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Establishment and Quality Control Criteria for Population Culture Collection - Promising Strategy for Animal Genetic Resource Preservation

Yuehui Ma et al.¹

*Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing
PR China*

1. Introduction

Animal genetic resources top the list of the most fundamental properties for animal husbandry and medical sciences, play an irreplaceable role in human survival and social prosperity, constitute an invaluable substrate for scientific researches, and have an enormous bearing on sustainable development of economy. Biodiversity takes an underlying part in ecological balance. The havoc people wreaked on nature had been aggravating ever since agricultural civilization, accelerating the extinction of animal species and breed incomparable than gradual natural loss, which became more and more apparent upon the advent of industrial age. The statistics of Food and Agriculture Organization (FAO) in 1995 revealed that approximately 15% of the total 738 registered livestock and poultry breeds in Sub-Saharan Africa were on the brink of extinction. The situation has been aggravating ever since. To date, the proportion of livestock breed in danger has increased from 8% to 19%, while that of poultry has risen from 20% to 34%. Among the 1251 registered breeds in Asia, 10% are severely endangered. From 1995 to 1999, livestock breeds about to be extinct grew from 11% to 14%, and the proportion of poultry was 32% to 37%. Owing to economic pressure, some low yield breeds are being subjected to marketing elimination and shrinkage in population, for instance, the production of poultry and swine depends heavily on only a few breeds. The trend is extremely obvious in Eastern Europe, which is further worsened by political instability. Similarly, sustaining intensification of animal husbandry makes the food production rely more and more on a small number of high yield breeds, thereby exacerbating the animal diversity crisis. In Latin America, the number of endangered breeds accounts for 20% of the whole. As was reported by the FAO in 2000, livestock and poultry throughout the earth are disappearing at the rate of 2 breeds per week. Worse still, 1350 breeds are next to imminent extinction. For all that matter, animal genetic resources are confronted with a progressive narrowing in diversity. Accordingly, it's absolutely exigent to protect and preserve them with effective measures.

¹ Weijun Guan, Xiangchen Li, Xiaohong He, Yabin Pu, Qianjun Zhao, Dapeng Jin, Shen Wu, Taofeng Lu, Xiaohua Su, Chunyu Bai
Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, PR China

In the context of biodiversity crisis, no country around the world stands indifferent regarding the preservation of animal genetic resources. The contest for genetic resources, the basis for animal husbandry and sustainable development, is nearly incandescent.

Scientists from all around the world have been endeavouring to preserve and to make use of animal genetic resources, which are now stored in terms of individual animals, semen, embryos, genomic libraries, cDNA libraries, etc. Unfortunately, these alternative methods remain problematic for several reasons: i) endangered species and breeds are incredibly diversified, rendering the costs for preservation of individual animals unaffordable; ii) some core techniques for semen and embryos are still immature; iii) genomic DNA or organ preservation are not applicable in long term because of their finite proliferative capabilities; iv) genomic libraries and cDNA libraries are not the basic unit of cellular activities, moreover, their biological function can only be represented in transgenic techniques. Accordingly, preservation of animal genetic resources in terms of somatic cells is essentially an effective and appealing procedure to protect vulnerable mammalian and avian species, as well as all other kinds of animals. In comparison, somatic cell line, by virtue of its low costs, large capacity, convenient application, proliferative potential and so on, is supposed to be a promising strategy for storage of animal genetic resources.

Consistent with this notion, culture collections of animal materials, mainly identified cell lines, have been established and developed over time. American Type Culture Collection (ATCC), for instance, endeavours to isolate, collect, preserve and supply reliable cell lines, with its all identified type culture applicable for register, preservation, instant use, and even commercial provision. European Collection of Animal Cell Culture (ECACC), jointly run by England and Switzerland, has collected and identified some 1600 cell lines. Established in 1986, Kunming Institute of Zoology, Chinese Academy of Sciences has collected cell stains, tissues and germ cells of numerous precious species of wild life.

The lab of Animal Genetic Resources, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, by virtue of its solid technical platform, advantages in animal genetic resources, and persistent efforts in the past decade or so, has established Animal Population Culture Collection of China (APCCC), the most massive animal population culture collection in the world, comprising somatic cell lines with different tissue origins, including ear margin, blood, kidney, heart, brain, muscle, stomach and so on, from 88 animal breeds primarily in China, e.g. chicken (*Gallus gallus*), duck (*Anseriformes Anatidae*), goose (*Anser cygnoides orientalis*), sandpiper (*Scolopacidae*), cattle (*Bos taurus*), sheep (*Ovis aries*), goat (*Capra hircus*), pig (*Suidae*), ferret-polecat (*Mustela Pulousius Furot*), raccoon dog (*Nyctereutes procyonoides*), horse (*Equus caballus*), mule, red deer (*Cervus elaphus*), sika deer (*Cervus nippon*), fox (*Vulpinae*), wolf (*Canis lupus*), bactrian camel (*Camelus bactrianus*), tiger (*Panthera tigris*), etc., and further endeavours to conserve other animal species and breeds in the world. A well-orchestrated series of standardized technical lines and quality control criteria is steadily ameliorated in this process.

This chapter will introduce the preservation of animal genetic resources in terms of somatic cells and the quality control criteria by detailed experimental description and technical line.

2. Isolation, *in vitro* culture and identification of somatic cell lines

2.1 Sampling and cell culture

Tissue pieces (about 1 cm³ in size) were sampled from animals and placed into sterile tubes containing Dulbecco's modified Eagle's medium (DMEM, Gibco) (for livestock breeds)/

Modified Eagle's Medium (MEM, Gibco) (for poultry breeds) medium supplemented with ampicillin (100 U/ml) and streptomycin (100 µg/ml). The samples were immediately brought back to the laboratory for further experiments.

The tissues were rinsed and chopped to 1 mm³ pieces, which were then plated onto the bottom of a tissue culture flask in an incubator at 37°C with 5% CO₂ for 2 h until the tissue pieces spontaneously adhered to the flask surface, and then DMEM/MEM containing 10% fetal bovine serum (FBS, Gibco) was added. Cells were harvested when they reached 80%–90% confluence and were passaged into more flasks at the ratio of 1:2 or 1:3 (Freshney 2000).

After three passages, the cells in logarithmic phase were harvested and resuspended at the concentration of 4 × 10⁶/ml in cryogenic media containing 40% DMEM/MEM, 50% FBS and 10% DMSO (Sigma), aliquoted into cryovials, and kept at 4°C for 20–30 min to equilibrate the DMSO. Then they were put into a programmed cryopreservation system with controllable temperature dropping rate, and finally transferred to liquid nitrogen for long-term storage (Jenkins, 1999).

2.2 Trypan Blue exclusion test

Viabilities before cryopreservation and after resuscitation were determined using Trypan blue exclusion test. The cells were plated in 6-well plates at 10⁴/well and counted with a hemocytometer (Qi et al., 2007).

2.3 Growth dynamics

According to the method of Gu et al. (Gu et al., 2006) and Ikeda Y et al. (Ikeda Y, 1990), cells at the concentration of 1.5 × 10⁴/ml were plated into 24-well plates. Three wells were counted each day until the plateau phase. Based on the numbers, the mean values of cell density were then calculated and plotted against the culture time. The population doubling time (PDT) was calculated accordingly.

2.4 Microbial detection

- Tests for contamination with bacteria, fungi and yeasts: the cells were cultured in antibiotic free media. Bacterial, fungal and yeast contamination was assessed within 3 days as described by Doyle et al. (1990).
- Test for viruses: the cells were subjected to Hay's hemadsorption protocol using phase-contrast microscopy to detect cytopathogenesis (Hay, 1992).
- Test for mycoplasmas: cells were cultured in antibiotic free media for at least 1 week, and then fixed and stained with Hoechst 33258 (Sigma) according to the method of Masover and Becker (1998) and Freshney (2000). The ELISA Mycoplasma Detection kit (Roche, Lewes, East Sussex, UK) was used to confirm the results of the DNA fluorescent staining.

2.5 Karyotyping and chromosomal indices

The cells were harvested upon 80%–90% confluence. Microslide preparation and chromosome staining were performed as described by Suemori et al. (2006). Fifty to 100 spreads were sampled for counting chromosome numbers of diploid cells. There are three important parameters for chromosomal analysis, i.e. relative length, arm ratio, and centromere index, which were determined according to the protocol of Kawarai et al. (2006).

2.6 Isoenzyme analysis

Isoenzyme profiles of lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) were identified by vertical slab non-continuous polyacrylamide gel electrophoresis (PAGE). The cells were harvested, pelleted and resuspended in protein extraction solution (0.9% Triton X-100, 0.06 mmol/L NaCl:EDTA in volume ratio of 1:15) at the density of 5×10^7 cells/ml. Then the suspension was centrifuged and the supernatant was stored in aliquots at -70°C . Isovolumic 40% (m/v) sucrose and 2.5 ml loading buffer were added to each sample to get loading solution (Zhongxiao and Shuzheng, 1999). Subsequently the electrophoresis was performed at the voltage of 120 V. When the bromophenol blue migrated into the separation gel, the electrophoresis voltage was modified to 220 V. The electrophoresis was terminated when the bromophenol blue migrated to the bottom (0.5 - 1 cm to the margin). Different mobility patterns were differentiated by the relative mobility front (RFs), which was calculated as the ratio of the migration distances of the isozyme bands to that of the bromophenol blue.

2.7 Expression of exogenous genes

According to the method of Tsuchiya et al. (2002), the fluorescent plasmids pEGFP-C1, pEGFP-N3, pEYFP-N1, pDsRed1-N1, pECFP-N1 and pECFP-mito were transfected into the cells with Lipofectamine™ 2000 transfection reagent (Invitrogen Corp., Carlsbad, CA). The plasmid DNA (μg) to Lipofectamine 2000 (μl) ratio was 1:3. After 8 h, the serum-free transfection media were replaced with complete media. To evaluate the transfection efficiency, the cells were observed under a confocal microscope (Nikon TE-2000-E, Japan) at 24 h, 48 h and 72 h after transfection, respectively. The test data were subjected to multiple comparisons to analyze statistical difference. For each sample, images were captured from ten visual fields, and the total and positive cells were counted in each field to determine the transfection efficiency.

3. Results

3.1 Morphological observation

The somatic cells sprouting from tissue explant pieces grew rapidly and migrated from the tissues with a different time and speed according to the species origin. Fibroblasts were initially mingled with epithelial cells, but the fibroblasts, by virtue of their proliferative superiority, would outgrow the epithelial cells gradually after 2-3 passages, and prevail in the population (Fig. 1). Then, purified fibroblast lines were obtained. The cells had fibrous contour with plump cytoplasm, and during growth they were morphologically fibroblast-like with radiating, flame-like or whirlpool migrating patterns. The cells were then subjected to programmed cryopreservation. Trypan blue exclusion test showed non-significant difference ($P > 0.05$) in viability upon proper freezing procedures, and resuscitated cells displayed good morphology and proliferative activities.

3.2 Growth dynamics

The growth curves of the somatic cell lines in APCCC before cryopreservation and after cryopreservation displayed a typical "S" shape (Fig. 2) and the PDT was approximately 24 h to 48 h, which varies from species to species or even between subspecies. There is usually a lag time or latency phase of about 24 h to 48 h after plating, corresponding to the adaptation and recovery of the cells from trypsinization, and then the cells proliferate rapidly and enter

exponential phase. As the cell density increased, proliferation slows down due to contact inhibition. The cells subsequently enter the plateau phase and begin to degenerate. The growth curves before cryopreservation is generally consistent with that after resuscitation.

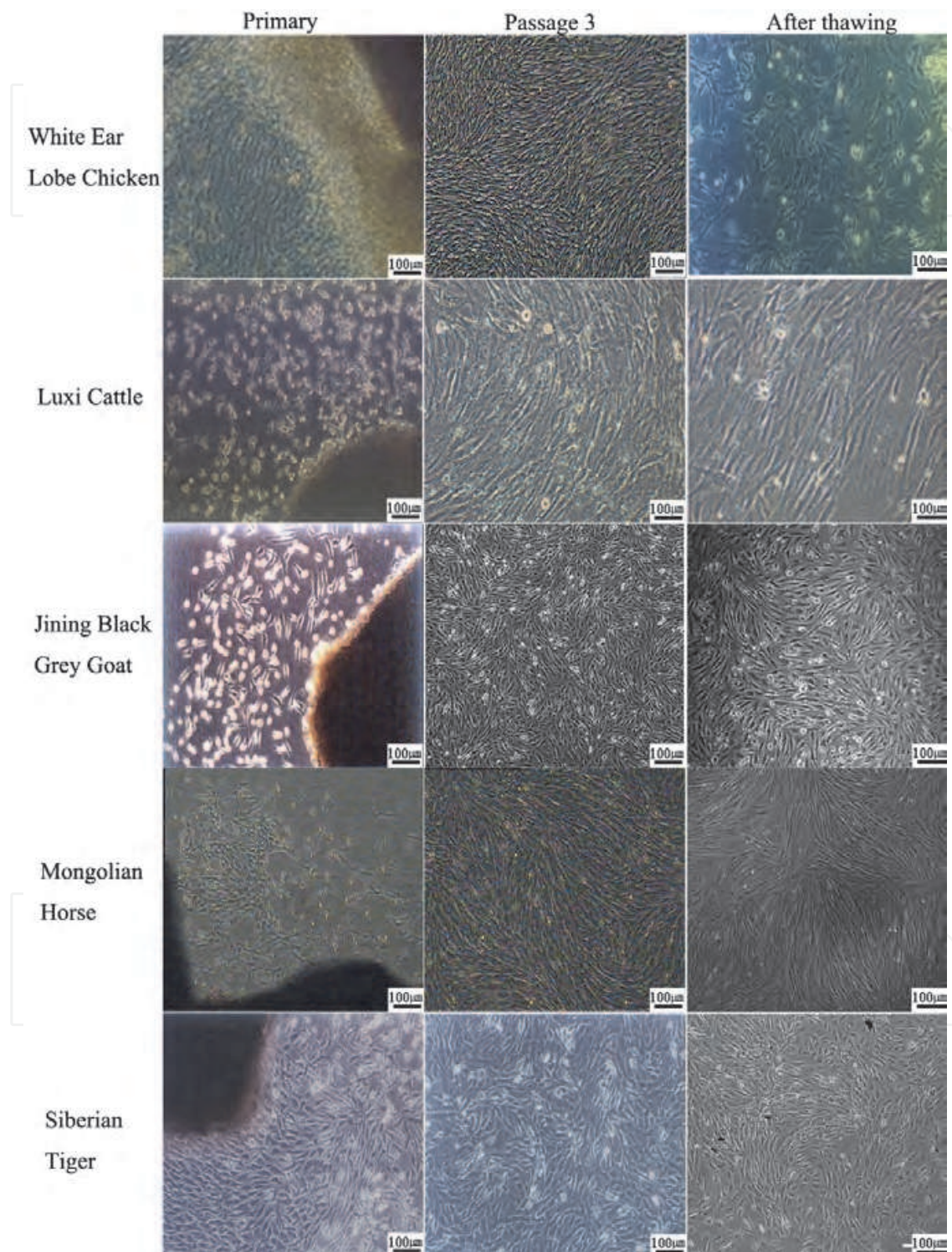


Fig. 1. Morphology of somatic cells of White Ear Lobe chicken, Luxi cattle, Jingning Black Grey goat, Mongolian horse and Siberian tiger in primary culture, before cryopreservation and after resuscitation.

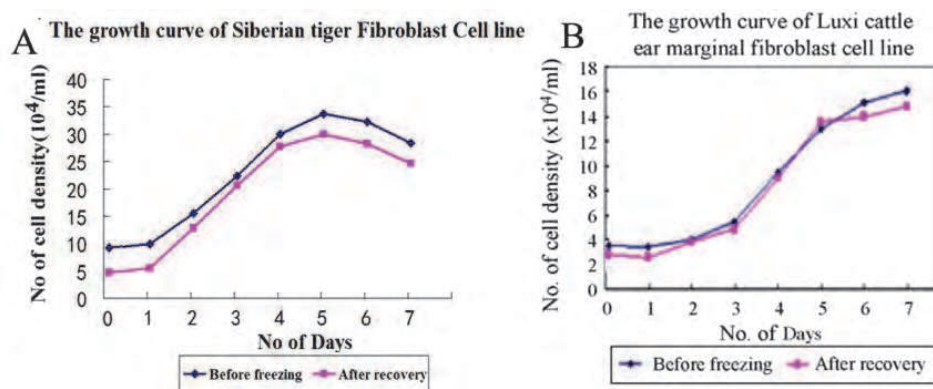


Fig. 2. Growth dynamics. Growth curves of (A) Siberian Tiger fibroblast line, and (B) Luxi Cattle ear marginal fibroblast line before cryopreservation and after resuscitation. A representative growth curve consist of latency phase, exponential phase, plateau phase and decline phase.

3.3 Microbial detection

In a sharp contrast with infections by bacteria, fungi and yeasts, characterized by turbidity, colony or hypha, which can be observed by unaided eyes, the mycoplasma contamination, usually undistinguishable, is only accompanied with slightly slower growth and increased cell fragmentation. As a result, Hoechst 33258 staining or molecular assays are required further. Would there be abundant punctiform and filiform blue fluorescence in the nucleoli, it could be concluded that the cells were contaminated by mycoplasmas (Fig. 3B).

In APCCC, all the somatic cells are subjected to microbial detection prior to cryopreservation to ensure they are free of bacterial, fungal and yeast contamination. No microorganisms were observed in the culture media. No viruses were indicated either by the hemadsorption test. Mycoplasma testing by both the ELISA detection kit and Hoechst 33258 staining (Fig. 3A) was negative. Through microbial detection, the safety and reliability of future commercial and experimental applications of the cell lines are to a large extent ensured.

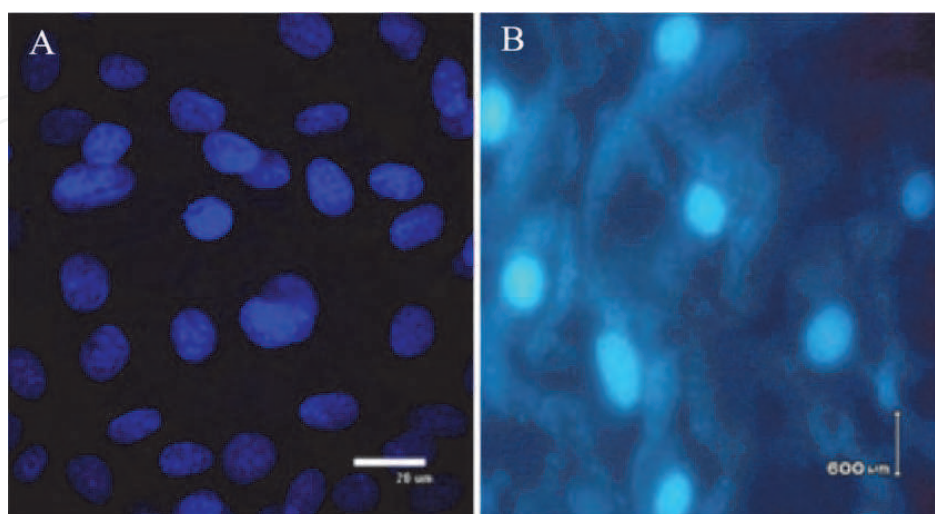


Fig. 3. Hoechst 33258 staining for (A) the detection of mycoplasma in the Siberian tiger fibroblasts; and (B) a positive control of mycoplasma contamination.

3.4 Karyotyping and chromosomal indices

Diploid cells of a given species possess a characteristic chromosome number, shape and structure, which remain very stable in normal cells (Fig. 4). Therefore, karyotype analysis is a major method for distinguishing normal cells from mutants. The percentage of diploid cells tends to decrease with increasing passage number. However, the fact that the diploid proportion is normally higher than 90% warrants the hereditary stability.

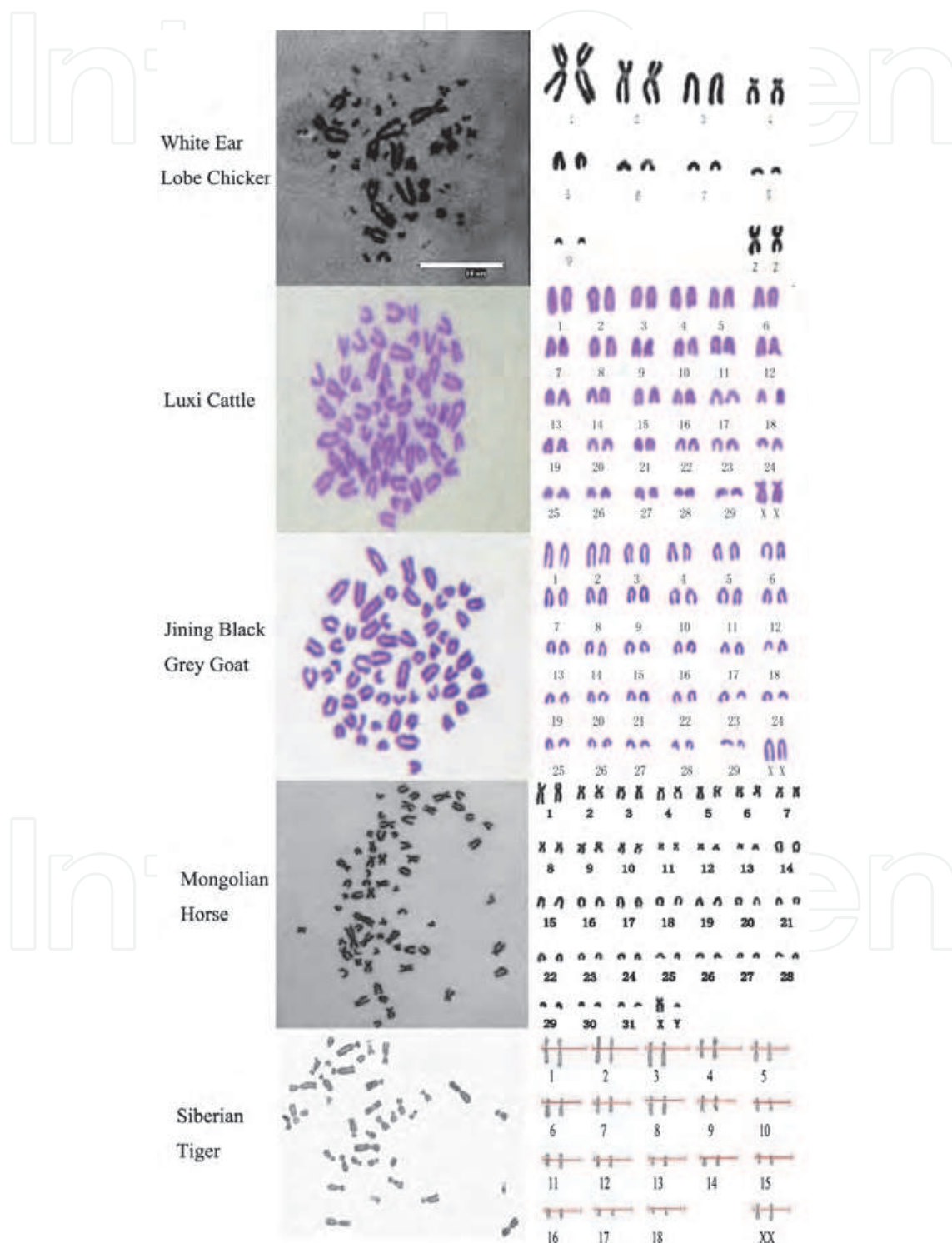


Fig. 4. Chromosome at metaphase (left) and karyotype (right).

Chromosome number	Relative length (%)	Centromere type
1	5.58 ± 0.26	T
2	5.12 ± 0.16	T
3	4.68 ± 0.34	T
4	4.49 ± 0.41	T
5	4.23 ± 0.12	T
6	4.05 ± 0.45	T
7	3.87 ± 0.38	T
8	3.86 ± 0.57	T
9	3.81 ± 0.04	T
10	3.76 ± 0.22	T
11	3.61 ± 0.11	T
12	3.56 ± 0.19	T
13	3.41 ± 0.33	T
14	3.36 ± 0.20	T
15	3.27 ± 0.41	T
16	3.26 ± 0.32	T
17	3.01 ± 0.09	T
18	2.97 ± 0.19	T
19	2.97 ± 0.06	T
20	2.71 ± 0.31	T
21	2.70 ± 0.24	T
22	2.60 ± 0.12	T
23	2.58 ± 0.27	T
24	2.21 ± 0.19	T
25	2.14 ± 0.22	T
26	2.09 ± 0.53	T
27	2.07 ± 0.10	T
28	1.85 ± 0.35	T
29	1.75 ± 0.32	T
X	4.47 ± 0.11	SM

Note: Relative length, 1.0-1.6, Metacentric chromosome (M); 1.7-2.9, Submetacentric chromosome (SM); 3.0-6.0 Subtelocentric chromosome (ST); ≥7.0 Telocentric chromosome (T).

Table 1. Chromosomal parameters of White ear lobe chicken(♀).

The chromosome number of Luxi cattle was $2n = 60$, comprising 58 autosomes and two sex chromosomes, XY or XX. All the autosomes are acrocentric, and only the two sex chromosomes (XY) were submetacentric (Table 1). The chromosome numbers were counted for 100 spreads of passages 1, 3 and 4 respectively, and the frequencies of cells with $2n = 60$ were 92.2%, 91.6% and 90.7% accordingly.

The chromosome number of Siberian tiger is $2n=38$, consisting of 36 autosomes and two sex chromosomes, XY or XX. The karyotype composition of the Siberian tiger is 12 (M) + 16 (SM) + 4 (ST) + 4 (T), XY (M, M) (Table 2). The chromosome numbers were counted for 100 spreads of passages 1, 3 and 4 respectively, and the frequencies of cells with $2n=38$ were 91.6%, 91.2% and 90.2% accordingly.

Chromosome No.	Relative length (%)	Centromere type
1	10.13±0.93	SM
2	9.48±1.05	M
3	8.43±0.92	ST
4	6.65±0.85	M
5	6.31±0.81	SM
6	5.85±0.75	ST
7	5.66±0.70	M
8	5.34±0.67	SM
9	5.22±0.71	SM
10	4.47±0.61	SM
11	4.11±0.75	M
12	3.51±0.66	SM
13	3.54±0.44	M
14	3.34±0.69	T
15	3.18±0.67	T
16	2.84±0.25	SM
17	2.43±0.32	SM
18	2.25±0.64	M
X	5.54±0.62	M

Table 2. Chromosomal parameters of Siberian tiger.

3.5 Isoenzyme analysis

Isoenzyme profiles of at least 5 kinds of animals were analysed simultaneously. Each kind of animal has its specific bands. The LDH bands obtained from Siberian tiger, for instance, were compared with those of other species or breeds, and five breed-specific isoenzyme bands (LDH-1, -2, -3, -4, -5) were observed (Fig. 5A). Enzymatic activities were in the order of LDH-3, LDH-2, LDH-4, LDH-5, LDH-1. LDH-2, LDH-3 and LDH-4 were dominant, while LDH-1 and LDH-5 were scarcely observable. In the MDH patterns of Siberian tiger and other breeds, two MDH bands (s-MDH, m-MDH) were observed (Fig. 5B), with the m-MDH band near the cathode and the s-MDH band (comprise two subbands but hardly identified) near the anode (Fig. 5B). Similar activity was seen from both m-MDH and s-MDH. There were significant differences in the isoenzyme patterns of LDH and MDH between the Siberian tiger fibroblasts and other cell lines in APCCC. These animals have their distinctive bands with different relative mobility. These results showed that there was no cross-contamination between different cell lines.

3.6 Expression of exogenous genes

Six fluorescent protein genes with stable structures, high expression levels and species-independent efficiency (Baird et al., 2000) have been used as marker genes to observe the expression, distribution and function of target proteins in live cells and organisms (Heim et al., 1995; Genyang et al., 2003). At APCCC, the 6 kinds of fluorescent genes were introduced into the preserved cells to evaluate the expressibility of exogenous genes. Positive cells were usually the most abundant and with the strongest fluorescence at 24 h-48 h after transfection. While the transfection efficiency decreased, strong expression levels were observed after a week, indicating that the exogenous genes can be replicated, transcribed,

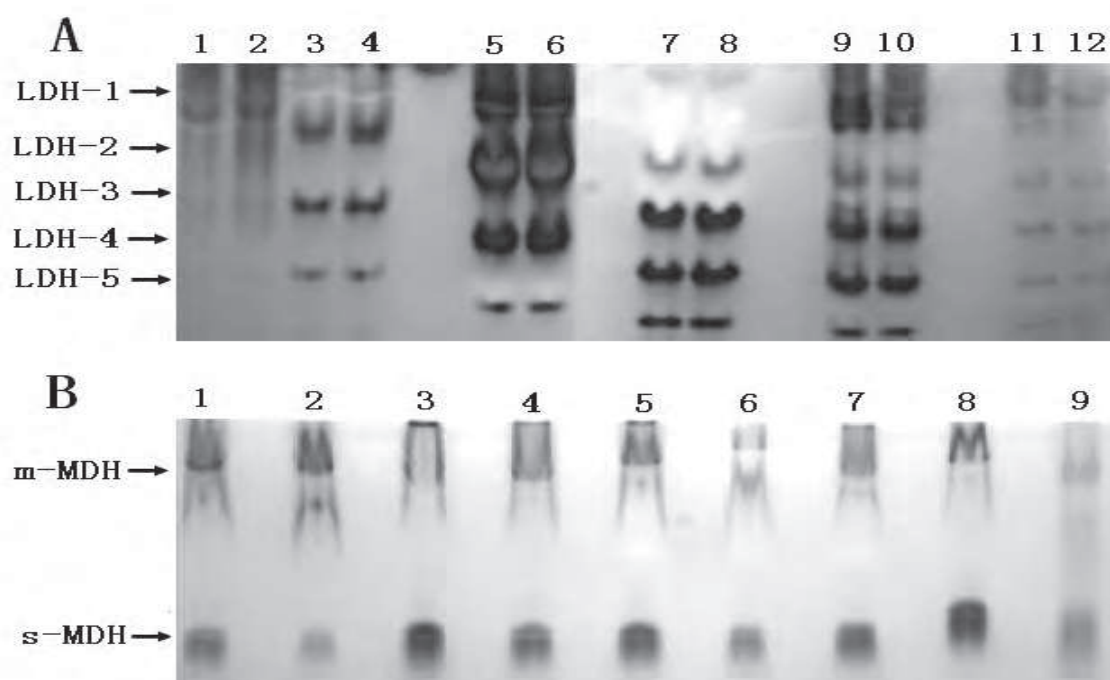


Fig. 5. LDH zymotype and MDH zymotype of several cell lines. A, SDS-PAGE electrophoresis of LDHs, from up to down, there were LDHs-1, 2, 3, 4 and 5. Panel A: 1,2 Simmental cattle, 3,4 Zhiwei goat, 5,6 Jining black goat, 7,8 Mongolian horse, 9,10 Bengal tiger, and 11,12 Siberian tiger; Panel B, MDHs from up to down were mMDH and sMDH. 1 Siberian tiger, 2 Bengal tiger, 3 Large white pig, 4 Songliao Black pig, 5 Jining Black goat, 6 Mongolian sheep, 7 Saf sheep, 8 Simmental cattle.

translated and modified within the cells. The transfected cells were not significantly less viable than the control cells ($P > 0.05$), showing that the expression of fluorescent proteins had no obvious effect on the growth and proliferation of the transfected cells.

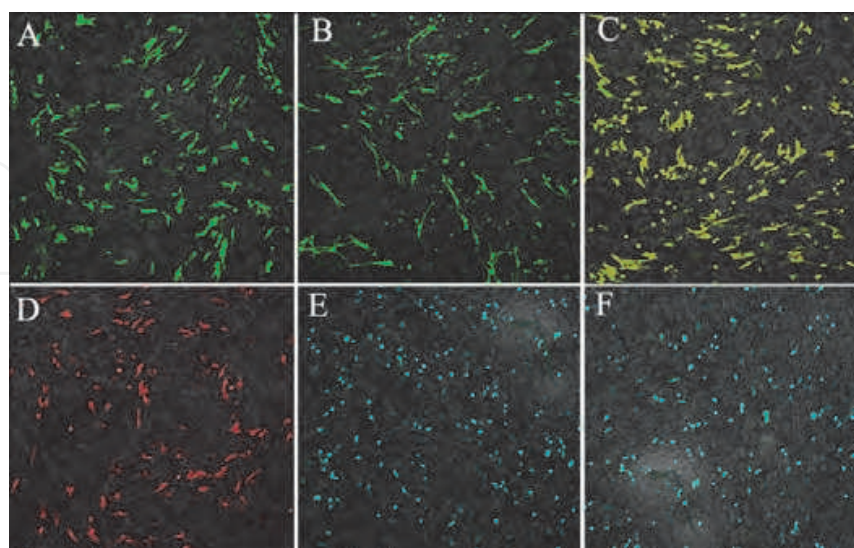


Fig. 6. Comparative figures of six fluorescent proteins in White Ear Lobe chicken fibroblasts at 24 h after transfection ($\times 10$). A, B, C, D, E and F were the transfection results of pEGFP-C1, pEGFP-N3, pEYFP-N1, pDsRed1-N1, pECFP-N1 and pECFP-mito, respectively.

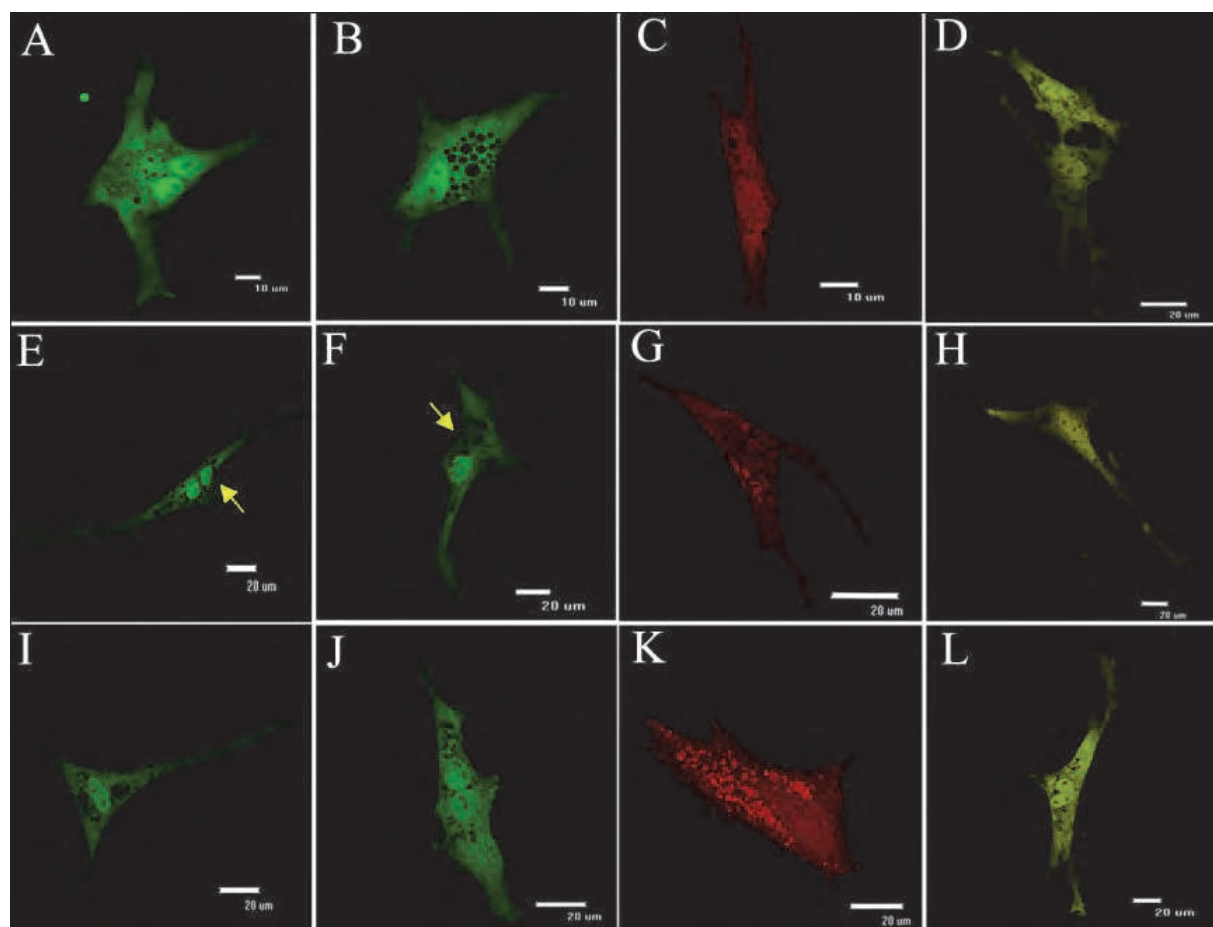


Fig. 7. The expression and distribution of pEGFP-C1, pEGFP-N3, pDsRed1-N1 and pEYFP-N1 in White Ear Lobe chicken fibroblasts ($\times 40$). A, B, C and D are the expression of pEGFP-C1, pEGFP-N3, pDsRed1-N1 and pEYFP-N1 at 24 h; E, F, G and H at 48 h; and I, J, K and L at 72 h after transfection.

4. Conclusion

Animal resources, a fundamental respect of agriculture and industry in close correlation with production and social stability, supply human beings with meat, eggs, milk, furs, medicinal materials, products for athletic and ornamental purposes, etc. In most developed countries, scalization of animal husbandry has restricted animal feeding to high yield breeds or crossbreeds with an intensified operating system, greatly compromising the diversity of local animal breeds. In the meanwhile, despite the abundance of animal genetic resources in developing countries, the lack of efficient preservation strategies and blind introduction of exotic breeds for hybridization also has reduced the animal variety. Emerging evidence has revealed that owing to the interference from human activities, species extinction has sped up for about 1000 fold, 100 million times faster than speciation, or in other words, 1 species per day. The total 7176 livestock and poultry breeds throughout the earth are disappearing at the rate of 2 per week, and 690 are on the edge of extinction.

Species extinction signifies a perpetual loss of the precious hereditary information, and will be an irreparable defeat of world genetic resources and biological theoretical repositories. Haven't the genetic resources been preserved in any forms before their extinction, not only the genetic resources will be lost evermore, but also it becomes impossible to investigate the

unknown cell and molecular mechanisms, let alone to regenerate corresponding species via cloning technique. Therefore, it is exigent to employ practical measures to conserve endangered animal species.

Therefore, the APCCC has as yet preserved, for each cell line, 45-1250 cryovials of somatic cells from 30-212 individuals using primary explantation, serial passage and programmed cryopreservation. Each vial contains approximately 1.5×10^6 cells. The cells are cryopreserved within 3 passages, and are subjected to evaluation in terms of morphology, growth kinetics, viability, microbial detection, karyogram and isoenzyme analyses according to quality control standards of ATCC. The purified fibroblasts are fusiform, displaying flame-like or swirl-like patterns. The growth curves are sigmoidal with characteristic PDTs. Trypan blue exclusion test suggests that programmed preservation exerts a non-significant effect ($P > 0.05$) on cell viability compared with that before freezing. Tests for bacteria, fungi, viruses and mycoplasmas are unanimously negative. Karyograms of peripheral blood lymphocytes and the in vitro cultured cells are photographed, according to which the mode of chromosome numbers are determined as that of the diploid cells, and indices including relative length, arm ratio and centromeric index and kinetochore type are calculated or determined. Comparison between peripheral blood lymphocytes and the in vitro cultured cells in respects of chromosome number and non-banding karyotype reveals no perceptible differences, manifesting the genetic stability of the cell lines established. Isoenzyme patterns of LDH and MDH are detected using vertical slab non-continuous PAGE assay, the breed specific bands of which rule out cross-contamination amongst the cell lines, and in the meanwhile further evince the hereditary stability.

Aforementioned results indicated that the APCCC conforms to all the ATCC criteria for somatic cell lines. In addition, plasmids of pEGFP-N3, pEGFP-C1, pECFP-N1, pECFP-mito, pDsRed1-N1, and pEYFP-N1 encoding the corresponding fluorescent proteins are transfected into the cells using lipofectin mediated protocol to study the expression of exogenous genes. By observation or detection of spatiotemporal expression of the fluorescent proteins, proliferation and growth of positive cells, apoptotic rate and viability, the ability and characteristics to accommodate exogenous genes are initiatively adopted as a constitutional index for cell line quality control.

The establishment of the APCCC is technically and theoretically conducive to preserve genetic resources of animals at somatic cell level, and definitively has a profound and long-lasting influence on biological and biomedical research in the future. The quality control standards it's been adopting will definitely provide insights for future development of culture collections.

5. Abbreviations

APCCC - Animal Population Culture Collection of China

ATCC - American Type Culture Collection

DMEM - Dulbecco's modified Eagle's medium

ECACC - European Collection of Animal Cell Culture

FAO - Food and Agriculture Organization

LDH - lactic dehydrogenase

MDH.- malic dehydrogenase

MEM - modified Eagle's medium

PAGE - polyacrylamide gel electrophoresis
PDT - population doubling time

6. References

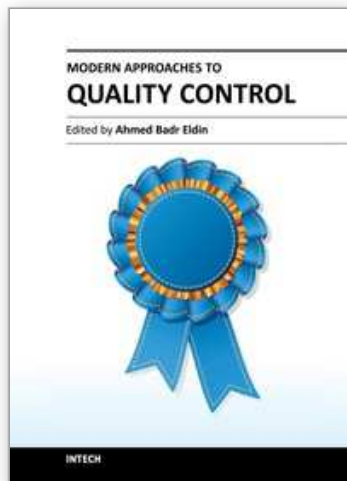
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Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
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Phone: +86-21-62489820
Fax: +86-21-62489821

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