Research Article

From endangered to preserved: Biobanking of the Katchaikatti Black sheep through dermal fibroblast cryopreservation

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ABSTRACT

The present study was carried out with the aim of *ex-situ* conservation of the elite germplasm of Indian sheep breeds including Katchaikatti Black sheep in the form of cryo cell bank repository. The Katchaikatti Black sheep are distributed in the villages of Vadipatti block of Madurai district in Southern Tamil Nadu and belong to the meat type sheep with extremely coarse and hairy wool. As on date, Katchaikatti black sheep breed is at the risk of extinction and considered to be endangered. It is therefore imperative to conserve the highly valuable sheep germplasm of Indian origin. In this connection, as a preliminary step, a total number of eight ear pinna samples were collected from eight individual sheep (four males and four females from Katchaikatti village) using the sterile surgical blade, washed once in 70 % alcohol, saturated once in washing medium, stored in complete cell culture medium and transported under cold chain. Once in six hours, the complete medium was changed. After bringing to the laboratory at ICAR-CSWRI, Avikanagar, standard cell culture protocol was followed for the initial tissue explant culture. Subculturing of the resultant somatic fibroblast cell was done up to the passage four level at the periodical intervals. In order to use the skin fibroblasts in downstream processing, karyotyping was also carried out through outsourcing, as a gold standard. Various parameters such as viability assay, population doubling time were also carried out. As a final step, cryopreservation of the skin fibroblast cells derived from Katchaikatti black sheep was done using LN_2 protocol, after the completion of the standard characterization regimen.

Key words: Cryopreservation, Endangered, Karyotyping, Katchaikatti Black sheep, Skin fibroblast cells

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INTRODUCTION

As per the report of the International Union for Conservation of Nature (IUCN), India is known as a megadiverse country covering about 4% of the globe's total land area, and possesses 7-8% of all recorded species, including species of plants and animals. In the conservation of biodiversity, the genetic resources of both livestock and poultry contribute a pivotal role owing to their precious and non-renewable strategic resources for a nation's livestock wealth (Notter, 1999). As per the 20th Livestock census of Govt. of India during the year 2019, total sheep population in the country is 74.26 million and there is an increase of 14.13% of total sheep population in India, when compared to previous Livestock Census during the year 2012 and sheep shares about 13.8% of the total livestock in the nation. As far as the biodiversity of indigenous ovine breeds is concerned, India is known to have 45 registered indigenous breeds. In the nation, sheep is being considered as multi-purpose animal species and explored for meat, wool, milk, skin and manure. Sheep husbandry is one of the precious components of the Indian rural economy, particularly in the arid, semi-arid and mountainous areas and shares around 7-8% in meat and 100% in wool production. Based on the geographical locality, Indian sheep breeds are divided into four agro ecological regions (Aggarwal *et al.,* 2022).

1. Southern Peninsular Region
2. North Western arid and semi-arid region
3. North Temperate Region and
4. Eastern Region

The state Tamil Nadu comes under Southern Peninsular Region and is known to have 10 registered breeds of sheep namely Madras Red, Mecheri, Ramand White, Kilakarsal, Vembur, Coimbatore, Trichy Black, Nilgiri, Katchaikatti Black and Chevvadu (nbagr.icar.gov.in). Among the 10 registered sheep breeds of Tamil Nadu, the Katchaikatti black sheep is peculiar in terms of its cultural importance and it is commonly used in Ram fighting (baiting), which is a famous traditional event in and around the villages of Madurai district of Tamil Nadu state, India (George et al., 2024). With an individual farmer, the flock strength of Katchaikatti black sheep is around 45, having one or two rams on an average (Pathak *et al.,* 2020). Ravimurugan *et al.,* (2012) reported that Katchaikatti black sheep breed is being reared by *Konar* and *Pallar* communities, which have the dwellings in the villages of Vadipatti block of Madurai district and Melaneelithanallur block of

Tirunelveli district in Southern Tamil Nadu. Katchaikatti sheep is of meat type and is a hairy breed. In the year 2022, As per the breed-wise report of Livestock and Poultry based on 20th Livestock Census published by the Ministry of Fisheries, Animal Husbandry and Dairying, Dept. of Animal Husbandry and Dairying, Government of India, Krishi Bhavan, New Delhi, the total number of Katchaikatti sheep was 1506 only.

In recent times, the activities such as invasion of the grazing area by the process of urbanization, non-systematic breeding plans and lack of appropriate breeding and management policies have seriously led to erosion of the genetic diversity among the sheep breeds of southern agroclimatic zone of Tamil Nadu (including the Katchaikatti black sheep) and there is an urgent need for the conservation of the precious sheep breeds of this geographical locality (Ravimurugan *et al.,* 2012). In addition to the *in-situ* conservation of livestock genetic resources, there are two approaches under *ex-situ* conservation of the elite germplasm of valuable animal species. 1. *ex situ in vivo*, which involves the conservation of living animals in the environment, wherein they have been raised and 2. *ex situ in vitro*, which involves the preservation of the biological materials from the living animals (e.g. semen, oocyte, embryos, cells or tissues) in an artificial environment commonly under ultra-low temperature (Pathak *et al.,* 2020). In case of the conservation of endangered animals, breeds or species including both wild and domestic animals, somatic cells are the excellent materials, which follow the cryopreservation protocols for posterity. For rescuing endangered mammalian breeds and species, somatic cell cloning technology and the eventual establishment of somatic cell banks are playing an important role (Sharma *et al.,* 2018). Due to the drastic reduction in the population of Katchaikatti black sheep, it is mandatory to take up the immediate steps on priority basis for the conservation of such elite germplasm. Furthermore, while searching the public research domain, little information is available about the *ex-situ in-vitro* conservation of the genetic materials obtained from Katchaikaati black sheep. Keeping the aforementioned information in view, the present study was carried out with the objectives of isolation of dermal fibroblasts derived from Katchaikatti Black Sheep, characterization of the somatic cells by karyotyping and preservation of the fibroblast cells under cryogenic conditions.

MATERIALS AND METHODS

Cells and Reagents

Fresh marginal ear tissue samples of sheep (Katchaikatti Black) for isolation of primary fibroblasts cells. DMEM medium (4.5 g/l D-Glucose, 110 mg/L Sodium Pyruvate; without L-Glutamine) phosphate-buffered saline (PBS, pH = 7.2), trypsin-EDTA solution (0.25%), Fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), penicillin, and streptomycin (Antibiotic-Antimycotic solution 100×) (Sigma-Aldrich, USA).

Area of sampling

For the present study, sampling was done at Katchaikatti village located in Vadipatti taluka of Madurai district in Tamil Nadu, India. It is situated 5 km away from sub-district headquarter Vadipatti (tehsildar office) and 30 km away from district headquarter Madurai. The total geographical area of village is 2402.35 hectares. Vadipatti is nearest town to Katchiakatti village for all major economic activities. Its coordinates are 10.1139° N latitude and 78.0616° E longitude.

Identification of Katchaikatti black sheep

Through the interaction with the members of the society established exclusively for Katchaikatti sheep breeders in the Katchaikatti village, Tamil Nadu and the team of earlier reports on Katchaikatti Black sheep, the farmers were identified. Upon the survey and interaction with the said farmers from the village, Katchaikatti sheep was identified by black color, moderate size with long tail, medium sized head, short and stumpy ears. Rams are known for their twisted horns, whereas ewes are polled and five percent of the ewes possess thin small horns. Thorax is thicker and broader in males and slender in case of females. The limbs are straight, medium-sized and squarely formed beneath the body. The average body weight is 34.42 ± 1.97 kg and 28.14 ± 0.7 kg, in rams and ewes, respectively (Fig.1), as reported by Ravimurugan *et al.,* (2012).

Fig. 1: *Flock of Katchaikatti Black sheep in the breeding tract*

Isolation and culture of primary skin fibroblasts of sheep

The inner side of the ear was cleaned with 70% ethanol swabs, and the skin samples were excised aseptically

from a total number of eight adult sheep (four males and four females), washed once in 70% alcohol, saturated once in washing medium (complete cell culture medium without serum), stored in complete cell culture medium (DMEM medium with 10 % FBS and 1X concentration of antibiotic-antimycotic solution) and transported under cold chain to ICAR-Central Sheep and Wool Research Institute (ICAR-CSWRI), Avikanagar, Rajasthan, India. Once in six hours, the complete medium was changed. Upon the arrival at the biotechnology laboratory at ICAR-CSWRI, Avikanagar, excised external ear pinna samples were chopped into 2-3 mm size pieces (explants) aseptically and allowed to adhere onto 25 cm^2 diameter flasks. Each flask contained 4 explants placed at a uniform distance. Adhered explants were cultured in complete media, at 37°C, 5% CO_2 in a humidified environment. The medium in the flask was changed on every third day. At the same time, the flasks were also observed for any microbial or fungal contamination, explant dislodging, and for outgrowth of cells around explants, under a ZOE Fluorescent Cell Imager (Bio-Rad; Hercules, California, USA). Throughout the cell culture procedure, the culture media was changed every third day. Trypsin/EDTA (0.25%) was used to passage the cells once they attained confluence.

Generating secondary cultures

Primary outgrowing cells (80-90% confluence) around the explants were trypsinized (0.25% trypsin/EDTA) and secondary cultures were established and at passage four (P4) cells were cryopreserved. Briefly, the cells in flasks were washed twice with 3.0 mL of the Dulbecco's Phosphate Buffered Saline with Calcium Chloride and Magnesium Chloride (Gibco@ Life Technologies, Grand Island, NY, USA), and incubated with 2.0 mL of 0.25% trypsin for 1-3 min at 37°C. Five volumes of neutralized medium (DMEM containing 5% FBS) were used to neutralize the trypsinized cells, and they were then pelleted at 1800 X g for 10 min. Then, the supernatant was discarded, and the pellet was dissolved in the complete medium before being divided into two flasks with the same volume of medium and aseptically kept at 37°C, 5% CO_2 in a humidified environment to encourage continued development.

Cell viability count through trypan blue exclusion method

Cell culture flasks were trypsinized in duplicate for every time point at the P4 stage, and the trypan blue exclusion method was used to count all the viable cells. Briefly, cells were centrifuged in neutralized medium and supernatant was discarded. Cell pellet was resuspended in DMEM. Equal volume of cell suspension and 0.4% trypan blue (Gibco Carlsbad, CA, USA) were mixed and incubated for 2 min at room temperature. A drop (10 microlitre) of the trypan blue/cell mixture applied to a Countess™ 3 cell counting chamber slides (Thermo Fisher Scientific Inc.), and examined under the Countess™ 3 Automated Cell Counter (Thermo Fisher Scientific Inc). Separately, the viable (unstained) and nonviable (stained) cells were counted in the Countess 3.

Generating growth curves

Four-well plates were used to construct growth curves from the cultures at the stage of P4 level. In brief, the culture was started by inoculating 50,000 cells per well with 0.2 ml of growth medium. The wells were trypsinized (in triplicate) after 12, 24, 36, 48, 60, 72, 84, and 96 hours of culture. Till reaching the plateau phase, the cells in the wells were observed and the results were recorded for the analysis of cell density. Based on the recorded data, the growth curve was drawn and the population doubling time (PDT) was determined as per the growth curve pattern (Gu *et al.,* 2006).

Chromosome harvesting for karyotyping

Chromosome preparation from the somatic fibroblast cells of Katchaikatti black sheep for karyotyping purpose was done as per the protocol described by Howe *et al., (*2014) with minor modifications. Briefly, upon the achievement of 80% confluency, or log phase, Colcemid (Gibco® KaryoMAX® Colcemid™ Solution) was added to the cell culture flask at the concentration 10 µl/ml. Cells were cultivated for 40 minutes at 37 °C in an incubator with 5% CO_2 and the media from the cells were poured into a 15 ml conical tube using a sterile pipette. The flask was filled with 2 ml of HBSS buffer and the cells were washed gently, the buffer was swirled and then discarded. About 2-3 ml of trypsin was added into the flask and it was incubated for 2 mins. Once the majority of the cells have detached, the media was pipetted back onto the cells and the cell suspension was transferred in 10 ml aliquots into 15 ml conical tubes, centrifuged at 1800X g for 10 min. Then the supernatant was removed and the pellet was resuspended with 10 ml of 0.075 M KCl, vortexed at medium speed for proper mixing of KCl and cells. The cells were incubated at 37°C for 10 min, centrifuged at 1800 x g for 5 min at 25°C, the supernatant was removed leaving 0.5 ml and the pellet was resuspended. About 5 ml of fresh Carnoy's Fixative (3:1 ratio of methanol:glacial acetic acid) was carefully added to the cells while vortexing. Then 5 ml more fixative was added without vortexing to make a total volume of 10 ml and was centrifuged at 1800X g for 5 min. The supernatant was discarded and cells were resuspended with 5 ml of fixative solution. For the purpose of karyotyping, the cell pellets from one male and one female Katchaikatti Balck sheep were shipped

under cold storage condition to M/S Gene Lab, Surat, Gujarat, India for the chromosomal analysis.

Cryopreservation

Upon the achievement of P4 level of subculturing of dermal fibroblasts of sheep, the cells in flask (25 cm^2 diameter cell culture flasks, Nunc) were washed twice with 3.0 ml of the Dulbecco's Phosphate Buffered Saline containing Calcium chloride and Magnesium chloride (Gibco@ Life Technologies, Grand Island, NY, USA), and incubated with 2.0 ml of trypsin (0.25%) for 2 min at 37° C. Five volumes of neutralization medium (DMEM with 5 % of FBS) were used to resuspend the trypsinized cells, and they were then pelleted at 1800X g for 10 min at room temperature. The supernatant was discarded and the cell pellet was suspended in growth medium containing 30% FBS and 10% DMSO and the cell suspension of 1 ml volume (concentration between 1-2 X 10^6 cells/ml) was aliquoted into cryogenic vials (Cat # 5000-0020; Nalgene) and kept overnight at -80˚C in an isopropanol freezing container (Mr. Frosty™ Freezing (Thermo Scientific™). The vials were moved to a liquid nitrogen tank on the following day, where they were kept until their usage in downstream applications.

RESULTS AND DISCUSSION

Across the globe, India is one among the top ten species-rich countries and one of the 17 mega biodiversity nations. Further, in India, about 10% of the recorded wild flora and fauna is included in the threatened list and most of them are known to be at the level of extinction (Balasubramanian, 2017). India is also rich in elite livestock gene pool, affording enormously in the filed of augmented animal productivity at the international level. High level of endemism is mainly responsible for the richness of biodiversity among sheep in India. Alterations in agroclimatic conditions of the various geographical areas of the nation have resulted in the generation of different sheep breeds/strains, which have good adaptation traits for a particular environment. Wherever the agriculture and dairy farming are not cost effective, sheep husbandry is the major source of livelihood for the major proportion of small, marginal and landless farmers. But, due to the non-scientific breeding plans, exploration of exotic breeds and shift in the farming systems, there is a reduction in the number of pure breeds and dilution of the gene pool. The conservation of elite ovine genetic resources is not only important at the national level, but also at the international level. The ideal approaches have to be undertaken on priority basis for the conservation of the precious ovine biodiversity within the country (Bhatia and Arora, 2005). Katchaikatti black is one of the sheep breeds coming under Southern Peninsular Region and its major breeding tract is located at Katchaikatti village, Vadipatti block in Madurai district, Tamil Nadu, India. This breed is famous for Ram fighting, which is a well-known cultural event in and around the villages of Madurai district. The total number of Katchaikatti black sheep were 1350 and 2170, during 2009 (Kumarasamy *et al.,* 2009) and 2012 (Ravimurugan *et al.,* 2012), respectively. But, George *et al.,* (2024) reported that the total number of Katchaikatti sheep was 1506 only during the year 2022.

In situ and/or ex situ is also one of the components of conservation approaches for sheep genetic resources. Fibroblast cell lines derived from the somatic tissues of an animal is an *ex-situ in vitro* conservation approach commonly used for the preservation and exploration of its genetic resources. The present study is also aimed to follow the *ex-situ* conservation of Katchaikatti black sheep by utilizing the platform of establishment of fibroblast cell lines derived from the dermal fibroblasts and external ear pinna tissue was used. A sheep farmer possessing the Katchaikatti black sheep flock size of 65 (including the lambs), was chosen for the present study and out of 65, there were six breedable rams. Fibroblasts from ear tissues are the well-established platforms for the harvesting and passaging purposes, when compared to other cells and there could be a minimal chance of risk to the health of the donor animal (Hosseini *et al.,* 2008). Further, detachment of fibroblast cells from the epithelial cells would happen at a faster rate and even with the minimal quantity of biopsy material derived from an endangered animal species, it is easier for the generation of viable cryo-preserved cell lines (Sharma *et al.,* 2018). Collection of the ear tissue was done under the local anaesthetic conditions by spraying mild level of commercial preparation of topical anaesthetic agent over the ear tissue. Healthy rams and ewes were used for the experimentation and it was critically ensured that the animals were subjected to minimal pain in the process of ear tissue collection. Considering the long distance of transport all the way from Tamil Nadu to Rajasthan and maintaining the high level of viability of the ear tissues derived from sheep, the complete medium was changed once in every six hours during the period of shipping the biological materials under cold storage conditions. Upon the adherence of the tissue explants to the surface of the culture flask, cells were observed for migration from the margins of the tissue pieces (Fig. 2) as reported earlier (Pei *et al.,* 2015).

The duration for sprouting of the cells from tissue explants in the present study was five to seven days. Once the confluence (Fig. 3) achieved, passaging of the primary culture was done. Further, it was observed that there was no outgrowth from the floating tissues in the flasks.

Fig. 2: *Outgrowth from the primary tissue explant*

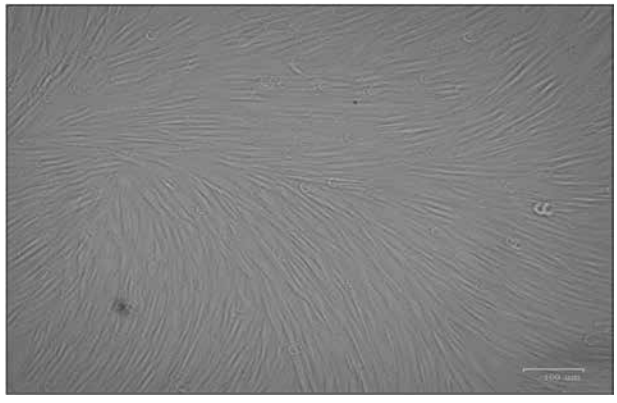

Fig. 3: *70-80% confluent fibroblasts.*

The primary outgrowth of fibroblasts from the tissues depends on many factors namely (i) Size of the tissue: bigger size of processed tissue showed faster outgrowth than the smaller one. (ii) Percentage of Serum (FBS) in the complete medium: Ideally 10 % of FBS was used for complete medium but there was more outgrowth upon the addition of double the volume of actual FBS concentration. In the present study, the antibiotic-antimycotic solution was used in the cell culture medium at the final concentration of 1X and it resulted in the growth of cell lines without any fungal contamination. But the usage of antibiotic-antimycotic solution at the total concentration below 1X in the cell culture medium led to the fungal contamination in some of the cell culture flask. Subculturing of the cells from the primary outgrowth in the tissues was done using the standard cell culture protocols involving trypsinization and it was repeated up to the P4 level. The time needed for the growth of the cells having 70-80 % confluency, from the secondary culture was shorter (within 3-4 days) when compared to the time required for the tissue overgrowth from the primary culture (five days to one week). The most plausible reasons for the faster growth of secondary culture could be due to the biochemical and technical factors like cell adaptation and selection. When compared to secondary culture, primary culture was conditionally subjected to the stress for the survival in the growing environment and selection of proliferative cells. Secondary cultures were usually having the homogenous population of fast-growing cells, when compared to the primary cultures having the mixed cell population, reduced cellular stress, stabilization of the culture conditions, cell cycle synchronization, improved nutrient availability and reduced cellular debris and contaminants (Freshney, 2015). Cell viability was calculated in the cell counter (Countess 3, Invitrogen) using Trypan blue dye exclusion method. At Passage P4, growth kinetics of the cells was determined at 12 hourly intervals and it was ranging between 24 to 60 hrs. The growth was gradual up to 72 hrs and there was a decline after 84 hrs, which was appreciated in the growth curve (Fig. 4). Before doing the freezing process of fibroblast cells, karyotyping was carried out through outsourcing. As far as the karyotyping analysis was concerned, it was as found that the diploid number of chromosomes of Katchaikatti black sheep was 54 and there were 26 pairs of autosomes and one pair of allosomes. The sex of the fibroblast cells derived from sheep was further confirmed by the presence of XY and XX chromosomes in the samples of male and female, respectively (Fig. 5A & B).

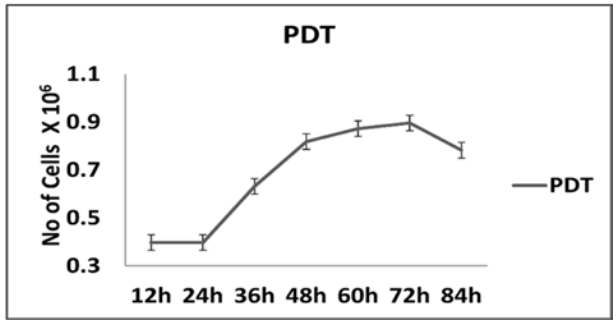

Fig. 4: *Population doubling time or growth curve.*

A total number of 20 metaphase spreads were analysed and karyotyped using G-banding technique. The X and Y chromosomes were the tallest and shortest, respectively. Further, there were four metacentric chromosomes viz., 1, 2, 3 & Y and 23 telocentric chromosomes namely 4 to 26 and X. The results of the present study are in accordance with that of Liu *et al.,* (2011). The chromosome spreads obtained at the arrest level of metaphase were free from aberrations and/or abnormalities. The number of ovine specific chromosomes (2n = 54) and the absence of aberrations revealed that the fibroblasts obtained out of the present study were of ovine origin and suitable for further experimentation studies. At each and every stage of *in vitro* culture assays starting from the chopping of ear tissue to the stage of cryopreservation of fibroblasts, the absence of

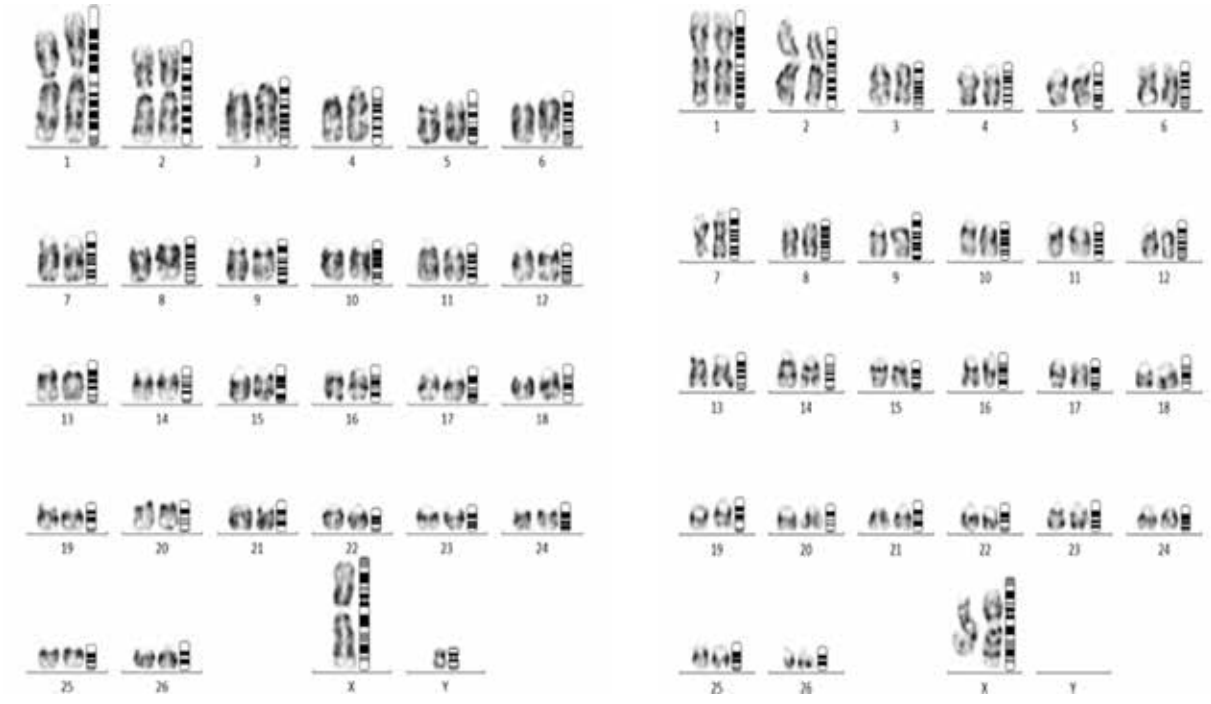

Fig. 5: *Karyogram A. Male Sheep; B. Female sheep.*

mycoplasma contamination was ensured by using the EZdetect PCR (Cat# CCK009-25R, Himedia) kit (data not shown). At P4 level, a total number of 120 samples were preserved in LN as per the standard protocols. Finally, the frozen fibroblast cells stored in cryovials were shipped under liquid nitrogen temperature conditions and submitted to ICAR-National Bureau of Animal Genetic Resources, Karnal for the purpose of their downstream applications. Due to the drastic reduction in the elite sheep germplasm from Southern Tamil Nadu, SEVA (Sustainable Agriculture and Environmental Voluntary Action) in association with Tamil Nadu Veterinary and Animal Science University initiated the conservation programme of Katchaikatti black sheep by means of lamb distribution, provision of elite rams, mineral mixture, consultancy on farm set-up, insurance coverage, health care and organization of awareness programmes, etc (Pathak *et al.,* 2020).

CONCLUSIONS AND FUTURE PROSPECTS

The present study demonstrated the successful establishment of fibroblast cell lines derived from ear pinna of Katchaikatti black sheep, which is known to be at the peril of extinction. The established ovine somatic cell lines could be used for its regeneration to the forthcoming generations. It is further recommended that other than karyotyping, other parameters need to be carried out for further characterization of the said cell lines derived from Katchaikatti Black sheep.

ACKNOWLEDGEMENTS

The authors gratefully acknowledged the facilities rendered by the Director, ICAR-Central Sheep and Wool Research Institute, Avikanagar, Rajasthan for carrying out the present study. The authors are extending their heartfelt thanks to Mr. Vivekananda & Mr. Kishore, from Madurai and Mr. Jyothi Ranga, a sheep farmer from Katchaikatti village for their valuable support in the identification and interaction with the farmers who have Katchaikatti sheep flock at the field level. The authors are also thankful to Mr. Raju, the Katchaikatti black sheep owner, Katchaikatti village, for his valuable services in the provision of biological samples from the sheep. Further, the help rendered by Mr. S. Rathinakumar in the collection of ear pinna samples from Katchaikatti sheep at the field level, is also gratefully acknowledged.

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