptide emulsified with Freund's complete adjuvant. Two booster injections (250 μg) of antigen emulsified with Freund's incomplete adjuvant was then administered 14 days apart. The serum was then collected and used for immunohistochemistry after checking titre and antibody specificity using Western blot and preadsorption with antigen.

2.7. IHC and IF

IHC was carried out in catfish testis sections to localize Sycp3 using the using anti-Sycp3 antibody of catfish. In brief, testis from catfish was fixed in Bouin's fixative (saturated picric acid: formaldehyde: glacial acetic acid, 15:5:1) for 3–4 h. This was then embedded in paraplast (Sigma) after dehydration with a gradient series of alcohol and xylene. Paraplast sections of 6 μm were cut using a rotary microtome (Leitz 1512, Holly, MI, USA) and fixed on to glass slides. The tissue sections were then deparaffinized in xylene and hydrated through a graded series of alcohol. Endogenous peroxidase was blocked with 0.1% H_2O_2 added to the slides for 10 min at room temperature. The sections were washed in 0.1 M phosphate-buffer saline (PBS) pH 7.4 for 10 min and then blocked with 10% normal goat serum (Bangalore Genei) in 0.1 M PBS for 1 h at room temperature. Anti-Sycp3 polyclonal antibody (1:1000) or pre-adsorbed antibody with excess (Sycp3) antigen (for negative control) was then added to the slides, and incubated in a humid chamber at 4 °C overnight. Following incubation, the slides were washed with 1 × PBS with 0.1% Tween (PBST). The sections were then incubated with HRP conjugated secondary antibody (Bangalore Gene I) for 2 h at room temperature. VECTASTAIN® Elite ABC reagent (Vector Laboratories, Burlingame, CA, USA) was added to the slides, and incubated for approximately 30 min at room temperature. The slides were washed with PBS and developed with 3′3'-diaminobenzidine (DAB) as chromogen and H_2O_2 (Vector Laboratories) as a substrate. Counterstaining was done with hematoxylin (Qualigens Fine Chemicals, Worli, Mumbai, India), followed by dehydration with a gradient alcohol series, xylene, and mounted using DPX Mountant. Images were acquired using Q capture Pro 6 software (Quantitative Imaging Corporation) with Micropublisher 3.3 RTV-CCD camera in CX-41 Olympus Microscope (Olympus Corporation, Tokyo, Japan).

For IF, testis sections were collected from adult catfish and washed in 0.1 M PBS twice and then fixed using 4% paraformaldehyde in 0.1 M PBS for about 2–3 h. Repeated washing was done with 0.1 M PBS. The testis tissues were then immersed in OCT compound (Leia, Buffalo Grove, IL, USA) and then stored in − 80 °C freezer for about 2–4 h. Tissue sections of 7 μm were cut using cryostat (Leica CM1850). After blocking with normal goat serum and then incubating with primary antibody Sycp3 overnight, the sections were incubated with FITC-conjugated anti-rabbit secondary antibody. Pre-adsorbed antibody with excess antigen was used as a negative control. The IF images were then taken with an IX81 Olympus Microscope (Olympus Corporation), containing Cell Sens dimension software.

2.8. ISH

ISH was performed based on Rajakumar and Senthilkumaran (2014) to localize the mRNA transcripts of *sycp3* in adult catfish testis. In brief, fixation of testis and sectioning was done in freezing medium as described for IF analysis. Sense and antisense 'cRNA' probes were synthesized using digoxigenin (DIG) RNA labelling mix (Roche) as per the manufacturer's instructions and then purified using UFC3LKT column (Millipore) and then stored at − 80 °C. The testis sections were washed with PBST (PBS with Tween 20-DEPC) and treated with proteinase K and 4% PFA. About 200 μl of hybridization buffer was used to dilute 1 μl each of purified sense and antisense cRNA probes and heat denatured at 80 °C for 5 min. The diluted probes were then added onto the slides and incubated overnight at 50 °C in a sterile RNase free incubator. After incubation, the slides were washed briefly with blocking buffer solution (Roche). Anti-DIG-ALP antibody, diluted 1:1000 in maleic acid buffer (Roche) were added to the slides and kept at 4 °C overnight. The slides were washed with DIG washing buffer (Roche) several times and then incubated with detection buffer (Roche). The sections were colour developed using BCIP-NBT (Roche) as substrates with nuclear red as a counter stain. Once the colour had developed, the slides were washed and dehydrated using a gradient alcohol series and mounted using DPX mountant. Images were obtained using Leica DM6 B digital research microscope (Leica Microsystems Gmbh, Wetzlar, Germany).

2.9. In vitro esiRNA-PEI transfection

Using the cDNA of *sycp3* cloned from catfish, endonuclease prepared small interfering RNA (esiRNA) was commercially synthesized (Sigma). Testicular cell culture of catfish was prepared as per the protocol described by Murugananthkumar and Senthilkumaran (2016). In brief, the testicular culture was prepared prior to the day of transfection and 1 × 10^6 cells of the culture were plated into several wells of Corning® Costar® cell culture plates, 24 well (Sigma). Culture medium containing DMEM, 10% FBS, 1 × Glutamax and antibiotic and antimycotic was prepared and then incubated at 30 °C supplied with 5% CO_2. The culture medium was carefully pipetted out and 100 μl of OPTI-MEM® I solution was added to each well. Control siRNA (MISSION siRNA Fluorescent Universal Negative Control #1, Cat. no. SIC007 conjugated with 6-FAM, Sigma, 10 ng/μl) and different concentrations of *sycp3*-esiRNA (10, 20, 40 and 100 ng/μl) were complexed with branched PEI in sterile HEPES-NaCl buffer, pH 7.4 by incubation for 20 min at RT. The control siRNA/*sycp3*-esiRNA-PEI complex of different concentrations were then added to the respective wells and incubated for 12 h. PEI alone as well as *sycp3*-esiRNA with no PEI was also added as additional controls to rule out any off target effects. The OPTI-MEM® I and esiRNA-PEI mixture were then discarded after the transfection and replaced with cell culture medium as stated before. The cells were allowed to grow for another 24 h and then collected for further analysis. Total RNA was prepared from control siRNA/esiRNA/PEI/*sycp3*-esiRNA-PEI treated samples. Relative expression of certain testis related genes post-transfection were analysed, and calculated as explained

before. To analyse the transfection efficiency, the cells transfected with control siRNA were microphotographed in an IX81 Olympus Microscope (Olympus Corporation) with Cell Sens dimension software.

2.10. In vivo esiRNA-PEI transfection

Branched PEI (MW 25 K, Sigma) was dissolved in sterile HEPES-NaCl pH 7.4, and was then combined with 3 μg of *sycp3*-esiRNA as per the protocol described by Höbel and Aigner (2010). It was then kept at room temperature for 30 min to form an esiRNA-PEI complex and then used for transfection (Falco et al., 2009; Zhou et al., 2012). Adult (one year and ten months old) catfish ($n = 5$) during prespawning phase were taken for the experiment. The fishes were briefly anesthetized with 100 mg/L of MS-222 (Sigma). The ventral region of the catfish was sterilized with ethanol, and a small incision of about 10 mm was made before the pelvic fin junction with a sterile scalpel so as to expose the testis. The esiRNA-PEI complex was then slowly injected directly into the testis with a sterile 1 ml syringe. Control siRNA-PEI complex or PEI was injected for control groups. The incision was carefully sutured with sterile 30 mm catgut and treated with antibiotics. The animals were monitored for 3 days in isolated tanks, and tissue samples were taken at different intervals by sacrificing the fishes at approximately 48 and 72 h post-transfection (to analyse the duration of the esiRNA silencing that occurred *in vivo*) for qPCR analysis (snap frozen using liquid nitrogen and stored in − 80 °C).

2.11. Statistical analysis

All the data were expressed as mean ± standard error of mean (SEM). Significance among groups was tested by ANOVA followed by Student's–Newman–Keuls' test using SigmaPlot 12.0 software (Systat Software Inc., Chicago, IL, USA). Differences among groups were considered significant at $P < 0.05$.

3. Results

3.1. Identification of Sycp3 by 2D gel electrophoresis and MALDI-TOF/TOF-MS analysis

To screen the differentially expressed proteins during gonadal development, 2D-electrophoresis was used to isolate the differentially expressed spots. The 2D analysis was done a minimum number of three times and adult fish used were approximately 1 year old with fully matured testis, while for juvenile fish, testis of < 100 dph fish were pooled and used since the testis is still not fully matured at these stages. This was important in identifying factors which come into play during the process of germ cell and gonadal development. For this particular experiment, juvenile fish testis of 70-100 dph were pooled ($n = 20$) for protein extraction and for comparative analysis between adult and juvenile testis, the same concentration of total protein extracted from each tissue were used. The comparative analysis between the 2D gel images of adult and juvenile testis tissues showed many differentially expressed protein spots (Fig. 1). Differential expression was calculated in fold changes, and only the protein spots which had fold changes > 2 were considered significant and taken for MALDI analysis. Protein spots with lower molecular weights (< 100 kDa) were favored, to narrow down our search for factors essential for gonadal development considering encoded nucleotide sequence length. Among the total spots detected with Image Master 2D Platinum (GE Healthcare), we isolated four spots which had significant differential expression in juvenile testis, taking adult testis as control. Among these, two spots were downregulated (DR1, DR2) in juvenile testis, and two spots were upregulated (UR1, UR2). The list of proteins identified using MALDI-TOF analyses are shown in Table 1. Among these, one upregulated spot in juvenile testis (UR1) was identified as Sycp3, which was selected for further investigation as it is related to meiosis as per the aim of our study. The three other sports identified are P2X purinoceptor 1-like protein, annexin A and hemoglobin cathodic subunit alpha, which showed little or no evidence for their role in gametogenesis. The data on ovary samples were not provided as meiosis-related proteins could not be identified.

3.2. Molecular cloning of sycp3 from catfish testis

To investigate expression profile of the protein upregulated in juvenile testis, we characterized the *sycp3* gene of catfish. A partial cDNA of 372 bp of *sycp3* was cloned from juvenile catfish testis by RT-PCR by using degenerate primers based on the Sycp3 peptide sequence data obtained by 2D MALDI-TOF/TOF mass spectrophotometry. RACE strategy was used to obtain the 5′ and 3′ ends, to get the full length cDNA of 723 bp. The ORF was found to encode a putative protein of 241 amino acids. The nucleotide sequences of the partial and RACE cDNAs were aligned with lasergene software in order to generate the complete cDNA. The primers used for cloning were specified in Table 2. Sequence analysis revealed the presence and high homology of the peptide sequence identified through 2D proteomic and MALDI-TOF analysis.

3.3. Sequence and phylogenetic analysis

Multiple sequence alignment of deduced amino acid sequences was done based on the Sycp3 sequences of various mammals and teleost species using the ClustalO alignment software from European Bioinformatics Institute website and phylogenetic analysis was done using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny) by neighbor-joining method (Fig. 2 and 3). The GenBank accession numbers used for Sycp3 are: *Homo sapiens* (NM_001177949.1), *Mus musculus* (NM_011517.2) *Xenopus tropicalis* (NM_001078758.1), *Danio rerio* (NM_001040350.1) *Monopterus albus* (AJP00088.1), *Oreochromis niloticus* (XP_003439417.1), *Onchorynchus mykiss* (NP_001117979.1), *Oryzias corvinotus* (BAE47002.1). The multiple sequence alignment revealed considerable homology only at the C-terminal region of the protein sequences while the same is not evident in the DNA binding (N-terminal) region (Fig. 2). Phylogenetic analysis (Fig. 3) revealed that the catfish Sycp3 is closely related to rainbow trout, *Onchorynchus mykiss* and forms a separate clade.

3.4. Tissue distribution, reproductive cycle and ontogeny expression analysis of sycp3 in catfish

Tissue distribution of *sycp3* revealed exclusive expression in gonads of both male ($P < 0.01$) and female ($P < 0.01$) when compared to other tissues analysed such as liver, kidney, brain, muscle and heart (Fig. 4A and B, respectively). The expression of *sycp3* was found to be significantly ($P < 0.05$) elevated during the preparatory phase of the reproductive cycle of catfish, and moderate expression was found in the prespawning and spawning, while there was minimal expression during the resting phase (Fig. 5). In the ontogeny study (Fig. 6), there was a stage-dependent increase in *sycp3* expression observed in testis at different age groups of catfish, with significant ($P < 0.05$) high expression was seen at 50 dph in the gonads of both male and female catfish. This high expression of *sycp3* is comparable to preparatory phase results where recrudescence of gonadal development is evident in annually reproducing teleosts.

3.5. Immunolocalization of Sycp3 in catfish testis

Localization of Sycp3 through IHC and IF were done in the testis of adult catfish during preparatory phase (Fig. 7 and 8, respectively). Sycp3 immunoreactivity was observed in the spermatocytes of testis (Fig. 7A–C and 8A–C), while mature sperm/spermatids did not show any immunoreactivity (counterstained with hematoxylin). The negative

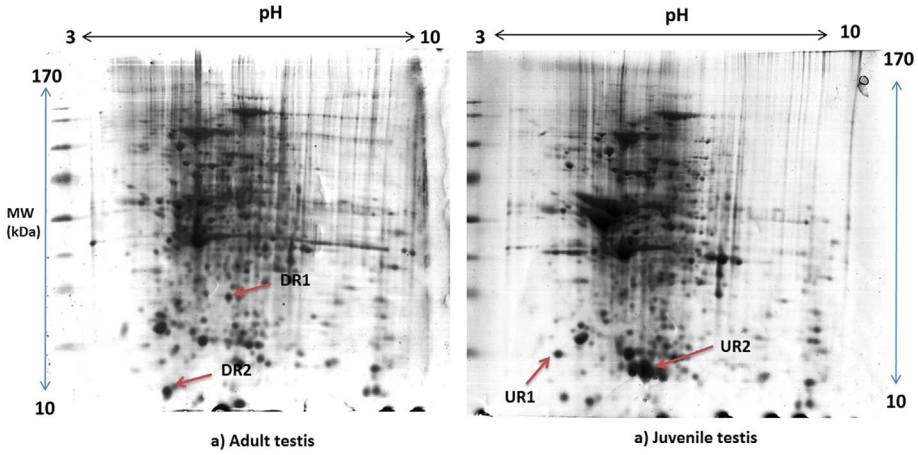

Fig. 1. Representative two-dimensional (2D) electrophoresis gels of adult and juvenile testis of catfish with adult testis taken as control. Spots indicated by arrows were either upregulated (UR1, UR2) or downregulated (DR1, DR2) in juvenile catfish. Spots identified are synaptonemal complex protein 3 (UR1), P2X purinoceptor 1-like protein (UR2), annexin A5 (DR1) and hemoglobin cathodic subunit alpha (DR2).

Table 2
List of primers used for cDNA cloning and qPCR analysis.

S. No	Primer name	Primer sequence 5′–3′	Usage	GenBank accession number
1.	*sycp3* F2	GAACATRGAGGARAYGGAG	RT-PCR	KY553233
2.	*sycp3* R	CCATCTCYTGYTGTTGHGYKTCC		
3.	5P	AGCAGAGCCATCGCCTTCTTCAGCTCC	RACE	
4.	5N	CTCCCACTGCTGAAGCACTGACAGCACC		
5.	3P	GGTGCTGTCAGTGCTTCAGCAGTGGGAG		
6.	3N	GGAGCTGAAGAAGGCGATGGCTCTGCT		
7.	*sycp3* RTF	CCCAGTATAGCCAGAGGCAGAAGCTG	qPCR	
8.	*sycp3* RTR	GAACATGGAGGAGATGGAGAAGAGTC		
9.	*18S rRNA* F	GCTACCACATCCAAGGAAGGCAGC	qPCR	KM018296
10.	*18S rRNA* R	CGGCTGCTGGCACCAGACTTG		
11.	*ckit* F	GCACTGTTGGAGGTCCGACTCTGGTG	qPCR	MF093750
12.	*ckit* R	GGTGCTGGCTCTTACACAGACTGTG		
13.	*vasa* F	GGTCTGAGTAAAGTTCGTTATCTG	qPCR	GU562470
14.	*vasa* R	GCCAACCTTTGAATATCCTCTG		
15.	*dmrt1* F	ATGGCCGCTCAGGTGGCTCTGCGG	qPCR	FJ596554
16.	*dmrt1* R	GCGGCTCCCAGAGGCAGCAGGAGA		
17.	*sox3* F	CACGGTATGAGTAGCCCACCA	qPCR	HQ680982
18.	*sox3* R	GCGATGGCAGGTGGTGGTGAG		
19.	*sox9* F	TCTGGCGGCTGCTGAATGAAGG	qPCR	HM149258
20.	*sox9* R	CTCGGTATCCTCGGTTTCACC		
21.	*wt1* F	ACGCGCACAGGGTGTTCGA	qPCR	JF510005
22.	*wt1* R	GGTACGGTTTCTCTCCTTGTG		

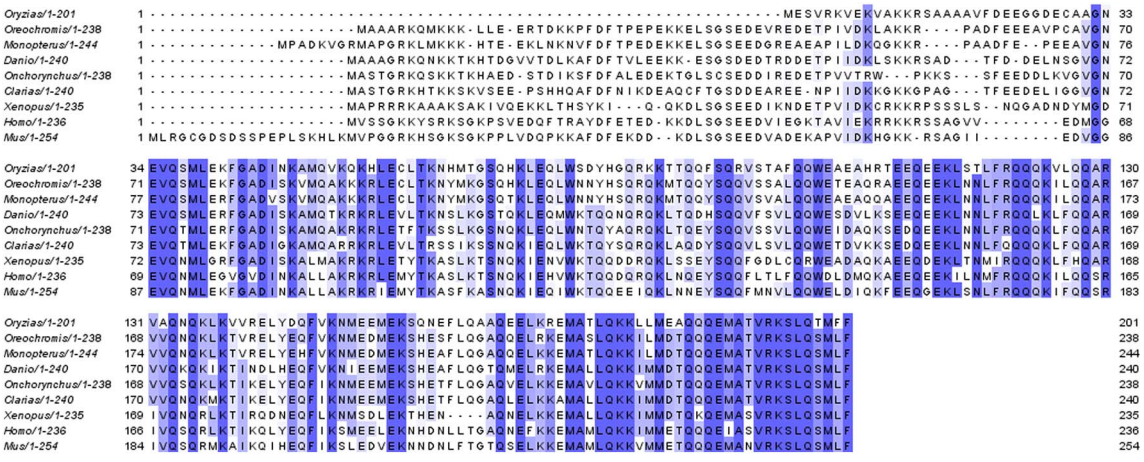

Fig. 2. Multiple sequence alignment of catfish Sycp3 with other mammalian and teleost species: *Homo sapiens* (NM_001177949.1), *Mus musculus* (NM_011517.2) *Xenopus tropicalis* (NM_001078758.1), *Danio rerio* (NM_001040350.1) *Monopterus albus* (AJP00088.1), *Oreochromis niloticus* (XP_003439417.1), *Onchorynchus mykiss* (NP_001117979.1), *Oryzias corvinotus* (BAE47002.1) using the ClustalO alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/).

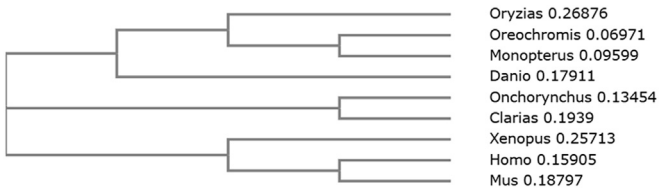

Fig. 3. Phylogenetic analysis of catfish Sycp3 with other mammalian and teleost species. The analysis was done using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny) by neighbor-joining method.

controls with pre-absorbed antibody of excess Sycp3 antigen did not show any positive signals (Fig. 7D and 8D). A similar result was also found in IF detection, where positive immunoreactivity were detected in the spermatocytes. The specificity of the antibody used was checked using Western Blot, which detected a positive band at ~27 kDa (Fig. 7E) that corresponds to the predicted size in testis of other fish species where it has been previously identified, while there was no signal observed in negative control (heart) which confirmed the specific binding of primary catfish Sycp3 antibody to testis.

3.6. ISH of sycp3 in catfish testis

ISH to localize *sycp3* mRNA transcripts was performed in adult catfish testis. Positive signals were detected in the spermatocytes (Fig. 9A–C) but not in mature sperm/spermatids counterstained with nuclear red. Sense probe as a negative control did not show any signal (Fig. 9D), thereby signifying the specificity of the antisense probe used.

3.7. Effects of in vivo and in vitro hCG induction on sycp3 expression

The expression of *sycp3* was found to be significantly upregulated ($P < 0.05$) after hCG induction in adult male catfish as compared to the control samples. In the *in vivo* induction, an elevated expression of *sycp3* was observed after the period of induction, when compared to control (Fig. 10A). For the *in vivo* study (Fig. 10B), a significant elevation of *sycp3* expression was detected from 6 h onwards when compared to the control samples analysed.

3.8. In vitro transfection of sycp3-esiRNA in catfish testicular culture

In the *in vitro* transfection analysis ($n = 5$), PEI complexed with *sycp3*-esiRNA showed significant ($P < 0.05$) decrease in the levels of *sycp3* in comparison with control and other groups (Fig. 11A). In addition, the expression levels *sycp3* post-esiRNA transfection showed that downregulation was dose-related. The expression level of *sycp3* was significantly ($P < 0.05$) decreased by 65% post-transfection with 100 ng/μl of *sycp3*-esiRNA when compared to control, and this dose was selected for further experiments. The transient knockdown of *sycp3* showed a significant ($P < 0.05$) decrease in the expression levels of other important testis-related genes when compared to control (Fig. 11B). The relative mRNA levels of *vasa*, *dmrt1*, *sox3*, *sox9* and *wt1* showed a decrease of 41, 71, 20, 23 and 82% respectively, while *c-kit* did not show any significant difference when compared to control. The injection of only PEI on catfish testicular cells did not show any

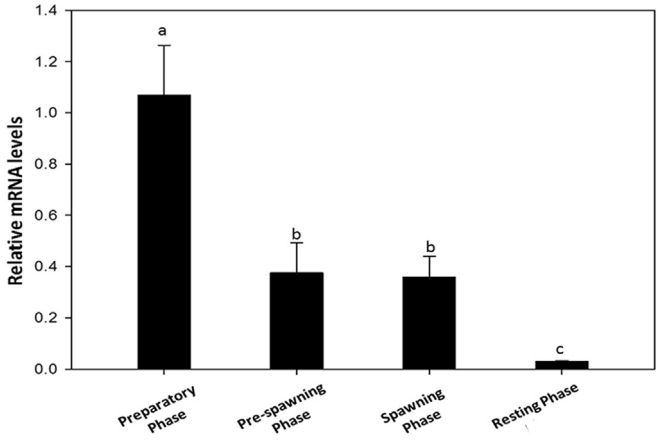

Fig. 5. qPCR analysis of *sycp3* expression during different phases of the testicular cycle in catfish, *Clarias gariepinus*. The relative expression of *sycp3* was normalized with *18S rRNA* and the values were calculated using $2^{-\Delta ct}$ method. Data ($n = 5$) were expressed as mean ± SEM. Mean with different letters differs significantly while mean with same letters are not significant ($P < 0.05$; ANOVA followed by Student-Newman-Keuls' *post-hoc* test).

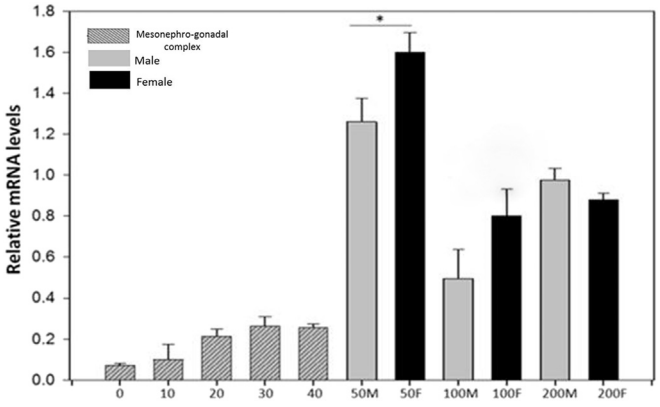

Fig. 6. qPCR analysis of *sycp3* expression during various stages of development of catfish. The relative expression of *sycp3* was normalized with *18S*rRNAand the values were calculated using $2^{-\Delta ct}$ method. Data ($n = 5$) were expressed as mean ± SEM. (*$P < 0.05$; ANOVA followed by Student-Newman-Keuls' *post-hoc* test).

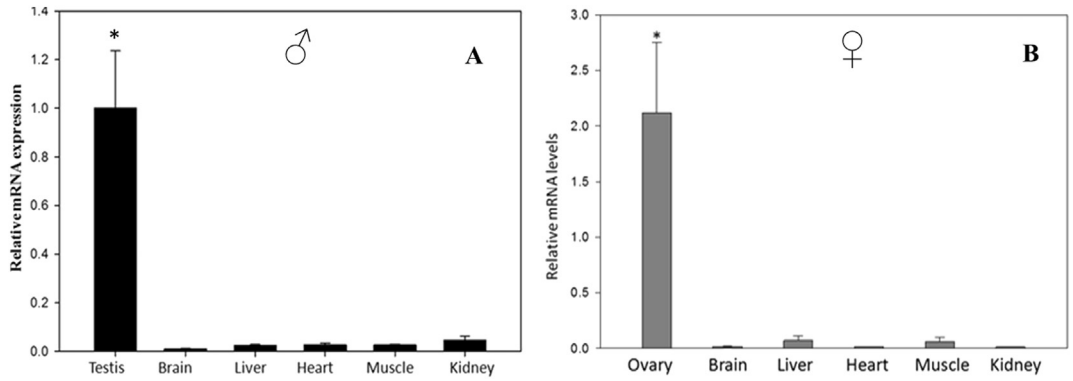

Fig. 4. qPCR analysis of *sycp3* expression in various tissues of catfish in male (A) and female (B). The relative expression of *sycp3* was normalized with *18S*rRNAand the values were calculated using $2^{-\Delta ct}$ method. Data ($n = 5$) were expressed as mean ± SEM. (*$P < 0.05$; ANOVA followed by Student-Newman-Keuls' *post-hoc* test).

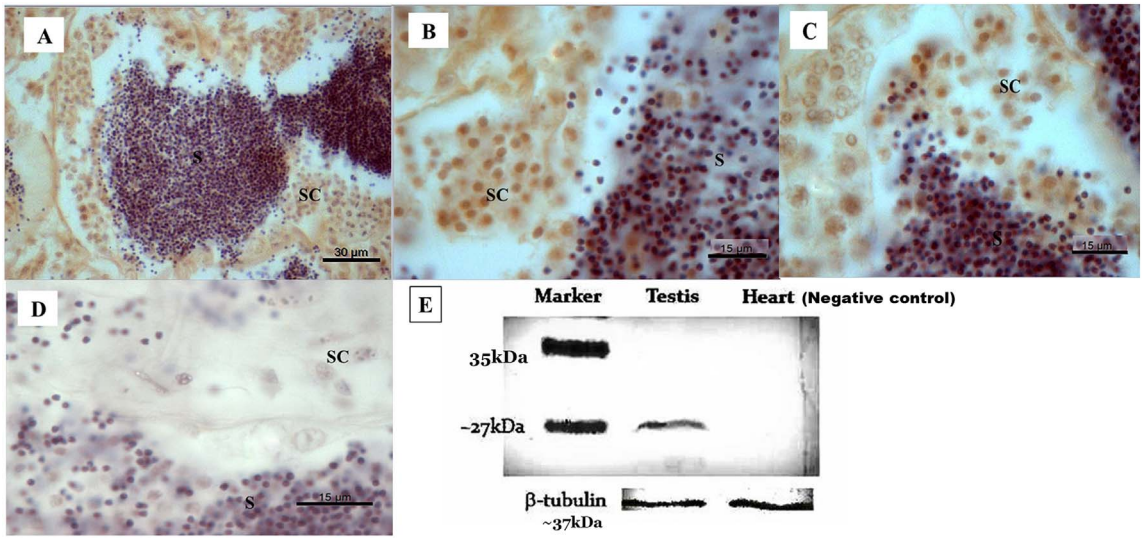

Fig. 7. Localization of Sycp3 protein in catfish testis. Immunoreactivity was observed in spermatocytes (A–C) but not in sperm/spermatids (counterstained with hematoxylin), while negative control (D) did not show any signal (pre-adsorbed antibody with excess Sycp3 antigen). Western blot analysis detected a positive band at ~27 kDa which confirmed the specificity of the antibody used (E). Abbreviation: SC-spermatocytes, S-sperm/spermatids. Scale bars indicate A: 30 μm, B, C and D: 15 μm.

significant change when compared to control. The primary testicular culture transfected with control siRNA survived normally (Fig. 12A) and transfection of control siRNA with PEI in catfish primary testicular culture showed fluorescent signals confirming siRNA uptake in the cells (Fig. 12B and C).

3.9. *In vivo transfection of sycp3-esiRNA in adult catfish*

Initially, PEI without any siRNA was injected into the testis of adult catfish, and tissue samples were collected for quantification of *sycp3* mRNA with qPCR. These were analysed and compared with the gene expression levels in control siRNA (Sigma) injected as well as *sycp3*-esiRNA-PEI complex injected animals. There were no significant changes observed in the levels of *sycp3* in the PEI-only injected catfish ($n = 5$) when compared to control which infers that neither PEI alone nor control siRNA did not alter the gene expression levels. However, a combination of PEI with *sycp3*-esiRNA injected into the testis significantly ($P < 0.05$) decreased the expression of *sycp3* mRNA expression levels when compared to the other groups (Fig. 13A). This indicated that esiRNA complexed with PEI injected into the testis was capable of silencing *sycp3* mRNA expression. Expression levels of *sycp3* were also quantified for the tissues collected at two separate time intervals, 48 and 72 h post-transfection and compared to control groups at each time point (Fig. 13B). The esiRNA-PEI complex injection resulted in significant decrease ($P < 0.05$) in the levels of *sycp3* expression both at 48 and 72 h post-transfection with 63 and 54% decrease respectively when compared to control. The percentage decrease had been found to be lowered after 72 h indicating that transcripts might get restored to normal levels as shown earlier by Murugananthkumar and Senthilkumaran (2016). Histological HE stained images of testis injected with esiRNA compared to control (data provided as Supplementary information) showed the transient nature of

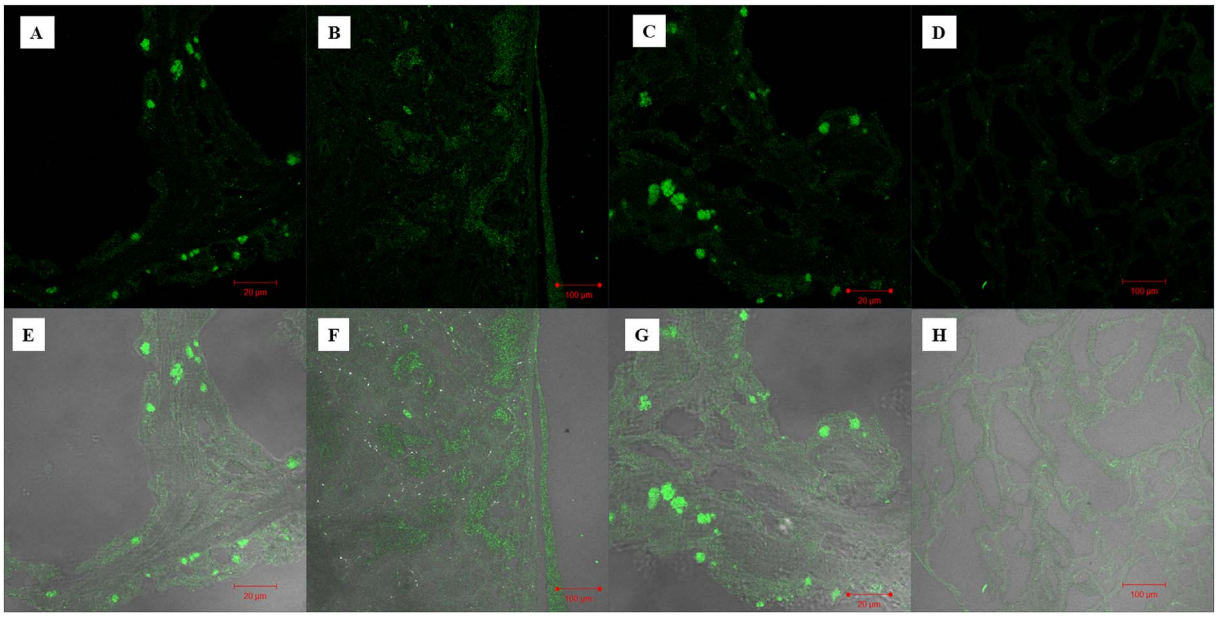

Fig. 8. Localization of Sycp3 protein in catfish testis using immunofluorescence stained with FITC which indicates positive signals for spermatocytes (A–C) compared to negative control (D). E–H represents the corresponding phase contrast images. Scale bars indicate, A, C, E, and G: 20 μm; B, D, F and H: 100 μm.

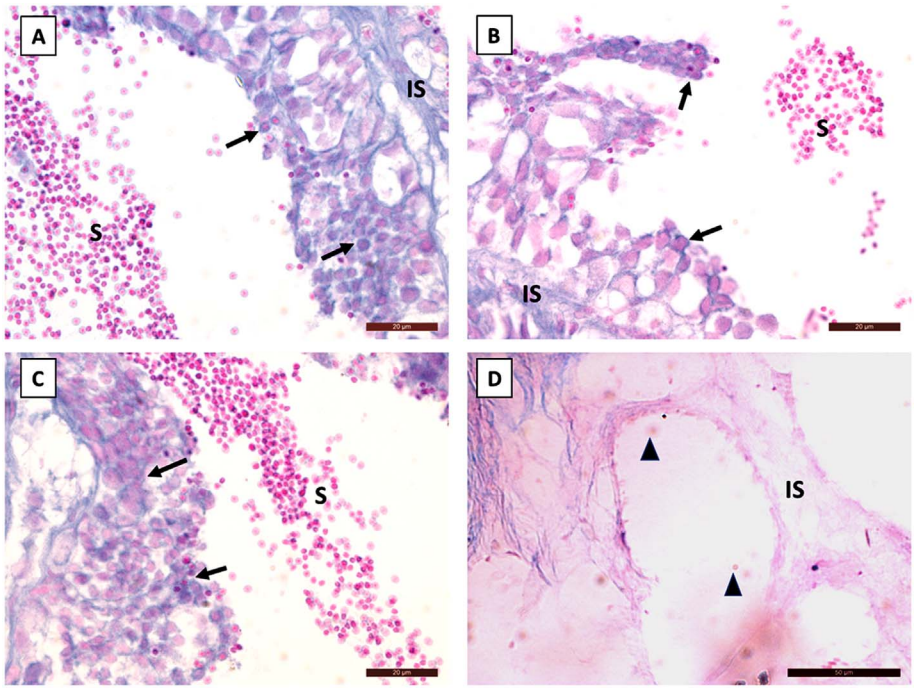

Fig. 9. Localization of *sycp3 m*RNA in adult testis of catfish by *in situ* hybridization. Testis of adult catfish displayed positive signals mainly in spermatocytes indicated by black arrows (A–C). Signals were not detected in mature sperm/spermatids (S). Sense probe of *sycp3* did not show any signal (D) and mature sperm are indicated by black arrowheads. Counterstain was done with nuclear red for antisense slides. Scale bars indicate A–C: 20 μm; D: 50 μm; IS–interstitial cells.

the PEI-esiRNA transfection was not enough to cause any significant phenotypic change post-transfection. Testis morphology was not affected significantly, and sperm/spermatids were observed to develop as normal. Hence, transient knockdown may not impart phenotypic changes unlike gene based permanent or conditional knockout that can impart phenotypic changes. However, molecular changes can be effectively seen by transient gene silecing (see below).

In addition, the expression levels of various genes which are crucial for germ cell development were also analysed with qPCR after 48 and 72 h *sycp3* esiRNA silencing in the catfish testis as well as in the control groups (Fig. 14). Various transcription factor genes such as *dmrt1*, *sox3*, *sox9* and *wt1* as well as RNA-binding protein *vasa*, which all play crucial roles in germ cell development and spermatogenesis, showed a significant decrease ($P < 0.05$) in expression levels at both 48 and 72 h post-transfection. Tyrosine kinase receptor *c-kit* however did not show any significant downregulation. There were not much difference seen in the expression levels between 48 and 72 h for most genes, except for *sox3* whose levels reverted back to normal at 72 h.

4. Discussion

Present study reports the identification of Sycp3 from catfish testis using 2D proteomic analysis, and then by using the peptide sequence data, the full length cDNA was cloned using RT-PCR and RACE strategy. Expression analysis was done during development stages, reproductive cycle and after hCG induction *in vivo* and *in vitro*. Using IHC and IF, localization of Sycp3 protein was found specifically in the spermatocytes. ISH also revealed the presence of mRNA transcripts in the same type of testicular cells.

A comparative proteomics approach was used to scan for key genes pertaining to germ cell development and meiosis during juvenile and adult stages of testis to get a more detailed insight into factors involved during critical stages of gonadal development, and spermatogenesis in particular (Chocu et al., 2012). Tissue distribution analysis demonstrated expression of *sycp3* only in the testis, when compared to other somatic tissues such as brain, liver, heart, kidney and muscles. A similar pattern of expression was seen in female catfish, where *sycp3* expression was restricted to ovary.

The elevated levels of *sycp3* expression during preparatory phase as well as 50 dph onwards was also in correlation with the onset and

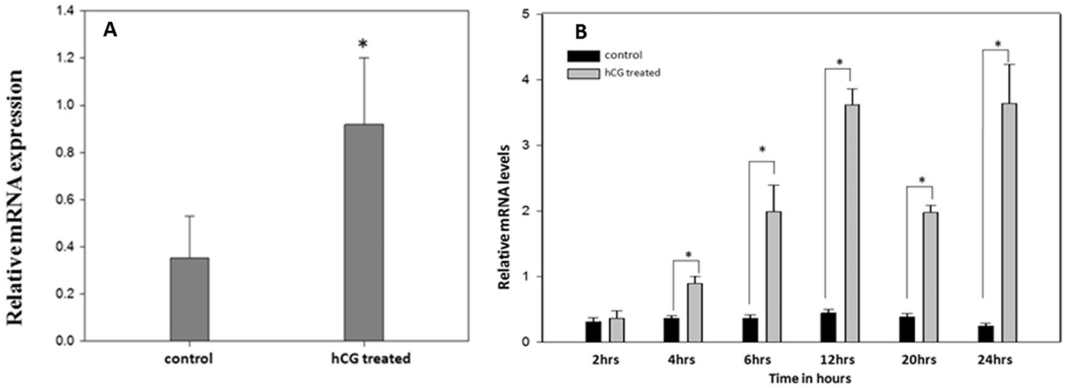

Fig. 10. qPCR analysis showing *sycp3* mRNA levels *in vivo* (A) and the testicular slices treatment with hCG *in vitro* (B) at different time intervals. *Sycp3* mRNA levels were significantly increased after hCG induction both *in vivo* and *in vitro* compared with the 0 h time point. The relative expression of *sycp3* was normalized with *18S rRNA* and the values were calculated using $2^{-\Delta ct}$ method. Data ($n = 5$) were expressed as mean ± SEM. (*$P < 0.05$; ANOVA followed by Student-Newman-Keuls' *post-hoc* test).

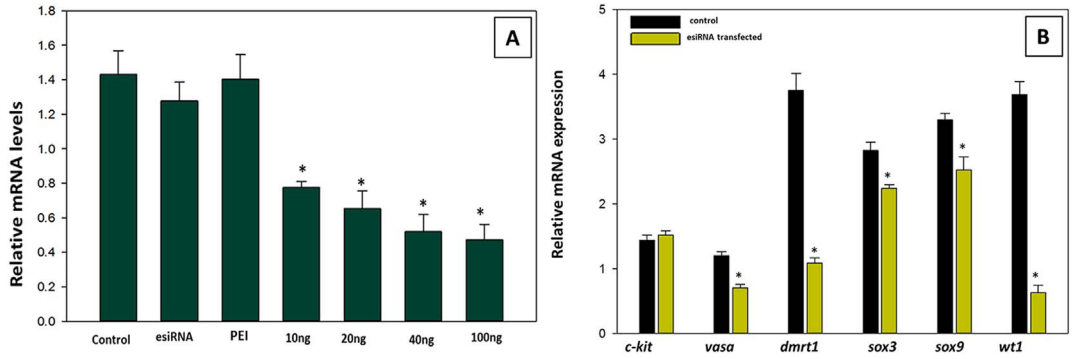

Fig. 11. *In vitro* transfection of PEI mediated *sycp3*-esiRNA in catfish testicular culture (mixed; $n = 5$). Relative expression of *sycp3* was analysed and was observed to be dose dependent after transfection with *sycp3*-esiRNA (A) and *sycp3* transcripts were quantified and compared between control siRNA, *sycp3*-esiRNA and PEI + esiRNA groups. Expression levels of certain testis related genes (B) were analysed between control and *sycp3*-esiRNA transfected cultures. (*$P < 0.05$; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

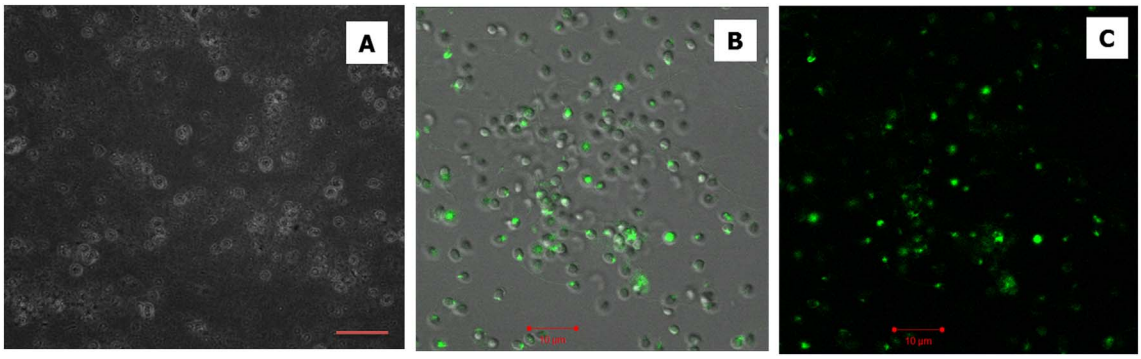

Fig. 12. Representative phase-contrast image of catfish primary testicular cells (A) and confocal microscope images (B and C) after PEI mediated transfection of control siRNA on catfish testicular culture (mixed) to check the efficiency.

progression spermatogenesis in catfish, which implies the involvement of this gene in germ cell development and gametogenesis during sexual development and recrudescence. There were minimally detectable levels of *sycp3* transcripts from 20 to 40 dph, and increased dramatically from 50 dph, which correspond to the stages of gonadal development and sex differentiation in catfish (Raghuveer et al., 2011) leading to an increase in the number of germ cells, that was also seen in the levels of germ cell marker *vasa* (Raghuveer and Senthilkumaran, 2010). In the teleost, *Oreochromis niloticus*, the entry of germ cells into meiosis *in vivo* was found to occur in females around 35 dph, while in male gonads, meiotic cells were found to be differentiated around 85 dph (Kobayashi, 2010). In the protandrous black porgy *Acanthopagrus schlegelii*, it was observed that during gonadal development, the germ cells located in the ovary entered meiosis earlier when compared to the testis, and treatment with estradiol-17β (E2) that induced *cyp26* expression was found to decrease *sycp3* mRNA in the gonads (Lau et al., 2013). Higher expression levels of *sycp3* in female catfish just after gonadal differentiation might indicate meiosis entry.

Sycp3 was localized through IHC and IF mainly in primary and secondary spermatocytes, but not in mature sperm/spermatids. The same pattern of localization was also evident for *sycp3* transcripts by ISH, with expression seen mostly in developing spermatocytes. This corroborates the results observed in zebrafish, where *sycp3* has been established as a molecular marker for spermatocytes in the testis, at

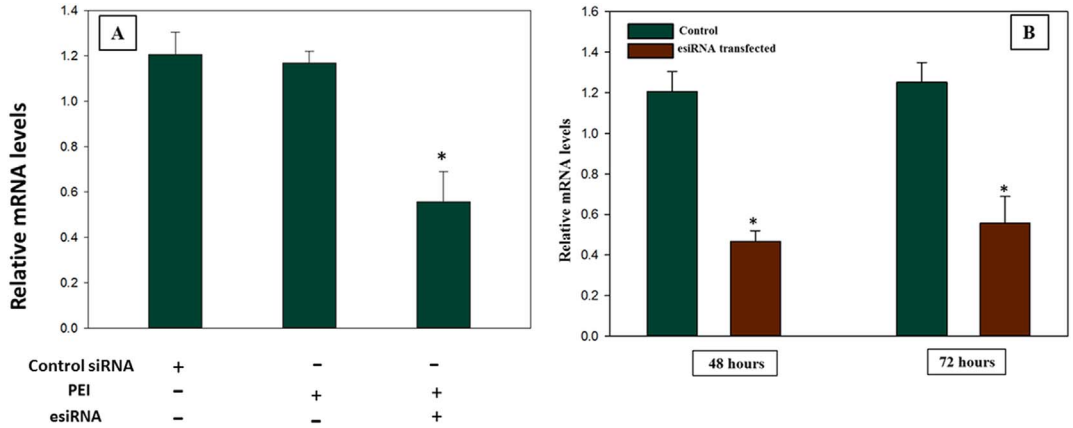

Fig. 13. qPCR analysis showing the *sycp3* levels after esiRNA transfection. (A) *In vivo* PEI mediated *sycp3*-esiRNA transfection in adult catfish ($n = 5$). qPCR analysis did not show any difference in the expression of *sycp3* in the PEI injected testis and control groups. However, a significant decrease in the expression was seen in the *sycp3*-esiRNA complexed with PEI.·(B) Comparison of *sycp3* gene expression levels between control and *sycp3*-esiRNA-PEI complex transfected samples at different time points, *i.e.*, 48 and 72 h post-transfection. (*$P < 0.05$; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

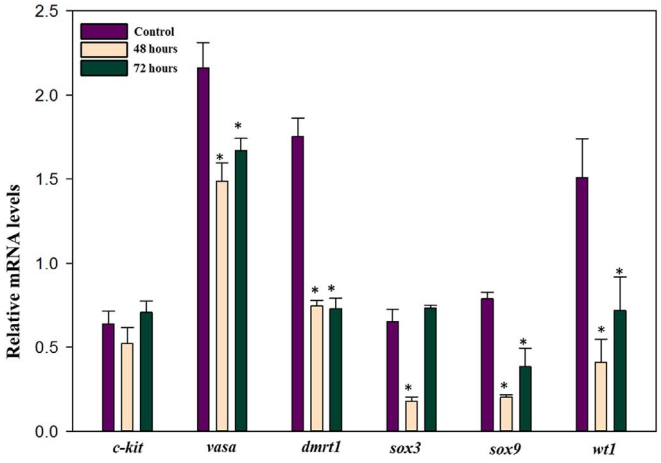

Fig. 14. Expression analysis of various genes which are crucial for germ cell development and spermatogenesis in the testis after *sycp3* esi-RNA silencing. Expression levels of each gene are compared to their respective control levels. (*$P < 0.05$; Mann–Whitney test one way ANOVA on ranks followed by SNK test).

different meiotic stages (Ozaki et al., 2011). Also in medaka, *sycp3* was expressed in primary spermatocytes but was not detected in secondary spermatocytes and spermatogenic cells, and signals were found to be diminished at the later stages of meiosis (Iwai et al., 2006) which shows a slight difference in the localization pattern observed in catfish. The expression patterns of *sycp3* have been examined in just a few fish species as most studies were done in mammals. It is essential to reveal the expression patterns of germ cell/meiotic regulators in the reproductive systems of lower vertebrates. Considering this, the present study is the first of its kind to perform a dual approach with proteomics and genomics to corroborate the role of *sycp3* in gonadal development and recrudescence spanning two years of reproductive cycle.

In order to observe the impact of gonadotropins on the expression of *sycp3*, hCG induction was done by both *in vivo* and *in vitro* methods. The fishes used for both induction experiments were in late preparatory/pre-spawning phase, and hCG was found to upregulate the transcript levels of *sycp3* even at those phases where the normal levels of expression of this gene was found to be low. This might serve as an indication to infer that gonadotropin stimulates germ cell proliferation during recrudescence in other fishes like *C. batrachus* which have a much shorter breeding season (Rajakumar and Senthilkumaran, 2014) and upregulation of *sycp3* expression indicate its correlative response. The influence of androgens on this gene is another exciting area of research. The expression level of the gene was found to be elevated after both types of induction. The processes of meiosis and the completion of various stages of spermatogenesis is dependent primarily on androgens, mainly 11-ketotestosterone in teleosts, which is produced by the Leydig cells under the influence of luteinizing hormone (LH), and secondarily dependent on the influence of follicle-stimulating hormone (FSH) which also facilitates pre-meiotic germ cell development (Schulz et al., 2001). However, the exact molecular mechanisms by which gonadotropins support the testicular production of steroids and gametes in lower vertebrates are less understood and the identification of the specific genes involved has proven difficult. Our study implies that *sycp3* may be directly or indirectly regulated by gonadotropins, although the exact mechanism is not yet elucidated. In addition, the expression of *sycp3* during the testicular reproductive cycle correlated well with the levels of gonadotropins observed in catfish (Joy et al., 2000; Kirubagaran et al., 2005).

Our analysis also demonstrated that transfection of catfish testis with PEI mediated esiRNA targeted to *sycp3* significantly downregulated its expression levels both *in vitro* and *in vivo*. Further, the transient knockdown of this gene also resulted in the alteration of expression levels of various genes critical for germ cell and gonadal development such as *vasa*, *dmrt1*, *sox3*, *sox9* and *wt1*. The germ cell marker gene, *vasa*, as well as transcription factors *dmrt1*, *sox9* and *wt1* showed a significant decrease in their expression levels after 48 and 72 h, while *sox3* was transiently downregulated at 48 h, but returned to normalcy at 72 h. However, *c-kit* did not show any change in expression. Most of the knockdown/mutation experiments towards investigating the impact of the absence of this gene during gametogenesis had been done in mammals, but very few attempts were made in lower vertebrates including teleosts. In one such study, the targeted deletion of this gene in mice resulted in perturbed processes such as chromosome synaptic failure at male meiotic prophase, extended meiotic chromosomes and male sterility (Kolas, 2004) and male mice with *Sycp3* null mutations have also resulted in complete sterility (Yuan et al., 2000). Consequently, the effect of the *in vivo* silencing on other testis related genes in the present study might highlight the importance of this role during fish spermatogenesis, and meiosis, as can be observed in the significant downregulation of *vasa* in particular, which is a germ cell universal marker, and disruptions in this gene expressions have been shown to result in impaired meiosis and germ line stem cell loss (Hartung et al., 2014).Studies in mice where a null mutation was generated on the *Sycp3* gene had been shown to cause massive apoptotic cell death resulting in sterility during meiotic prophase, failure of synapsis and formation of axial/lateral elements and SCs, and also affected expression of synaptonemal complex protein 1 (Yuan et al., 2000). Studies in human ovary and testis have also revealed the critical function of this gene during meiosis, and its relation to various reproductive problems (Yuan et al., 2002; Miyamoto et al., 2003; Wang and Höög, 2006). Taken together, the functional role of *sycp*3 during spermatogenesis and meiosis could be extrapolated to lower vertebrates such as teleosts where very little investigations have been done. The impaired expression levels of other testis-related transcription factors after *sycp3* esiRNA silencing might also imply a direct or indirect role of this gene in conjunction with other factors to impact germ cell development. These results also warrant direct or indirect interactions and regulatory mechanisms that occur between the testis-related genes analysed and *sycp3*.

In summary, present study aimed to classify factors involved in the crucial processes of germ cell development/meiosis through2D proteomic analysis which identified the Sycp3 protein due to significant expression during gonadal development stages of juvenile catfish. This may be one of the few identified markers of meiosis which have been reported in lower vertebrates such as teleosts, specifically in annual breeders. Full length cDNA of *sycp3* was then cloned using peptide sequence data and its expression profile was analysed during gonadal development, maturation as well as different stages of the reproductive cycle. The study presented the involvement of *sycp3* during the crucial stages of reproductive development *via* its expression pattern, as well as its specific localization only in the germ cells of catfish. In addition, the work also inferred the regulatory effects of gonadotropins on the process of meiosis perhaps by modulating *sycp3*. Transient silencing of *sycp3*, *in vivo* using esiRNA decreased the expression of various testis related genes which implied either a direct or indirect impact of this gene in testicular germ cell development and recrudescence.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.genrep.2017.10.009.

Conflict of interest

The authors listed in the manuscript have no affiliations with or involvement in any organization or entity with any financial interest.

Funding

A grant-in-aid (SR/SO/AS-06/2012) from the Department of Science and Technology, India to B. Senthilkumaran supported this work completely. BS received TATA innovation grant (BT/HRD/35/01/

02/2013) from Department of Biotechnology (DBT), India.

Acknowledgements

CL is thankful to the DBT, India for junior and senior research fellowships. BS is the recipient of TATA innovation fellowship from DBT, India.

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