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In vitro culture and characterisation of a new brain cell line from the spine cheek anemone fish *Premnas biaculeatus* (Bloch, 1790)

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Abstract

In this study, a new brain cell line designated PB1BrTr was derived from the maroon clownfish *Premnas biaculeatus* (Bloch, 1790) and characterised. The PB1BrTr cell line was developed by trypsinisation method using Leibovitz's L15 (L-15) medium supplemented with 20% FBS (foetal bovine serum) and subcultured over 100 times. Characterisation encompassed studies on optimal growth kinetics, chromosomal analysis and genotyping of the mitochondrial CO1 gene. A high revival rate (85-95%) and good attachment during seeding after a year of cryostorage demonstrated the high stability of the cell line. This cell line exhibited good seeding efficiency of 84% at 1.25 x 10^5 cells ml^{-1} and a range of plating efficiencies from 14-23% at varying cell densities. It was observed that 28˚C was the ideal temperature for its growth. Serum requirement decreased with increased passage and lowered to 2% FBS beyond 60 passages. However, higher serum concentration (2-20%) caused a concurrent increase in cell growth. The cell line displayed a fibroblast-type morphology, with immunotyping results revealing robust reactivity towards the fibroblast marker. Chromosome analysis of this cell line revealed aneuploidy and its authenticity was validated by mitochondrial cytochrome c oxidase subunit I (COI) genotyping analysis. This brain cell line demonstrated notably high transfection efficiency with pcDNA3-EGFP plasmid using Lipofectamine 3000 transfection reagent. This continuous cell line presents a valuable *in vitro* tool for diverse research applications, including gene transfer and expression studies.

Introduction

The spinecheek anemone fish, or maroon clown *Premnas biaculeatus* (Bloch, 1790), is the only species in the genus *Premnas*. The alluring colouration and tendency to live symbiotically with anemones make this reef fish a highly demanded commodity in the marine ornamental fish industry. This species occurs in reef-associated habitats across the Indo-Australian Archipelago including regions from India, where it thrives abundantly in the coral reef ecosystems of the Andaman and Nicobar Islands. The wholesale and retail markets for marine ornamental fish in the international trade have grown steadily over the past several years (Madhu *et al*., 2006; 2012). Overexploitation in response to rising demand and deteriorating natural habitats has led to a sharp decline of the natural populations of the species in recent years (Pirarat *et al.,* 2011). Consequently, captive breeding and seed production methods have been developed and standardised for the species for commercial and conservation purposes (Madhu *et al*., 2006; 2012). It has been reported that clown fishes are prone to marine viruses like lymphocystis disease virus (LCDV), which can lead to a population decline of affected species (Pirarat *et al.,* 2011; Yashwanth *et al*., 2020). *In vitro* culture of fish cells is an essential tool for researching a wide range of topics in life science, specifically for investigations on fish viruses that cause significant mortality and epizootic outbreaks. Fish cell lines are indispensable for isolation and characterisation of infectious viruses (Hasoon *et al*., 2011; Fu *et al*., 2015). Species-specific cell lines are essential for the successful isolation

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of marine fish viruses. Fish cell lines have also been utilised to investigate significant subjects like functional genomics, epidemiology, immunology, cytogenetics, molecular carcinogenesis and host-pathogen interactions as well as in toxicology to explore the potential effects of environmental contaminants on the health of fish populations (Speer *et al*., 2018).

Brain-derived cell lines have been reported in tilapia (TB2; Wen *et al*., 2008), grouper (GB; Lai *et al*., 2003) and barramundi (BB; Chi *et al*., 2005). Exogenous DNA delivery employing cultured cells is highly beneficial for both basic research and biotechnological applications. Fish cell lines offer a valuable resource for genetic manipulation and other gene expression research, as well as in facilitating the exploration of host-pathogen relationships (Fu *et al*., 2015). In this paper, we outline the methodology followed to establish a cell line derived from the brain tissue of *P. biaculeatus*, while also detailing its characterisation for optimum growth conditions, plating and seeding efficiency, documentation of morphology, chromosome number and authentication. The cell line was further assessed for *in vitro* gene transfection efficiency.

Materials and methods

Initiation of primary cell culture

Normal and healthy maroon clownfish *P. biaculeatus,* weighing 4±0.5 g (mean+SD) and having a body length of 7+0.1 cm were used for initiating primary culture. The fishes were maintained in the laboratory, in a glass aquarium holding seawater (30+2 ppt salinity) and fed on dry marine pellet feed for a week. Before initiating primary cell culture, fishes were starved for two days, to reduce the gut bacterial load. Subsequently, the fish were kept in sterile, aerated seawater overnight. Ahead of sacrifice, the fish was sedated by submerging in iced water for 1-2 min, then dipped in a solution containing sodium hypochlorite (500 ppm) for 10 s, followed by washing in sterile seawater and swabbing with 70% ethanol. Aseptically removed brain tissue was collected in sterile culture dishes holding serum-free Leibovitz' L-15 medium containing antibiotic-antimycotic solution (Penicillin - 500 IU ml^{-1}; Streptomycin - 500 µg ml^{-1} and Amphotericin B - 1.25 µg ml^{-1}). Using a sterile surgical scalpel, tissue pieces were minced into tiny fragments and again washed in serum-free L-15 medium supplemented with antibiotic-antimycotic solution. To initiate primary culture, the cells from the brain tissue were dissociated using trypsinisation method (Sobhana *et al*., 2008). The cells were harvested by centrifugation at 200 *g* for 10 min and the cell pellet was resuspended in fresh growth medium [L-15 medium supplemented with 20% foetal bovine serum (FBS); 100 IU ml^{-1} Penicillin; 100 µg ml^{-1} Streptomycin and 0.25 µg ml^{-1} Amphotericin B] and seeded into 25 cm^2 tissue culture flasks. The flasks were then incubated at 28±2°C and observed for growth, cell attachment, as well as the formation of monolayers using an inverted microscope (Nikon TS 100F).

Subculture and maintenance of cell monolayers

In primary culture, PB1BrTr cells formed a confluent monolayer in five to eight days with good cell adhesion at 28±2˚C. For subculture, the confluent cell monolayer was carefully dislodged from the flask surface by treatment with 1 - 2 ml of trypsin-EDTA solution (0.25% trypsin and 0.2% EDTA in PBS). The trypsinised cells were harvested in 5 ml of fresh growth medium, then split into two portions, transferred to new culture flasks and incubated at 28±2°C. After 20 passages, cells were subcultured at a ratio of 1:2 at 5 to 7 day intervals, with FBS being reduced to 10% and post-30 passages the FBS concentration was reduced to 5% in L-15 medium. Further after passage 60, the FBS concentration in the growth medium was reduced to 2%.

Storage in liquid nitrogen and revival

For cryopreservation, cells at 80-90% confluence at the logarithmic phase were trypsinised and harvested by centrifugation. PB1BrTr cells were resuspended in a cryopreservation medium containing 40% FBS and 10% dimethyl sulfoxide (DMSO). The cell suspension aliquots were dispensed into 2 ml sterile cryovials and processed for preservation in liquid nitrogen (-196˚C), after overnight storage at -80˚C in a freezing container. The PB1BrTr cells at different passages were used for long-term cryopreservation. To recover the cells, the cryovials were removed from liquid nitrogen, rapidly thawed in a water bath at 42˚C, following centrifugation and the cell pellet was resuspended in Leibovitz's L-15 medium supplemented with 20% FBS. The cell suspension was seeded into 25 cm^2 culture flasks and incubated at 28±2˚C. Cell viability was assessed after revival, by the trypan blue exclusion method.

Plating and seeding efficiency

The plating efficiency of PB1BrTr cells was tested at passage 62 and the seeding efficiency at the 66th passage. To determine the plating efficiency, the exponentially growing cells were trypsinised, counted and diluted in L-15 medium containing 10% FBS to the plate at different cell densities (1 x 10^2, 2 x 10^2, 5 x 10^2, 1 x 10^3, 2 x 10^3 and 4 x 10^3 cells per well) in 6-well cell culture plates. Triplicates were used for each cell density. The medium was replaced thrice a week. Following incubation at 28±2°C for 14 days, the medium was aspirated, the cell monolayers were rinsed, fixed and stained with Giemsa stain. The cell colonies were counted and cell plating efficiency was calculated using the formula:

$$\text{Plating efficiency} = \frac{\text{No. of colonies}}{\text{No. of cells seeded}} \times 100$$

Triplicate experiments were performed independently. To calculate the seeding efficiency, PB1BrTr cells at various densities (3 x 10^4, 1.25 x 10^5, 2.5 x 10^5, 5 x 10^5 and 1 x 10^6 cells per flask) were seeded onto T-25 flasks. After an incubation period of 6-8 h at 28°C, the attached cells were harvested by trypsinisation and cells were counted using a haemocytometer. Seeding efficiency was calculated using the formula:

$$\text{Seeding efficiency} = \frac{\text{No. of cells attached}}{\text{No. of cells seeded}} \times 100$$

Effect of temperature and FBS concentration on cell growth

The growth characteristics of PB1BrTr cell line at different temperatures and FBS concentrations were studied. PB1BrTr cells at

passage 70 were seeded in T-25 flasks at a concentration of 1×10^6 ml^{-1} and were incubated at selected temperatures of 20°C, 24°C, 28°C and 32°C for growth evaluation over 7 days. The cells from duplicate flasks at each temperature were trypsinised and counted using a haemocytometer on alternate days. The growth response to different concentrations of FBS (2, 5, 15 and 20%) was determined following the same procedure as mentioned above, incubating the cells at 28°C.

Chromosome study

To ascertain the chromosome number of PB1BrTr cell line, the cells at passage 72, grown in a T-25 flask for 24 to 36 h were used. After the incubation period, the cells at metaphase were arrested by adding 1 µg ml^{-1} colchicine for 2 h at 28 ±2°C. Then the cells were harvested by centrifugation (200 *g*, 7 min) and the cell pellet was resuspended in a hypotonic solution consisting of 0.075M KCl for 30 min. Freshly mixed 0.5 ml cold Carnoy's fixative solution (methanol:acetic acid at 3:1 ratio) was added to the cell suspension for fixation, followed by centrifugation. After three washes in fresh fixative, the fixed cells were resuspended in a small amount of fixative. Using the conventional drop-splash technique, slides were prepared, and stained with 5% Giemsa for 10 min and the chromosome numbers were counted under a compound microscope.

Authentication of PB1BrTr cell line

DNA extraction from the muscle tissue of *P. biaculeatus* as well as from the PB1BrTr cell line (at passage 75) was carried out by conventional phenol-chloroform and salting out methods, respectively (Sambrook and Russel, 2006). The universal primer pair, FISH F1 (5'TCAACCAACCACAAAGACATTGGCAC3') and FISH R1 (5'TAGACTTCTGGGTGGC CAAAGAATCA3') (Ward *et al.,* 2005) was used for the amplification of the mitochondrial CO1 gene. A 25 µl volume PCR was carried out in a Veriti thermal cycler (Applied Biosystems) using DreamTaq Green PCR Master Mix (Thermo Scientific). The cycling conditions involved an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, annealing at 50°C for 30 s, and 72°C for 45 s, with a final extension phase of 5 min at 72°C. PCR products were examined in ethidium bromide containing 1.8% agarose gel, visualised in a gel documentation system (Azure Biosystems) and were sequenced at the sequencing facility. The partial sequences of the CO1 gene of the cell line and the muscle tissue were compared to known sequences in the National Centre for Biotechnology Information (NCBI) database for species identity and the sequences were deposited in the NCBI GenBank.

Documentation of cell morphology

Giemsa staining

To visualise the cell morphology of PB1BrTr cell line, the confluent cells at passage 76 were stained with Giemsa. After removing the medium, the cells were rinsed in PBS and fixed in methanol, before staining with undiluted Giemsa for 2 min. The stained cells were washed with water to remove excess stains and inspected under an inverted microscope (Nikon TS100F). Photomicrographs were taken at different magnifications, using a digital camera attached to the microscope.

Immunofluorescence staining

PB1BrTr cells at the exponential growth phase at passage 78 were used for immunophenotyping. Cells grown in 4 well chamber slides to 90% confluency were fixed with 7.4% p-formaldehyde (20 min) and permeabilised in 0.1% Triton X-100 for 10 min. To reduce non-specific binding, cells were blocked with 5% normal goat serum (30 min) and incubated with primary antibody [monoclonal anti-vimentin (Sigma Aldrich, USA)/monoclonal anti-pancytokeratin (Invitrogen, USA)] for 1 h at room temperature (RT). Cells were then washed thrice with PBS, followed by incubation with the secondary antibody [goat anti-mouse IgG FITC (Sigma Aldrich, USA)], for one hour at RT, cell DNA in nuclei was stained using 4‘‘, 6‘‘- diamidino-2-phenylindole (DAPI, Sigma Aldrich, USA) for 15 min and mounted using Prolong Gold antifade reagent. Negative controls were kept, with the primary antibody replaced with blocking buffer. Stained cells were analysed using a fluorescence microscope (Nikon TS100F).

Gene transfection

The PB1BrTr cell line at passage 87 was seeded at a density of 5 x 10^5 cells per well in a six-well plate. Monolayers with 80% confluence were transfected with pcDNA3-EGFP plasmid (Addgene) using Lipofectamine 3000 transfection reagent (Thermo Scientific, USA) according to the manufacture's protocols. Briefly, for transfection with Lipofectamine 3000, 1.5 µg of DNA was diluted in 50 µl of Opti-MEM (Invitrogen) and 3 µl of P3000 reagent was added. Lipofectamine 3000 reagent (0.75 µl) was also diluted in 25 µl of Opti-MEM. The transfection solutions were mixed, incubated at room temperature for 15 min and directly added to confluent monolayers of PB1BrTr cell line. After 48 h, the medium was completely removed and mounted with Prolong gold antifade reagent and the cells were visualised for green (EGFP) fluorescent signals under a fluorescence microscope (Nikon TS100F).

Results and discussion

Initiation of primary cell culture

The dispersed cells yielded from the dissociated brain tissue showed a gradual progression in growth, attachment and proliferation during the first month. The initial growth of the cells began on day 2 when analysed under an inverted microscope and continued through the formation of many colonies or incomplete cell monolayers. In primary culture, most cells were heterogeneous and consisted of epithelial and fibroblastic cells (Fig. 1a and b).

Subculture and maintenance of cells

When the primary cell cultures proliferated and formed a complete monolayer, cells were passaged to new flasks by trypsinisation. The trypsinised cells successfully formed monolayers in the following subcultures and showed good adhesion to the flask surface. Cells were subcultured in L-15 medium with 20% FBS for the initial 20 passages. After that, cells were subcultured every 5-7 days

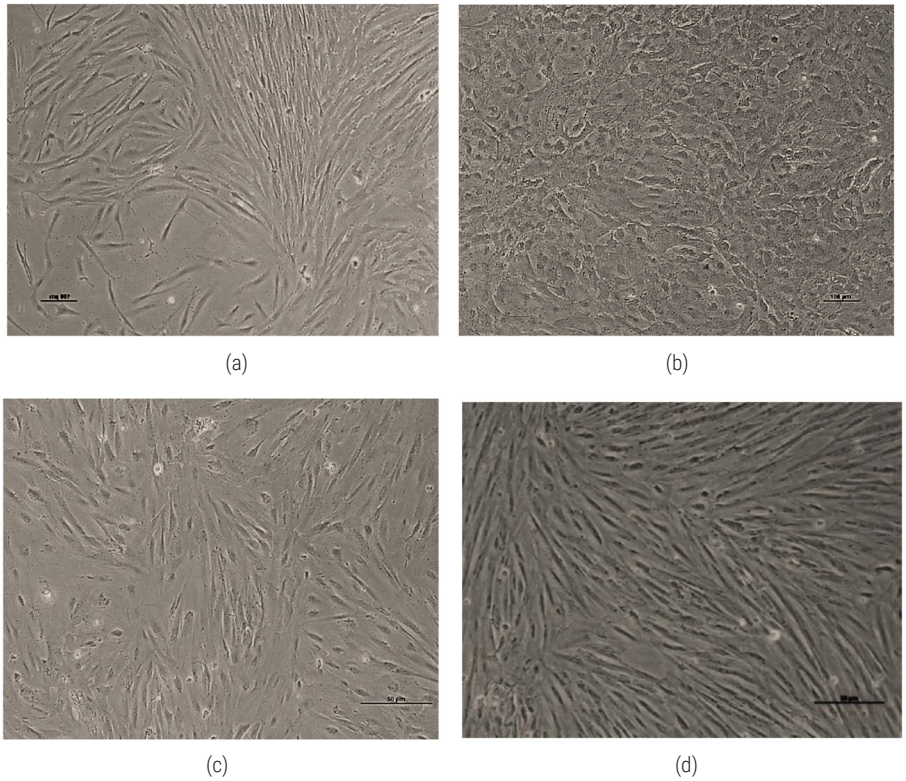
with media containing 10% FBS, which was reduced to 5% post-30 passages. The FBS supplementation in the growth medium was further reduced to 2% once the cells crossed 60 passages. As the cell passage progressed with routine subcultures, the fibroblast-like cells predominated in the monolayers (Fig. 1c and d). Several studies have successfully initiated primary cultures using the trypsinisation method (Sobhana *et al*., 2008, 2009, George *et al*., 2018). The bipolar and epithelial-like cells vanished after successive subculturing, leaving a comparatively homogeneous population of fibroblast-like cells. In the same way, Swaminathan *et al*. (2012) derived a fibroblast-like cell line (RTF) from the fin tissue of freshwater ornamental fish, *Puntius denisonii*. Related studies (Chi *et al*., 2005; Wen *et al*., 2008) also revealed the existence of cells that resembled fibroblasts and epithelial cells, in the cell line TB2 isolated from adult tilapia brain tissue and primary culture of BB cell line from the brain tissue of the barramundi, *L. calcarifer*. Earlier researchers reported (Hasoon *et al*., 2011; Lakra *et al*., 2011; Yan *et al*., 2011) that in fish cell cultures, fibroblastic cells predominate over epithelioid cells. This study thus established a new cell line designated as PB1BrTr from the brain tissue of maroon clownfish *P. biaculeatus* by trypsinisation method. To date, the newly developed brain cell line PB1BrTr has crossed 100 passages.

Storage in liquid nitrogen and revival

The newly established cell line was cryo-stored at different passage levels in liquid nitrogen (-196˚C) in L-15 medium containing FBS and DMSO. The viability of these stored cells was checked at various time intervals by rapid thawing at 42˚C. The cells showed 85-95% viability after revival and attained confluency within 2-4 days of seeding. No alterations in morphology or growth pattern were observed after cryopreservation. Lakra *et al*. (2011) observed a 75% revival rate for the PSCF cells after the storage period which retained the ability to attach, with no noticeable changes in morphology or growth pattern. Chang *et al*. (2001) revealed 80-85% average viability for cryostored SF cell line established from Asian seabass.

Plating and seeding efficiency of cells

The plating efficiency of the PB1BrTr cell line at passage 62 ranged from 14 to 23% at varying cell densities. Plating efficiency was 16% at a cell density of 4000 cells per well, whereas cells showed increased plating efficiency of 22 and 23% at 500 and 2000 cells per well, respectively, without any notable variations between replicates. The seeding efficiency in a 25 cm^2 tissue culture flask after 6 h for an initial inoculum density of 2 ml of 2.5 x 10^5 cells ml^{-1} was 50%. For 2 ml of 1.25 x 10^5 cells ml^{-1}, the seeding efficiency was 84% and for 3 x 10^4 cells ml^{-1} it was 66%. Transformation of the cell line or genotypic change is indicated by the high rates of cell division and plating efficiencies. Yashwanth *et al*. (2020) demonstrated a plating efficiency of 2% at a cell density of 1×10^3 cells in OCF cells at the 16th passage. Xu *et al*. (2019) reported plating efficiencies of 8-18% for CrCB and GFB cell lines at 200, 500, or 1000 cells per flask. Cell plating at a greater seeding density was found to be more effective compared to those at a lower density, as they encountered obstacles in replication.



(a) (b) (c) (d)

Fig. 1. Phase contrast images of PB1BrTr cells. (a) and (b) monolayers in primary culture showing epithelial-like and fibroblastic cells; Cell monolayers at (c) 56 p and (d) 86 p

Effect of temperature and FBS concentration on cell growth

The PB1BrTr cell line showed varying growth rates in response to temperature. Though substantial growth was seen at temperatures ranging from 20 to 32°C; 28°C was determined to be the ideal temperature for maximal growth (Fig. 2a). Similarly, in the case of BB, a new cell line derived from barramundi, *L. calcarifer* brain tissue, and also in PSCF cell line developed by Lakra *et al*. (2011), it was observed that 28°C was the ideal temperature for maximal growth, but able to grow at temperatures from 20 to 30°C (Chi *et al*., 2005), whereas the optimum temperature for maximum growth of the immortal cell line TB2 derived from adult tilapia brain tissue was 25°C.

When the FBS proportion increased from 2 to 20% at 28°C, the growth rate of PB1BrTr cells increased correspondingly. At early passages, the cells exhibited poor growth at 2 and 5% concentrations of FBS, a moderate increase at 10%, and the highest growth at 15 and 20% FBS concentrations. For later passages, PB1BrTr cells demonstrated good growth as the FBS concentration increased from 2 to 20%. However, 2% FBS concentration was found to be sufficient to support the growth of the cells (Fig. 2b). Hasoon *et al*. (2011); Yan *et al*. (2011) and Fu *et al*. (2015) reported similar results for FBS concentrations in growth media.

Chromosome study

PB1BrTr cell line at passage 72, revealed chromosome counts ranging from 45 to 65 per 100 metaphase plates. The modal number was 55 (Fig. 3a). Lai *et al*. (2003) reported different ranges of modal chromosome numbers ranging from 48 to 86 for the four different cell lines derived from the eye, fin, heart, and swim bladder of the grouper *Epinephelus awoara*. The same pattern of results of an asymmetric chromosomal distribution and aneuploidy were observed in a continuous cell line, CPB established from *Siniperca chuatsi* brain (Fu *et al*., 2015).

Authentication of cell line

The newly established brain cell line PB1BrTr was authenticated by mitochondrial CO1 gene sequence analysis. The amplified DNA extracted from *P. biaculeatus* muscle tissue and PB1BrTr cell line at passage 75, using PCR primers FISH F1 and FISH R1 yielded PCR products of 699 and 685 bp, respectively. The sequences showed >99% similarity to the mitochondrial CO1 sequences of *P. biaculeatus* available in NCBI GenBank. The CO1 gene sequences of both the cell line and its corresponding tissue, were submitted to the NCBI GenBank with the accession numbers OM131593 for tissue and OR290980 for the PB1BrTr cell line. Researchers have unequivocally displayed the utilisation of the mitochondrial gene, COI as a universal barcode, for the genetic recognition of animal life and have been successfully used for the authentication of fish cell lines (Lakra *et al*., 2011; Goswami *et al*., 2014; Yashwanth *et al*., 2020).

Documentation of cell morphology

Giemsa staining

The morphology of cells in the cell line monolayers was analysed by Giemsa staining under light microscopy. PB1BrTr cell monolayers

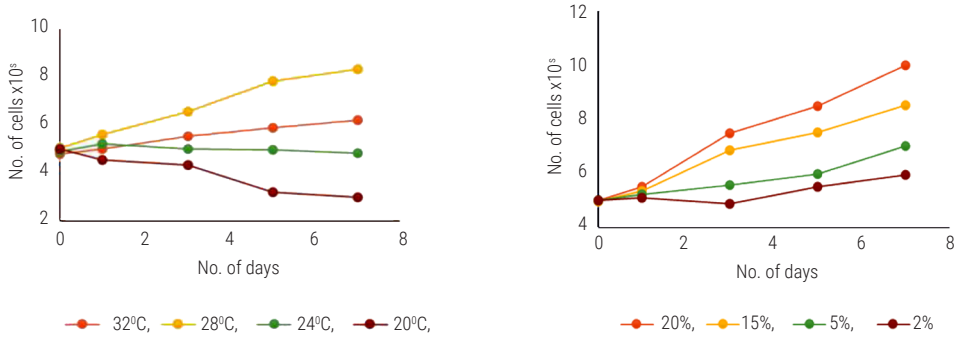


Fig. 2. Growth response of PB1BrTr cell line (at passage 70) to varying (a) temperatures and (b) foetal bovine serum concentrations

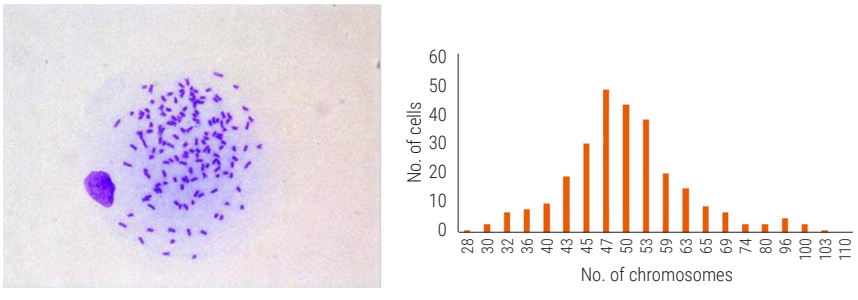


Fig. 3. (a) Chromosome spread (x 1000) and (b) Chromosome number distribution of PB1BrTr cell line (at passage 72)

at passage 76 stained with Giemsa appeared to have comparatively larger spindle shaped morphology, forming characteristic parallel arrays and whorls (Fig. 4). ASG-13 gill cell line derived from Atlantic salmon, cells showed a bipolar or multipolar elongated spindle shape suggesting a fibroblastic nature (Gjessing *et al*., 2018). Based on the density of the cells, the arrangement of the cells in the cell monolayers was found to be in random orientation. An indication of the fibroblastic nature of the cells is their characteristic spindle-like morphology, which grows attached to the surface in parallel arrays.

Immunofluorescence staining

PB1BrTr cell line was characterised by immunofluorescence staining using a fibroblast marker, mouse monoclonal anti-vimentin antibody and an epithelial marker, mouse monoclonal anti-cytokeratin, pan antibody. Cell monolayers at the 78th passage demonstrated a strong immunoreactivity to the antibodies against vimentin, a fibroblast marker and no immunoreactivity was observed to the epithelial marker, cytokeratin. These results, therefore, validate the cell line's fibroblastic nature as shown in Fig. 5. Goswami *et al*. (2014) reported similar results, being positive for vimentin and negative for cytokeratin in PCF cells derived from the caudal fin tissue of dark mahseer. Likely, the ASBB cell line derived from the brain tissue of Asian sea-bass and CPB a continuous cell line from *Siniperca chuatsi* were also composed of fibroblast-like cells (Hasoon *et al*., 2011; Fu *et al*., 2015).

Gene transfection

Green fluorescence signals were observed 36-48 h after the pcDNA3-EGFP plasmid was successfully transfected into PB1BrTr cells at passage 87 (Fig. 6). Moderate transfection efficiency was shown by this cell line using the transfection reagent lipofectamine 3000. The findings demonstrated that genetic manipulation of this cell line is feasible, which indicates potential for *in vitro* gene transfection, gene targeting and expression applications using commercially accessible transfection reagents. Researchers have reported on successful transfections using fish cell lines and the system can be used effectively as an *in vitro* tool for assessing promoter efficiency in different recombinant constructs and expression projects (Lai *et al*., 2003; Goswami *et al*., 2014; Fu *et al*., 2015; Yashwanth *et al*., 2020).

This newly established and characterised *P. biaculeatus* cell line PB1BrTr, derived from brain tissue, represents the first reported *in vitro* cell culture system from this species. This cell line may provide a valuable biological tool for future detection and diagnosis of marine fish viruses as well as for genetic manipulation and host-pathogen interaction studies.

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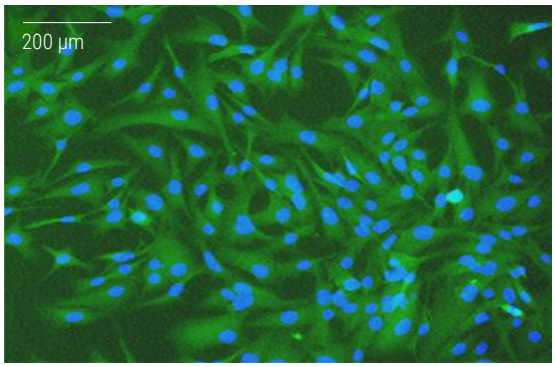


Fig. 5. PB1BrTr cell line (78 p) showing positive reactivity towards the fibroblast maker, Vimentin under fluorescence microscope (x400)

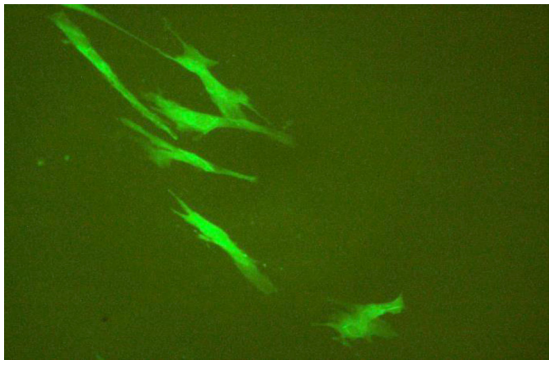
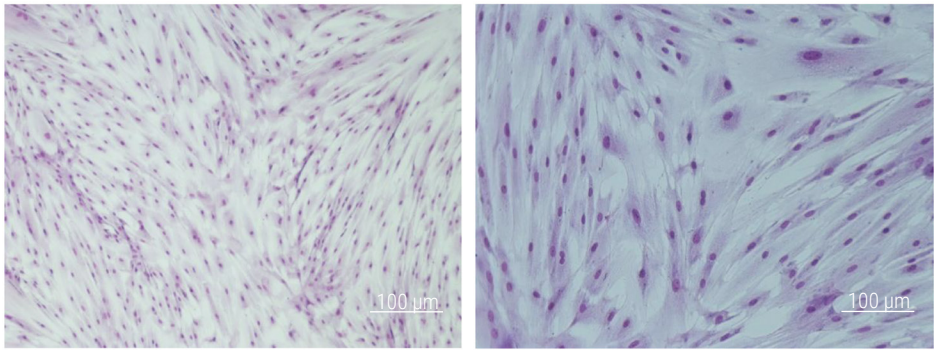


Fig. 6. GFP gene expression in PB1BrTr cell line (87 p) transfected with pcDNA3-EGFP (x 400)



(a) (b)

Fig. 4. Photomicrographs of Giemsa stained monolayers of PB1BrTr cell line (at passage 76). (a) x100 and (b) x200

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