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Establishment and characterization of Caspian horse fibroblast cell bank in Iran

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Abstract Caspian horse, a rare horse breed found in 1965 by Louise Firouz in northern Iran, is a small horse which is reported to be in danger of extinction in its original homeland. There seems to be a great need to prevent extinction of this valuable horse. In this study, 51 fibroblast cell lines from Caspian horse ear marginal tissue were successfully established by sampling 60 horses using primary explant technique. Cells were authenticated and growth curve was plotted. According to results obtained, population doubling time (PDT) was calculated 23 ± 0.5 h for all cell lines. Multiplex polymerase chain reaction (multiplex PCR) revealed that cell lines had no cross-contamination with other species. Bacteria, fungi, and mycoplasma contamination were checked using standard methods such as PCR, direct culture, and Hoechst staining. In addition to providing a valuable source for genomic, postgenomic, and somatic cloning researches, the established cell lines would preserve Caspian horse genetic resources. It will also create an accessible database for researchers.

Keywords Caspian horse · Fibroblast cell line · Conservation · Cell banking

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Introduction

Horses are economically important and valuable for agriculture, transportation, warfare, and recreation. According to reports, there are more than 58 million horses with nearly 500 different breeds worldwide (Petersen JL, et al. 2013). Caspian horse is one of the rare animals with high environmental and economic values. Caspian horse is an ancient breed of small horses native to northern Iran. There have been very limited studies on Caspian horse due to its rareness. According to bottleneck studies on Caspian horse, this valuable breed is at risk of extinction (Amirinia C, et al. 2007).

Conservation of animal genetic resources is not a new debate and has approximately 60 yr of history. Despite remarkable progress in recent years, its development has been slow. Importance of native livestock species and breed conservation as farm animal genetic resources (FAnGR) seems to be due to prevention of genetic erosion of valuable animal populations, preservation of genetic diversity in response to current and future challenges and needs, deliver options to meet the demands of new markets for livestock products, and finally preservation of historical and cultural values (Gibons J, et al. 2006). According to Food and Agriculture Organization (FAO), it is estimated that from 7600 breeds of farm animal species worldwide, about 20% are at risk and about 1% becomes extinct every year. Diversity of cattle, sheep, goat,

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horse, poultry, and other farm animal species represents an irreplaceable source of traits for livestock development in response to changes in environment and human needs. Diversity in farm animal species can be divided in between and within breed diversity (Wright S 1969). However, these genetic resources are being eroded due to alterations in agricultural practices, economic issues, and environmental factors (Gibons J, et al. 2006).

There are a number of options to conserve the animal genetic resources including in situ, ex situ, and in vitro. These three strategies serve different objectives and are also different in terms of costs and efforts required. In situ conservation of FAnGR has several benefits; however, many developing countries have not reached advanced levels to support maintenance of FAnGR yet (Gibons J, et al. 2006). Therefore, there seems to be a great need for somatic cell collection as a way to conserve genetic resources of this breed. Somatic cell banking has been reported to be an appropriate technology for conservation of endangered breeds (Gupta N, et al. 2005). Somatic cells could be collected from skin, tissue, or blood sample of animals. Therefore, collection of samples can be inexpensive, fast, and easy. It is also possible to reconstitute the animal from every sample of somatic cells. Given that FAnGR are rapidly endangered, a new strategy is needed which can deploy quickly, sufficiently cheap, and simple to carry out in countries with limited infrastructure. Here, storage of somatic cells may open an option (Groeneveld E 2007).

Cryopreservation comprised material such as semen, embryos, and oocytes. Fast freezing can be applied for limited number of species. Furthermore, it requires substantial infrastructure which might not be available at all countries. Hence, this might not be an appropriate option (Woolliams J and Wilmut I 1999).

In this study, we aimed to produce more than 50 Caspian horse cell lines in order to protect genetic resources of Caspian horse and produce the related DNA bank.

Materials and Methods

Caspian horse selection Initially, Caspian horses geographic distribution areas and breeding centers were studied for sampling of registered horses. About 40 and 25 heads of Caspian horses are kept in Agriculture & Natural Resources centers in Tehran province (Khojir research complex) and Gilan (Caspian horse preservation of genetic resources complex), respectively. These horses are offspring of foundation flock. Number of criteria was considered for horse selection such as registration in the Caspian stud book, reliable pedigree, morphological characteristics, and achieving appropriate genetic diversity. Respectively, 34, 16, and 10 horses were selected for sampling from Khojir, Gilan, and Azmoon stations with a total number of 60. Sampling location, quantity, and gender of horses are listed in Table 1.

Ear sample collection method Ear margin tissues (about 1 cm^2 in size) were collected with an "O" shape ear notcher (AESCULAP Co., Center Valley, PA) from samples. The procedure was as follows:

1. Select a suitable horse based on the mentioned factors.
2. At the station, horses should be fixed by a rope.
3. Measure morphological characteristics such as back, head, neck, and ear height and write in the form.
4. Shave both sides of ear skin by disposable razor or shaver. Shaving hair makes it easy to anesthetize and disinfect the animal's ear.
5. Anesthetize the ear by spraying both sides with 10% lidocaine.
6. Disinfect sampling place carefully with 70% ethanol and wait until dried.
7. Take tissue samples using sterilized ear notcher.
8. Transfer ear biopsy to a falcon tube containing 4 ml DMEM (Dulbecco's Modified Eagle Medium) with antibiotic 1X (Penicillin–Streptomycin) and keep cold.
9. Transfer sample to the lab 1 to 5 d following the sample collection.

Somatic cell culture Primary culture In laboratory, primary culture process integrates after several actions. The ear tissue sample was treated with 70% ethanol and washed and kept in phosphate buffer saline (PBS) solution for 1 min. Skin was then removed from ear tissue and washed with PBS for 1 min.

Table 1. Characteristics and number of Caspian horses in each center and number of sampling.

Item	Location of sampling	Number of horses		Total	Number of samples		Total	Number of cell lines produced
		Male	Female		Male	Female		
1	Khojir research complex	24	17	41	19	15	34	29
2	Gilan research complex	11	15	26	6	10	16	14
3	Azmoun Equestrian Club, Tehran	5	10	15	2	8	10	8
Total		40	42	82	27	33	60	51

The sample was transferred to DMEM media. Subsequently, it was cut into approximately 1 mm^3 pieces and seeded in a 35-mm^2 tissue culture dishes with a sterile 22-mm^2 glass cover slip over it.

Afterwards, 3 ml DMEM (Gibco, Grand Island, NY) with 10% horse serum (Tehran University, Tehran, Iran), 1% L-glutamine (200 mM, 100X, Gibco), and Penicillin–Streptomycin 1X (Sigma, St. Louis, MO) were added (Takashima 2001). The dishes were cultured in a 37°C incubator with 5% CO_2 and 95% air for approximately 2 wk until they reached 80% confluency. The medium was changed every 3 d, and cultures were screened daily for substantial outgrowth of cells from the tissue pieces.

Subculture Media, sterile glass, and tissue pieces were removed from dishes. Cells were thoroughly washed with PBS and were detached by 0.25% Trypsin–0.02% EDTA (Gibco, 10X) solution at 37°C incubator for 3 min. After inactivation of trypsin, the cell suspension was centrifuged at 250*g* for 7 min and supernatant was discarded. The harvested cells were counted and viability was checked by trypan blue staining. The cells were split into new culture flasks containing DMEM with 10% fetal bovine serum, 1% L-glutamine (2 mM) and cultured at 37°C with 5% CO_2.

Growth curve The cells were seeded into 24-well plates, with a density of 5×10^4 cells/well. Cell growth and density data were monitored and recorded for 7 d. A cell growth curve was then plotted and the population doubling time (PDT) was calculated (Gu Y, et al. 2006, Kong D, et al. 2007).

Chromosome analysis Once cell cultures reached 75% confluency, colcemid was added in final concentration of 0.01 μg/ml and incubated at 37°C for 3 h. The medium was then transferred to a centrifuge tube. Cells were washed with PBS. After 30 s, PBS was removed and transferred to a selfsame tube. Cells were then trypsinized and transferred into a selfsame tube containing medium and PBS. After centrifugation, 10 ml of pre-warmed (37°C) hypotonic solution (0.075 M KCl) was added to the pellet and mixed gently and incubated for 30 min at 37°C. Next, 1 ml of cold fixative (3:1 solution of methanol to acetic acid) was added and centrifuged for 10 min at 250*g*. Cell pellet was suspended by flicking the bottom of the tube. Fixation step was repeated three times. After final fixation, 1 ml fixative was added to the suspended pellet. Two drops of the cytogenetic suspension was placed on a slide. Once the slides dried, they were aged at 65°C for 18 h. The slides were then placed in trypsin solution (0.025%) for 30 s. After washing with PBS, slides were stained using Giemsa for 10 min. The slides were rinsed afterwards and air dried for analysis. At least 10 to 20 metaphases were scored and analyzed (Moore CM and Best RG 2001).

Contamination During cell culture steps, cells were screened by microscope for fungal and bacterial contamination. The cells were cultured without antibiotic for three passages. Cultured cells and supernatant were inoculated to thioglycolate broth and tryptone soya broth and incubated for 14 d in 22 and 32°C, separately (Freshney RI 2010). Mycoplasma contamination was detected with three methods: fluorescent DNA staining of DNA using Hoechst dye 33258 (Sigma B1155), multiplex PCR evaluation, and microbial direct culture as gold standard for efficient mycoplasma detection (Volokhov DV, et al. 2011).

Cell line identification Genomic DNA was extracted from primary cells with column-based DNA extraction kit (IBRC MBK0021). The authentication of primary cell was determined with amplification of cytochrome C oxidase subunit I (COI) mitochondrial gene by multiplex PCR method with specific primer of different animal species such as mouse, rat, rabbit, camel, horse, cow, sheep, cat, dog, guinea pigs, pig, rhesus monkey, African green monkey, Chinese hamster, human, and chicken. Each species reveals a unique band size (Cooper JK, et al. 2007).

Cross-contamination identification was assessed using 17 microsatellites markers recommended by the International Society for Animal Genetics. Markers including VHL20, HTG4, AHT4, HMS7, HTG6, AHT5, HMS6, ASB23, ASB2, HTG10, HTG7, HMS3, HMS2, ASB17, LEX3, HMS1, and CA425 were studied. All primers were premixed and analysis was conducted under optimized PCR condition, according to procedure recommended by the manufacturer. All loci were amplified by multiplex PCR reactions and then loaded with GeneScan-500 LIZ size standard into the ABI PRISM 310 DNA Sequencer.

Immunocytochemistry After adding the cell suspension in 6-well plates, they were kept in CO incubator at 37°C until they reached 80% confluency. Then, cells were fixed in 10% paraformaldehyde for 30 min at room temperature. Cells were then washed with PBS three times. Block the cells in 2% bovine serum albumin (BSA) in PBS at room temperature. Thereafter, cells were incubated overnight in the primary antibody (Anti-Collagen I antibody, Abcam, Cambridge, UK) at 4 °C. Cells were rinsed in 2% BSA in PBS. Cells were incubated in the fluorophore-conjugated secondary antibodies (Anti-C3c antibody (FITC), Abcam) for 2 h at room temperature in the dark. After that, incubation was done in the diluted DAPI for 10 min in the dark.

Cryogenic preservation and recovery To ensure optimum health and good cell recovery, cells were freezed in their log phase (actively growing phase). The culture medium was changed 24 h prior to harvesting. The cell suspension was acquired by digesting cells in a 0.25% Trypsin–0.02% EDTA solution (at third passage). The suspension was centrifuged at 250*g* for 7 min and supernatant was discarded. The harvested cells were counted with a hemocytometer and viability was checked by trypan blue staining. Cells were resuspended in sufficient media for freezing (10% DMSO plus

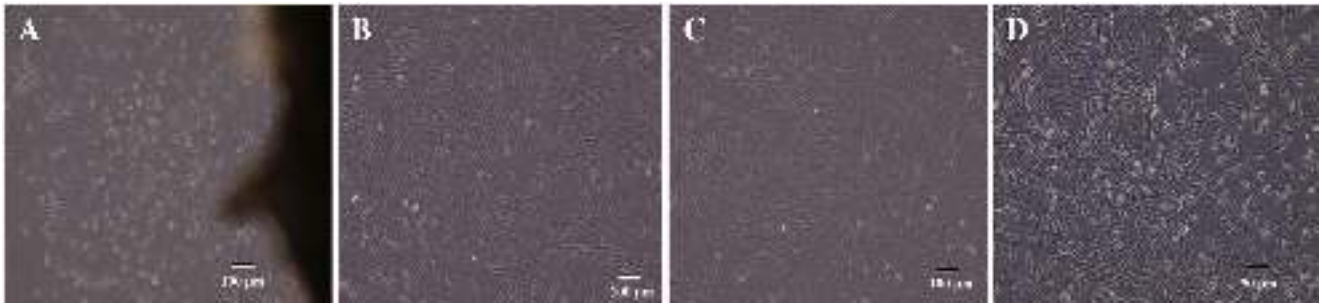

Figure 1. Cell morphology of Caspian horse fibroblast cells. *a* Primary cells (×100). *b* Subcultured cells (×100). *c* Cells prior to cryopreservation (×100). *d* Cells in recovery after thawing (×50).

90% horse serum) to reach a final density of 1–2 × 10^6 cells/ml. Subsequently, 1 ml of the cell suspension was allocated into one sterile cryogenic vial labeled with required information such as cell line name, accession number, and date of freezing. The vials were sealed and placed into cryoboxes. These boxes were placed in a −20°C freezer for 1 h and then transferred to a −80°C freezer overnight and finally stored in a liquid nitrogen (−196°C) storage system.

Transfection efficiency Transfection is a routine method to produce genetically modified cells which is basically introducing foreign nucleic acids (DNAs or RNAs) into cells. Transfection is known to be an influential analytical tool for study of gene and protein function (Recillas-Targa F 2006). In the current study, The HIV packaging (pCMVDR8.91), VSV-G (pMDG) plasmids and the lentiviral pCSGW vector were provided by IBRC. Lentivirus carrying GFP (green fluorescent protein) was used as a control in gene transfer study. For production of lentivirus, 293T cells were seeded into a 10-ml petri dish and transfected with 15 μg of transfer vector pCSGW carrying GFP, 10 μg of pCMVDR8.91, and 10 μg of pMDG using polyethylenimine. The viruses were harvested 48 h after transfection, passed through a 0.45 μm filter, and concentrated by ultracentrifugation at 100,000*g* for 90 min. Viral particles were resuspended in serum-free DMEM, snap-frozen in liquid nitrogen, and stored at −80°C. For infection, the cells were transduced at various multiplicities of infection (MOIs of 3 and 6) for 16 h (Song J, et al. 2007).

Results

Caspian horse characterization: Eventually, 51 cell lines were successfully produced. Nine samples were lost due to nonproliferation and contamination during sampling and laboratory steps (Table 1).

Cells morphology & viability Once ear samples were cultured for 5–7 days, migration of fibroblast and epithelial-like cells was detected (Fig. 1*a*). Cells continued to proliferate and were sub-cultured when they reached 80–90% confluency (Fig. 1*b*). Fibroblasts grew rapidly, gradually outgrowing and excluding other cells such as epithelial cells and only fibroblasts were observed after three passages in most cell lines (Fig. 1*c*, *d*). Morphology of fibroblast cells was mostly spindle like and a few triangles or multiangular shapes were also observed. Fibroblast phenotype was confirmed using collagen synthesis imaging which is shown in Fig. 2. Viability for all cell lines was calculated 95–98% before freezing as well as post recovery.

Growth curve Caspian horse skin fibroblasts were seeded in tissue culture dishes containing DMEM media with all necessary supplements. The number of cells was assessed every 24 h for 7 d post-seeding. The results indicated that Caspian horse fibroblast cells had similar growth patterns to other animal skin fibroblast with an obvious "S" shape growth curve. Culture conditions were optimal for Caspian horse fibroblast cells. The population doubling time (PDT) was approximately 23 h for all cell lines (Fig. 3).

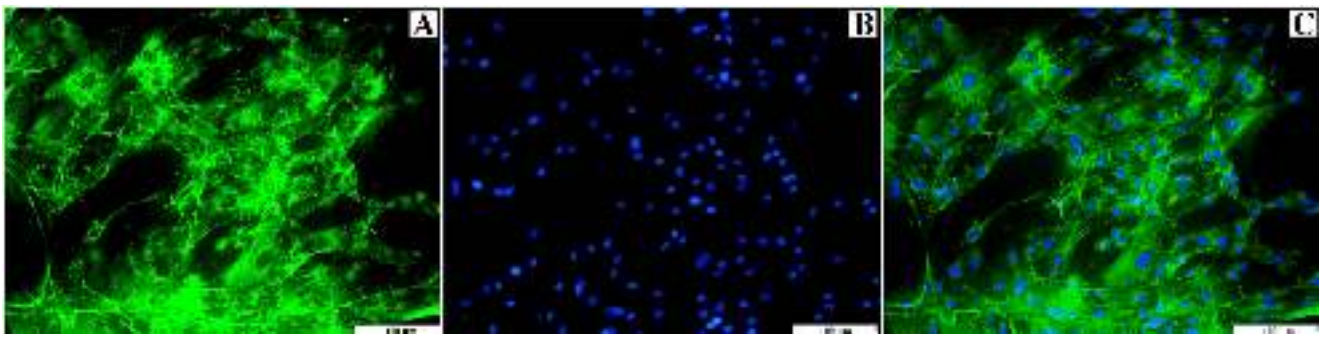

Figure 2. (*A*) Cells stained with collagen antibody and FITC stain. (*B*) Nucleus of cells stained by DAPI. (*C*) Double staining of collagen and DAPI. Nucleus can be observed as *blue* and collagen as *green*.

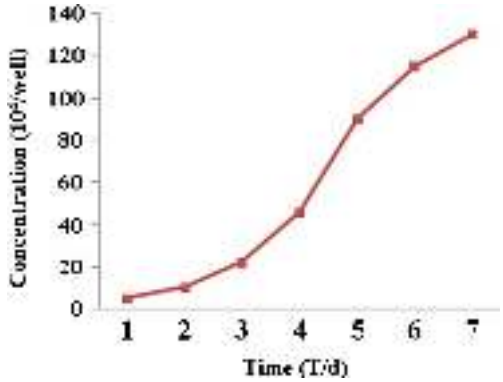

Figure 3. The CaHo cell lines growth curve. The growth curve pattern had typical "S" shape including three phases of growth (lag phase, exponential phase, and stationary phase). The average of population doubling time was approximately 23 ± 0.5 h.

Chromosome analysis The chromosome number of domestic horse *(Equus caballus*) was investigated in 10 samples. Chromosome count in all samples was 2n = 64 of which 13 pairs are submetacentric (chromosome 1–13) and 18 pairs acrocentric (chromosome 14–31). The X chromosome is submetacentric and the Y is acrocentric (Fig. 4). After 20 passages, cells were still showed 80% diploid chromosomes.

Contamination Fifty-one out of total 60 samples were negative for bacteria, fungi, mycoplasma, and yeasts. Other contaminated cells were discarded. The results were confirmed by Hoechst 33258 staining, multiplex PCR method, and microbial direct culture. After staining with Hoechst 33258, fluorescence microscopy revealed no contamination in samples.

Cell line identification For authentication, one sample of each Caspian horse fibroblast cell lines was analyzed for species identification. All Caspian horse fibroblast cell lines were showed single and specific band with 243 bp in length which was specific for horse species compared with other species. Analysis of microsatellites markers revealed no cross-contamination between cell lines. Relationship between parents and their offsprings was demonstrated by Parentage test (Fig. 5). Results obtained from these two tests confirmed cell lines authentication.

Immunocytochemistry Results obtained from immunocytochemistry is illustrated in Fig. 2. As can be seen in Fig. 2, the cells showed to express high levels of collagen. Therefore, by considering the fibroblast morphology and collagen expression, it can be concluded that cells were fibroblasts.

Establishment of Caspian horse cell line After culture, subculture, and cell proliferation, suitable samples were frozen and stored in nitrogen tank. Cell lines were kept at low passage number (two passages without antibiotic) (Fig. 1*c*, *d*). Ten vials containing 1 × 10^6 cells/ml of each cell line were successfully stored in nitrogen tank.

Accession number and cell line name were assigned to each cell line (from CaHo-01 to CaHo-51). Meanwhile, quality control test including viability and contamination testing were examined at intervals of 2 wk, 6 mo, and 1 yr post freezing.

Animal and cell certificates were prepared for all cell lines. Horses' characteristics were recorded in animal certificate, including photo, name/code, microchip number, sex, age, color, year and place of birth, sire, dam, date of sampling, location of sampling, withers height, back height (horizontal distance between withers and loin), head height, neck height, ear height, and breed specification.

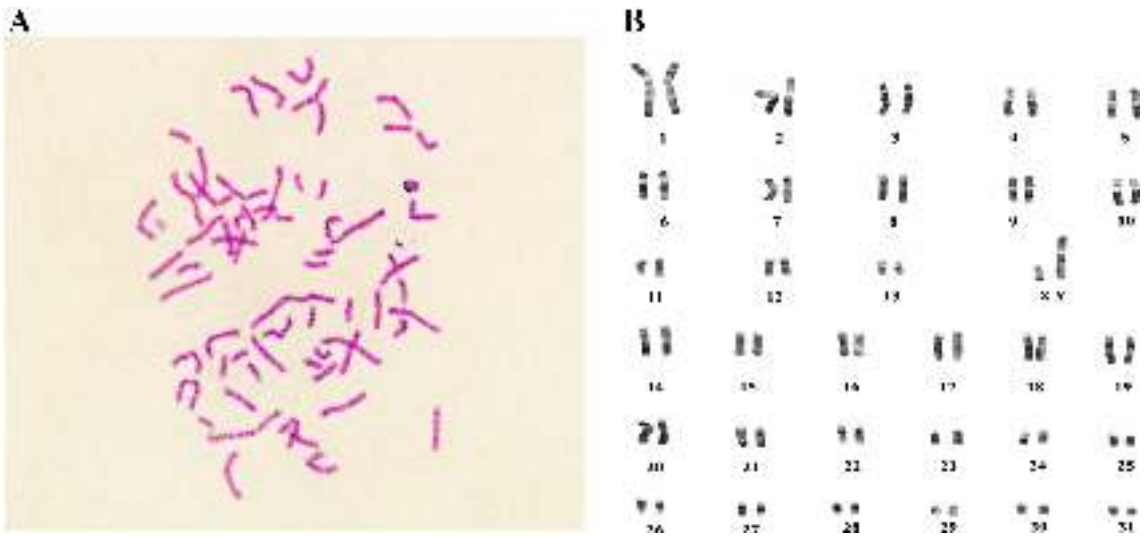

Figure 4. Chromosome analysis. Metaphase chromosome plate (*A*) and karyotype analysis of CaHo cells (male Caspian horse) (×1000) (*B*). Caspian horse karyotyping indicated 2n = 64 chromosomes.

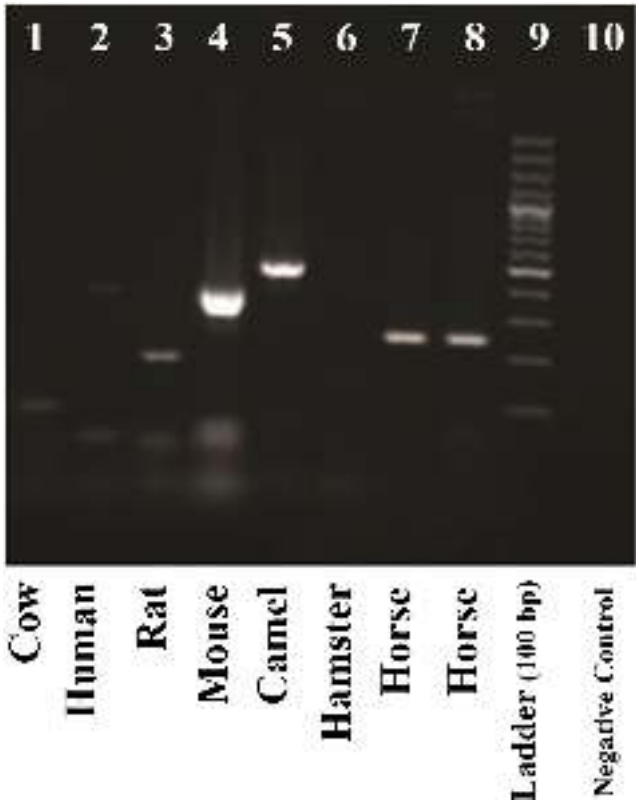

Figure 5. Identification of species by multiplex PCR using specific primers. Lines *7–8* showed the CaHo cell lines showing no cross-contamination for horse species. Lines *1–6* was cow, human, rat, mouse, camel, hamster (cross contaminated with human cell line), and horse samples, respectively. Line *10* was considered as PCR negative control.

Cell line characteristics were recorded in cell certificate, including cell line name, accession number, type, morphology, culture condition, and mycoplasma, bacteria and fungi contamination tests results. Samples were taken from 1- to 23-yr-old horses in different colors (black, white, chestnut, and bay).

Transfection efficiency The HIV packaging (pCMVDR8.91), VSV-G (pMDG) plasmids, and the lentiviral pCSGW vector were used for transfection. CaHo-01 cells were transiently transfected. Phase contrast fluorescence inverted microscope evaluation showed more than 50% transfected cells at various multiplicities of infection (MOIs of 3and 6) (Fig. 6).

Discussion

Results of the current study confirmed that animal ear tissue is suitable to achieve an animal fibroblast cell line which is in accordance with other studies conducted by Groeneveld (Groeneveld E 2007). Moreover, Lima-Neto et al. has reported fibroblasts obtained from equine to be an appropriate source to establish equine cell lines (Lima-Neto JF, et al. 2010). Somatic cell banking has been reported to be an alternative technology for endangered sheep breeds by Gupta et al. (2005) which indicates hope for using somatic cells to use in cell therapy, cloning, and other methods to prevent these breeds from extinction (Yee J 2010). In the future, stem cell differentiation into sperm will provide us with animal restoration, reproduction, and transgenic production (Easley CA 4th, et al. 2012). Equine cloning has been conducted for the first time in 2003(Woods GL, et al. 2003), and due to fibroblast potential ability to be used in cloning technology, it is confirmed that fibroblast cell bank is vital for breed restoration (Hinrichs K 2006).

According to our results, the established cell lines had normal growth rate and chromosome stability. Species were confirmed as horse, correctly and no cross-contamination was observed. The cells were cryopreserved at low passage number with 1×10^6 cell/ml density, to conserve cells from DNA damage caused by several passages. The fibroblast cells were obtained using explant technique which has higher efficiency than enzymatic technique (Klingbeil MF, et al. 2009).

As can be seen in Fig. 4, complete karyotype of Caspian horse is shown. Horse karyotype has been studied by several researchers up until now. Karyotype of Caspian horse observed in the current study was in accordance with past reports conveyed by Richer and Ruvinsky (Richer CL, et al. 1990, Ruvinsky A and Bowling AT 2000). Hatami-Monazah et al. particularly investigated Caspian horse karyotype which was in accordance to our findings (Hatami Monazah H and Pandit RV 1979).

Transfection efficiency is dependent on many factors including plasmid DNA concentration and incubation time, and serum in medium (Escriou V, et al. 2001, Rui R, et al. 2006). The CaHo cell lines were screened for their potential to

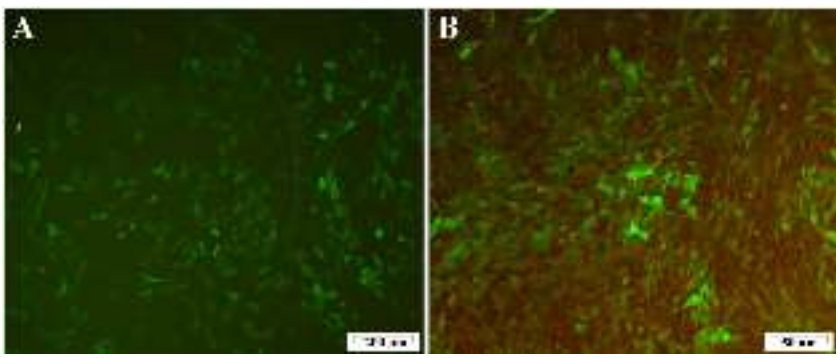

Figure 6. Transfected Caspian horse cells. (*A*) MOI 3. (*B*) MOI 6 Transfection efficiency in CaHo-01 cells (×200). Cells were observed 72 h after transfection, and the results showed that CaHo cells have the ability to express genes encoding fluorescent protein.

express exogenous genes such as GFP. Results obtained in the current study revealed that the established cell lines can be used in further studies in transgenic cloning.

In conclusion, considering the need to prevent extinction of endangered animals, it can be gathered that established fibroblast cell bank with approved transfection potential can be a great resource for further studies on Caspian horse. Further study is needed to characterize the established cell lines at the molecular level and to evaluate genetic diversity and phylogenetic analysis using molecular markers such as microsatellites and mtDNA.

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Conflict of interest The authors declare that they have no conflict of interest.

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