Research

Preserving the Vanishing Biodiversity of Indian Donkeys via Fibroblast Cell Cryobanking

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ABSTRACT

India possesses four officially registered indigenous donkey breeds, namely Halari, Kachchhi, Spiti, and Ladakhi, which represent unique genetic resources adapted to diverse agro-climatic conditions. The steep decline in donkey populations due to mechanization and changing socio-economic conditions necessitates the development of complementary *ex situ* conservation strategies. The present study aimed to establish and cryopreserve skin fibroblast cell lines from ear pinnae tissues of all four registered Indian donkey breeds. Ear tissue biopsies of at least 2 male and 2 female animals of each breed were collected from their representative breeding tracts. Primary cell cultures were established within 10-14 days. Cultures were expanded, characterized morphologically, and cryopreserved in liquid nitrogen using dimethyl sulfoxide-based freezing medium. Population doubling time of cells selected for freezing varied within a narrow range of 22.4-25.1 hours across all the 4 breeds. Successful preservation of viable fibroblast cultures was evident from the post-thaw viability of (>90%) after 6 months and >85% after a year. The established cell lines exhibited typical spindle-shaped morphology, rapid attachment following thawing, and sustained proliferative capacity under *in vitro* conditions. The generated fibroblast repository represents a secure backup of the complete diploid genome of all registered Indian donkey breeds and provides a valuable resource for future applications including genomic characterization, induced pluripotent stem cell generation, somatic cell nuclear transfer, and breed restoration programmes. This study also demonstrates the utility of somatic cell banking as a strategic component of national animal genetic resource conservation programmes.

Keywords: Animal genetic resources, Fibroblast, Somatic cell banking

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INTRODUCTION

The conservation of animal genetic resources has emerged as a global priority owing to increasing rates of breed erosion and biodiversity loss (FAO, 2015). Indigenous livestock breeds embody unique combinations of adaptive traits that have evolved under specific ecological and management conditions, making them invaluable reservoirs of genetic diversity for future breeding and climate resilience programmes (FAO, 2012). Donkeys (*Equus asinus*) have historically played an indispensable role in supporting rural livelihoods through transportation, agriculture, and pack animal services (Zhu *et al.,* 2025). In India, four indigenous donkey breeds have been officially recognized (https://nbagr.res.in/): Halari (INDIA_DONKEY_0400_HALARI_05002), Kachchhi (INDIA_DONKEY_0400_KACHCHHI_05003), Spiti (INDIA_DONKEY_0600_SPITI_05001), and Ladakhi (INDIA_DONKEY_3800_LADAKHI_05004). The Halari and Kachchhi breeds are adapted to the hot arid and semi-arid regions of western India, whereas the Spiti and Ladakhi breeds thrive under the harsh environmental conditions of the trans-Himalayan cold desert ecosystem. Despite their utility, Indian donkey populations have experienced substantial declines during the past few decades due to mechanization, urbanization, and reduced dependence on animal power. According to livestock census data, India's donkey population has declined by more than 61% between 2012 and 2019. From about 3.2 lakh, their numbers have fallen to around 1.2 lakh only (Livestock census, 2019). Experts fear the decline has continued thereafter. Consequently, the preservation of their genetic diversity has become an important national priority.

Cryoconservation has become an essential tool for *ex situ* conservation of livestock biodiversity (Saadeldin *et al.,* 2019). Although semen and embryo cryobanking are well-established approaches, they are constrained by reproductive status and sex of donor animals. In contrast, somatic cell banking preserves the entire diploid genome irrespective of age, sex, or reproductive capability (Sharma *et al.,* 2018a). Skin-derived fibroblasts are particularly attractive because

they can be obtained through minimally invasive biopsy procedures, cultured efficiently *in vitro*, and potentially used in advanced reproductive technologies including somatic cell nuclear transfer (SCNT), induced pluripotent stem cell (iPSC) generation, and genome editing (Fernandes *et al.,* 2025).The present investigation reports the establishment of a cryobank of ear pinnae skin fibroblasts representing all four registered indigenous donkey breeds of India.

MATERIALS AND METHODS

Ethical approval

All procedures involving animals were conducted in accordance with institutional guidelines for animal care and use. Tissue collection was performed under appropriate veterinary supervision following owner consent.

Animals

Representative donor animals belonging to the four registered Indian donkey breeds (Halari, Kachchhi, Spiti, and Ladakhi) were selected from their respective breeding tracts. Healthy animals exhibiting typical breed characteristics were included for tissue sampling. Sampling was ensured from a minimum of 2 male and 2 female animals per breed. A total of 23 samples were collected of which 8 (3M+5F) belonged to Ladakhi, 6 (2M+4F) to Kachchhi, 5 (2M+3F) to Halari and 4 (2M+2F) to Spiti.

Collection and transportation of ear pinnae tissue

All reagents and culture media utilized in this protocol were procured from Sigma-Aldrich (St. Louis, MO, USA) and Gibco-BRL (Carlsbad, CA, USA). Approximately 1 cm × 1 cm skin biopsies were collected aseptically from the outer margin of the ear pinna after disinfection with 70% ethanol and povidone iodine. Tissue samples were immediately transferred into sterile transport medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotic-antimycotic solution (100 IU/mL penicillin, 100 µg/mL streptomycin, and 200 µg/mL neomycin) and transported under cold conditions (4°C) to the laboratory (Sharma *et al.,* 2018).

Establishment of primary fibroblast culture

Tissues were washed several times in phosphate-buffered saline containing antibiotics. Upper skin layer (Epidermis) and subcutaneous fat were removed aseptically, and the skin was minced into approximately 1 mm^3 explants (Sharma *et al.,* 2024). Explants were placed in 60mm petri plates containing DMEM supplemented with 20% fetal bovine serum, antibiotic-antimycotic solution (100 IU/mL penicillin, 100 µg/mL streptomycin, and 200µg/mL neomycin). These were cultured at 37°C, 5% CO_2 in a humidified incubator. Next day, 3 ml of additional media was added to the petriplates without disturbing the explants. Media was replaced every third day. Explants were observed for migration of cells under inverted microscope every alternate day after one week. Cellular outgrowth from explants was monitored daily using an inverted phase-contrast microscope.

Subculture and expansion

Fibroblast cultures were expanded through successive passages for cryopreservation. Upon reaching 80- 90% confluency, cells were detached using 0.25% trypsin-EDTA solution and passaged at a split ratio of 1:2 for the first passaging. For subsequent passaging cell pellet was re-suspended in 1 ml of media, cells were counted, and viability was estimated with trypan blue dye exclusion method (Freshney 2011). During serial passaging of fibroblasts, FBS was reduced to (10%), antibiotic-antimycotic solution to antibiotic solution (50 IU/mL penicillin, 50 µg/mL streptomycin) and cell seeding density was 80,000 cells/ 25cm^2 flask). All cultures were routinely examined for bacterial and fungal contamination. Fibroblasts at the 4th passage were used for assessing their growth characteristics by making the growth curve. For this, 10,000 cells were seeded in each well of 6 well culture plates. These were cultured for 7 days at the previously culture conditions (37°C, 5% CO_2, 95% humidity). Cells were counted every day (3 wells). The mean cell numbers of every day were adopted to plot a growth curve and to calculate the population doubling time (PDT). The population doubling time (PDT) of the donkey fibroblast cultures during the log phase was calculated using the formula: PDT = (t x ln2) / ln (Nt/ No), where t represents the cultivation time interval, and No and Nt represent the initial and final cell counts at the beginning and end of the exponential growth phase, respectively.

Cryopreservation

Cells harvested during logarithmic growth phase were centrifuged and resuspended at approximately 1×10^6 cells/mL in freezing medium comprising 70% DMEM, 20% fetal bovine serum, and 10% dimethyl sulfoxide (DMSO). Cryovials were cooled gradually at approximately −1°C/min before storage in liquid nitrogen at -196°C.

Post-thaw recovery and viability assessment

Cryovials were rapidly thawed in a 37°C water bath and immediately diluted with pre-warmed culture medium. Cell viability was determined by the Trypan Blue dye

exclusion method. Morphological recovery and cell attachment were monitored microscopically.

Statistical analysis

Cell viability and growth parameters were expressed as mean ± standard deviation.

RESULTS

The unique adaptive traits inherent in the four indigenous donkey breeds of India represent a valuable reservoir of genetic variations that warrant urgent conservation. The establishment of fibroblastic lines involved4 major steps (i) Tissue sample collection (ii) Cell isolation and multiplication (iii) Cryopreservation of fibroblasts; and (iv) Cell culture after cryostorage. At each stage, optimizing species-specific conditions in the species of interest represents the first step toward its successful use for animal conservation.

Sample collection and primary culture initiation

Ear pinnae skin biopsies were collected from 23 animals, both male and female of all the 4 indigenous donkey breeds (Halari, Kachchhi, Spiti, and Ladakhi) from true to the breed type animals from their respective breeding tract.

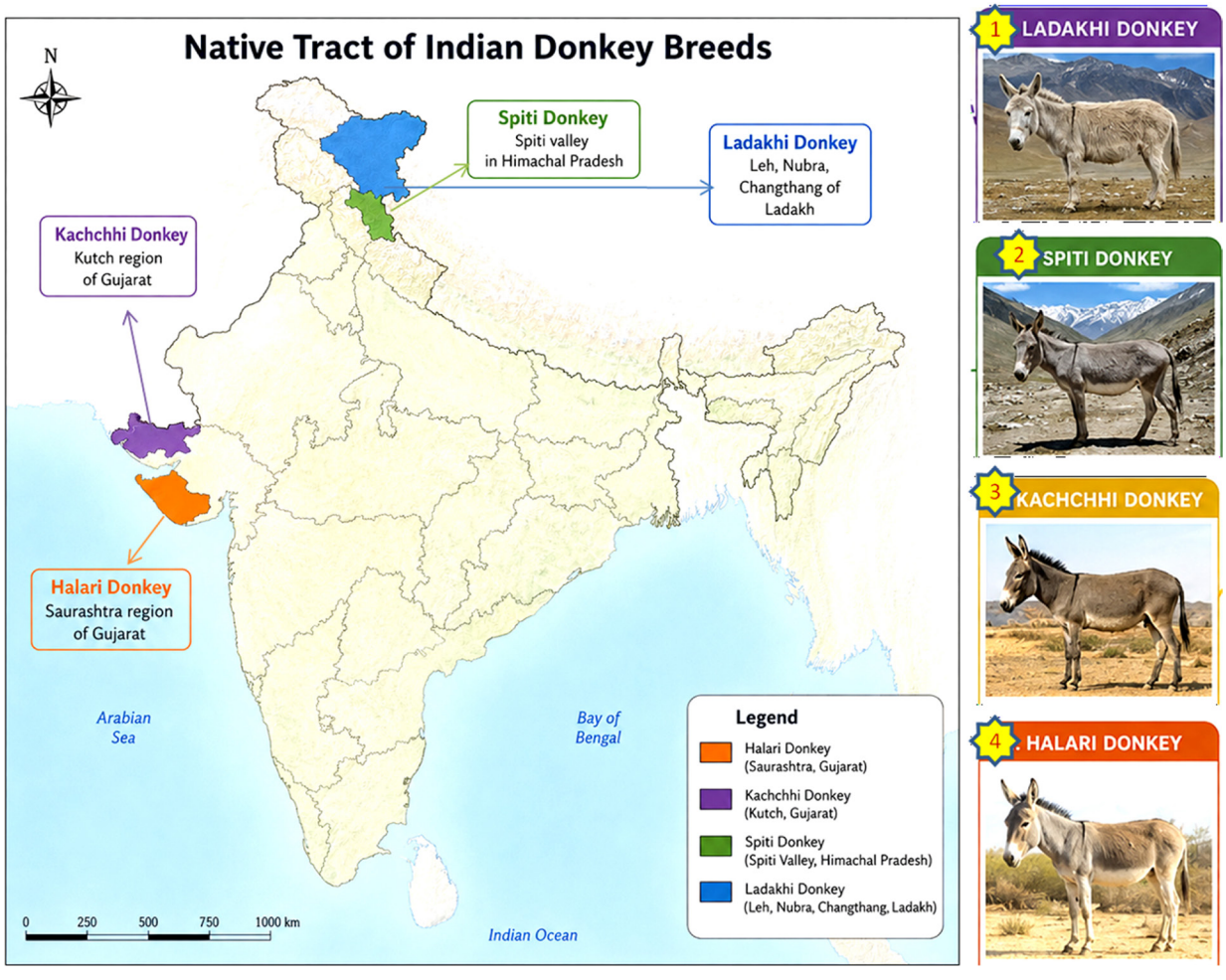

Fig. 1: *Distribution area of the 4 donkey breeds from where sampling was done and their representative animals.*

The Halari and Kachchhi breeds are adapted to the harsh climatic conditions of western India, whereas the Spiti and Ladakhi breeds possess remarkable physiological adaptations to high-altitude cold desert ecosystems (Fig. 1).

Primary cultures were initiated using mechanical minced tissue explant culture. Primary fibroblast cultures were successfully established. Fibroblast outgrowth from the margins of the tissue explants was observed between Days 4 and 6 post-seeding across all groups. Initial cell emigration did not differ significantly among the breeds, though Spiti explants exhibited a slightly tighter, compact adherence profile during the first 72 hours. Complete cellular monolayers (80–90% confluence) in primary culture (Passage 0, P0) were achieved within 10-14 days across all the 4 donkey breeds (Fig. 2). Thus the explant culture method employed in this study proved effective for establishing primary fibroblast cultures while minimizing enzymatic damage.

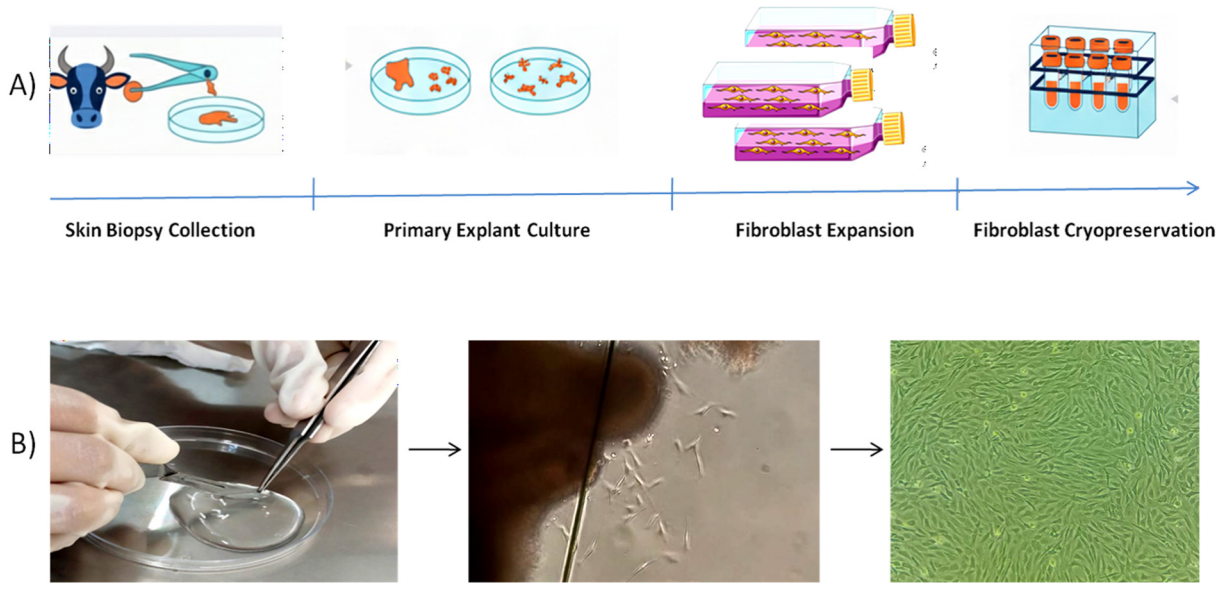

Fig. 2: *Generation of donkey fibroblast cells A) Schematic representation of obtaining cells from skin tissue. B) Primary culture, outgrowth of cells from tissue explant (dark) on day 4 and confluent fibroblast culture (All photos of cells were taken under the phase imaging captured with the 10X objective).*

Cellular expansion and *in vitro* growth dynamics

Somatic cells were sequentially expanded up to Passage 3 (P4) to obtain the sufficient number of cells required for cryostorage. Microscopic examination of the established cultures across all four breeds revealed a highly homogenous population of cells exhibiting classic fibroblast morphology. The cells displayed a characteristic elongated, spindle-shaped, or fusiform architecture with prominent oval nuclei and long cytoplasmic extensions. Serial passaging demonstrated sustained proliferative ability without noticeable morphological alterations. The cultures remained free from bacterial, fungal, and mycoplasma contamination throughout the study period. Growth curve kinetics was plotted over a continuous 8-day observation window following a standard seeding density of 80,000 cell/ 25cm^2 flasks (Fig. 3).

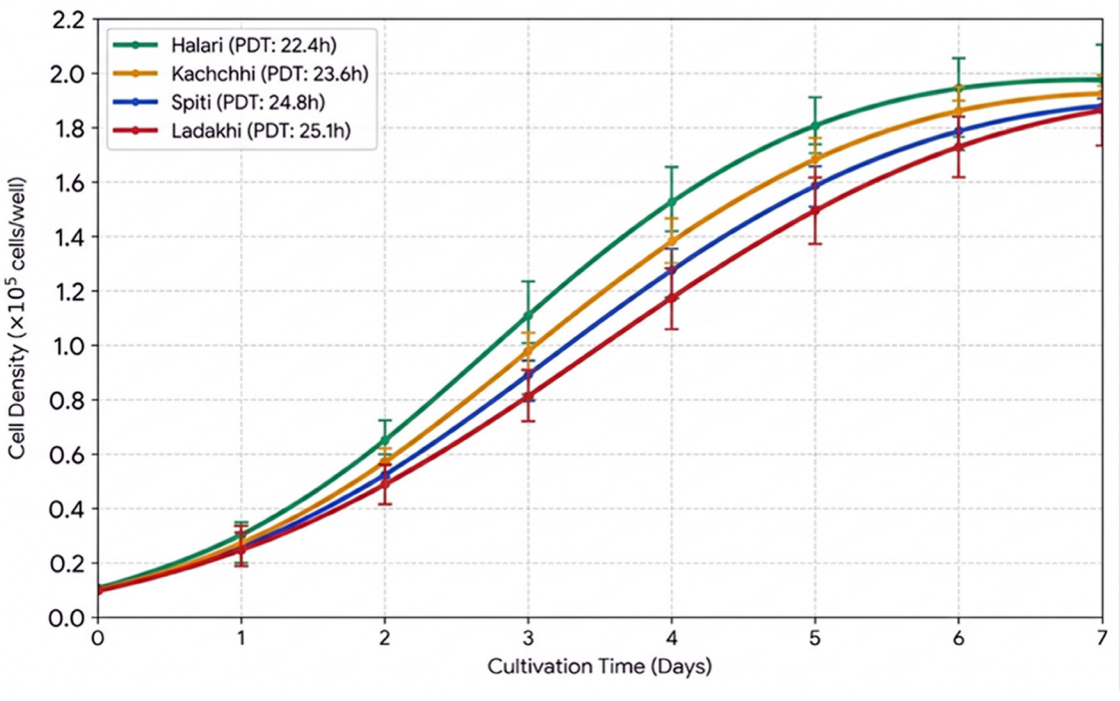

Fig. 3: *In vitro growth kinetics of 4th passage fibroblast cells across all the 4 donkey breeds of India (data represent the mean ± SD; n = 3).*

All four breeds generated a highly uniform, sigmoidal growth trajectory comprising an initial lag phase (Days 1–2), an exponential log growth phase (Days 3–5), and a plateau/stationary phase (Days 6–7) as contact inhibition took effect. The estimated population doubling times (PDT) ranged from 22.4 to 25.1 hoursduring the exponential phase (Table 1).

Table 1: Cell growth performance of fibroblast cells derived from Indian donkey breeds.

Breed	Lag phase (days)	Maximum cell count (10^5 cells/well)	Population Doubling Time (hrs)
Spiti	1.5±0.2	1.85±0.12	24.8±1.1
Halari	1.2±0.1	1.98±0.09	22.4±0.8
Kachchhi	1.4±0.2	1.92±0.15	23.6±1.3
Ladakhi	1.4±0.3	1.89±0.11	25.1±1.2

Value are Mean ± SD (n=3)

Post-thaw viability and functional recovery

Cryopreservation of the donkey fibroblast cells was performed using a freezing medium containing 20% Fetal Bovine Serum (FBS), 10% Dimethyl Sulfoxide (DMSO) and 1% Penicillin-streptomycin. Slow-rate cooling (1°C/minute) was achieved by putting filled cryovials in the Mr. Frosty container (Thermo Fisher Scientific, Waltham, MA) and keeping them in -80°C deep freezer for 12 hrs and subsequently long-term immersion in liquid nitrogen (-196°C). Cells were cryo-banked for minimum of 60 days for post-thaw evaluation. Cells were recovered after rapid thawing in a 37°C water bath followed by immediate cell viability quantification using the Trypan Blue exclusion assay. All four indigenous breeds exhibited exceptional post-thaw survival metrics, significantly exceeding the standard target baseline of 80% for robust somatic biobanking (Table 2).

Table 2: Mean post-thaw viability estimates after 1 year of cryofreezing at -196°C across Indian donkey breeds.

Breed	Animals (M+F)	Date of sample collection	Sample Freezing	Total vials freezed (1x10^6 cells/ ml/vial)	Post thaw viability after of cryofreezing	
					6 months	1 year
Ladakhi	8 (3M+5F)	20.04.2018	June 2018	384	95%	93.7%
Kachchhi	6 (2M+4F)	17.12.2018	February 2019	180	93%	89%
Halari	5 (2M+3F)	16.12.2019	February 2020	180	90%	85%
Spiti	4 (2M+2F)	24.10.2024	December 2024	120	98%	95%

The observed viability was more than 90% after 6 months of cryofreezing for all the four donkey breeds.It ranged between 90-98% with maximum viability recorded for the Spiti cells (98%). The viability after one year was also more than 85%, and once again the highest viability was depicted by the Spiti cells. Furthermore, within 24 hours of re-seeding, thawed cells successfully attached to the surface of tissue culture flasks. Cells re-established their typical bipolar/spindle morphology, and reached confluence within 4 to 5 days without displaying signs of accelerated senescent degeneration (Fig 4).

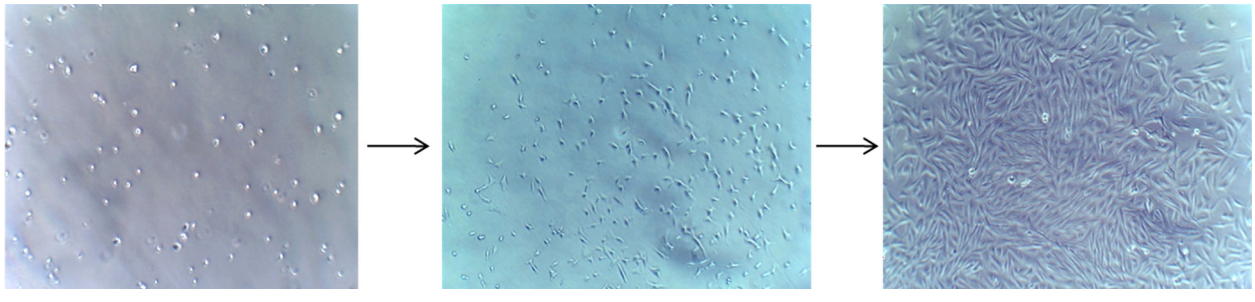

Fig. 4: *Cells (>1 year of cryofreezing)- after thawing, after attachment and after 80% confluency (All photos of cells were taken under the phase imaging captured with the 10X objective).*

DISCUSSION

The conservation of endangered livestock diversity increasingly relies upon the integration of conventional breeding strategies with modern biotechnological approaches. Somatic cell cryobanking has emerged as an effective *ex situ* conservation tool because it captures the complete genomic constitution of individual animals independent of reproductive status (Sharma *et al.,* 2017). The loss of indigenous donkey biodiversity at an alarming rate has encouraged us to cryopreserve skin derived fibroblast cell lines, which are amongst the established tools for the mammalian conservation. Fibroblasts are mesenchymal cells that constitute fundamental components of connective tissues and are essential for maintaining tissue homeostasis (Bihuniak *et al.,* 2026). These cells have potential to drive their reproduction through their use in cloning by somatic cell nuclear transfer (SCNT) and induction of cells to pluripotency. While domestic equids like horses have enjoyed extensive biotechnological coverage throughout the world, the genetic resources of indigenous asinine populations largely remain neglected (Khan *et al.,* 2024; Zhu *et al.,* 2026). Indian donkey has remained critically under-preserved and vulnerable to genetic erosion. This investigation successfully provides a standardized roadmap for isolating, characterizing, and long-term cryopreserving viable dermal fibroblasts from the ear pinnae of all the 4 registered donkey breeds of India.

Ear pinnae skin biopsies provide a highly practical, minimally invasive pathway for harvesting somatic cells. This procedure can be executed under localized field conditions with minimal stress to the animal (Deng *et al.,* 2024). The overlapping performance of explant migration timelines (4-6 days) across all four breeds demonstrates that despite their vast geographical and environmental differences, ranging from the high-altitude, cold arid conditions of the Spiti valley to the hot, semi-arid maritime landscapes of Gujarat for Halari and Kachchhi, their dermal tissues maintain a relatively uniform *in vitro* metabolic baseline. The majority of literature reports a period of 7-10 days for the appearance of cells during the primary culture (Selokar*et al.,* 2018). Thus, the method described here was efficient in the generation of primary cells. The removal of outer skin layer, which was most likely epidermis comprising mainly of keratinocytes, might have helped in obtaining the fibroblasts at a faster pace (Lendahl *et al.,* 2022). The uniform morphology observed in our expanded cells aligns seamlessly with classic mammalian fibroblastic hallmarks. Because mixed primary cultures run an inherent risk of containing epithelial or endothelial cells, cell verification gave added advantage. It is non-negotiable for downstream genetic rescue efforts, such as Somatic Cell Nuclear Transfer (SCNT), where epithelial cross-contamination reduces blastocyst development rates.

The growth kinetics verified that *in vitro* expansion did not induce early replicative senescence or alter cellular vitality prior to freezing. The cells in senescence decrease the cell application for the conservation of animals, where the cells cannot divide and may undergo DNA damage, resulting in less success with SCNT (Azuma *et al.,* 2020). The calculated population doubling times (22.4 - 25.1 hours) reflect robust proliferative velocity. The classic sigmoidal progression across all breeds showed that contact inhibition mechanism remains fully intact, meaning the cells retain their normal homeostatic response profiles in culture (Verma *et al.,* 2020).

Cryopreservation consists of the use of low temperatures to inhibit metabolism and check biochemical processes responsible for the degradation of living cells (Marcantonini *et al.,* 2022). It permits the formation of biobanks, increasing the possibility of developing assisted reproduction technologies (ARTs) by enabling the use of biological materials with their genetic variability preserved irrespective of time and space. Thus, cryopreserved cell lines provide a viable and expandable source of material genetic, facilitating the protection of endangered species in the future. Therefore, it is crucial to monitor the quality of the cells, efficiency and reliability of fibroblastic lines. The high post-thaw viability averages (>88%) across all four donkey breeds confirm that the cryofreezing protocol (10% DMSO + 20% FBS) coupled with a slow controlled cooling gradient (-1°C/min) provides exceptional cryo-tolerance. Slow cooling minimizes the formation of lethal intracellular ice crystals by facilitating controlled extracellular dehydration, while DMSO prevents excessive cell shrinkage via membrane stabilization (Prasad *et al.,* 2025). The fact that the post-thaw cells displayed capacity to adhere, proliferate, and retain standard spindle-like architecture, proves that structural protein networks and enzymatic mechanisms were undamaged by liquid nitrogen storage. High post-thaw viability and continued proliferative activity indicated the effectiveness of the cryopreservation protocol. Successful cryopreservation and post-thaw recovery demonstrate the suitability of skin fibroblasts for long-term germplasm banking. Similar approaches have been reported for several domestic and wild species, highlighting the broad applicability of fibroblast repositories for biodiversity conservation (Arantes *et al.,* 2021).

The established repository currently preserves fibroblast cell lines representing all four registered indigenous donkey breeds of India. Results provide boost towards establishing the somatic cell repositories for other livestock

species of India (Sharma, *et al.,* 2017). Unlike gamete banking, somatic cell repositories preserve both maternal and paternal genomes and can be established from males, females, juveniles, aged animals, and even deceased individuals if tissues are collected promptly. Furthermore, fibroblast cell lines can serve as donor nuclei for SCNT, substrates for genome editing technologies, and source material for induced pluripotent stem cell production. The establishment of fibroblast cell lines from Halari, Kachchhi, Spiti, and Ladakhi donkeys therefore represents an important milestone in the conservation of Indian equine genetic resources.

CONCLUSION

This study reports the successful establishment and cryopreservation of ear pinnae skin fibroblast cell lines from all four registered indigenous donkey breeds of India. The resulting somatic cell repository safeguards the complete diploid genome of these valuable breeds and provides a robust platform for future conservation and biotechnological interventions. The integration of fibroblast cryobanking into national germplasm conservation programmes will significantly strengthen long-term preservation efforts for threatened livestock biodiversity. This effort contributes to national and international biodiversity conservation commitments under the Convention on Biological Diversity and the FAO Global Plan of Action for Animal Genetic Resources.

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