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Development and characterization of a continuous cell line PSCF from *Puntius sophore*

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A continuous *Puntius sophore* caudal-fin (PSCF) cell line of the pool barb *Puntius sophore*, an important freshwater food and ornamental fish of Asia and South East Asia, was developed from the caudal fin for the first time. The cell line was optimally maintained at 28° C in Leibovitz-15 (L-15) medium supplemented with 10% foetal bovine serum (FBS). The cytogenetic analysis revealed a diploid count of 50 chromosomes at 25th and 50th passage and 52 chromosomes at passage 70, 85 and 100. The viability of the PSCF cell lines was 75% after 6 months of storage in liquid nitrogen (−196° C). The most striking feature of the PSCF cells was its high increased growth ratio as evident from the population doubling time of 25 h at passage 100. The origin of the cell lines was confirmed by the amplification of 581 and 655 bp fragments of 16 S rRNA and cytochrome oxidase subunit I (COI) of mitochondrial DNA (mtDNA) genes, respectively. The PSCF cells were successfully transfected with green fluorescent protein (GFP) reporter plasmids and the expression of GFP gene in the cells indicated the potential utility of the cells in gene expression studies.

Key words: Cell line; food and ornamental fish; mtDNA; *Puntius sophore*.

INTRODUCTION

The physiology and blood plasma constituents of teleosts are similar with those of terrestrial vertebrates; therefore, the methodology for culture of cells is also similar. Nevertheless, fish cell culture differs somewhat from mammalian cell culture in having a wider temperature range for incubation. Because of lower metabolic rates than eurythermic cells, fish cells can be maintained with little care for long periods of time. Thus, permanent fish cell lines, in contrast to the mammalian cells, are easier to maintain and manipulate, and unlike primary cultures, produce highly reproducible results (Wolf & Quimby, 1976). Cell lines from fishes are particularly useful in detecting viruses and studying the molecular and cellular basis of physiological processes and toxicological mechanisms (Fryer & Lannon, 1994; Bols *et al*., 2001). In recent years, cell lines from aquatic animals have attracted considerable attention as a means of expediting disease diagnosis. Teleost cell lines have been developed from a broad range of tissues such as ovary, fin, swimbladder,

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heart, spleen, liver, eye muscle, vertebrae, brain and skin. Embryonic and larval cells are the most easy to cultivate being mitotically activated. In recent years, a number of embryonic stem-like cell lines have been established by various workers from fish species (Sun *et al*., 1995; Hong *et al*., 1996, 2000; Chen *et al*., 2003; Parameswaran *et al*., 2006*a*, *b*; Lakra, 2010). Currently, *c*. 283 cell lines have been established from finfishes around the world (Lakra *et al*., 2010*a*). Most of the cell lines have been developed from temperate fishes in the past (Fryer & Lannon, 1994) except some recent reports on golden mahseer *Tor putitora* (Hamilton) (Lakra *et al*., 2006*a*), Barramundi *Lates calcarifer* (Bloch) (Lakra *et al*., 2006*b*; Parameswaran *et al*., 2006*a*), Orange-spotted grouper *Epinephelus coioides* (Hamilton) and milkfish *Chanos chanos* (Forsskål) (Parameswaran *et al*., 2007) and rohu labeo *Labeo rohita* (Hamilton) (Lakra *et al*., 2010*b*).

The pool barb *Puntius sophore* (Hamilton) is a tropical freshwater fish of high ornamental and food value belonging to the family Cyprinidae. The species is reported from Pakistan, India, Nepal, Bangladesh, Myanmar, Bhutan, Afghanistan and Yunnan of China (Talwar & Jhingran, 1991; Petr, 1999).

There have been several incidences of mass mortality of carps in culture systems suspected to be caused by microbial diseases, particularly of viral aetiology (Roberts *et al*., 1993; Mohan & Shankar, 1994). Hence, the development of cell line from tropical fish species for identifying pathogenesis of viral diseases and for vaccine production against viral and bacterial diseases is imperative. The development of cell lines from *P. sophore* would facilitate pathological studies related to the epizootic ulcerative syndrome (EUS) and other fish diseases of carps. This paper reports a continuous and characterized cell line for the first time from the caudal fin of *P. sophore*.

MATERIALS AND METHODS

PRIMARY CELL CULTURE

Healthy juveniles of *P. sophore* (15–20 g in mass and 5–10 cm in total length) were obtained from the farm maintained by the National Bureau of Fish Genetic Resources, Lucknow, India. Prior to sacrifice, the specimens were starved for a day and allowed to swim in well-aerated sterile water. The specimens were euthanized by keeping them on ice for 5–10 min and surface sterilized by dipping in an iodophore (0·5% w/v iodine, Betadine; Pharmabutors Pvt Ltd, Rajasthan, India) for 5 min. Primary cell cultures were initiated by aseptically collecting caudal fin tissues from the fish. The tissues were transferred to phosphate-buffered saline (PBS) (Invitrogen; www.invitrogen.com), containing antibiotic and antimycotic solution (1000 U penicillin, 1000 µg streptomycin and 25 µg amphotericin B ml^{-1}) (Invitrogen). The tissue samples were then minced with sterile dissecting blades and scissors at room temperature and washed four times with PBS containing antibiotic and antimycotic solution. Approximately 25 tissue fragments (1–2 mm^3) were individually explanted into 25 cm^2 tissue culture flasks (Nunc; www.nuncbrand.com) in 50 µl of foetal bovine serum (FBS) (Invitrogen). After allowing the tissue to attach for 8 h at room temperature, 5 ml of Leibovitz-15 (L-15) medium containing 20% FBS was added to each flask.

MORPHOLOGICAL OBSERVATIONS

The flasks were observed daily for attachment of explants, spreading and proliferation of cells, morphological details using an inverted microscope (Olympus Optical Co., Ltd; www.olympus.co.jp).

MORPHOLOGICAL CONFIRMATION BY CELL-SPECIFIC MARKER

Confirmation of the fibroblast morphology of the *Puntius sophore* caudal-fin (PSCF) cells was done by immunocytochemical staining with monoclonal antibodies directed against vimentin and cytokeratin (C-18) at the 100th passage. In brief, cells were grown to confluency in Lab-Tek chamber slides (Nunc). Cells were washed with PBS and were fixed in methanol at 4° C for 20 min. After fixation, the cells were washed in PBS and were blocked with 3% BSA in PBS for 15 min at 37° C. Block was removed and 100 µl of either a 1:40 dilution antivimentin clone V9 (Sigma-Aldrich; www.sigmaaldrich.com) or a 1:400 dilution of antipan cytokeratin clone-11 (Sigma-Aldrich) was added. Slides were incubated for 1 h at 37° C. Cells were washed with PBS and were incubated for 1 h with 100 µl of a 1:300 dilution of fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG. Cells were washed in PBS, were covered with 50% glycerol in PBS under a coverslip and were observed under a fluorescence microscope. Appropriate controls for autofluorescence and secondary antibodies were included.

SUBCULTURE

Upon reaching 95% confluency, the cells were trypsinized using trypsin, versene, glucose (TPVG) solution [0·1% trypsin, 0·2% ethylenediaminetetra-acetic acid (EDTA) and 2% glucose in 1× PBS]. The subcultured cells were grown in fresh L-15 with 15% FBS. In the initial 10 subcultures, 50% of the culture medium was replaced with the fresh medium. The concentration of FBS in the L-15 medium was reduced to 10% for further subculturing.

GROWTH STUDIES

Growth characteristics of the cell lines in L-15 media were assessed at five different incubation temperatures (16, 20, 24, 28 and 32° C) for 7 days. A seeding concentration of 1×10^5 cells ml^{-1} at passage 15 and subsequent passages was used in 25 cm^2 tissue culture flasks. On alternate days, three flasks from different temperatures at which they were incubated were withdrawn, trypsinized and cell counting performed (four counts per flask) using a haemocytometer. Analogous procedures were performed for the effects of various concentrations of FBS (5, 10, 15 and 20%) on cell growth at 28° C for 7 days.

MEASUREMENT OF CELL DOUBLING TIME

Each dish (60 mm diameter; Terumo; www.terumo.co.jp) was seeded with $5{\cdot}0 \times 10^6$ PSCF cells in 5 ml of the medium. The medium was renewed every third day. At the time of the first change of medium, a half of the volume was renewed. The number of the cells per dish was determined at various times. Cell counts were made on triplicate plates with a haemocytometer. Cell doubling time was confirmed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazdium bromide (MTT) assay (Hansen *et al*., 1989).

CELL-PLATING EFFICIENCY

Cell lines at the 30th passage were used to determine the plating efficiencies. Plating efficiency for the cell line was determined at seeding concentrations of 200, 500 and 1000 cells per flask (25 cm^2 tissue culture flask) in duplicate. The cells were incubated at 28° C in L-15 medium with 10% FBS. After 12 days, the medium was discarded and the cells were fixed with 5 ml of crystal violet (1%)–formalin (25%) stain fixative for 15 min, rinsed with tap water and air-dried. The colonies were then counted (x) under the microscope, and plating efficiency (y) was calculated using the formula: $y = 100x\ z^{-1}$, where z = number of cells seeded (Freshney, 1994).

CRYOPRESERVATION

The ability of cells to survive in liquid nitrogen (LN_2) and their stability were assessed in three replicates in freezing medium at 20, 30, 50 and 70 passages using previously described methods (Freshney, 1994). Cells growing logarithmically for PSCF were harvested and resuspended at densities of 3×10^6 to 4×10^6 cells ml^{-1}. The cell suspensions were carefully mixed with an equal volume of recovery medium (Invitrogen), as per the manufacturer's instruction. Aliquots (1·0 ml) were dispensed into 1·5 ml sterile cryovials (Nunc) held at 4° C for 2 h, −20° C for 1 h, at −70° C overnight and then transferred into LN_2 (−196° C). The frozen cells were recovered after 6 months of post-storage by thawing at 37° C in a water bath. Following removal of the freezing medium by centrifugation, cells were suspended in L-15 with 10% FBS. The viability of cells was measured by trypan blue staining and the number of cells was counted using a haemocytometer. The viable cells were seeded into 25 cm^2 tissue culture flasks for further subculturing.

CHROMOSOME ANALYSIS

Chromosomal counts were established at passage 25, 50, 70, 85 and 100. Cells were seeded in duplicate 75 cm^2 tissue culture flasks in L-15 medium with 10% FBS. After 24 h incubation, spent medium was replaced with 10 ml of fresh medium containing 0·1 ml colcemid solution (1 μg ml^{-1}), (Sigma-Aldrich) into the 1 day-old cell culture for 2 h at 28° C. After harvesting by centrifugation (70 *g*, 5 min), the cells were suspended in a hypotonic solution consisting of 0·5% KCl for 10 min and fixed in methanol:acetic acid (3:1). Slides were prepared following the conventional drop-splash technique (Freshney, 1994). The chromosomes were counted under a microscope (Leica; www.leica-microsystems.com), after staining with 5% Giemsa for 10 min.

MOLECULAR CHARACTERIZATION (16S rRNA AND COI mtDNA GENES)

DNA extraction from PSCF cells at passage 50 was accomplished following Ruzzante *et al*. (1996) with minor modifications. Briefly, samples were homogenized separately in incubation buffer (10 mM Tris–HCl and 10 mM EDTA, pH 8·0), centrifuged at 9168 *g* at 4° C after which the supernatants were digested with lysis buffer (10 mM Tris–HCl, 10 mM EDTA, pH 8·0, 0·5% SDS and 50 μg ml^{-1} proteinase K). After incubation at 37° C overnight, the digests were deproteinized by successive phenol–chloroform and iso-amyl alcohol extraction and DNA was recovered by ethanol precipitation, drying and resuspension in TE buffer. The concentration of isolated DNA was estimated at wavelength of 260 nm using a UV spectrophotometer. The DNA was diluted to get a final concentration of 100 ng μl^{-1}.

The 581 bp fragment of mitochondrial 16S rRNA gene was amplified in a 50 μl reaction volume with 5μl of 10× Taq polymerase buffer, 0·2 mM of each dNTP, 0·4 μM of each primer, 2·5 U of Taq polymerase and 5 μl genomic DNA using the thermal cycler PTC 200 (MJ Research; www.gmi-inc.com). The primers used for the amplification of the partial 16S rRNA gene were 16SAR (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SBR (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi *et al*., 1991). The thermal profile used was 36 repetitions of a three-step cycle consisting of denaturation at 94° C for 1 min, annealing at 55° C for 1 min and extension at 72° C for 1·5 min including 4 min for initial denaturation at 94° C and 7 min for the final extension at 72° C.

The 655 bp fragments of cytochrome oxidase subunit I (COI) was also amplified in a final concentration of 50 μl volume with a final concentration of 5 μl of 10× Taq polymerase buffer, 2 μl of $MgCl_2$ (50 mM), 0·25 μl of each dNTP (0·05 mM), 0·5 μl of each primer (0·01 mM), 0·6 U of Taq polymerase and 5 μl of genomic DNA. The primers used for the amplification of the COI gene were FISHF1-5′TCAACCAACCACAAAGACATTGGCAC3′ and FISH1-5′TAGACTTCTGGGTGGCCAAAGAATCA3′ (Ward *et al*., 2005). The thermal regime consisted of an initial step of 2 min at 95° C followed by 35 cycles of 40 s at 94° C, 40 s at 54° C and 1 min 10 s at 72° C followed by final extension of 10 min at 72° C.

The PCR products were visualized on 1·2% agarose gels and the most intense products were selected for sequencing. Products were labelled using the BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc.; www.appliedbiosystem.com) and sequenced bidirectionally using an ABI 3730 capillary sequencer following manufacturer's instructions. The obtained sequences of PCR fragments were compared to the known sequences of the species.

MICROSATELLITE ANALYSIS

A set of nine polymorphic microsatellite loci (*PS113*, *PS116*, *PS65*, *PS85*, *PS93*, *PS145*, *PS131*, *PS122* and *PS127*) developed using cross-species amplified primers at the institute were used for unequivocal identification of the PSCF cell line at individual level. The matching probability (P_M) was calculated in 16 randomly selected samples.

TRANSFECTION WITH GFP REPORTER GENE

Subconfluent monolayers (70–80% confluency) of PSCF cells at the 50th passage were transfected with pEGFP-C1 plasmid using lipofectamine LTX and Plus Reagents (Invitrogen). In brief, the cells of PSCF were seeded at a density of 1×10^5 in a 24 well plate individually and incubated for 18 h at 28° C in normal atmospheric incubator. Before transfection, cells were rinsed and supplemented with 500 µl of fresh L-15 medium devoid of serum and antibiotics at pH 7·4. The plasmid (200 ng of pEGFP-C1) was dissolved in 100 µl of Optimum and then 0·5 µl of Plus Reagent was added. The mixture was incubated for 5 min at 30° C and then 2 µl of LTX was added and incubated for another 30 min. Then, the entire mixture was added dropwise on 70–80% confluent PSCF cells in the 24 well plate. The medium was changed after 6 h and fresh medium added. The green fluorescence signals were observed after 16 h under a fluorescence microscope.

DNA CONTENT ANALYSIS

Fluorescence-activated cell sorter (FACS) analysis was carried out for PSCF cells in 24 and 48 h with a plating cell count of 2×10^5 in each flask. Cells with 80% confluency were dislodged with TPVG solution to obtain a single cell suspension and washed twice with PBS. The cells were then fixed in 80% methanol and kept overnight at 4° C. The suspension was centrifuged at 100 *g* for 5 min and the pellet was gently washed with PBS twice. To the final pellet, 4 µl of propidium iodide (500 µg ml^{-1}) (Sigma-Aldrich) were added and each sample was kept at 4° C overnight. The samples were then assessed on a Becton Dickinson FACS Vantage system (www.bd.com). They were excited with an argon laser and the emission (e_{may}) was collected at 615 nm. Each of the samples for 24 and 48 h was repeated four times with an average of 6000 cells counted in each assessment and one representative result for each day was chosen for further analysis. The data were analysed by ModFitLTv100 (MAC) software (www.vsh.com).

Statistical Analysis

Data were expressed as mean ± S.E. The number of cells was analysed with independent samples test and statistical analysis was carried out using SPSS software (www.spss.com). A value of $P < 0{\cdot}05$ was considered as significant.

RESULTS

MORPHOLOGY OF PSCF CELLS

The initial subcultures of PSCF cell line consisted of both epithelial and fibroblast-like cells. Morphologically, PSCF comprises fibroblast-like cells (Fig. 1). Radiation

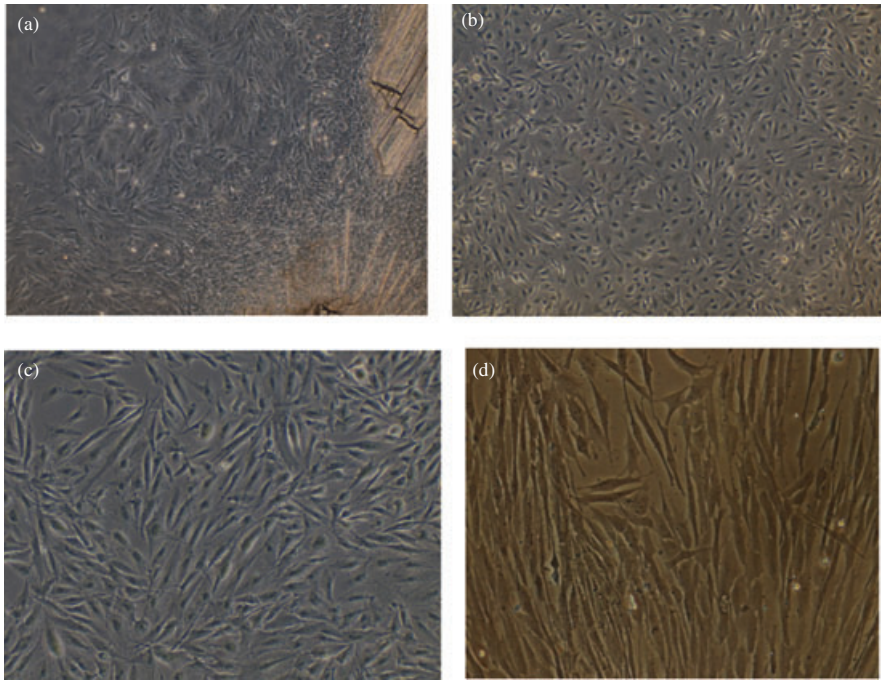

FIG. 1. Phase-contrast photomicrographs of PSCF cells derived from the caudal fin of *Puntius sophore*. (a) Primary culture on day 5 following seeding tissue explant (×100), (b) subcultured cells at passage 30 (×100), (c) subcultured cells at passage 70 (×100) and (d) subcultured cells at passage 100 (×200).

of cells from the explants started after 2–3 days in 25 cm^2 tissue culture flasks for PSCF, where a confluent monolayer reached in 5–7 days. The confluent monolayer was achieved at a split ratio of 1:2. The cells adhered well to the substratum and achieved confluency in 5–7 days at 28° C in L-15 medium containing 15% FBS during subsequent subcultures. The PSCF cell line was subcultured 104 times. During the initial 10 subcultures at 5–7 day intervals, a combination of 50% each of new and old medium was used. In subsequent subcultures, cells were subcultured in L-15 with 10% FBS in a ratio of 1:3 at 2–3 days of interval. After the 70th passage, cells are subcultured at 1–2 days of interval as PSCF cells were growing very fast. During the initial subculture, the cells were mostly fibroblastic with few epithelial cells [Fig 1(b)]. The later cultures were dominated by fibroblast cells only [Fig.1(c), (d)].

MORPHOLOGICAL CONFIRMATION BY CELL-SPECIFIC MARKER

The PSCF cells showed positive reaction for vimentin and negative reaction for cytokeratin (Fig. 2).

GROWTH STUDIES

The growth rate of the cells in medium containing 20% FBS was higher than that of cells in medium containing 5–15% FBS [Fig.3(a)]. The cells were able to grow

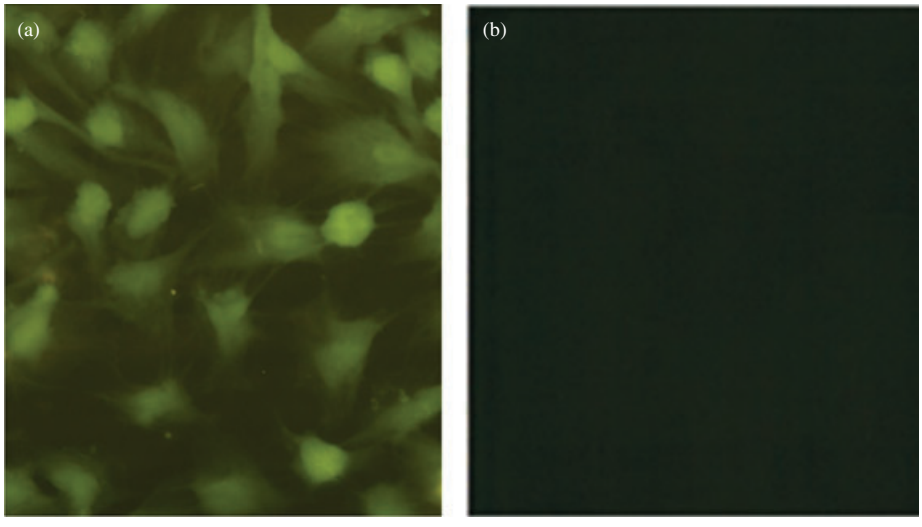

FIG. 2. Characterization of PSCF fibroblast cells of *Puntius sophore* by immunocytochemistry, (a) vimentin-FITC and (b) cytokeratin-FITC labelled fibroblast cells.

at temperatures between 24 and 32° C. Maximum growth, however, was obtained at 28° C for the PSCF cell line [Fig.3(b)]. No significant growth was observed at 16 and 20° C in the cell lines.

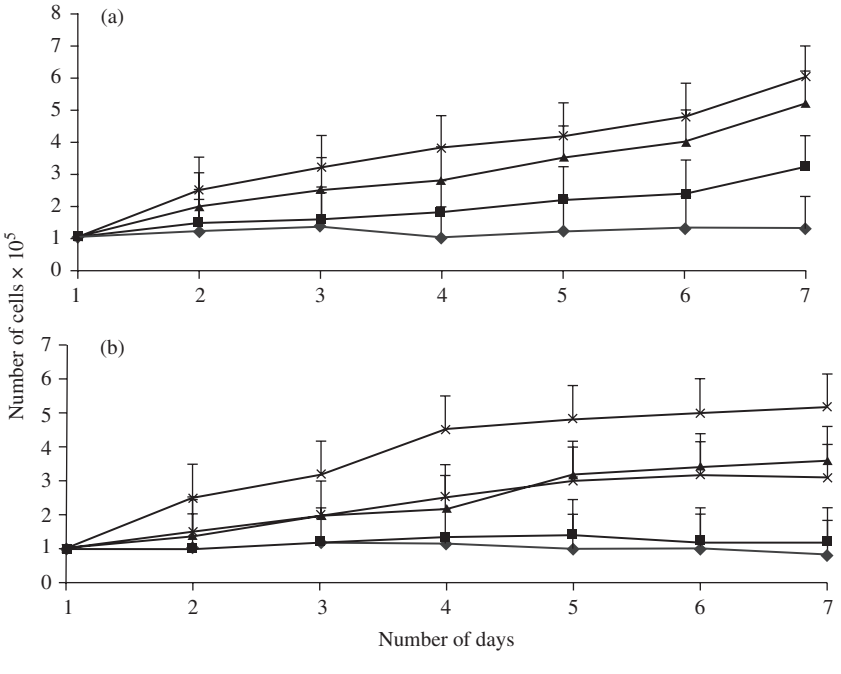

FIG. 3. *In vitro* growth curves of PSCF cells of *Puntius sophore* at (a) selected concentration of foetal bovine serum (FBS) [5 (◆), 10 (■), 15 (▲) and 20 (×) %] and (b) temperatures [16 (◆), 20 (■), 24 (▲), 28 (×) and 32 (×) ° C]. Values were significantly different ($P < 0{\cdot}05$). Values are means ± S.E. ($n = 3$).

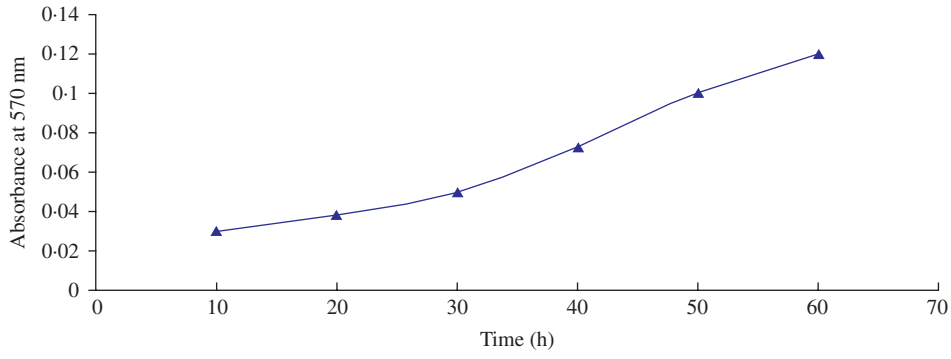

FIG. 4. Graphical representation of PSCF cells of *Puntius sophore* at passage 100 with cell doubling time.

CELL DOUBLING TIME

Cell doubling time of PSCF cells was estimated at 25 and 100 passages. The cell doubling time estimated at the 25th passage was 48 h. The growth potential of the PSCF cells estimated by MTT assay revealed that the cells were growing very fast with a cell doubling time of 25 h at the 100th passage (Fig. 4).

PLATING EFFICIENCY

Plating efficiency for PSCF cell line was determined at seeding concentrations of 200, 500 and 1000 cells. PSCF cells showed high plating efficiency, *i.e.* 64·20% with no significant differences between replicates.

CRYOPRESERVATION AND REVIVAL

Evaluation of the viability of PSCF cells stored in liquid nitrogen (-196° C) established the capability of cells to survive following 6 months of storage. Seventy-five per cent of the PSCF cells from each vial remained viable after the storage period and retained the ability to attach and grow at 28° C. Following storage, no obvious alterations in morphology or growth pattern were observed in the cells.

CHROMOSOME ANALYSIS

The results of chromosome counts of 112 metaphase plates at passage 25 and 50 revealed that the number of diploid chromosomes in PSCF cells ranged from 34 to 60 and 35 to 62, respectively, with a modal peak at 50 chromosomes [Fig. 5(a)]. The chromosome number distribution based on 124 metaphase plates displayed a 2n value ranging from 33 to 64 (70th passage), 41 to 72 (85th passage) and 43 to 74 (100th passage) with the modal value at 52 chromosomes [Fig. 5(b)]. A standard chromosome spread of *P. sophore* at passage 50 is shown in Fig. 5(c).

MITOCHONDRIAL GENE ANALYSIS

Analysis of mitochondrial 16S rRNA and COI was performed to verify the origin of the cell line. Amplification from the 16S rRNA and COI genes for PSCF cell

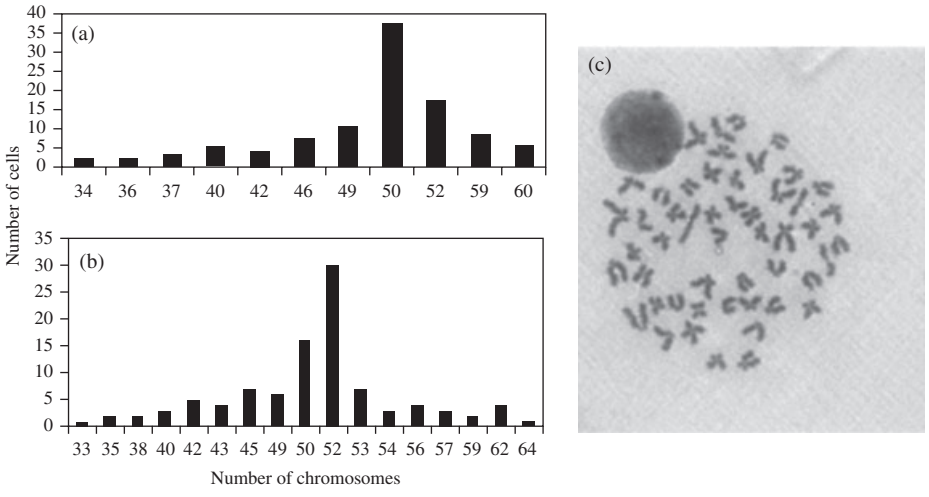

FIG. 5. Chromosome number distribution of PSCF cell lines at (a) passage 25 and (b) passage 70. (c) A standard chromosome spread of *Puntius sophore* at passage 50.

line revealed the expected PCR products of 581 and 655 bp, respectively (Fig. 6). Subsequent comparative analysis of the sequences of 16S rRNA and COI derived from PSCF cells demonstrated a 99 to 100% match with the known mitochondrial DNA sequences from the *P. sophore* voucher specimens. GenBank accession number for 16S rRNA and COI of PSCF cell line were HM057187 and HM057188, respectively.

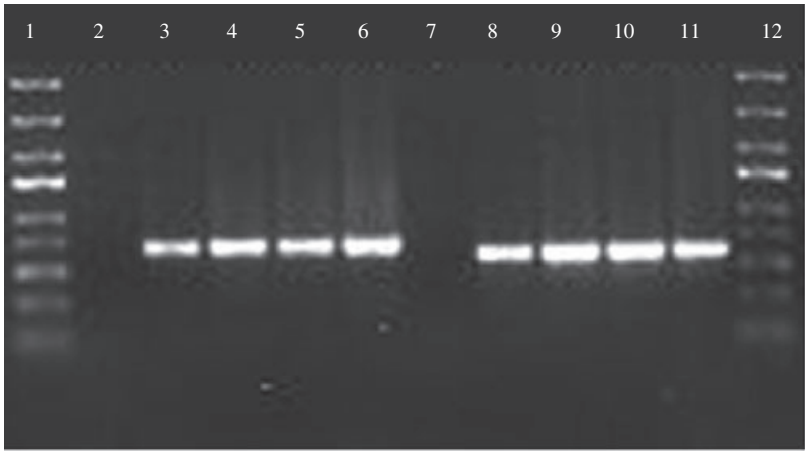

FIG. 6. PCR amplification of 655 and 581 bp fragment of the *Puntius sophore* genome using oligonucleotide primers from the conserved portions of COI and 16S rRNA region. Lane 1, generuler express DNA ladder (Fermentas; www.fermentas.com); lane 2, COI negative control; lane 3, positive control COI; lane 4, CCH COI; lane 5, CCF COI; lane 6, 16S rRNA negative control; lane 7, 16S rRNA positive control; lane 8, CCH 16S rRNA; lane 9, CCF 16S rRNA; lane 10, generuler express DNA ladder (Fermentas) (negative control: without template; positive control: muscle tissue of *P. sophore*).

TABLE I. Microsatellite analysis of the PSCF cell line from *Puntius sophore* with matching probability value

Locus	Genotype (correspond to allele sizes of PSCF cells)	Matching probability
PS113	141/143	0·000214
PS116	131/139	0·000751
PS65	182/212	0·002347
PS85	135/136	0·001341
PS93	182/184	0·000168
PS145	134/136	0·001654
PS131	175/178	0·001476
PS122	114/134	0·000346
PS127	118/132	0·001843

MICROSATELLITE ANALYSIS

The microsatellite genotyping of PSCF cell line is given in Table I. All alleles of PSCF cell line were widely represented in the individuals of the donor species *P. sophore* from which PSCF cell line was developed. The power of discrimination of the set of loci was extremely high ($P_M = 1{\cdot}06026 \times 10^{-21}$).

TRANSFECTION EFFICIENCY

The PSCF cell lines were successfully transfected with pEGFP-C1 plasmid using lipofectamine LTX and Plus Reagents (Invitrogen). The expression of EGFP in the PSCF cell line could be detected as early as 16 h after transfection (Fig. 7). The estimated transfection efficiency was 10%. This indicates the ability of the PSCF cells for transfection and cytomegalovirus (CMV) promoter can drive the expression of EGFP gene in PSCF cells.

DNA CONTENT ANALYSIS

The DNA content of PSCF cells was determined in 24 and 48 h. The histogram obtained from flow cytometry of PSCF cells is shown in Fig. 8. The histograms of

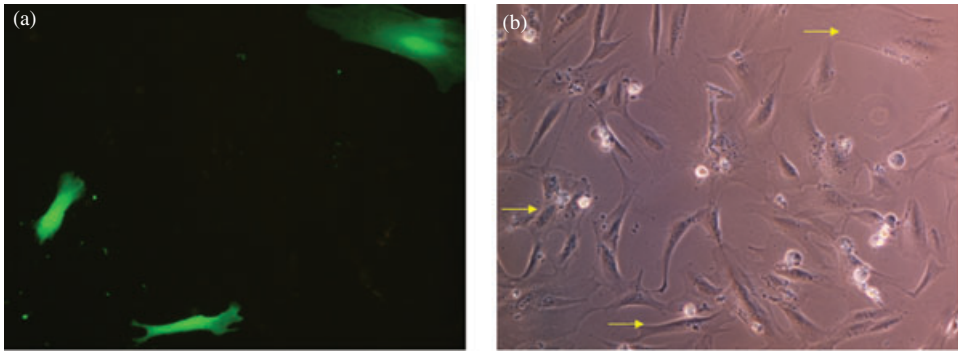

FIG. 7. (a) Green fluorescent protein expression in transected PSCF cells of *Puntius sophore* at 50 passage and (b) non-fluorescent control.

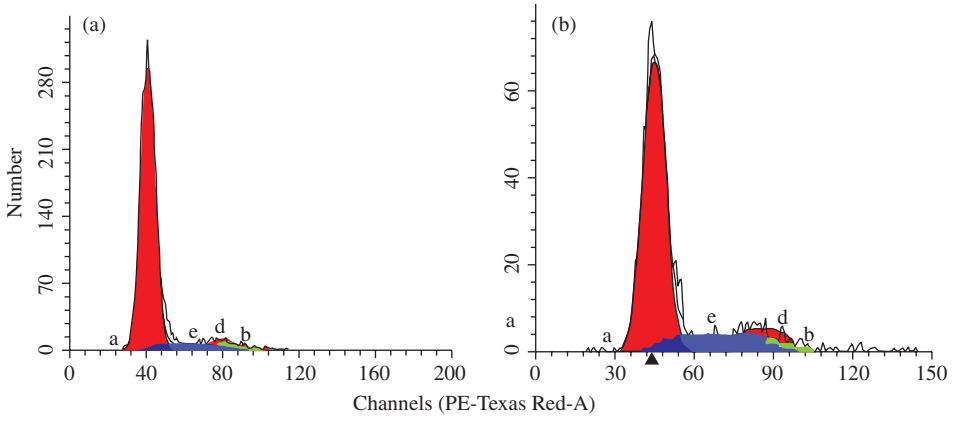

FIG. 8. DNA content analysis of PSCF cells of *Puntius sophore* at different time intervals: (a) 24 and (b) 48 h with the peak marked on the *x*-axis [a (▭) debris; b (▭) aggregates; c (▭) Dip G1; d (▭) Dip G2; e (▭) Dip S]. The large peak represents G0–G1 and the small peak to G2-M population in each case, M3 represents the S-phase.

the PSCF cells revealed one main distinct peak corresponding to the G0–G1 fraction and a small peak corresponding to the G2-M fractions of the cells. The second lower peak corresponding to the G2-M fraction of the histogram in 48 h was higher than that of day 2. The percentage of the mitotic cells was higher in the 48 h cultured cells (15·25%) with maximum cells (57·24%) in the G0–G1 phase and minimum cells (27·51%) in the S-phase (Table II).

TABLE II. Frequency distribution of the phases of cell cycles of the PSCF cell line from *Puntius sophore* estimated by the profile of fluorescence-activated cell sorter in 24 and 48 h

Time (h)	Dip G0–G1 (%)	Dip G2-M (%)	Dip % C.V.	Total S-phase (%)
24	80·19	0·55	11·40	19·26
48	57·24	15·25	6·80	27·51

C.V., coefficient of variation; Dip, diploid; G0–G1, gap 0 to gap 1 phase; G2-M, gap 2 to mitosis phase; S-phase, syntheses phase.

DISCUSSION

In the present study, an *in vitro* cell culture system from the caudal fin of *P. sophore*, namely the PSCF cell line, was established by means of the explant technique. The explant technique has many advantages over the trypsinization method in terms of speed, ease and maintenance of cell interactions and the avoidance of enzymatic digestion which can damage the cell surface (Avella *et al*., 1994). The PSCF cell line was grown in L-15 medium supplemented with 10% FBS. The PSCF cell line was subcultured 104 times, respectively, over a period of 1·5 years. Initially, fibroblastic and epithelial cells were found in PSCF cell, but after several subcultures, fibroblast cells predominated in the PSCF cell line. The fibroblast morphology of the PSCF cells was supported by cell-specific staining. Usually, a predomination of

fibroblastic cells over epithelioid cells in cell cultures from fishes has been reported (Bejar *et al*., 1997; Lai *et al*., 2003; Lakra *et al*., 2006*a*). Ye *et al*. (2006) developed a fibroblast-like cell line (LJH-2) from sea perch *Lateolabrax japonicus* (Cuvier). In contrast, SPH (*L. japonicus* heart) cells migrating from heart tissue have been reported to be epithelioid in morphology with no change during successive propagation (Tong *et al*., 1998).

The temperature ranged from 24 to 32° C for the growth of PSCF cells with an optimum growth at 28° C, which conforms with other fish cell lines reported earlier (Tong *et al*., 1997; Lakra *et al*., 2006*a*). One of the advantages of cell lines that grow over a wide temperature range is their potential suitability for isolating both warm-water and cold-water fish viruses (Nicholson *et al*., 1987). The growth rate of the present cells increased as the FBS concentration increased from 5 to 20%. A 10–15% concentration of FBS, however, also provided relatively good growth, and this is an advantage to maintain the cell line using 10% FBS at low cost. Relatively good growth of the fish were observed at 10% FBS, but maximum growth was observed with the concentrations of 15 and 20% (Lakra *et al*., 2006*b*; Hameed *et al*., 2006; Ye *et al*., 2006).

One of the important features of the continuous cell line is the increased growth ratio. The growth potential of PSCF cell line was assessed by MTT assay which revealed fast growth potential of PSCF cells with a cell doubling time of 25 h at 100 passages. The result showed that cell doubling time of PSCF cells was reduced considerably to 25 h at the 100th passage from 48 h at the 25th passage. This reflects that the PSCF cells showed high potential to grow very fast with a high increased growth ratio. The cell doubling time reported in the continuous cell line SAF-1 from gilthead sea bream *Sparus aurata* L., RTG-2 cell line, PSM-1 cell line from a hybrid between southern platyfish *Xiphophorus maculatus* (Günther) and *Xiphophorus hellerii* Heckel was 48 h (Plumb & Wolf, 1971; Wakamatsu, 1981; Bejar *et al*., 1997). The TP-1 cell line developed from *T. putitora* showed population doubling time as 28 h (Lakra *et al*., 2006*a*). Hameed *et al*. 2006 reported population doubling time of 36 h in a continuous cell line (SISK) from *L. calcarifer*.

The feasibility of cryopreservation of the present cell lines was demonstrated where the recovery rate of the cells after 6 month of cryopreservation was over 75%. It was 50% for SAF-1 (*S. aurata*) (Bejar *et al*., 1997), 73% for GF-1 (*E. coioides*) (Chi *et al*., 1999) and 80–85% for SF (*L. calcarifer*) (Chang *et al*., 2001).

The PSCF cell line showed high plating efficiency, *i.e.* 64% in L-15 medium with 10% FBS. Bejar *et al*. (1997) recorded 65% for the continuous cell line SAF-1 at 10% FBS. Chi *et al*. (1999) recorded the plating efficiency of 21% of the GF-1 cells seeded at a density of 100 cells per flask at subculture 50 and this increased to 80% at subculture 80. The modal chromosome number (2n) in *P. sophore* was found to be 50 at passage 25 and 50, which is identical to the modal chromosome number, reported earlier (Nayyar, 1964; Klinkhardt *et al*., 1995; NBFGR, 1998). In the present study, PSCF cells at passages 70, 85 and 100 showed a distribution of diploid chromosomes with a modal peak at 52 chromosomes. Hameed *et al*. (2006) reported that the continuous cell line (SISK) developed from *L. calcarifer* showed a modal value at diploid chromosome number 48 and 46 at passage 37 and 61, respectively.

The capacity to authenticate a cell line is very useful to check the originality and cross-contamination with other cell lines. Species identification of cell lines is

crucial for scientific research accuracy and reproducibility. To confirm that the cell line originated from *P. sophore*, an amplification of 581 and 655 bp fragments of 16S rRNA and COI for all cell lines were performed. The sequence analysis of both 16S rRNA and COI fragments showed 99 to 100% similarity with respective gene fragment of *P. sophore* voucher specimens. The results indicated that the PSCF cell line was of *P. sophore*. Hebert *et al*. (2003) have demonstrated the utility of COI gene as a universal barcode, referred as DNA barcoding for the genetic identification of animal life. Recently, Cooper *et al*. (2007) used COI region for identification of 67 cell lines used for barcode analysis. The present analysis also proves the utility of COI gene in the identification of the cell line from *P. sophore*. The microsatellite analysis also revealed unequivocal identification of the PSCF cell line at individual level as the power of discrimination of the set of loci was extremely high ($P_M = 1{\cdot}06026 \times 10^{-21}$).

It is essential to estimate the transfection efficiency and gene expression capability of newly developed cell lines for exogenous gene manipulation. The present study clearly demonstrated that the PSCF cell line could be transfected with p-EGFP plasmids. This implies that PSCF cell lines could serve as an *in vitro* system for exogenous gene manipulation in fish. The estimated transfection efficiency (10%) of PSCF cell line was identical to other fish cell lines (Sha *et al*., 2010; Wang *et al*., 2010). Zhou *et al*. (2008) reported 2% transfection efficiency in a CSTF cell line developed from Chinese sturgeon *Acipenser sinensis* Gray.

The FACS analysis revealed that the percentage of DNA content was more in the S-phase in 48 h of culture. The result showed conformity with a continuous cell line SAF-1 developed from *S. aurata* by Bejar *et al*. (1997).

The lack of established cell culture systems from tropical fish species is a major constraint in virus isolation and identification. The success in establishing new cell lines from *Puntius* sp. in India would facilitate *in vitro* research on virology, genetics and genomics ultimately leading to improved health management and conservation of aquaculture species.

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