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Establishment and Molecular Characterization of a Liver-derived Cell line from *Schizothorax esocinus* (Chirruh) for Cold Water Fish Research

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Authors' contributions

This work was carried out in collaboration among all authors. Author KK conceptualized the study and wrote the original draft of the manuscript. Author TAT reviewed and edited the manuscript and provide final approval to publish. Author FAS performed the methodology and did visualization. Author SSNQ did visualization. Author IQ did data analysis and interpretation. Author BAB supervised the work. Author AA reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Schizothorax esocinus, commonly known as Chirruh, is an important indigenous coldwater fish of the Kashmir. The new cell line was developed from the liver tissue of *Schizothorax esocinus*, and was characterized by molecular technique. The developed cell line was maintained and passaged 14 times in Leibovitz's L-15 medium. The cell line was standardized using L-15 media by testing at various FBS (fetal bovine serum) concentrations and temperature ranges. Cell numbers increased more rapidly in 20% FBS, compared to other concentrations ranging from 5% to 25%. The tissue got fully adhered after 72hrs when seeded at 24°C as compared to other temperatures ranging from 8 to 28°C. The tissue preparation approach was more reliable than the enzymatic dissociation approach. Cell colonies of various sizes were formed after 1 week and 60-70% confluent cell monolayer were achieved after 20 days. The cell morphology was found to be of epithelial-like cells. The origin of the cell line was confirmed at passage 12 by the amplification of 629 bp of cytochrome oxidase subunit I (COI) similar to the original liver issue. The current cell line developed from liver tissue of *S. esocinus* has tremendous potential to enhance the generation of cold-water fish cell lines could and potentially be used as a important approach for conservation genetics and biotechnological applications.

Keywords: In-vitro; Schizothorax esocinus; cell line; fetal bovine serum; cytochrome oxidase subunit I.

1. INTRODUCTION

India's cold water fishing resources include lakes, rivers, streams, tributaries, and reservoirs that are dammed over high and mid-altitude rivers (Ayyappan et al., 2014). There are around 22 species of fish in Kashmir water bodies, including exotic carp (*Cyprinus carpio*) and native schizothoracids (*Schizothorax spp*.). Both *C. carpio* var. *communis* and *C. carpio* var. *specularis* are phenotypes that are representative of *C. carpio*. The primary indigenous group in the valley with significant commercial value is of Schizothoracids (Shah & Balkhi 2020). *Schizothorax esocinus* (Heckel, 1838) is a species belonging to family Cyprinidae. It is primarily found in mountain streams, rivers fed by snow, and rivers with gravel bottoms. In Kashmir, this fish is locally referred as "Chirruh." Its silvery body is speckled with many tiny, asymmetrical black dots on its sides and back. A variety of related stresses, including as overcrowding, frequent handling, grading, and declining water quality, have a detrimental effect on the health of cultured fishes as a result of efforts to produce more fish per unit volume of water (Khati et al., 2018). Given the available literature, no cell line has been developed so far from *S. esocinus* creating immense necessity for establishment of stable cell line for pathological and virological studies which can also serve as an *In-vitro* model for other schizothoracids.

With the establishment of the RTG-2 cell line from rainbow trout gonads, fish cell line research achieved diagnostic significance for the first time (Wolf & Quimby 1962). Numerous tissues, including the ovary, fin, kidney, swim bladder, heart, spleen, liver, eye muscle, vertebrae, brain, and skin, have been used to develop teleost fish cell lines (Thangaraj et al., 2021). According to Bairoch's (2018) Cellosaurus database, 944 fish cell lines have been developed originating from 211 species and hybrids as of 28 February 2024 (Friesen et al. 2024). Cell lines are one of the most commonly used *in-vitro* techniques for virology, toxicology, carcinogenesis, and transgenic studies. Fish cell lines have become increasingly relevant model systems in embryology, neuroscience, endocrinology, and environmental biology (Qayoom et al., 2024; Hafeez et al., 2022; Braga et al. 2006). In past years, researchers have shown a strong research interest in piscine primary cell cultures because of the wider incubation temperature range, easy handling and sustenance of cells for longer periods in contrast to the mammalian cell lines (Goswami & Lakra 2012). The recently developed RTgutGC cell line on the permeable

membranes from rainbow trout (*Oncorhynchus mykiss*) form the basis for epithelial barrier model of the fish intestine (Drieschner et al., 2019).

In recent years, cell lines from aquatic animals have attracted considerable attention as a means of speeding up the disease diagnosis. Fish cell lines can be cultured in two-dimensional (2D) or three-dimensional (3D) culture systems, and they can be sourced from primary cell cultures or commercial cell lines (Nguyen et al., 2024). These cell lines allow for in-depth research on how cells react to different stimuli since they preserve a large number of the original tissue's genetic and metabolic traits (Freshney 2015).

2. MATERIALS AND METHODS

The present study was conducted at Cell culture lab of Division of Aquatic Animal Health Management, Faculty of Fisheries, SKUAST-K. The following methodology was used to accomplish the study goals:

2.1 Primary Cell Culture and Subculture

Healthy live fingerlings of *S. esocinus* weighing about 50±1gm were taken from fishermen of river Jhelum near Shadipora. The collected live fish specimens were transported to the cell culture facility of AAHM division and were acclimatized in the freshwater aquarium tanks having temperature of 18℃ and fitted with continuous aeration, where one-third of the water was changed every alternate day. The donor fish *S. esocinus* was starved for one or two days to minimize the chance of contamination from excrement and regurgitated food (Khurshid et al., 2024). For the purpose of lowering the microbial contamination from the skin and gills, fish were let to swim in well-aerated, autoclaved water added with 1ml of antibiotic-antimycotic solution (Himedia) containing 100 IU/ml penicillin and 1000µg/ml streptomycin per 100ml of water for 24 hours. The acclimatized fish samples were dissected aseptically by euthanization using ice for 5-10 min and wiped with 70% ethanol to remove the liver weighing about 1.6gm. Then dissected organ was washed twice with phosphate buffer saline 1X (PBS, pH-7.4) (Gibco) containing 30µl of antibiotic-antimycotic solution followed by one wash with Leibovitz L-15 (SLCM8709 Sigma, Life Science) medium without L-glutamine. After washing, the tissue was chopped and diffused into small pieces called explants of 1mm size using sterile scissors. Then the chopped tissue was seeded into tissue culture flask of 25cm² (Nunclon, Delta Surface) containing 200µl fetal bovine serum (FBS, Qualified, Gibco), and then the flasks were kept in a vertical position for 2hr to allow the culture to attach to the surface of flask. Later, 3ml of Leibovitz L-15 medium supplemented with different range of FBS concentration (5-20%) and 20µl of antibiotic- antimycotic solution were added to the flask and incubated at 8ºC to 28ºC in CO_2 incubator for growth (Kumar et al., 2020). The culture flasks were observed daily for the attachment of explants, proliferation and spreading of cells with the help of inverted microscope. Live cells adhere to the substrate and multiply effectively, but dead cells were unable to attach the substrate of the flask and hence float. During this period half the medium was changed every 2-3 days. The dead cells were removed during the subsequent medium exchange.

Once attaining 70-80% confluency, the subculture was done at a ratio of 1:2 according to standard trypsinization method using 0.25% trypsin-EDTA (Ethylenediaminetetraacetic acid) solution (Himedia). The cells were resuspended in L-15 growth medium with 5-20% FBS. Following the dividing of the flasks, no antibiotics were administered, and the passage number was always noted (Guo et al., (2015).

2.2 Growth Studies

In order to estimate the ideal conditions required for cell growth and maintenance, cells were grown at distinct temperatures and FBS concentrations in L-15 media. The flasks were incubated at 8, 12, 16, 20, 24, 28ºC for 15 days at seeding concentration of 1×10^5 cells in 25cm² tissue culture flasks to ascertain the optimum temperature for cell growth. Flasks at each temperature were trypsinized and cell density was measured using hemocytometer (Majeed et al., 2013). The effects of various concentrations of FBS (5, 10, 15, 20, 25%) on cell growth at 24ºC for 7 days were estimated by carrying same standard procedure as for temperature (Meena et al., 2020).

2.3 DNA Isolation

DNA was extracted from liver cells of *S. esocinus* at passage 12 according to the method followed by Majeed et al. (2013). Briefly, after trypsinising and homogenizing the samples independently in sodium chloride-tris-EDTA (NTE) buffer (0.2M NaCl, 0.02M Tris-HCl, and 0.02 M EDTA, pH 7.4) and centrifuging them at 3000 g at 4℃, the supernatants were put in new centrifuge tubes

Fig. 1. Map location of sampling site

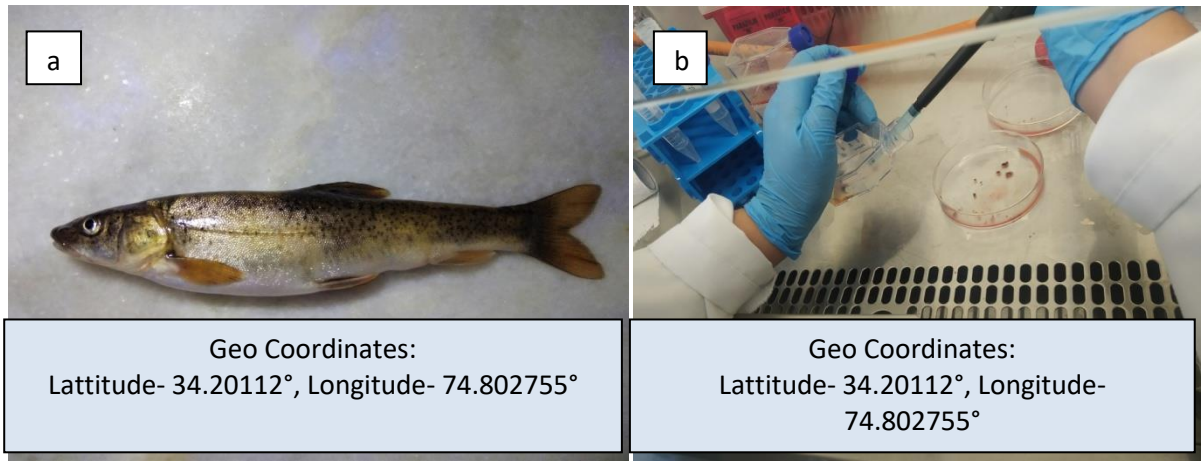

Fig. 2. (a) Specimen of *Schizothorax esocinus*; (b) Seeding of liver-derived cells

with the proper quantity of digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulphate, 0.1 mg/ml proteinase K). After two hours of incubation at 65℃, the digests were deproteinized using phenol/chloroform/iso-amyl alcohol extraction one after the other. Ethanol precipitation, drying, and resuspension in Tris-EDTA (TE) buffer were used to recover the DNA.

2.4 Molecular Characterization

The primers FISHF1 **(5'-TCAACCAACCACAAAGACATTGGCAC-3')** and reverse FISHR1 **(5'-TAGACTTCTGGGTGGCCAAAGAATCA-3')** were used for amplifying Cytochrome Oxidase Subunit 1 of *S. esocinus* (Gene Bank accession No. KP795426-KP795430) (Bashir et al., 2015). The forward and reverse primers (10 µM, 0. 5µL each), $MgCl_2$ (25 mM, 1.5 µL), dNTPs (2 mM, 2.0 µL), PCR buffer (10 ~ 2.5 µL), Taq-DNA (1U, 0.5 µL) (Thermo Scientific), template DNA (0.3 –0.4 µL), and nucleic acid-free water were all included in each 25µL PCR process. A 5min initial denaturation at 95℃ was followed by 30 cycles of 40s at 95℃, 45s at 55℃, and 45s at 72℃, with a final extension of 10min at 72℃. UV transilluminators were used to visualize the amplified products on 1% agarose gels that had been stained with ethidium bromide (Goswami et al., 2013).

2.5 Data Analysis

The data collected was subjected to analysis for descriptive statistics and ANOVA using Statistical software SPSS (version 16).

3. RESULTS AND DISCUSSION

For the first time, cell line for the native schizothoracid fish, *Schizothorax esocinus*, was developed as part of the study entitled "Establishment and Molecular Characterization of a Liver-Derived Cell line from *Schizothorax esocinus* (Chirruh) for Cold Water Fish Research". Additionally, it develops a research plan for further investigation in this field.

3.1 Explant Preparation and Primary Culture

Explants were prepared from selected tissue of *S. esocinus* viz., liver to develop primary culture. The explants exhibited variation in the degree of attachment to the culture flasks. The average attachment time was recorded as 24hrs after adding different FBS concentrations (5%, 10%, 15%, 20% and 25%) during incubation at different temperature ranges like 8°C, 12°C, 16°C, 20°C, 24°C and 28°C. Morphologically, initial subcultures of cell line consisted of both epithelial and fibroblast cells but after passage14 only epithelial like cells were seen. Our findings about the two cell types in the cell lines are consistent with those found in the *O. mykiss* spleen cell line (Flano et al., 1998) and the *Tor tor* caudal fin cell line (Yadav et al., 2012).

3.2 Growth Studies

3.2.1 *Schizothorax esocinus* liver cell proliferation in relation to varying FBS concentrations

All the explants were found to be attached properly after observing for 72hrs of incubation at

different FBS concentration. The radiation of cells started after 1 week of explant preparation from the liver tissue. Confluent monolayer around the explants was observed after 11 days. The cells exhibited poor growth at 5% and 10% concentration of FBS and didn't propagate at all over a period of experiment as illustrated in Fig. 3; relatively good growth was seen at 15%. Cell numbers increased more rapidly and higher proliferation was seen with 20% FBS concentration. Some colonies consisted of cells which were tightly packed together, whereas other colonies had a loose arrangement of cells at 20% of FBS concentration. The shape of these cells grown was a mixture of fibroblastic-like and epithelial-like, but as cultures became confluent after 1 month, epithelial cells became predominant (Fig. 4). Similar results were achieved by the study carried by Sultan et al. (2024) where 18-20% FBS was found to be optimum for the development of primary cell culture from heart of *Schizothorax niger*. The development of intestinal epithelial cell lines from rainbow trout was reported by Kawano et al. (2011) using FBS at the maximum concentration of around 30%. However, the growth of rainbow trout eye, kidney, and spleen cell lines was shown to be best maintained using FBS at a 15% concentration. Likewise, Ahmad et al. (2008) and Babu et al. (2012) developed cell lines from *Catla catla* and *Etroplus suratensis*, respectively, using 20% and 15% FBS. In order to develop fish cell lines, fish serum (< 1%) was combined with FBS (Chen et al., 2004). These results suggest that the kind of tissue determines the ideal FBS concentration needed for cell line development. The findings of Suryakodi et al. (2021), used L-15 media supplemented with 15% FBS to create a primary cell culture from several rainbow trout (*O. mykiss*) organs, are consistent with these results. Their findings are likely consistent with the current findings because both of the species under study were cold water fishes that thrived at temperatures lower than 20 to 25°C (Boyd 2018). However, these results show some difference with results of Khurshid et al. (2022) where in the primary cell culture of *S. esocinus* has been developed using FBS concentration of 15%.

3.2.2 *Schizothorax esocinus* liver cell proliferation in relation to varying temperature

Liver cells were grown in L-15 medium supplemented by 20% FBS, and examined at different temperatures like 8, 12, 16, 20, 24 or 28°C. It was observed that the tissue got fully adhered 72hrs after seeding at 24°C. The cell growth was optimal at 24°C whereas, no confluency was obtained at 8°C and 28°C. During the following days, cells show insignificant growth on 12-16°C while exponential growth was observed from 20-24°C as shown in Fig. 5. Cell colonies of various sizes were formed after 1 week and 60-70% confluent cell monolayer were achieved after 20 days. Cells lines established from 'cold water' fish appear to have lower optimum growth temperatures as nine cell lines from salmonids showed best growth between 21 and 24°C (Lannan et al., 1984). The cell line did not develop at low temperatures, but other researchers that developed cell lines from

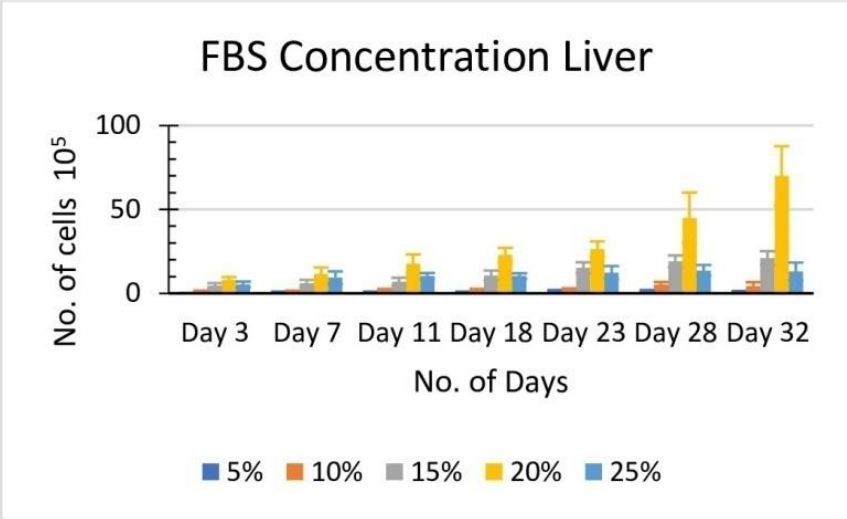

Fig. 3. A bar-graph reflecting the effect of different FBS concentration on the growth of cells in liver tissue (Each bar represents average triplicate values for each FBS concentration)

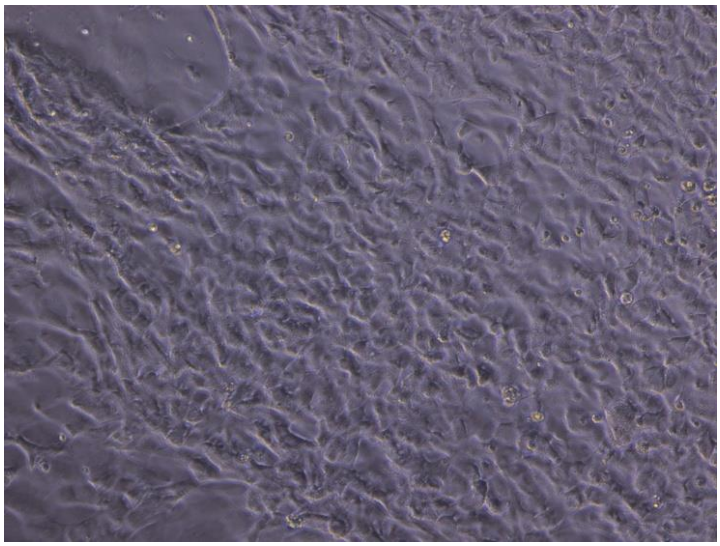

Fig. 4. Confluence layer of epithelial-like cells in liver tissue of *Schizothorax esocinus* (4X magnification)

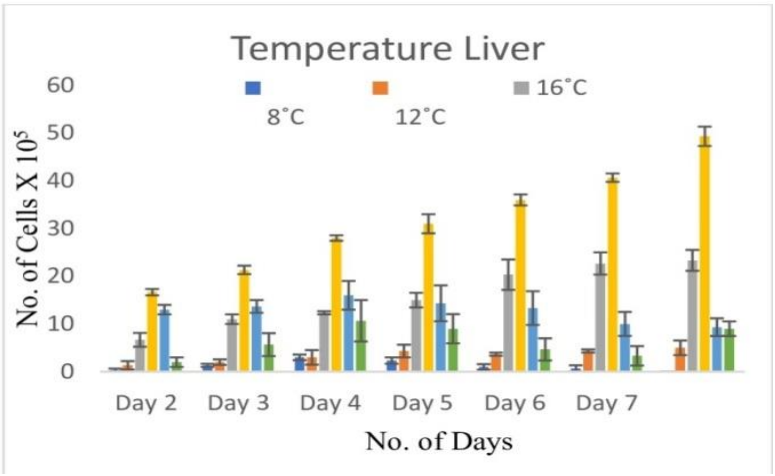

Fig. 5. A bar-graph reflecting the effect of varying temperatures on the growth of cells in liver tissue (Each bar represents average triplicate values for each tested temperature)

other schizothorax species discovered that growth was significantly impacted by high temperatures over 24°C (Goswami et al., 2013; Kawano et al., 2011; Luque et al., 2014). Chen et al. (2004) investigated the *Paralichthys olivaceus* cell line that was grown at a temperature between 24 and 30°C. At temperatures lower than 18ºC, a slower growth rate was noted. However, at 25ºC, a marine fish cell line (EAGS) was created from *Epinephelus akaara* (Haung et al., 2009). Goswami et al. (2013) reported similar results on the creation of a caudal fin cell line from the cold water schizothoracid fish *Schizothorax richardsonii*; they found that 24ºC was the ideal temperature for cell proliferation. According to Kumar et al. (2020), the ideal temperature for the growth of a cell line derived from the eye of *S. richardsonii* was determined to be 28ºC. These results conflict with the present findings about temperature optimization. Ahmad et al. (2009) also created and maintained the *Labeo rohita* cell line at 28ºC. A primary cell culture from the heart tissue of *Schizothorax plagiostomus* using the explant culture method demonstrated the best proliferation at 24°C in L-15 medium supplemented with FBS (Tantry et al., 2024), which was similar to our findings.

3.2.3 Development of cell line from liver

After 72 hours of explant preparation, morphological examination using an inverted

florescent microscope showed that every explant taken from the liver was correctly attached. After 1 week of explant preparation, the cells began to be radiate in cell culture. After 15 days, a confluent monolayer was seen around the explants (Fig. 6a and 6b), while as 70-80% confluency was seen after 20 days. Furthermore, the cells were taken to 14th passage which morphologically mostly composed of epithelial-like cells after which they showed complete death. Similar results were demonstrated by Suryakodi et al. (2021) where a monolayer was formed within 15 days in *O. mykiss* and cells consisted of heterogeneous population of both fibroblastic and epithelial like cells in early stages. As per Goswami et al. (2013), the radiation of cells started after 72 hours of implantation from the fin explant of *S. richardsonii and* a confluent monolayer was obtained after 7 days. The cells of initial subcultures consisted of morphologically both epithelial and fibroblast like cells and only fibroblast cells after 20 passages.

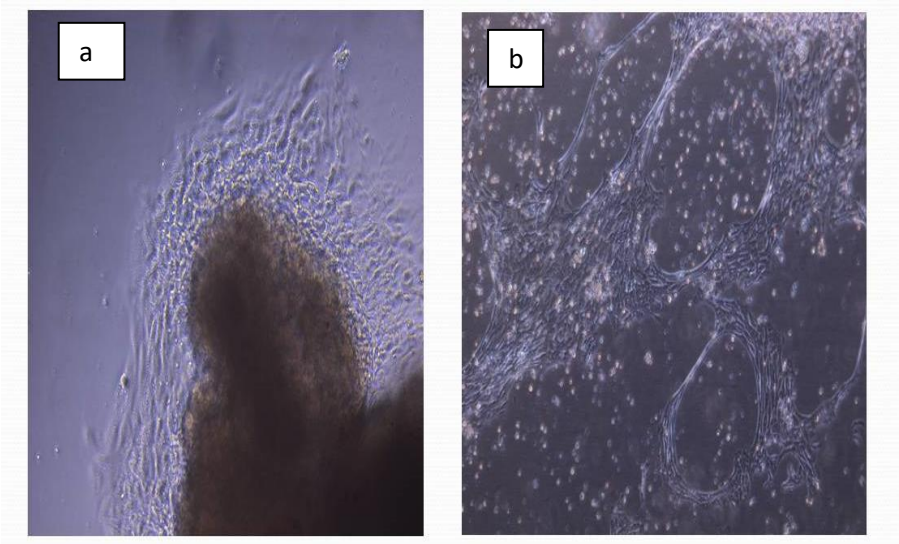

Fig. 6. (a) Proliferation of cells around the explant (4X magnification); (b) 70-80% confluency after passage 8 in liver tissue (4X magnification)

Fig. 7. Origin of cell line by Molecular Characterization

3.3 Molecular Analysis

The origin of the cell line was confirmed at passage 12 by the amplification of 629 bp of cytochrome oxidase subunit I (COI) as depicted in Fig. 7. The ensuing comparison analysis of the identified sequence revealed a 100% match with the original sample of *S. esocinus*, therefore, clearly showed that the cell line originated from *S. esocinus*. The mitochondrial COI gene is a highly conserved region within species but has sufficient variability between species. According to Hebert et al. (2003), studying the COI gene sequence is thought to be a helpful DNA marker for confirming the cell line's origin and identifying the species of established fish cell lines. In the present research work, the resulting amplicons were analyzed through gel electrophoresis to confirm successful amplification of 629 bp, ensuring the integrity of research by confirming the species identity and detecting cross-contamination. Our findings are in accordance with Bashir et al. (2015) in which findings helped identification of snow trout species in India based on their taxonomy. The origin of rainbow trout cell lines was verified by amplifying and sequencing a 695 bp region of the mitochondrial cytochrome oxidase I gene, as done by Cooper et al. (2007) to identify sixty-seven cell lines and Kawano et al. (2011) to confirm the intestinal epithelial cell line of *O. mykiss*. Similarly, by amplifying 655bp of cytochrome oxidase subunit I (COI) gene, the origin of *S. richardsonii* cell line was verified (Goswami et al., 2013). Other alternative marker such as 16S ribosomal RNA gene sequence was used to confirm the origin of muscle and fin cell lines of bluefin trevally (*Caranx melampygus*) (Zhao & Lu 2006).

4. CONCLUSION

Since fish cell culture in India is evolving so quickly, there has been a constant attempt to develop cell culture methods employing different organs and tissues of different species. For aquaculture and fisheries management, it would be essential to develop cell lines from commercially important and endangered species. The establishment of this cell line provides a valuable tool for in vitro studies, including toxicology, disease modeling, and conservation genetics, which are critical for the preservation and sustainable management of indigenous fish populations. By utilizing molecular techniques to confirm the cell line's origin and optimizing culture conditions, this research lays the groundwork for future advancements in cold-water aquaculture biotechnology. Additionally, the cell line can serve as a resource for genetic studies and biotechnological applications, contributing significantly to the field of aquatic research and conservation.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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