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Short Communication

Development and characterization of cell culture systems from *Puntius (Tor) chelynoides* (McClelland)

M. Goswami [a,*], B.S. Sharma [a], A.K. Tripathi [c], Kamalendra Yadav [a], S.N. Bahuguna [c], N.S. Nagpure [a], W.S. Lakra [b], J.K. Jena [a]

[a] National Bureau of Fish Genetic Resources, Canal Ring Road, PO Dilkusha, Lucknow- 226002, India
[b] Central Institute of Fisheries Education (CIFE), Versova, Andheri (W), Mumbai- 400061, India
[c] HNB Garhwal University, PO Box 70, Srinagar/Garhwal, Uttarakhand, India

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ABSTRACT

Puntius (Tor) chelynoides, commonly known as dark mahseer, is a commercially important coldwater fish species which inhabits fast-flowing hill-streams of India and Nepal. Cell culture systems were developed from eye, fin, heart and swim bladder tissues of *P. chelynoides* using explant method. The cell culture system developed from eye has been maintained towards a continuous cell line designated as PCE. The cells were grown in 25 cm^2 tissue culture flasks with Leibovitz' L-15 media supplemented with 20 % fetal bovine serum (FBS) at 24 °C. The PCE cell line consists of predominantly fibroblast-like cells and showed high plating efficiency. The monolayer formed from the fin and heart explants were comprised of epithelial as well as fibroblast-like cells, a prominent and rhythmic heartbeat was also observed in heart explants. Monolayer formed from swim bladder explants showed the morphology of fibroblast-like cells. All the cells from different tissues are able to grow at an optimum temperature of 24 °C and growth rate increased as the FBS concentration increased. The PCE cell line was characterized using amplification of mitochondrial cytochrome oxidase subunit I (COI) & 16S rRNA genes which confirmed that the cell line originated from *P. chelynoides*. Cytogenetic analysis of PCE cell line and cells from fin revealed a diploid count of 100 chromosomes. Upon transfection with pEGFP-C1 plasmid, bright fluorescent signals were observed, suggesting that this cell line can be used for transgenic and genetic manipulation studies. Further, genotoxicity assessment of PCE cells illustrated the utility of this cell line as an *in vitro* model for aquatic toxicological studies. The PCE cell line was successfully cryopreserved and revived at different passage levels. The cell line and culture systems are being maintained to develop continuous cell lines for further studies.

1. Introduction

Fish cell cultures have both fundamental and practical importance. Several short-term and continuous cell cultures from a variety of fish species have been reported since the development of first fish cell line from rainbow trout over 25 years ago (Wolf and Ahne, 1982; Wolf and Quimby, 1969). Some of the areas in which fish cell lines have made significant contributions are fish immunology (Bols et al., 2001; Clem et al., 1996), toxicology (Babich and Borenfreund, 1991; Segner, 1998), ecotoxicology (Castano et al., 2003; Fent, 2001; Schirmer, 2006), endocrinology (Bols and Lee, 1991), virology (Wolf, 1988), biomedical research (Hightower and Renfro, 1988), disease control (Villena, 2003), biotechnology and aquaculture (Bols, 1991) and radiation biology (Ryan et al., 2008). Cell culture systems also offer the best potential source of chromosome preparation for the application of banding techniques (Blaxhall, 1983). Fish cell lines have been used widely as *in vitro* models in aquatic toxicity studies.

Most of the established cell lines have been derived from cold water fish of European origin (Lakra and Bhonde, 1996). But very few cell culture systems have been developed from coldwater fishes of Asia and South East Asia. With fast changing status of fish cell culture in India, there have seen consistent efforts towards development of cell culture systems from different organs and tissues of different species. Since some pathogenic viruses are known to be organ or tissue-specific, the establishment of additional cell lines from different organs and tissues of a host species will be valuable for studying species-specific responses to viral infection at the cellular level. Recently cell culture systems have been developed from *Tor putitora* (Lakra et al., 2006a), *Etroplus suratensis* (Swaminathan et al., 2010), *Epinephelus coioides* and *Chanos chanos* (Parameswaran

Abbreviations: FBS, fetal bovine serum; PCE, *Puntius chelynoides* eye cell line; COI, cytochrome oxidase subunit I gene; 16S rRNA, 16S ribosomal RNA gene; NBFGR, National Bureau of Fish Genetic Resources; TE buffer, Tris EDTA (ethylenediaminetetraacetic acid) buffer; H_2O_2, hydrogen peroxide; PBS, phosphate buffered saline; DMSO, dimethyl sulphoxide; LN_2, liquid nitrogen.

* Corresponding author. Fax: +91 522 2442441.
E-mail address: mukugoswami@gmail.com (M. Goswami).

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et al., 2007), *Lates calcarifer* (Lakra et al., 2006b; Parameswaran et al., 2006), *Labeo rohita* (Lakra et al., 2010a), *Puntius densonii* (Lakra et al., 2010b) and *Puntius sophore* (Lakra and Goswami, 2011).

Puntius (Tor) chelynoides, commonly called as dark mahseer belonging to the family Cyprinidae, inhabits fast-flowing hill streams of India and Nepal and is listed as vulnerable in the IUCN Red List, 2010. The species is reported to be locally extinct from Assam and Kumaon lakes (Menon, 2004), and is also reported as very rare species (Ranjan et al., 2007). Mahseer (*Tor* spp, Family Cyprinidae) is the predominant group of indigenous fish captured from streams and rivers. The genus *Tor* with several species presents valuable food as well as sport fish in the mountain arc, which is threatened because of wreck and other destructive fishing, human interference, loss of habitat and pollution (Lakra et al., 2010c). Thus, much attention is needed for their biodiversity conservation using genetic and biotechnological tools. Development of cell lines from different tissues will open new vistas of *in vitro* research in pathogenesis and conservation of coldwater fish species. The paper aimed at developing and characterizing culture systems from eye, fin, heart and swim bladder of *P. chelynoides*.

2. Materials and methods

2.1. Specimens

Live fingerlings of *P. chelynoides* of 10–20 g were collected from the River Alaknanda, Garhwal, Uttarakhand, India and its tributaries and were maintained in the wet lab of National Bureau of Fish Genetic Resources (NBFGR), Lucknow, India. Before experimentation healthy fish were anaesthetized in ice-cold water containing 1000 IU/ml penicillin and 1000 μg/ml streptomycin, dipped in 5% Sodium hypochlorite for 5 minutes and wiped with 70% alcohol.

2.2. Explant preparation

Eye, fin, heart and swim bladder tissues were taken out aseptically and washed with PBS containing 500 IU/ml penicillin, 500 μg/ml streptomycin and 2.5 mg/ml Fungizone. The tissues were minced into small pieces and seeded into 25 cm^2 cell culture flasks with about 50 μl fetal bovine serum (FBS) and allowed to attach to the surface of the flask overnight. The flasks were thereafter incubated at 24 °C in L-15 medium supplemented with 20% FBS.

2.3. Primary cell culture and subculture

The primary cell cultures were maintained at 24 °C using L-15 medium supplemented with 20% FBS in 25 cm^2 tissue culture flasks. Upon reaching to 90–95% confluency, the cells were trypsinized using TPVG solution (0.1% trypsin, 0.2% ethylenediaminetetraacetic acid-EDTA and 2% glucose in 1X PBS). The subcultured cells were grown in fresh L-15 media with 20% FBS and as the culture progressed, the serum concentration was reduced to 15%. In the initial subcultures, half of the growth medium was changed after an interval of five days.

2.4. Morphological observation

The flasks were observed daily for morphological details viz. attachment of explants, spreading and proliferation of cells using an inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

2.5. Growth studies

To determine the optimum temperature and serum concentration, the cells were grown at different temperature range and FBS concentration. Temperature effect was determined by seeding cells in 1×10^5 range and incubated at 24 °C for 24 h and subsequently at selected temperatures of 20 °C, 24 °C and 28 °C in triplicates. Every other day, triplicate flasks at each temperature were trypsinized and cell density was measured with haemocytometer for a total of seven days.

The growth response to various concentrations of FBS (10, 15, and 20%) was carried out using the same procedure as mentioned above, at 24 °C.

2.6. Cell plating efficiency

Plating efficiency of the PCE cell line was determined at seeding concentrations of 200, 500, and 1000 cells per flask (z) (25 cm^2 tissue culture flask) in duplicate at passage 14. Following 14 days of incubation, the medium was removed and cell colonies were fixed with formalin (25%) and stained with crystal violet stain (1%). Colonies were then counted (x) in each flask under the microscope and plating efficiency (y) was calculated using the formula $y = 100xz^{-1}$ (Freshney, 1994).

2.7. Cytogenetic characterization

Cells from fin and PCE cell line were used to make chromosome spreads at 10th & 15th passage respectively. The cells were inoculated in a 25 cm^2 culture flasks and incubated for 24 to 36 h. Upon reaching 70–80% confluency, colchicine (0.05%) was added to the flasks and incubated overnight. Cells were trypsinized and transferred to a conical centrifuge tube. After centrifugation at 600 ×*g* for 5 min, excess medium was removed, pellets were gently resuspended in 0.027 M KCl and incubated at 30 °C for 30 min. Centrifugation was again carried out at 600 ×*g* for 5 min then cells were fixed with freshly prepared chilled Carnoy's fixative (1:3, Acetic acid-Methanol). Again cell suspensions were centrifuged at 600 ×*g* for 5 min, supernatant were discarded and cells were resuspended in fixative. These fixed cells were then washed two to three times with fresh fixative, and then resuspended in a small amount of fixative. The slides were made by dropping the suspension onto glass slides, air dried and stained with 5% Giemsa (pH 6.8) for 15–20 min. Finally, the slides were observed under microscope and chromosome counts were performed in more than 100 metaphase plates for both the cells.

2.8. Molecular characterization

2.8.1. DNA isolation

DNA extraction from *P. chelynoides* tissue and PCE cells at passage 18 were completed according to the previously described method (Lo et al., 1996). Briefly, the samples were trypsinized and homogenized in lysis buffer (100 mm NaCl,10 mm Tris–HCl pH 8.0, 25 mm EDTA pH 8.0, 0.5% sodium dodecyl sulphate and 0.1 mg ml^{-1} proteinase K) then incubated at 65 °C for 1 h, 5 M NaCl was added to a final concentration of 0.7 M followed by a slow addition of 1/10th volume of N-cetyl N,N,N-trimethyl ammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl). After incubation at 65 °C for 2 h, the digested samples were deproteinized by successive phenol/chloroform/iso-amyl alcohol extraction. Finally the DNA was precipitated with absolute ethanol, washed with 70% ethanol and dissolved in TE buffer. The amount of isolated DNA was quantified by using a UV spectrophotometer at wavelength of 260 nm and DNA was diluted to get a final concentration of 100 ng μl^{-1}.

2.8.2. Amplification and sequencing

Tow genes 16S rRNA and cytochrome oxidase subunit I (COI) of mitochondrial DNA were amplified. The 16S rRNA fragment was amplified using the following primers: forward 16SAR (5′-CGCCTGTTTATC AAAAACAT-3′) and reverse 16SBR (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi et al., 1991). The cytochrome oxidase subunit I (COI)

fragment was amplified using the following primers: forward FISHF1 (5′-TCAACCAACCACAAAGACATTGGCAC-3′) and reverse FISHR1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) (Ward et al., 2005).

16S rRNA gene was amplified in a 50 μl reaction volume contained 5 μl of 10X Taq polymerase buffer, 0.2 mM of each dNTP, 0.4 μM of each primer, 2.5U of Taq polymerase and 100 ng genomic DNA using the C-1000 thermal cycler (Bio-Rad). The PCR cycling conditions involved an initial denaturation step at 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min with a final extension phase at 72 °C for 10 min and a holding temperature of 4 °C after completion. Cytochrome oxidase subunit I (COI) gene was also amplified in a 50 μl reaction volume contained 5 μl of 10X Taq polymerase buffer, 0.25 mM of each dNTP, 0.4 μM of each primer, 2.5U of Taq polymerase and 100 ng genomic DNA using the C-1000 thermal cycler (Bio-Rad). The PCR cycling conditions involved an initial denaturation step at 95 °C for 2 min followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min 10 s with a final extension phase at 72 °C for 10 min and a holding temperature of 4 °C after completion.

The amplicons were checked on 1.2% agarose gel and the products with high intense bands were selected for sequencing. Products were labeled using the BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc) and sequenced bidirectionally using an ABI 3730 capillary sequencer following manufacturer's instructions. The obtained sequences of PCR fragments were compared to known sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST).

2.9. PCE cell line transfection with GFP reporter gene

pEGFP-C1 plasmid was used with transfection reagents lipofectamine LTX and Plus Reagent for transfecting the sub confluent monolayers of PCE cells at passage 22. In brief, the PCE cells were seeded at a density of 1×10^5 in a 12 well plate individually and incubated for 18 h at 28 °C in normal atmospheric incubator. Before transfection, cells were washed with PBS and supplemented with 400 μl of fresh L-15 medium devoid of serum and antibiotics. The plasmid DNA (200 ng of pEGFP-C1) was dissolved in 100 μl of optimem and then 0.5 μl of plus reagent was added. The mixture was incubated for 5 min at room temperature. 2 μl of lipofectamine LTX was added to the mixture containing plasmid DNA and incubated for 30 min at room temperature. Finally the mixture was added dropwise on 70-80% confluent PCE cells in 12 well plate. The medium was changed with fresh medium after an incubation of 6 h at 28 °C. The green fluorescence signals were observed after 18 h under a fluorescent microscope (Olympus).

2.10. Genotoxicity assessment in PCE cell line by Comet assay

PCE cells at 25th passage were treated with hydrogen peroxide (H_2O_2), a genotoxic model compound, in order to assess the efficiency of the comet assay for estimating genotoxicity on the cells. For the assessment, cells were first grown in 24 well plate in 1 ml medium for 24–48 h. The medium was then removed and replaced with 1 ml of medium containing serial dilutions of the tested genotoxic compound (H_2O_2). Exposure was carried out for 24 h. After exposure cells were washed with PBS, trypsinized and processed for Comet assay.

The Comet assay (single cell gel electrophoresis assay) was performed following previously described protocol (Singh et al., 1988) with minor modifications. In brief, the cell suspension was mixed with 0.6% low melting agarose and placed on a slide pre-coated with 1.0% normal melting agarose. Third layer of 0.5% low melting agarose was added and left to solidify. Subsequently, the cells, not the nuclei, were lysed in a lysis buffer (100 mM EDTA, 2.5 M NaCl, 10 mM Tris base, 1% Triton X-100, pH adjusted to 10) at 4 °C for at least 1 h. Single-strand DNA was prepared by unwinding the DNA in electrophoresis buffer (1 mM EDTA, 10% DMSO, 300 mM NaOH, pH 13) for 30 min and then subjected to micro-electrophoresis in the same electrophoresis buffer (25 V, 300 mA) for 15 min. After electrophoresis, the alkalis in the gels were neutralized by rinsing the slides in a neutralization buffer (0.1 M Tris, pH 7.5) for 5 min followed by drying and fixing of slides in methanol. After staining with ethidium

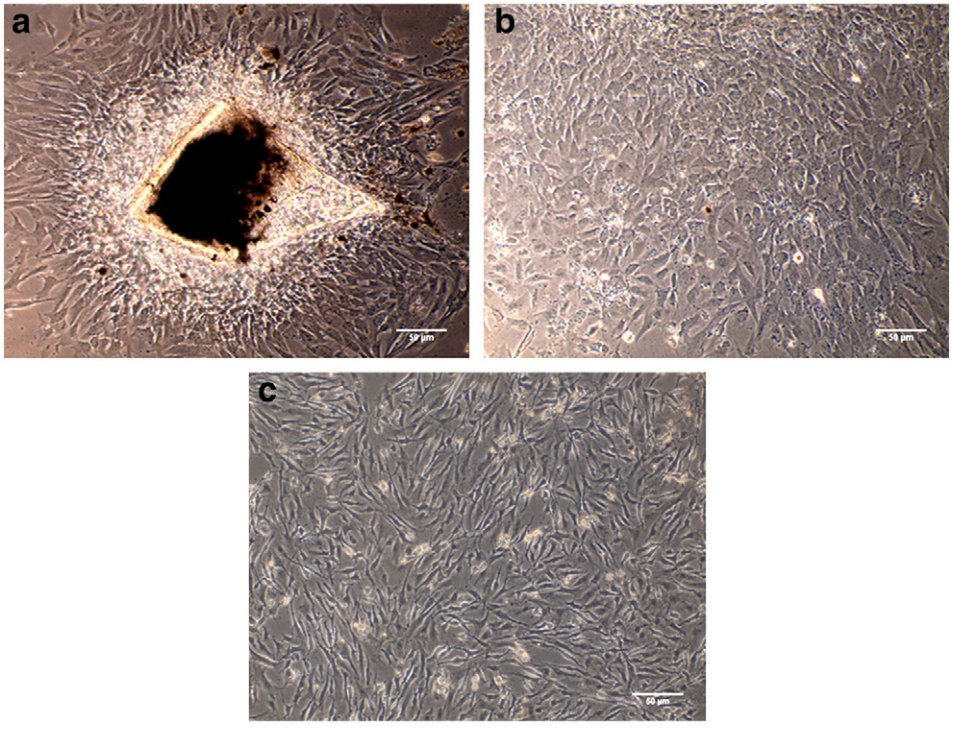

Fig. 1. Derivation of PCE cell line from eye (100x) (a) PCE cells primary culture at day 7 (b) Cells at passage 5 (c) Fibroblast-like cells at passage 25.

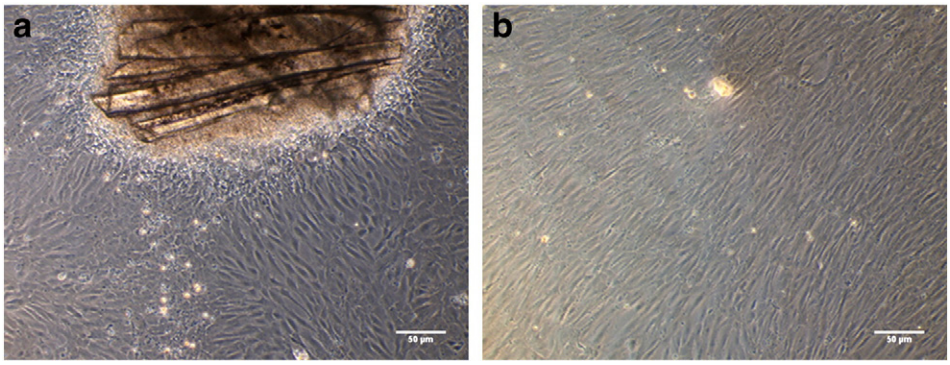

Fig. 2. Phase contrast micrographs (100×) of (a) Fin explant (b) Confluent monolayer of subcultured cells from fin.

bromide (20 μg/ml) for 10 min, the slides were viewed under a fluorescent microscope (Olympus).

2.11. Cryopreservation

The viability of PCE cells after freezing in liquid nitrogen (LN_2) was evaluated. The slow freezing method was used to cryopreserve cells. For cryopreservation, 72 h old cultures of PCE cells were used at passage 25 and 30. In brief, cells were removed from the flask surface, concentrated by centrifugation, and resuspended in a freezing medium consisting of L-15 medium containing 20% FBS and 10% dimethyl sulphoxide (DMSO) at a density of 1×10^6 cells per ml. The cell suspensions were poured into 2 ml sterile cryovials and tubes were placed in a −80 °C freezer overnight then transferred to a liquid nitrogen tank (−196 °C) for storage. After 3 months of post-storage the cells were recovered. For recovery the vials were thawed in running water at 24–28 °C. Following removal of the freezing medium by centrifugation, the cells were suspended in L-15 with 15% FBS and tested for viability by haemocytometer counting after trypan blue staining. The viable cells were seeded into 25 cm^2 cell culture flask and observed.

3. Results

3.1. Primary cell culture and subculture

Explants were found to be attached properly after 18–24 h of explant preparation for all the tissues. Radiation of cells started in cell culture flasks (25 cm^2) within 48–72 h (Figs. 1a, 2a, 3a and 4a) from all the explants of different tissues. Confluent monolayer around the explants of eye, fin and heart tissues was observed after seven days of implantation. Radiated cells from swim bladder tissue reached to confluency after 11 days. Cell culture systems were designated as PCF, PCH and PCS for fin, heart and swim bladder tissues, respectively.

The majorities of cells proliferating from eye explants were fairly heterogeneous in nature and were composed of both epithelial and fibroblast-like cells (Fig. 1b). After 15 subcultures, the cell line showed fibroblast-like morphology (Fig. 1c). The density of eye cells reached maximum on the 5th day on one passage. The cell line was designated as PCE and has successfully been subcultured up to 31 passages.

The primary cells from the fin and heart explants also showed epithelial and fibroblast-like morphology. In addition, prominent heart beat was observed in 40% of heart explants, though the frequency of beating declined gradually with the culture time and ultimately stopped. The longest duration of heart beat observed was for 29 days. After few passages of PCF cells the fibroblast-like cells dominated over epithelial cells (Fig. 2b). The cell culture system developed from fin tissue has successfully been subcultured upto 15th passage. In the case of PCH cells, although epithelial as well as fibroblast-like cells were present in the primary culture and initial subcultures, the epithelial cells predominated as the culture progressed (Fig. 3b). The subcultured cells from heart explants remained in good condition upto 9th passage after which they slowly started detaching from the surface and ultimately could not survive. The cells obtained from swim bladder explants were mostly dominated by fibroblast-like morphology and retained their morphology even after subculturing (Fig. 4b). Cell monolayer obtained from swim bladder tissue has successfully been maintained in good condition upto 7th passage.

3.2. Growth studies

The cells from all the tissues exhibited similar growth at different temperatures. However, optimum growth temperature was found to be 24 °C (Fig. 5). No significant growth was observed at 20 °C. At 28 °C, initially good growth was observed but as the culture time progressed, cell growth was not as good as was at 24 °C. During first 3 days cells

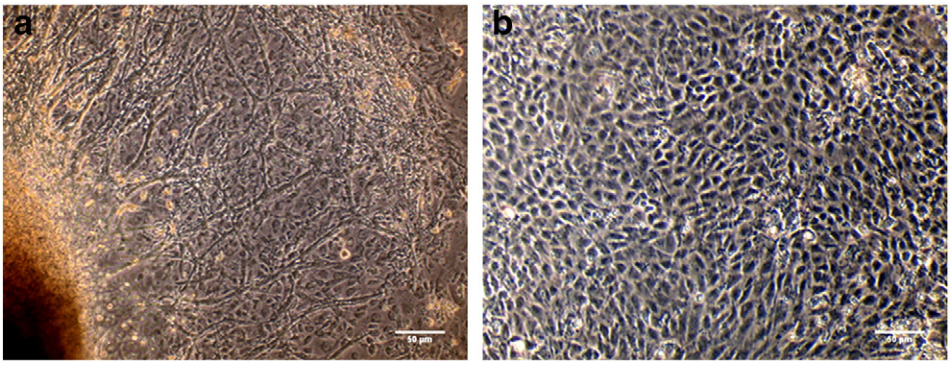

Fig. 3. Phase contrast micrographs (100×) of (a) Heart explant (b) Confluent monolayer of subcultured cells from heart.

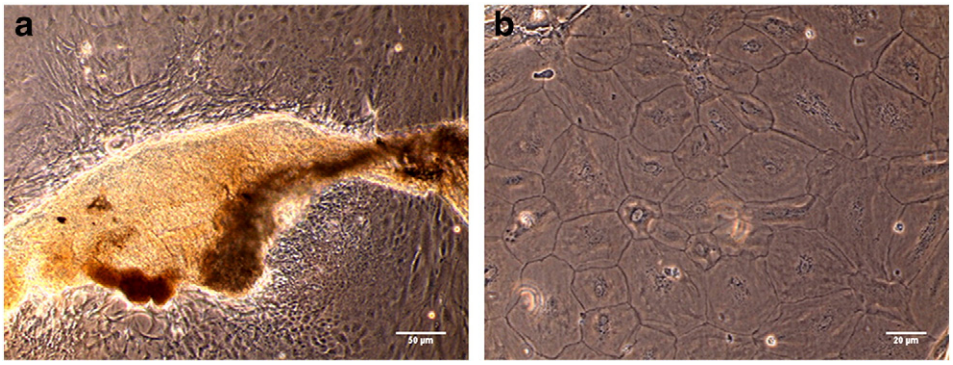

Fig. 4. Phase contrast micrographs of (a) Swim bladder explant (100×) (b) Confluent monolayer of subcultured cells from swim bladder (200×).

had a very good proliferation in L-15 media supplemented with 15% and 20% FBS (Fig. 6). Cell growth decreased a little in 15% FBS concentration in next few days. At much lower concentration (10%) of FBS, the cell growth decreased intensely.

3.3. Cell plating efficiency

The plating efficiency of PCE cells seeded at a density of 200 cells per flask was 41%, at 500 and 1000 cells per flask the plating efficiency increased to 64% and 88% respectively with no significant differences between replicates.

3.4. Cytogenetic characterization

Chromosomes spreads made from fin and PCE cells revealed the modal diploid chromosome number 100 though they ranged from 42 to 115 and 43 to 113 respectively (Figs. 7a, b and c). The observed modal chromosome number is similar to the standard chromosome number of *P. chelynoides*. The result demonstrated that PCF cell culture system and PCE cell line are indeed truly derived from *P. chelynoides*.

3.5. Molecular characterization

An analysis of mitochondrial COI and 16S rRNA genes was performed to verify the origin of the PCE cell line. Amplification from the COI gene for PCE cells revealed the expected PCR product of 655 bp. Subsequent comparative analysis of the identified sequences demonstrated a 99% to 100% match for COI to known *P. chelynoides* mitochondrial DNA sequence (GenBank Acc No. EU714105). Similar comparative analysis was performed for amplified mitochondrial 16S rRNA sequence. Cell line sequences were submitted to GenBank and accession numbers were JQ435840 and JQ435839 for COI and 16S rRNA genes, respectively.

3.6. PCE cell line transfection with GFP reporter gene

The PCE cell line transfected with pEGFP-C1 plasmid using lipofectamine LTX and Plus Reagent showed clear and strong green fluorescent signals. The expression of EGFP in PCE cells could be detected as early as after 6 h post transfection (Figs. 8a and b).

3.7. Genotoxicity assessment in PCE cell line by Comet assay

Comet is shown derived from the exposure of PCE cells with H_2O_2 (Fig. 9a). There was a significant difference between the comet tail-length of PCE cells exposed to H_2O_2 compared with the control (Fig. 9b).

3.8. Cryopreservation

The viability of PCE cells recovered after cryopreservation with the dimethyl sulfoxide (DMSO) slow freezing procedure yielded significant results. The revived cells recovered well and grew to confluency within 7 days. Ability of cryopreserved cells to adhere after thawing was good and most of the cells could be recovered after freezing. However, cell loss was noted in terms of the amount of viable cells recovered after cryopreservation compared to the

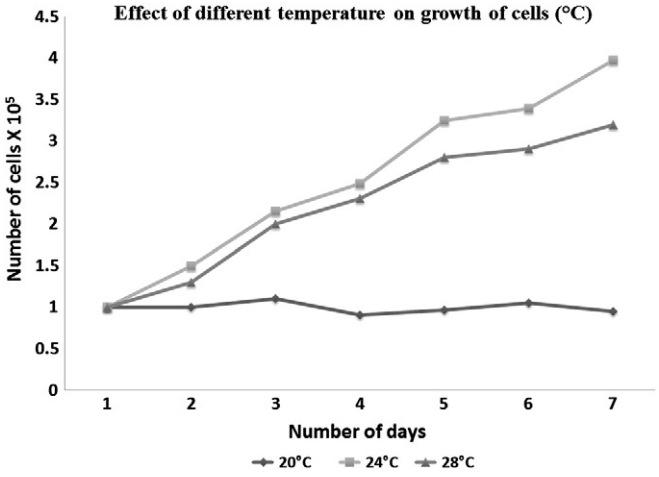

Fig. 5. Similar effect of different temperatures on growth of cells.

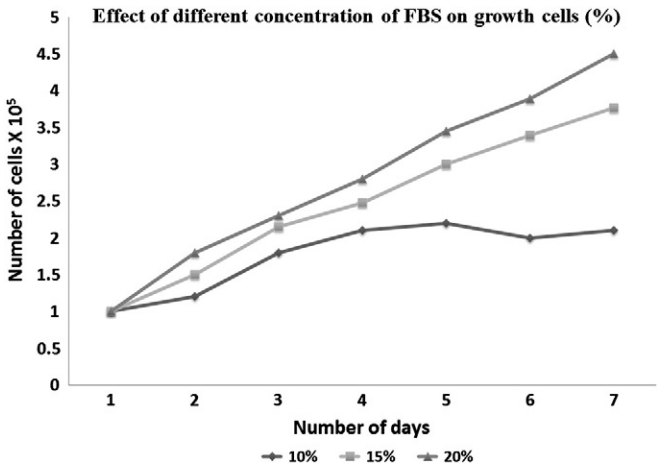

Fig. 6. Similar effect of different concentrations of FBS on growth of cells.

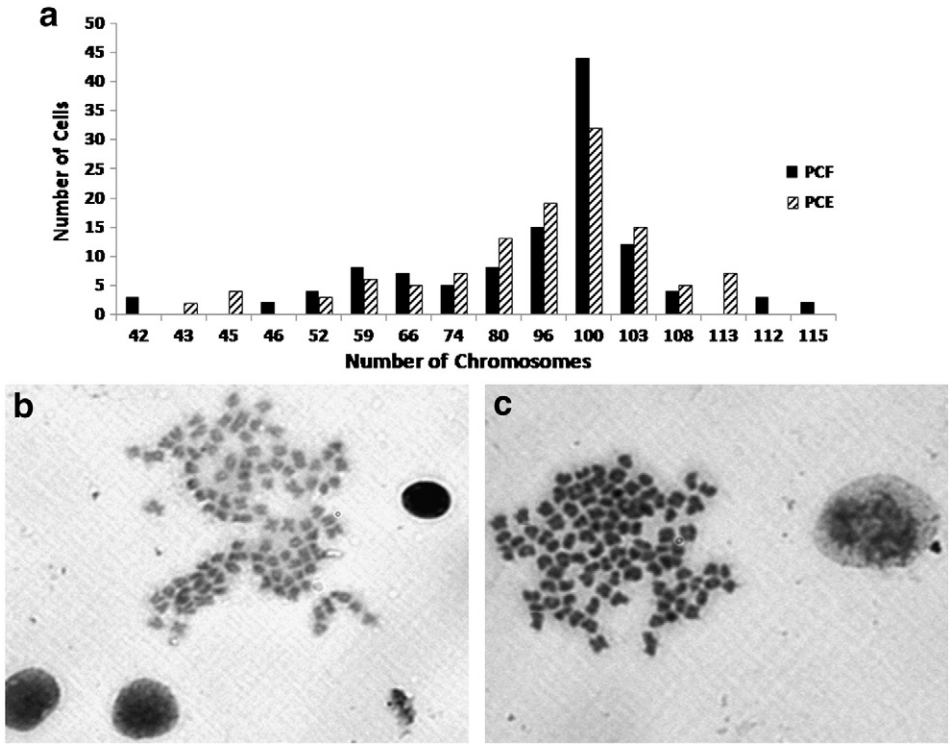

Fig. 7. Cytogenetic characterization (a) Chromosome number distribution of PCF and PCE cells (b) standard chromosome spread of PCF cells (c) standard chromosome spread of PCE cells.

amount of cells before the treatment. Average estimated recovery percentage was 80% to 85% of the initial cell population.

4. Discussion

Explant techniques has many advantages over the use of cell suspensions, such as speed, ease, maintenance of cell interactions and the avoidance of enzymatic digestion which can damage the cell surface (Avella et al., 1994; Parkinson and Yeudall, 1992). Considering such advantages, in the present study a cell line designated as PCE from eye and cell culture systems from different tissues of *P. chelynoides* fingerlings were developed by explant method.

Mixed population of cells was present in primary monolayers from eye, fin and as well as heart tissues as has been reported by many researchers during early cell cultures (Lakra et al., 2010a; Parameswaran et al., 2006). Generally, a predominance of fibroblast cells over epithelial cells in cell cultures from fishes has been reported (Bejar et al., 1997; Lai et al., 2003; Lakra et al., 2006a). A similar morphological change was also observed in the cells subcultured from eye and fin tissues of *P. chelynoides*. In contrast, Ahmed et al. (2008) reported presence of both epithelial-like cells and fibroblast-like cells during initial culture from eye cells of *Catla catla* and predominance of epithelial-like cells over fibroblast-like cells in subsequent subcultures. Heart cells from *P. chelynoides* obtained epithelial morphology after subsequent subcultures. Tong et al. (1998) also reported epithelial morphology of cells from heart tissue of Sea perch. In contrast, primary culture developed from heart explants of Indian major carps comprised mainly of fibroblast like cells (Rao et al., 1997). Similarly Lai et al. (2003) and Wang et al. (2010) reported fibroblast-like cells from heart tissue of *Epinephelus awoara* and *Cynoglossus semilaevis* respectively.

Rougée et al. (2007) described that the morphology of GFSB cells obtained from swim bladder of *Carassius auratus* remained consistently fibroblastic from their initial inception to the subsequent passages which showed conformity with the cells obtained from swim bladder tissue of *P. chelynoides* in the present study where the cell morphology remained fibroblast-like, Lai et al. (2003) and Lakra et al. (2010a) however reported epithelial-like morphology of cells in swim bladder of *L. rohita* and *E. awoara* respectively.

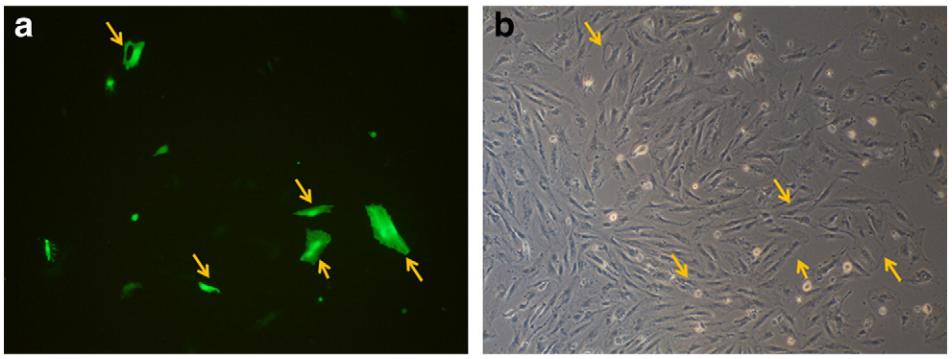

Fig. 8. Expression of green fluorescent protein in PCE cells transfected with pEGFP-C1 vector (a) Fluorescent view (b) Phase contrast view.

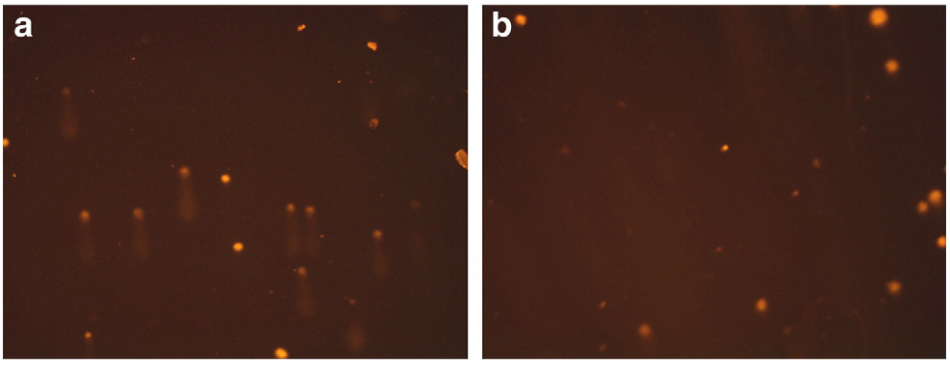

Fig. 9. Comets after single-cell gel electrophoresis of PCE cells (a) H_2O_2 treated PCE cells (b) Untreated PCE cells (control).

Cells from swim bladder took about 11 days to reach to confluency which is in accordance with GFSB cell line derived from swim bladder of *C. auratus* (Rougée et al., 2007).

The optimum growth was observed in the L-15 medium with 20% FBS, which showed conformity with the observations made by Lakra et al. (2006b). Suitability of L-15 in supporting growth of fish cell lines compared to that of other media has been reported by many researchers (Fernandez et al., 1993; Hameed et al., 2006; Kumar et al., 2001; Lai et al., 2000, 2003; Lakra et al., 2006b; Qin et al., 2006; Ye et al., 2006). Leibovitz L-15 medium has been designed to maintain pH in the physiological range under the normal atmospheric conditions without CO_2. Faster growth and better proliferation of cells was noticed in cells cultured with L-15 medium at pH 7.4. The flasks were incubated at 20, 24 and 28 °C to determine optimal growth temperature. Being a cold water fish, the optimum growth temperature must be low and in the present study the optimum growth temperature was found to be 24 °C. The result shows conformity with Alvarez et al. (1991), who also reported the optimum *in vitro* growth temperature a few degrees above from the preferred environmental level for live fish. Wolf and Ahne (1982) suggested that the difference between *in vivo* and *in vitro* temperature can be due to selection or adaptation of cells to growth at higher than normal temperature.

The PCE cells showed moderately higher plating efficiency and decreased contact inhibition as the passage number increased. Since high plating efficiency is one of the general characteristic of transformed cell cultures (Freshney, 1994), this might suggest that PCE cells transformed in the later passages for which they were tested and this finding strengthens by the fact that chromosome instability was seen in PCE cells in the form of occurrence of changes in chromosome number. However, PCE cell line and as well as PCF cell culture system were evidenced by modal diploid chromosome count of $2n = 100$ which has been documented earlier for this cold-water species (Mani et al., 2009).

Species-specific DNA markers have been used to identify species origin of established cell lines. Hence, cell line authentication using molecular marker is essential especially when contamination with other cell lines is suspected. COI gene sequence is a useful DNA marker and its analysis has demonstrated it as the core of global bio-identification systems for animals (Hebert et al., 2003). It has been used to identify species and to study relationships among organisms (Song and Toh, 1994; Ward et al., 2005). Recently, COI region has been used for identification of cell lines (Lakra and Goswami, 2011; Lakra et al., 2010d). This suggests the utility of COI sequence as a valid and universal marker for species identification of established fish cell lines. Other alternatives such as 16S ribosomal RNA gene sequence has also been used to confirm the origin of muscle and fin cell lines of bluefin trevally (Zhao and Lu, 2006). Identity of PCE cell line was confirmed by COI and 16S rRNA genes amplification.

The successful transfection of PCE cells with pEGFP-C1 plasmid points to the possibility for this cell line to be genetically manipulated for gene targeting and expression studies (Fernandez-Alonso and Coll, 1999; Liu et al., 1990). After exposure to H_2O_2 significant increase of DNA damage was observed in the PCE cells compared to cells that were not exposed to genotoxic compound in the Comet assay. This suggests that Comet assay can be applied successfully in fish cell lines, for genotoxic monitoring of toxic compounds. The Comet assay detects primary DNA lesions such as DNA strand breaks, but may also detect genomic instability, repair of double-strand breaks, DNA-adduct formation and DNA cross-links (Hartmann and Speit, 1995).

Cryopreservation of cell lines is necessary for long-term storage. DMSO is the most commonly used cryoprotectant for cultured cells, including fish cells (Wolf and Quimby, 1969) due to its low molecular weight and penetration capacity. Because of the slow penetration of the cryoprotectant into the cells, slow freezing method of cryopreservation proved to be highly efficient. However, decreased percentage recovery of cells demonstrates the mild toxic effect of DMSO (Mauger et al., 2006). For cryopreservation of PCE cells, the optimal concentration of DMSO used was 10%. However, the concentrations of DMSO used for frozen cell suspensions are 10% to 20% (Donahoe et al., 1977; Jezek et al., 2002).

In conclusion, an eye derived cell line PCE was developed and characterized from coldwater fish *P. chelynoides* that potentially could serve as a useful tool for coldwater fish conservation genetics and biotechnological applications. Good growth around the explants and formation of confluent monolayer of cells after subculturing from fin, heart and swim bladder explants indicates the possibilities of developing continuous cell lines from *P. chelynoides*.

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