



Establishment and Characterization of the Fibroblast Line from Silkie Bantam

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ABSTRACT : A Silkie Bantam embryo fibroblast line (named SBF59 line) was successfully established by using direct explant culture and cryopreservation techniques. Cell morphology, viability, dynamic growth and contamination were tested and the karyotype and levels of isoenzymes of lactic dehydrogenase and malic dehydrogenase were analyzed. Four kinds of fluorescent protein extro-genes, including pEGFP-N₃, pECFP-N₁, pEYFP-N₁ and pDsRed1-N₁ were transfected into the cells. The results showed that the cells were healthy and possessed a fibrous structure without a change in morphology. The average viability of the cells was 96% before freezing and 90.5% after thawing. The growth curve appeared as typical "S" shape and the cell growth passed through a detention phase, a logarithmic phase and a platform phase; the estimated population doubling time (PDT) was 38.5 h; assays for the presence of bacteria, fungi, viruses and mycoplasmas were negative; the cell line showed no cross contamination when assessed by isoenzyme analysis; the chromosome number was $2n = 78$ on more than 88% of occasions; four kinds of fluorescent protein extro-genes appeared to be expressed effectively with a high transfection efficiency between 18.3% and 42.3%. The cell line met the required quality control standard. It not only preserves the genetic resources of the important Silkie Bantam at the cellular level but also provides valuable materials for genomic, post-genomic, somatic cell cloning research and other applications. (**Key Words :** Silkie Bantam, Embryo Fibroblast Line, Establishment, Identification)

INTRODUCTION

The genetic diversity of livestock and poultry plays an important part in overall biological diversity, as well forms the basis for the survival and sustainable development of the human beings. Therefore, the preservation of genetic resources from endangered species is of important scientific significance. At present, the preservation of individual animals, semens, embryos, genomic libraries and cDNA libraries are all practical approaches. Nevertheless, the establishment of cell line using cryopreservation technique is another effective approach (Shi, 1989). Most cell banks emphasize conservation and utilization of animal resources, especially animal generative cells and embryos (Ho et al., 1997; Oishi, 1997; Simon, 1999; Park et al., 2009). In addition to these methods, modern somatic cell cloning technique has made somatic cells become attractive resource in the conservation of animal genetic materials

(Wu, 1999; Hong et al., 2005; Lee et al., 2007; Park et al., 2007; Yun et al., 2008). Many informations were recently published on fibroblast line development in different animals including the Debao pony (Ma et al., 2004), Beijing fatty chicken (Zhou et al., 2005), sheep (Chen et al., 2006; Pan et al., 2006; Lu et al., 2007), Taihu pig (Zhang et al., 2008), Luxi cattle (Liu et al., 2008) and white ear lobe chicken (Wu et al., 2008).

The Silkie Bantam is an ancient Chinese breed. It is also named as black-bone chicken. The breed originated in Jiangxi Taihe County. It was listed among the 138 nationally protected domestic animals by the Chinese government in 2006. The Silkie Bantam is famous for its black skin, skeleton, muscle and viscera. The bone, flesh and viscera can be made from various patent medicine and prescription, and the nutritive value of cocked chicken is abundant.

In the present study, we used the combined methods of cell viability, microorganism detection, chromosome analysis, isoenzyme analysis and fluorescent protein genes transfection to detect the established cell line. The average viability of cells detected that the cells were healthy in culture conditions and that freezing had little influence on

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the viability of the cells. Microorganism detection ensured that the cell line was free the presence of bacteria, fungi, viruses and mycoplasmas. Chromosome analysis indicated that the cell line was reproducibly diploid. Analysis of LDH and MDH isoenzymes ruled out cross-contamination between the breeds. In order to study exogenous gene expression, the fluorescent protein genes were transfected into the cells as reporter genes. In a word, we attempted to identify the cell line and describe its characteristics to allow the future preservation of the cell line at the cellular level. Our object is to cryopreserve this nationally protected genomic resource for the purposes of reviving the endangered breed by somatic cell cloning technology, and supplying convenient and effective resource for genomic research. Moreover, with the development of science and technology, the roles of limited cell lines will become increasingly prominent and there will be currently unforeseen applications.

MATERIALS AND METHODS

Primary culture and subculture

Nine days old chicken embryos were rinsed and cropped into samples measuring 1 mm 3 in size. The surface of a tissue culture flask was seeded with these tissue pieces and medium (MEM) containing 10% fetal bovine serum was added. The flasks were inverted and incubated at 37°C for 2 h in an incubator with 5% CO₂ until the tissue pieces spontaneously adhered to the flask surface after which the flasks were turned (Ma et al., 2004).

The medium was changed after 2-3 d. The cells were harvested at 80%-90% confluence. The cell sheet was rinsed twice with PBS to remove all traces of trypsin inhibitors in the serum supplied to the media and 0.05% trypsin solution was added. The flasks were inverted and incubated for 3 min at 37°C before being turned over. Subsequently, the flasks were shaken gently to detach cells from their walls and the medium was added to the cell suspension to stop the trypsinization. The cells were split into new culture flasks under the ratio 1:2 or 1:3 and incubated at 37°C with 5% CO₂.

Cryogenic preservation and recovery

Prior to freezing, the culture was maintained in an actively growing state (log phase or exponential growth) to ensure optimum health and good recovery. The culture medium was changed 24 h prior to harvesting and the harvested cells were treated in the same means as described for the subculture. Cells were enumerated with a hemocytometer and their viability was checked by Trypan Blue staining. The cells were then centrifuged at 1,000 rpm for 8 min to form a pellet, the supernatant was removed and

the cell pellet was re-suspended in a freezing media (10% DMSO+50% fetal bovine serum+40% MEM) to reach a final cell concentration of 3-5×10⁶ viable cells/ml. 1 ml samples of cell suspension were transferred into sterile plastic cryogenic vials labeled with animal name, gender, age, passage number and the date. The sealed vials were then placed into the boxes that filled with an appropriate amount of isopropyl alcohol, froze overnight at -80°C and then the cells were transferred into a liquid nitrogen storage system (Werners et al., 2004).

The frozen tubes were taken from the liquid nitrogen and plunged into 42°C water bath to quickly thaw them. The cells were subsequently transferred into a flask with the complete medium and cultured at 37°C with 5% CO₂. The medium was renewed 24 h later (Freshney, 1992).

Estimation of cell viability

Assays on cell viability before freezing and after recovering were performed by using the Trypan Blue vital stain method. Cells were seeded into 6-well plates and the viability of 1,000 cells was counted (Qi et al., 2007).

Growth curve

Following reported method (Gu et al., 2006), 24 well plates were seeded with cells with a concentration of 2.5×10⁴ cells/ml and cultured for 7 d. The growth and concentration were recorded from 3 wells per day until the plateau phase was reached. The cell growth curve was plotted and the population doubling time (PDT) was calculated based on this curve.

Microorganism detection

The Doyle (Doyle et al., 1990) and Freshney method (Freshney, 1992) were followed to detect potential bacteria, fungi and yeasts contamination. DNA fluorescent staining was achieved by using Hoechst 33258 (Sigma) to identify contamination by mycoplasma (Guan et al., 2005a).

An ELISA Mycoplasma Detection Kit (Roche Diagnostics Corp, Indianapolis, IN) that could detect the four most common Mycoplasma species (*M.arginini*, *M.hyorhinis*, *A.laidlawii* and *M. orale*) was used to confirm the results of the DNA staining.

Chromosome analysis

Cells were harvested at 80%-90% confluence during their exponential growth phase, microslides were prepared and chromosome staining was performed according to the approach of Suemori et al. (2006). The number of chromosomes in 50 to 100 spreads was counted. The three important chromosomal parameters, including relative length, arm ratio and centromere index were counted. Counts were determined according to the protocol of

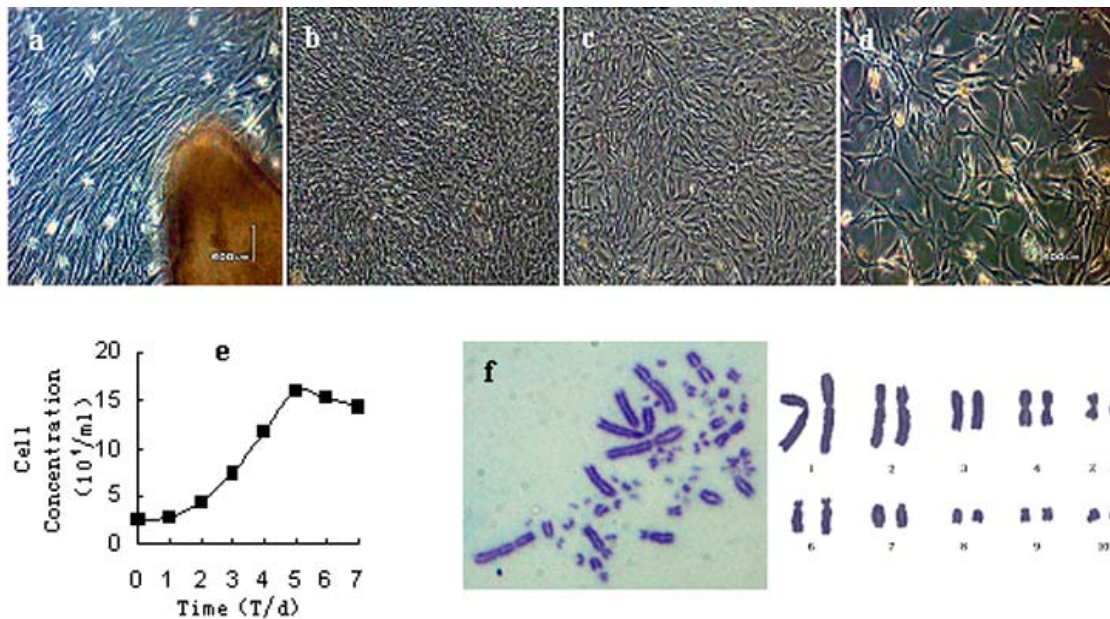


Figure 1. Morphology, growth curve and karyotype of SBF59 line. (a) Primary cells ($\times 100$), the cells were typical long spindle-shape with growth being slower. (b) Subcultured cells ($\times 100$), the cells were subcultured until they reached 90% confluence. After passage, growth accelerated and plateaued after 3-4 d. (c) cells prior to cryopreservation ($\times 100$), the cells were healthy and in mitotic phase (d) cells after recovery ($\times 100$), the cells were cultured for 48 h after thawing; (e) Growth curve of SBF59 line, the curve appeared as a typical "S" shape with cell density on the left axis. Growth curve included latency phase, exponential growth phase and stationary phase. The population doubling time calculated from the curve data was about 38.5 h. (f) Chromosome at metaphase (left) and karyotype (right) of SBF59 line (♀) ($\times 1,000$), the number of chromosomes in the Silkie Bantam was $2n = 78$, consisting of 10 pairs of macrochromosomes and 28 pairs of microchromosomes, whilst the sex chromosome type was ZW (♀).

Kawarai et al. (2006).

Isoenzyme analysis

Isoenzyme patterns of lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) were detected using a vertical slab non-continuous polyacrylamide gel electrophoresis (PAGE) assay. In brief, the cells were harvested and protein extraction solution (0.9% Triton X-100, 0.06 mmol NaCl:EDTA in mass ratio 1:15) was added after the cell concentration was adjusted to 5×10^7 cells/ml, then centrifuged and the supernatant was stored in aliquots at -80°C . 40% sucrose liquid and the samples (1:1) were mixed and then loaded in the individual lanes of the polyacrylamide gel (Zhang et al., 2000). Different mobility patterns were indicated by the relative mobility front (RF), which was calculated as the ratio between the distance of migration of the isozyme band and that of the bromophenol blue.

The detection of fluorescent proteins in SBF59 line

According to the protocol of Tsuchiya et al. (2002), the same quantity of fluorescent protein vectors pEGFP-N₃, pECFP-N₁, pDsRed1-N₁ and pEYFP-N₁ were transfected into the SBF59 cells with LipofectamineTM 2000 transfection reagent (Invitrogen Corp, Carlsbad, CA). The

ratio of plasmid DNA (μg) to Lipofectamine 2,000 (μl) was 1:3. After 8 h, the cells were removed from non-serum medium and transferred to the medium that contained serum. The cell morphology was observed, and the cells were dyed with Trypan Blue to estimate the cell viability. To estimate the transfection efficiency, the cells were observed after being transfected 24, 48 and 72 h (Nikon TE-2000-E, Japan), respectively. Multiple comparisons were made of the test data to judge the differences between the groups.

RESULTS

Cell morphology of SBF59 line

Two hours after embryo tissues attached, several types of epithelial-like and fibroblast-like cells moved from the tissues. As time progressed, fibroblasts moved out of the vicinity of the tissues in large numbers, multiplied rapidly and assumed a typical long spindle-shape. Primary cell growth was slow and the medium was changed only when it became yellow in colour. The cells were subcultured when they reached 90% confluence. After passaging, the cell growth was accelerated and within 2-3 d the cells covered the entire base of the flask (Figure 1a-d). The results indicated that the cells grew well, that the culture conditions were appropriate and that the survival rates were high and

Table 1. Chromosome number in SBF59 line

Generation	Chromosome number			Total cell score	Percentage of 2n (%)
	Hypodiploid	Diploid	Hyperdiploid		
2	1	47	2	50	94
3	1	46	3	50	92
4	2	44	4	50	88

not greatly affected by freezing.

Cell viability of SBF59 line

The average viability of the cells was 96% before freezing and 90.5% after thawing. These results were not significantly different ($p > 0.05$), so the cells were healthy in culture and freezing had little effect on their viability.

Growth curve (cell dynamics)

The growth curve of SBF59 line displayed a typical "S" shape (Figure 1e). A lag of around 24 h was apparent after the cells were seeded which was needed for recovery following damage by the protease. After this, the cells proliferated rapidly and entered the exponential growth phase until they reached the stationary phase after about 3 d. After 3 d, growth plateaued and the cells began to degenerate. The population doubling time (PDT) which calculated from the curve data was approximately 38.5 h.

Microbial analysis

The culture medium showed no increase in turbidity or other changes in the negative control or SBF59. Therefore the SBF59 appeared not to have been contaminated by bacteria, fungi or yeasts.

No plaque bacteriophage or lacunae was observed, and the hemadsorption result was negative. All these results demonstrated that the SBF59 line was not contaminated by viruses.

The fibroblast nuclei appeared as blue ellipses when studied under a fluorescent microscope after being stained with Hoechst 33258 (Sigma), this result indicate that the established cell line was mycoplasma negative. The finding that the established SBF59 line was negative for mycoplasma was confirmed by DNA staining with an ELISA Mycoplasma Detection Kit (Roche).

Chromosome analysis

Number of diploid chromosomes : As the chromosomes were small and difficult to differentiate, we sampled 50 cells and only calculated the numbers of hypodiploid, diploid and hyperdiploid chromosomes. The results indicated that the majority of chromosomes were diploid, but over time, the frequency of hyperdiploid chromosomes increased. By the 4th generation, the diploid proportion reached approximately 88% (Table 1), as a result of which it is recommended that only cells in the early generations

after culturing are frozen and stored.

Chromosome morphology of SBF59 line : The number of chromosomes in the Silkie Bantam was $2n = 78$, consisting of 10 pairs of macrochromosomes and 28 pairs of microchromosomes, whilst the sex chromosome type was ZZ (♂)/ZW (♀). Chromosomes 1, 4 and 9 were classified as type M; Chromosomes 2 and 6 were classified as type SM; 3, 7, 8 and 10 were classified as T and the sex chromosome was Z/W (male ZZ and female ZW). Z chromosome was classified as type M. W chromosome was classified as type SM. In size, the Z chromosome was as large as the chromosome 5, whilst the W chromosome was of similar size to the chromosome 8 (Figure 1f). These results are in agreement with studies on other chicken breeds, but there are discrepancies with other reports regarding chromosome type (Wang et al., 2003; Xu et al., 2004). These discrepancies relate to the ease of Robertsonian translocation, principally with microchromosomes of bird species. The exact cause, however, is yet to be fully elucidated.

Isoenzyme analysis

The pattern of distribution of isoenzyme polymorphisms may be characteristic of a species or tissue (MacLeod et al., 1999). Polymorphism analysis of isoenzymes is currently the standard method used during the quality control of cell line identification and interspecies contamination. Isoenzyme patterns of lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) from Silkie Bantam cells were obtained and compared with four breeds. Five bands that representing LDH1, LDH2, LDH3, LDH4 and LDH5 from anode to cathode were apparent (Figure 2a). The LDH activity of Wenchang chicken, White Leghorn Chicken, Qingyuanma Chicken and Silkie Bantam breeds were similar, whilst that of the Beijing Duck, White Ear Lobe Chicken and Silkie Bantam were significantly different.

The similarity in LDH activity between breeds is indicative of a similar ability to utilize lactic acid in different environments and implies a close genetic relationship. Simultaneous shading of an area represents the strength or weakness of the phenotype enzymatic activity. The Silkie Bantam progression from the highest to the lowest LDH activity was: LDH2, LDH3, LDH4, LDH5 and LDH1. Although the flow rate of the isodynamic enzyme bands of different breeds appeared to have little obvious differences, smaller differences were apparent. These results

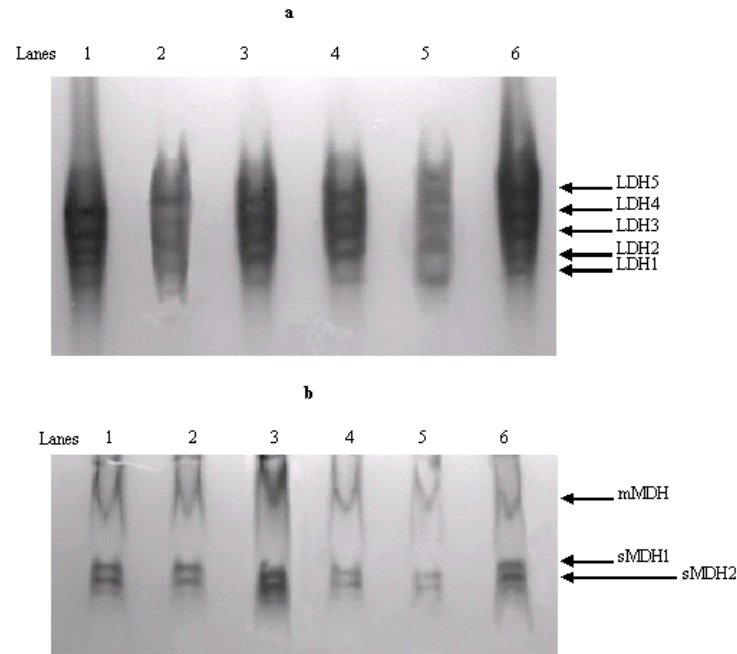


Figure 2. LDH zymotype and MDH zymotype of SBF59 line. These domestic animals have their own unique bands with a different relative mobility. These results showed that there was no cross-contamination between different breeds. (a) SDS-PAGE electrophoresis of LDH, from down to up, there were LDH-1, 2, 3, 4, 5. Panel a: 1. Beijing Duck; 2. Qingyuanma Chicken; 3. Silkie Bantam; 4. Wenchang Chicken; 5. White Leghorn Chicken; 6. White Ear Lobe Chicken. (b) MDH from down to up, there were sMDH-1, sMDH-2 and mMDH, whilst s-MDH2 had weaker activity. Panel b: 1. White Ear Lobe Chicken; 2. Silkie Bantam; 3. White Leghorn Chicken; 4. Wenchang Chicken; 5. Qingyuanma Chicken; 6. Beijing Duck.

indicate that no cross-contamination from different cell lines established in the laboratory during the same time occurred.

One band of m-MDH was located near the cathode and two bands of s-MDH were found near the anode. s-MDH1 and s-MDH2 had similar activity levels, whilst the activity of m-MDH was weaker (Figure 2b). Each chicken breed showed characteristic banding, and each band had a different relative mobility. These results confirm that there was no cross-contamination between different breeds.

Comparison of the expression of 4 fluorescent protein genes in SBF59 line

The expression of pEGFP-N₃, pECFP-N₁, pDsRed1-N₁ and pEYFP-N₁ at 24, 48 and 72 h were recorded using laser confocal microscopy after excitation by a specific wavelength of light.

The results indicated that all four fluorescent proteins were expressed in most positive cells, and the strongest fluorescence intensity and the highest transfection

efficiency of the exogenous genes appeared at 48 h after transfection. The expression efficiencies of the 4 fluorescent protein genes 24, 48 and 72 h after transfection were between 18.3% and 42.3% (Table 2). All positive cells appeared shrunken and were shed and disintegrated 24 h after transfection. The number of the positive cells increased at 48 h and there were many non-fluorescent vacuoles in the cytoplasm in most of the positive cells at 48 h and 72 h after transfection (Figure 3). The number of cells expressing fluorescent proteins decreased and the fluorescence intensity gradually faded, and disappeared 7 d after transfection, though some cells still expressed fluorescent proteins after 4-5 wk, indicating that the exogenous genes in the fibroblasts can be replicated, transcribed, translated and modified after correct translation. Through G418 resistance screening and monoclonal culture during 1 month, we gained 3 positive cell strains which can express EGFP, EYFP and DsRed stably. The viabilities of cells transfected with pEGFP-N₃, pECFP-N₁, pEYFP-N₁ and pDsRed1-N₁ were 86.5%, 88.2%, 89.6% and 87.6%

Table 2. Efficiency of transfection of four fluorescent proteins

Transfection time (h)	pEGFP-N ₃ (%)	pECFP-N ₁ (%)	pDsRed1-N ₁ (%)	pEYFP-N ₁ (%)
24	30.9	19.0	18.3	36.8
48	37.4	23.7	22.5	42.3
72	33.1	21.3	20.7	32.7

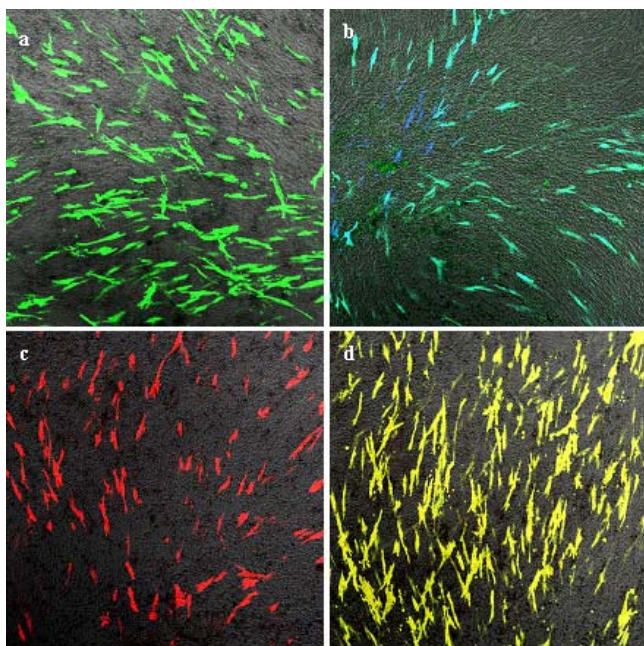


Figure 3. Comparative figures of pEGFP-N₃, pECFP-N₁, pDsRed1-N₁ and pEYFP-N₁ fluorescent protein expression (100×). (a) pEGFP-N₃, 48 h; (b) pECFP-N₁, 48 h; (c) pDsRed1-N₁, 48 h; (d) pEYFP-N₁, 48 h.

respectively; none of these was significantly different from controls (92.6%, $p > 0.05$). These results show that the fluorescent protein gene expression has no obvious effect on the growth and proliferation of the transfected cells and that the fluorescent protein gene can be safely used as a marker of foreign genes in animals transgenesis (Kim et al., 2008).

DISCUSSION

In this experiment, the technique of primary explant is particularly suitable for small amounts of tissue, such as skin. There are rather lower risks of losing cells than other methods. We suggest, based on our observations, that the technique of primary explant is a very applicable and effective technique for thick tissue samples such as ear marginal tissue, also for samples obtained from precious sources. Additionally, we have improved the protocols of electrophoresis, traditional chromosomal preparation and exogenous gene transfection by trying a local apparatus, different buffers, colchicine and hypotonic time, plasmid DNA incubation time and lipofectamine combination, thus make these techniques feasible under our conditions and greatly improve isoenzyme zymogram, karyotype quality and exogenous gene transfection efficiency. In a word, we explored a set of rational cell line detection approaches.

The morphological analysis indicated that both epithelial and fibroblast cells were present during the primary cultures and early passages. Due to their different

level of tolerance to trypsin, the fibroblast cells detached from the flask walls first when treated with trypsin and adhered again quickly after passaging, whilst most epithelial cells were unable to adhere, or only did so in an unstable manner and fell off when vibrated (Xue et al., 2001). For this reason, purified fibroblast cells were obtained after 2-3 passages (Guan et al., 2005b; Zhou et al., 2005).

The average motility rate after thawing was above 90% which indicate that freezing had little influence on the viability of the cells. Therefore it seems possible to conserve the genomic resources of the Silkie Bantam breed by the long-term freezing of fibroblasts in liquid nitrogen.

The chromosomes of poultry have been defined as possessing 8 pairs of macrochromosomes and 30 pairs of microchromosomes with the sex chromosomes Z and W. The range in frequency of diploid chromosomes varies notably between breeds with most having from 78 to 82. The macrochromosomes of *Gallus domestics* average 7.8 ± 0.9 in number but vary between 6 and 9, while there are 31.9 ± 2.5 microchromosomes with a range from 24 to 35. The present study sampled 100 cells and found 10 pairs of macrochromosomes and 29 pairs of microchromosomes with 92% of cells being diploid. Most chromosomes of the Silkie Bantam are therefore microchromosomes, and may be lost easily during section preparation and disruption of the purity of the chromatin. This makes it difficult to determine the number and morphology of the chromosomes whose number in the diploid state may vary from 78.

Isoenzymes show polymorphisms between diverse species, races, individuals and tissues, and intercellular pollution can be detected by isoenzyme analysis when 10% of cells are polluted (Nims et al., 1998). Our present study detected the isoenzyme zymograms of LDH and MDH and improved the traditional method of starch gel electrophoresis. Zeng et al. (1997) found 5-8 clear bands. Five such bands were found in samples of pectoral muscle. LDH isoenzymes have also been studied in samples of cardiac muscle, liver and blood of Chinese junglefowl, and for all three tissues, five bands were observed. In agreement with this, five LDH bands, LDH1, LDH2, LDH3, LDH4 and LDH5, were recorded in the Silkie Bantam samples in the present study.

The MDH of poultry is present in both a cellular solute form (s-MDH) and a mitochondrial form (m-MDH), and the movement rate of the former is higher than the latter. The s-MDH bands of 1-16 d old embryos, which appeared at day 3 and increased thereafter, all showed a deviation towards the positive electrode. The present results show that the MDH of the Silkie Bantam breed had also s-MDH and m-MDH forms, the movement rate of s-MDH was higher than that of m-MDH. This is in accordance with the MDH isoenzyme activity of embryos at the early and middle

stages of development (1-16 d) and indicates that the MDH enzyme activity of cells *in vitro* is similar to that of the original tissues. In the present study, the LDH and MDH isoenzyme bands from Silkie Bantam cells are distinct, suggesting that the cellular genetic characteristics are stable and that there is no cross-contamination from one breed to another in the laboratory.

CONCLUSION

In conclusion, the current results indicate that the newly established SBF59 line has genetical stability and normal biological characteristics. These characteristics suggest the SBF59 line provides a useful approach for preserving this unique breed in China and would be an effective experimental materials for further genetic studies on the Silkie Bantam as well.

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