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Development of Primary Cell Culture from Different Tissues of *Osteobrama belangeri*, a Near Threatened Fish Species of the North-Eastern Region of India

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ABSTRACT

Background: Primary cell culture closely matches the physiological and biochemical features of an in vivo system and it serves as a representative model for studying many key issues of disease, reproduction, genetics and biotechnology. The present study aimed to develop a primary culture system from the Gill, Liver and Kidney tissues of *Osteobrama belangeri*, which is an important candidate species for aquaculture.

Methods: The primary culture was established from tissues through the explant method. The cultured cells were maintained in Leibovitz's L-15 Medium supplemented with FBS (Fetal Bovine Serum) and Antibiotic Antimycotic Solution 100X Liquid. The growth optimization of cultured cells was performed at different incubation temperatures (25°C, 28°C, 30°C) and FBS concentrations (5%, 10% and 15%).

Result: The maximum growth rate of cultured gill cells was recorded at 30°C temperature and 15% FBS. The cultured cells were characterized for species authentication using DNA barcoding. The sequence analysis reveals and confirms the species of origin. This primary culture of different tissues provides a foundational *in vitro* platform for conservation genetics, disease diagnostics and biotechnological applications in *O. belangeri*.

Key words: Conservation biology, DNA barcoding, Explant method, *In vitro*, *Osteobrama belangeri*, Primary cell culture.

INTRODUCTION

The North Eastern Region of India harbours a rich and diverse aquatic ecosystem, supporting numerous fish species vital to regional biodiversity and the livelihoods of local communities. *Osteobrama belangeri* (Pengba), a Near Threatened fish species native to South and Southeast Asia, holds considerable ecological and economic importance (Deepti *et al*., 2023). Despite its significance, comprehensive studies on the cellular and molecular biology of Pengba remain scarce, limiting our understanding of its physiology and responses to environmental and biological stressors.

Establishing primary cell cultures from different tissues of Pengba provides a valuable approach for understanding its cellular physiology, host-pathogen interactions, toxicological responses and adaptive mechanisms. Primary cell culture offers a robust in vitro system that closely mimics in vivo conditions while allowing controlled experimental manipulation. Although fish cell cultures have been derived from various tissues, fin-derived cultures are most commonly reported because of their high regenerative capacity (Yashwanth, 2019). However, gill, liver and kidney-derived cultures offer distinct advantages because these organs are central to respiration, metabolism, osmoregulation, detoxification and immune function.

Gill, liver and kidney tissues are particularly relevant for cellular studies in threatened freshwater fishes. Gills serve as the primary interface with the environment and play key roles in respiration, osmoregulation and pathogen entry (Pampori *et al*., 2024), while the liver and kidney are critical for detoxification, metabolism and immune regulation. Consequently, primary cell cultures from these tissues enable tissue-specific investigations relevant to environmental stress, disease diagnostics and conservation physiology (Kamble *et al*., 2023). Fish cell culture systems differ markedly from mammalian models, particularly in terms of incubation temperature and osmotic requirements. Fish cells tolerate a wider temperature range and exhibit

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lower metabolic rates, enabling prolonged maintenance with minimal intervention (Ern *et al*., 2023). Consequently, fish cell cultures are widely used for virus isolation and for investigating tissue-and species-specific host-pathogen interactions (Gardenia *et al*., 2020). Intensification of aquaculture practices, including high stocking densities and uncontrolled inputs, has increased the incidence of viral diseases in cultured carps, often leading to mass mortalities (Nashiruddullah *et al*., 2021). In this context, well-characterised fish cell cultures are essential tools for advancing research in cellular physiology, immunology, molecular biology and aquaculture biotechnology (Hightower and Renfro, 1988).

This study reports the establishment and characterisation of primary cell cultures derived from gill, liver and kidney tissues of *Osteobrama belangeri*. These cultures provide a valuable platform for future applications in disease diagnostics, environmental toxicology, germplasm conservation and sustainable aquaculture management, contributing to species-specific conservation and comparative fish biology research.

MATERIALS AND METHODS

Maintenance of live fish and collection of tissue samples

The study was conducted at the Cell Culture Facility of the National Repository of Fish Cell Lines, ICAR-NBFGR, Lucknow and the Department of Fish Genetics and Reproduction, College of Fisheries, CAU(I), Lembucherra, Tripura. Six live and healthy Pengba (*Osteobrama belangeri*; 10-15 g) were acclimatised in a 200-L FRP tank with sterile, aerated water for one week. Before tissue collection, six donor fish were fasted for 24 h, anaesthetised with clove oil and externally disinfected with 70% ethanol. Gill, liver and kidney tissues were aseptically excised, rinsed thrice in 1 mL of phosphate-buffered saline (PBS) containing Antibiotic-Antimycotic solution (500 µg/mL streptomycin, 500 IU/mL penicillin and 2.5 µg/mL fungizone) and further cut into 1-2 mm^3 explants using sterile scissors, followed by two additional PBS washes.

Initiation and maintenance of primary culture

Primary cell cultures were established from gill, liver and kidney tissues using the explant culture method under aseptic conditions. For each tissue, 15-20 explants were prepared and seeded to establish a primary culture. Explants were placed in T-25 (25 cm^2) culture flasks and allowed to attach undisturbed. Afterwards, 50 µL of fetal bovine serum (FBS) (Gibco, USA) was gently added to enhance adhesion. Cultures were incubated at 25, 28 and 30°C with three FBS concentrations (5%, 10% and 15%). After 24 h, 4 mL of Leibovitz's L-15 (HiMedia, India) medium supplemented with the respective FBS concentrations and Antibiotic-Antimycotic solution (500 µg/mL streptomycin, 500 IU/mL penicillin and 2.5 µg/mL fungizone) was added. Cultures were maintained under static conditions, with 50% medium replacement every 3-5 days until a confluent monolayer developed.

Growth evaluation

Cell growth was assessed under varying incubation temperatures (25, 28 and 30°C) and FBS concentrations (5%, 10% and 15%) to determine optimal conditions for primary cell proliferation. Cultures were maintained in L-15 medium until a confluent monolayer formed and cellular morphology, attachment and proliferation were routinely monitored using an inverted phase-contrast microscope (Nikon TS100, Japan). At confluency, adherent cells were detached using 1× trypsin-EDTA solution (Himedia, India) (0.25% trypsin, 0.001% EDTA in PBS) and viable cells were quantified with a haemocytometer. Total cell numbers were calculated as the mean count across chambers multiplied by the dilution and correction factors (10^5).

Cell characterisation by DNA barcoding

Genomic DNA was isolated from cultured cells and fin tissue of *Osteobrama belangeri* using the Sambrook and Russell (2001) method. Species identity was confirmed by PCR amplification of the mitochondrial cytochrome c oxidase subunit I (COI) gene using universal fish primers FishF1 and FishR1 (Ward *et al*., 2005). PCR was performed in a 15 µL reaction containing dNTPs (2.5 mM each), Primer (10 мM) and Taq. Polymerase (0.75 U), Template DNA (100 ng/µl). The cycling conditions included initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation (94°C, 30 s), annealing (54°C, 30 s) and extension (72°C, 1 min), with a final extension at 72°C for 7 min. Amplicons were resolved on 2.0% agarose gels, visualised using a gel documentation system and sequenced on an ABI 3700 automated sequencer (Fig 1). Sequence assembly and editing were performed using BioEdit and species confirmation was achieved using BLASTn. The validated COI sequence was deposited in NCBI GenBank.

Statistical analysis

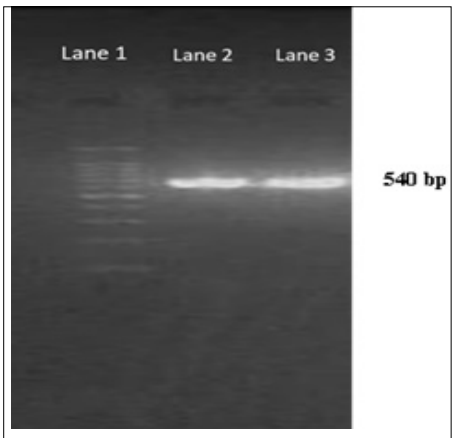
All experiments were conducted using a completely randomised factorial design in triplicate (n = 3). Data were expressed as mean ± standard error (SE). Statistical analysis was performed using MS Excel and SPSS software (version 23.0). Cell proliferation data (cell counts) were expressed as mean ± standard error (SE). For each tissue type (gill, liver and kidney), the effects of temperature (25, 28 and 30°C) and fetal bovine serum (FBS) concentration (5%, 10% and 15%) on cell proliferation were analyzed separately using two-way analysis of variance (ANOVA), with temperature and FBS concentration treated as fixed factors. The interaction effect between temperature and FBS concentration was also evaluated. When significant main or interaction effects were detected ($p<0.05$), post hoc multiple comparison tests were performed using Tukey's honestly significant difference (HSD) test to identify statistically significant differences among individual treatment means. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Development of primary cultures from gill, liver and kidney tissue

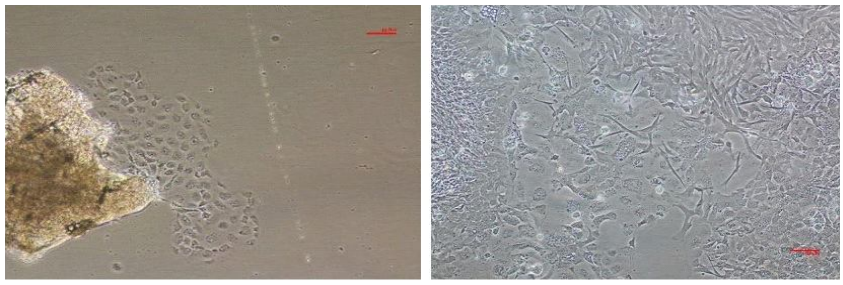
The present study successfully established primary cell cultures from gill, liver and kidney tissues of *O. belangeri*, a Near Threatened freshwater fish species of the North Eastern Region of India, using the explant method. In the case of gill tissue, approximately 75% of explants successfully attached and proliferated. The explants adhered firmly to the culture flask surface within 24 h of initiation. Cellular outgrowth from the margins of the explants was observed after approximately 36 h (Fig 2a) and a confluent monolayer was formed within 10 days (Fig 2b). For liver tissue, approximately 86% of explants successfully developed primary cultures. Attachment of explants occurred within 24 h and the initiation of cellular outgrowth was observed after 120 h (Fig 3a). A confluent monolayer was achieved after 12 days of culture (Fig 3b). Similarly, kidney tissue explants exhibited a successful attachment rate of approximately 71% within 24 h of seeding. Cellular outgrowth from the explant edges was observed after approximately 72 h and a confluent monolayer was formed after 12 days of incubation (Fig 4a, b).

Microscopic examination revealed all cultures contained a heterogeneous population of epithelial and fibroblast-like cells, with fibroblastic cells predominating (approximately 70-80%). A similar predominance has been widely reported in fish primary cultures and cell line development (Bejar *et al*., 1997; Lai *et al*., 2003; Lakra *et al*., 2006; Babu *et al*., 2011; Lakra and Goswami, 2011; Goswami *et al*., 2012), likely reflecting the faster attachment



Lane 1: 100 bp DNA ladder; Lane 2: Genomic DNA isolated from cultured cells; Lane 3: Genomic DNA isolated from fish tissue.

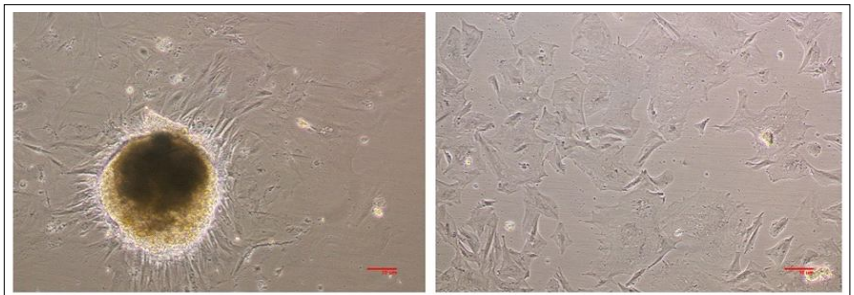
Fig 1: Agarose gel electrophoresis showing amplified COI gene fragment.



(a) **(b)**

(a) Radiation of cells started after 36 hours of explant preparation (10X).
(b) Confluent monolayer after 10 days of explant preparation (10X).

Fig 2: Primary cell culture development from the gill tissue of *Osteobrama belangeri*.



(a) **(b)**

(a) Radiation of cells started after 120 hr of explant preparation (10X).
(b) Confluent monolayer after 12 days of explant preparation (10X).

Fig 3: Primary cell culture development from liver tissue of *Osteobrama belangeri*.

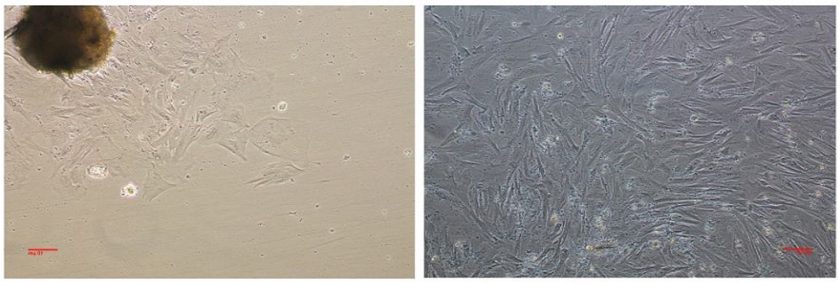
and proliferation of fibroblast cells under serum-supplemented conditions, whereas epithelial cells generally require more specific growth factors and proliferate more slowly in vitro (Bols *et al*., 2005). The explant technique offers advantages over enzymatic dissociation, including procedural simplicity, better preservation of cell-cell interactions and reduced cellular damage (Avella *et al*., 1994; Tyagi and Mani, 2023). Consistent with the present findings, earlier studies have reported efficient attachment and culture establishment using explant methods in teleosts and shellfish, such as *Cirrhinus mrigala* (Nanda *et al*., 2014), *Labeo calbasu* (Yadav *et al*., 2022) and other aquatic species (Toullec *et al*., 1996; Rathore *et al*., 2007; Wick *et al*., 2018; Khurshid *et al*., 2022).

Growth studies

Cell growth at different temperatures and FBS concentrations

Microscopic examination revealed tissue-specific growth responses in primary explant cultures derived from gill, liver and kidney tissues of *O. belangeri* under different incubation temperatures (25, 28 and 30°C) and fetal bovine serum (FBS) concentrations (5, 10 and 15%). Cell attachment and proliferation were significantly influenced by tissue type, temperature, serum concentration and their interactions ($p<0.05$).

Gill-derived cultures exhibited significantly higher growth at 30°C, followed by 28°C and 25°C, indicating a higher thermal optimum for gill cells. In contrast, liver and kidney cultures showed maximum proliferation at 28°C, with reduced growth at 30°C and 25°C (Fig 5-7). These findings suggest tissue-specific thermal preferences, likely reflecting differences in metabolic activity and physiological roles of individual tissues. Similar temperature-dependent growth patterns have been reported in other fish cell cultures, where optimal growth typically occurs between 24 and 32°C depending on species and tissue origin (Wolf, 1973; Tong *et al*., 1997; Lakra *et al*., 2006; Goswami *et al*., 2012; Dubey *et al*., 2014; Han *et al*., 2022). The observed variation in optimal temperature is consistent with reports that cells derived from warm-water fishes generally grow well between 25 and 30°C, whereas cold-water species



(a) (b)

(a) Radiation of cells started after 72 hr of explant preparation (10X).
(b) Confluent monolayer after 12 days of explant preparation (10X).

Fig 4: Primary cell culture development from kidney tissue of *Osteobrama belangeri*.

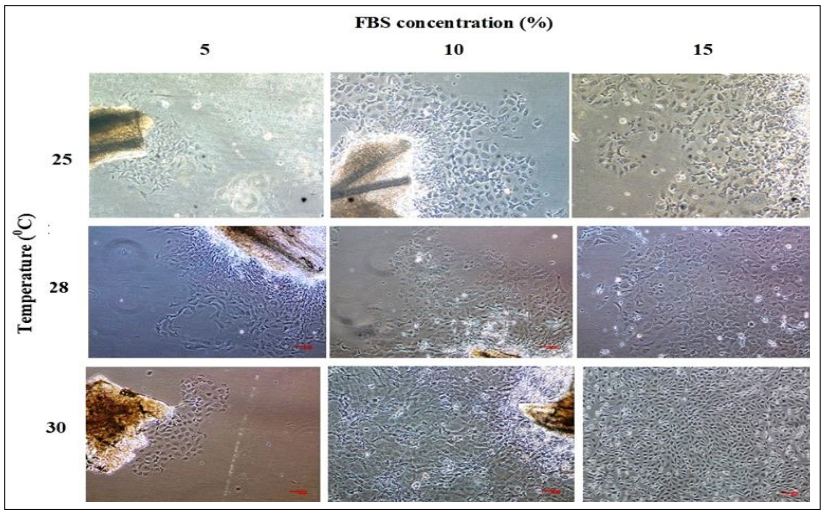


Fig 5: Cell growth of gill tissue at different temperatures (25°C, 28°C and 30°C) and FBS concentration (5, 10 and 15%) observed under inverted microscope (10X).

exhibit lower optima (Gabridge, 1985; Goswami *et al*., 2013; Murali *et al*., 2020).

Serum concentration also significantly influenced cell proliferation across all tissues. Gill cultures showed maximum growth at 15% FBS combined with 30°C, whereas liver and kidney cultures exhibited optimal proliferation at 15% FBS and 28°C. In all cases, growth at 15% FBS was significantly higher than at 10% and 5% FBS (Fig 8-10). This trend highlights the critical role of serum supplementation during the early stages of primary culture establishment. FBS provides essential growth factors, hormones, lipids, attachment-promoting proteins and anti-trypsin activity, which collectively enhance cell adhesion and proliferation (Freshney, 2005). Comparable serum-dependent growth responses have been widely documented in fish primary cultures and cell lines (Bols *et al*., 1994; Freshney, 2005; Hameed *et al*., 2006; Babu *et al*., 2011).

The requirement of higher serum concentrations (15-20%) during initial culture establishment has been widely reported in fish cell culture studies (Bols *et al*., 1994; Hameed *et al*., 2006; Babu *et al*., 2011; Beaulah *et al*., 2017). Similar observations of maximum growth at 15% FBS have been documented in fin and organ-derived fish cell lines, including *Clarias batrachu*s and other teleost species (Babu *et al*., 2011; Lakra *et al*., 2010). The present findings further support that serum requirements and thermal optima are both tissue- and species-specific, emphasising the importance of optimising culture conditions for each tissue type.

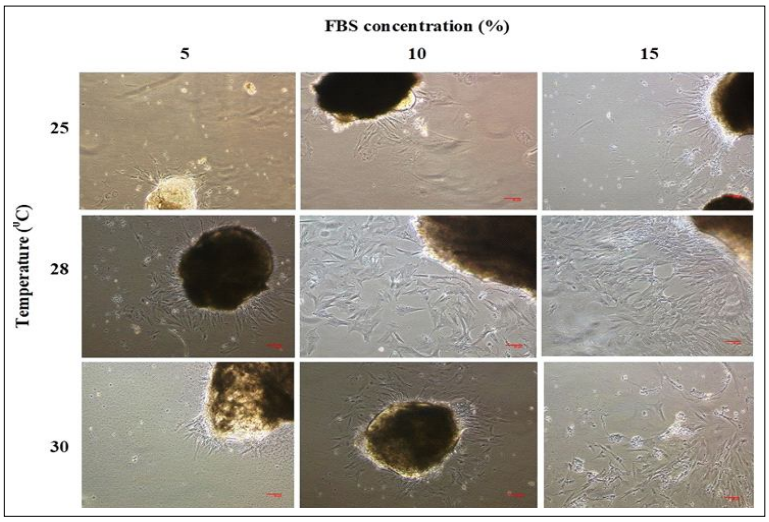


Fig 6: Cell growth of liver tissue at different temperatures (25°C, 28°C and 30°C) and FBS concentration (5, 10 and 15%) observed under inverted microscope (10X).

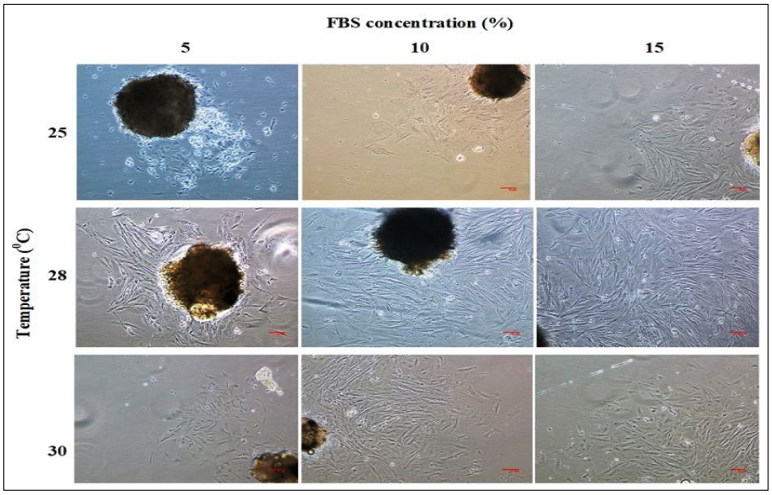


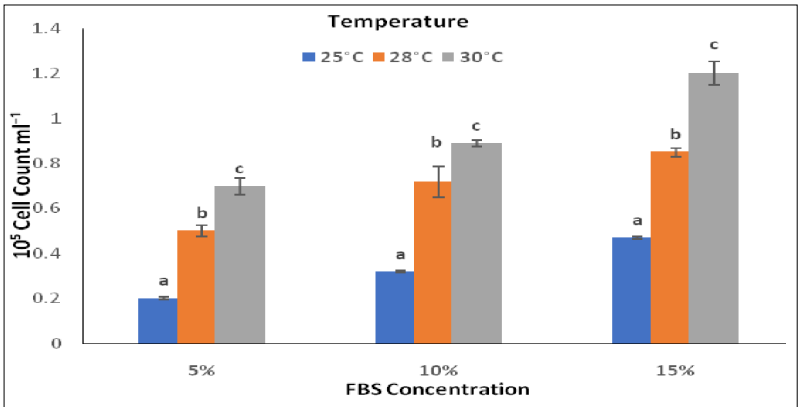
Fig 7: Cell growth of kidney tissue at different temperatures (25°C, 28°C and 30°C) and FBS concentration (5, 10 and 15%) observed under inverted microscope (10X).

Cell counting

Cell counts obtained using a haemocytometer were consistent with microscopic observations and revealed distinct tissue-specific growth responses under varying temperature and FBS conditions (Table 1). Gill-derived cultures exhibited a marked increase in cell density with rising temperature and serum concentration, reaching a maximum of 1.20×10^5 cells ml^{-1} at 15% FBS and 30°C. In contrast, liver and kidney cultures showed optimal proliferation at 28°C with 15% FBS, attaining peak densities of 0.76×10^5 and 0.90×10^5 cells ml^{-1}, respectively.

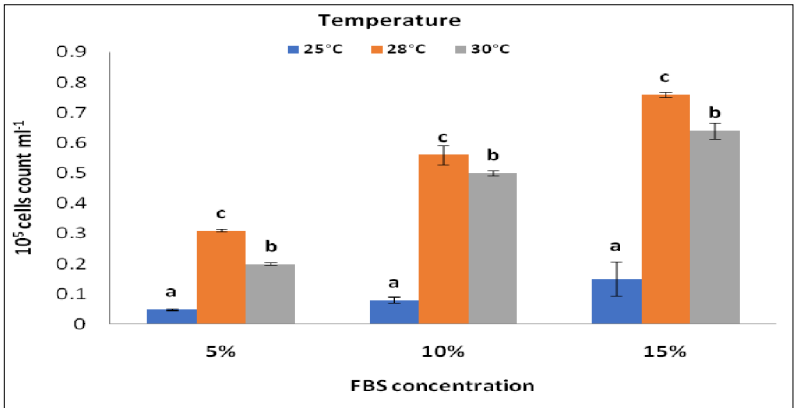
Two-way ANOVA revealed that temperature and FBS concentration had significant effects on cell proliferation in gill-derived cultures and a significant temperature × FBS interaction (Table 2). Gill cells exhibited maximum proliferation at 30°C, particularly at 15% FBS, whereas significantly lower cell counts were recorded at 25°C across all serum concentrations. Post hoc comparisons further confirmed that each temperature and FBS level differed significantly from one another, indicating a strong temperature-dependent growth response in gill tissue.

In liver-derived cultures, both temperature and FBS concentration significantly influenced cell growth, with a significant temperature×FBS interaction (Table 3). Maximum proliferation was observed at 28°C combined with 15% FBS, while growth declined at 30°C despite higher serum



Bar graphs represent cell counts (cells ×10 u ml^{-1}) at different incubation temperatures (25°C, 28°C and 30°C) and FBS concentrations (5%, 10% and 15%). Values are expressed as mean±SE (n = 3). Different letters above the bars indicate statistically significant differences among treatment groups (two-way ANOVA, $p<0.05$).

Fig 8: Quantitative analysis of cell growth in gill tissue-derived primary cultures of *Osteobrama belangeri* under different temperature and FBS conditions.



Bar graphs represent cell counts (cells ×10 u ml^{-1}) at different incubation temperatures (25°C, 28°C and 30°C) and FBS concentrations (5%, 10% and 15%). Values are expressed as mean±SE (n = 3). Different letters above the bars indicate statistically significant differences among treatment groups (two-way ANOVA, $p<0.05$).

Fig 9: Quantitative analysis of cell growth in liver tissue-derived primary cultures of *Osteobrama belangeri* under different temperature and FBS conditions.

supplementation. These findings indicate a narrower optimal thermal range for liver cells compared to gill cells.

Similarly, kidney-derived cultures showed significant effects of temperature, FBS concentration and their interaction on cell proliferation (Table 4). Optimal growth occurred at 28°C with 15% FBS, whereas both lower (25°C) and higher (30°C) temperatures resulted in significantly reduced cell counts. The moderate interaction effect suggests that kidney cells are particularly sensitive to deviations from optimal culture conditions.

These differential responses highlight inherent tissue-specific metabolic and physiological adaptations, wherein gill tissue, being directly exposed to environmental fluctuations, exhibits greater tolerance to elevated temperatures, while internal organs such as liver and kidney maintain narrower thermal growth ranges. Similar tissue-dependent temperature preferences have been reported in teleost primary cell culture systems (Wolf, 1973; Tong *et al*., 1997; Lakra *et al*., 2006; Goswami *et al*., 2012; de Almeida *et al*., 2022; Lahnsteiner, 2024).

Molecular characterization for species authentication

The origin of the cultured cells was confirmed using DNA barcoding based on the mitochondrial cytochrome c oxidase subunit I (COI) gene. The amplified COI fragment (~540 bp) was sequenced and deposited in the NCBI GenBank under accession number OR539611. Sequence homology analysis revealed >99% similarity with reference *Osteobrama belangeri* COI sequences available in GenBank, thereby confirming the species authenticity of the cultured cells.

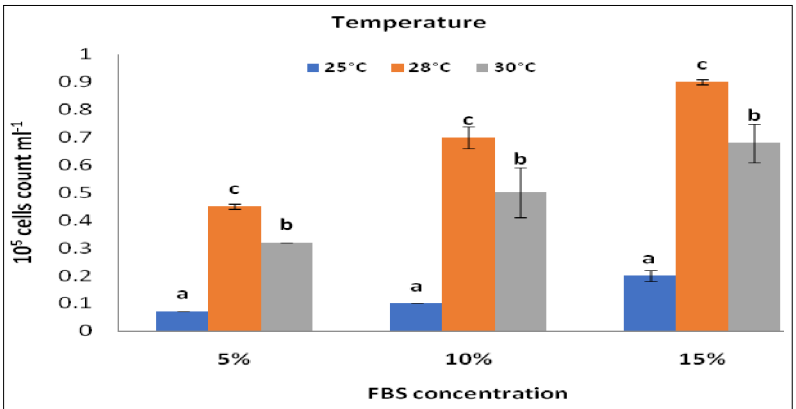
Species authentication is an essential quality control step in cell culture-based studies, particularly in conservation and biodiversity research, as it prevents misidentification and cross-contamination (Petit-Marty *et al*., 2021). The COI gene is widely recognised as a universal DNA barcode for animal identification (Hebert *et al*., 2003) and has been extensively used to authenticate fish cell lines and primary cultures (Cooper *et al*., 2007; Barman *et al*., 2014; Yashwanth *et al*., 2020). The standardised COI barcode region, typically amplified using universal primers, has proven effective for reliable species-level identification across diverse teleost taxa, including cultured fish cells.

The successful establishment of primary cell cultures from multiple tissues of *O. belangeri* provides a validated in vitro system with broad applicability in conservation genetics, environmental toxicology, disease diagnostics, viral susceptibility studies and germplasm conservation. The availability of gill, liver and kidney-derived cultures offers a controlled platform for tissue-specific investigations of cellular physiology, stress responses, immune function and host-pathogen interactions that are difficult to elucidate solely in vivo. In view of the species' increasing vulnerability to habitat degradation, climate variability and emerging diseases, these cultures constitute a non-lethal, sustainable tool for conservation-oriented research.

Table 1: Effect of temperature and fetal bovine serum (FBS) concentration on cell proliferation in primary cultures derived from gill, liver and kidney tissues of *Osteobrama belangeri*.

Temperature	Gill (5% FBS)	Gill (10% FBS)	Gill (15% FBS)	Liver (5% FBS)	Liver (10% FBS)	Liver (15% FBS)	Kidney (5% FBS)	Kidney (10% FBS)	Kidney (15% FBS)
25°C	0.20±0.00^{a}	0.32±0.00^{b}	0.47±0.00^{c}	0.05±0.00^{a}	0.08±0.00^{b}	0.15±0.00^{c}	0.07±0.00^{a}	0.10±0.00^{b}	0.20±0.02^{c}
28°C	0.50±0.02^{a}	0.72±0.06^{b}	0.85±0.01^{c}	0.31±0.01^{a}	0.56±0.03^{b}	0.76±0.00^{c}	0.45±0.01^{a}	0.70±0.04^{b}	0.90±0.01^{c}
30°C	0.70±0.03^{a}	0.89±0.01^{b}	1.20±0.05^{c}	0.20±0.05^{a}	0.50±0.01^{b}	0.64±0.02^{c}	0.32±0.00^{a}	0.50±0.09^{b}	0.68±0.07^{c}

This study thus establishes a foundational framework for the future development of continuous cell lines. It highlights the importance of species-specific cell culture systems for long-term applications in conservation biology, disease management and aquaculture biotechnology of *O. belangeri*. Nonetheless, long-term subculture stability and detailed characterisation of the heterogeneous cell populations were not assessed. Future work should prioritise the development and characterisation of continuous cell lines to support immunocytochemical profiling, cryopreservation and advanced applications in aquaculture and conservation research.



Bar graphs represent cell counts (cells ×10 u ml^{-1}) at different incubation temperatures (25°C, 28°C and 30°C) and FBS concentrations (5%, 10% and 15%). Values are expressed as mean±SE (n = 3). Different letters above the bars indicate statistically significant differences among treatment groups (two-way ANOVA, $p<0.05$).

Fig 10: Quantitative analysis of cell growth in kidney tissue-derived primary cultures of *Osteobrama belangeri* under different temperature and FBS conditions.

Table 2: Two-way ANOVA showing the effects of Temperature and FBS concentration on cell number for gill tissue.

Source variation	df	Sum of squares	Mean square	F-value	p-value
Temperature (°C)	2	16022390084.22	8011195042	7364.32	<0.001
FBS concentration (%)	2	6087808294.89	3043904147	2798.12	<0.001
Temperature × FBS concentration	4	426672406.22	106668101.60	98.06	<0.001
Error	18	19581103.33	1087839.07		
Total	26	22556451888.67			

Table 3: Two-way ANOVA showing the effects of Temperature and FBS concentration on cell number for liver tissue.

Source variation	df	Sum of squares	Mean square	F-value	p-value
Temperature (°C)	2	9959743579	4979871789.37	11,774.215	<0.001
FBS concentration (%)	2	5009733163	2504866581.60	5,922.409	<0.001
Temperature × FBS concentration	4	1290122830	322530707.54	762.579	<0.001
Error	18	7613050.67	422947.26		
Total	26	16267212622.74			

Table 4: Two-way ANOVA showing the effects of temperature and FBS concentration on cell number for kidney tissue.

Source variation	df	Sum of squares	Mean square	F-value	p-value
Temperature (°C)	2	5868383133	2934191567	9.93	0.001
FBS concentration (%)	2	6640681636	3320340818	11.23	<0.001
Temperature × FBS concentration	4	4896391941	1224097985	4.14	0.015
Error	18	5321293243	295627402.4		
Total	26	22726749953			

CONCLUSION

The present study successfully established primary cell cultures from gill, liver and kidney tissues of *Osteobrama belangeri* and defined tissue-specific optimal growth conditions. Gill-derived cultures exhibited maximal proliferation at 30°C, whereas liver and kidney cultures showed significantly higher growth at 28°C. Among the serum levels tested, 15% fetal bovine serum consistently supported the most significant proliferation across all tissues, followed by 10% and 5%. Species authentication of the cultured cells was verified by DNA barcoding of the mitochondrial cytochrome c oxidase subunit I (COI) gene, which showed >99% sequence identity with reference *O. belangeri* entries in the NCBI database, thereby confirming the species origin and reliability of the culture system. This work provides the first tissue-specific in vitro cellular resource for *O. belangeri*, a Near Threatened freshwater fish of conservation concern. The authenticated primary cultures offer a non-lethal, sustainable platform for investigating species-specific cellular physiology, thermal tolerance, toxicological responses, disease susceptibility and host-pathogen interactions without imposing additional pressure on wild stocks. Moreover, these cultures constitute a critical foundation for the development of continuous cell lines, enabling long-term applications in conservation biology, disease management and aquaculture biotechnology for *O. belangeri*.

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Disclaimers

The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Data availability

The data generated during the current study will be available upon request.

Informed consent

All the experiments were conducted following the animal ethics guidelines and the study was approved by the Institutional Animal Ethics Committee (IAEC) of ICAR-NBFGR, Lucknow and College of Fisheries (Central Agricultural University, Imphal), India.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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