Mitochondrial DNA Heteroplasmy in Calves Cloned by Using Adult Somatic Cell

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ABSTRACT Adult somatic cell cloned calves were produced by somatic cell nuclear transfer prepared by fusion of cultured ear fibroblast from a Holstein cow into enucleated oocytes of Luxi Yellow cow. In order to determinate the source of mitochondrial DNA of cloned calves, we designed the breed-specific PCR primers by aligning the known D-loop sequences of *Bos taurus* and analyzed the displacement loop sequences of five live cloned calves by breed-specific primers PCR. The results demonstrated that mtDNA originated from Holstein breed and that from Luxi breed co-exist in all five live calves. *Mol. Reprod. Dev. 67: 207–214, 2004.*
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Key Words: clone; bovine; nuclear transfer; mitochondria DNA

INTRODUCTION

Since the first somatic cell cloned lamb was reported in 1997 (Wilmut et al., 1997), successful somatic cell cloning has been achieved in several species (Cibelli et al., 1998; Wakayama et al., 1998; Wang et al., 1999; Polejaeva et al., 2000; Chesné et al., 2002; Shin et al., 2002) by somatic cell nuclear transfer (NT). The study of mitochondrial DNA (mtDNA) fate of somatic cell cloned animals has gradually become more important. But there were only a few reports of mtDNA fate of somatic cell cloning and the mtDNA fate is still controversial.

Eukaryotic cells contain two distinct genomes. One is located in the nucleus (nDNA) and the other is located in the mitochondria (mtDNA). In the process of somatic cell NT, nDNA originates from donor somatic cell, while mtDNA originated from donor somatic cell and mtDNA originated from recipient oocyte are mixed in the reconstructed embryos. Evans et al. (1999) reported that the mtDNA of ten nuclear-transfer sheep was derived exclusively from the recipient enucleated oocytes, with no detectable contribution from the respective somatic donor cells. Lanza et al. (2000) and Loi et al. (2001) reported that mtDNA of cloned animal were also exclusively derived from the recipient oocytes in genetically close interspecies cloning study. However, Steinborn et al. (2000) reported mtDNA heteroplasmy in cloned cattle produced by fetal and adult cell cloning using allele-specific real-time quantitative PCR. Very recently, mtDNA heteroplasm has been observed in cumulus cell cloned embryos of bovine (Takeda et al., 2003).

Displacement loop (D-loop) is the control region of mtDNA with high variability. In different breeds (Holstein breed and Luxi breed) of *Bos taurus* intraspecies cloning, we analyzed the mtDNA of cloned calves using D-loop as genetic marker and confirmed the mtDNA heteroplasmy in all five live cloned calves.

MATERIALS AND METHODS

Donor Cell

The donor cells were isolated from the ear of an adult Holstein cow (GN). The ear skin was cut into pieces and digested with 0.25% trypsin (GIBCO BRL, Life Technologies, Grand Island, NY) for 2 hr at 4°C and then for 30 min at 37°C. The digested cells were cultured in Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (Ham) = 1:1 (D-MEM/F-12, GIBCO) supplemented with 20% FBS (GIBCO).

Donor cells passed for 1–5 generations were used for NT. The 70–80% confluency cells were induced to enter quiescent stage by culturing in the D-MEM/F-12 containing 0.5% FBS for 2–7 days. To obtain donor cells from adherent cell layer, the ear fibroblast cells were digested with 0.25% trypsin at 39°C for 2–3 min.

In Vitro Maturation of Oocytes

Recipient oocytes were collected from ovaries of sex mature Luxi Yellow cow obtained from a slaughterhouse. Cumulus–oocyte complexes (COCs) were collected and cultured in maturation medium which comprised

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tissue culture medium 199 (GIBCO) supplemented with 10% FBS, 0.5 μg/ml FSH, 0.01 IU/ml LH, 1.0 μg/ml estradiol at 38.5°C in the humidified 5% CO_2 in air atmosphere for 19 hr. Oocytes with the first polar body were selected for NT after culture.

Nuclear Transfer, Embryo Culture, and Embryo Transfer

NT was conducted as previously reported (Chen et al., 1999). Briefly, oocytes were enucleated and one ear fibroblast cell was transferred into the perivitelline space of an enucleated oocyte in M2 medium with 7.5 μg/ml cytochalasin B (Sigma Co., St. Louis, MO). Fusion of cell–oocyte couplets was accomplished by two direct current pulses (2.0 kV/cm, 10 μsec each, 1 sec apart) in the electrical fusion solution which contained 0.28 M mannitol (Sigma Co.), 0.5 mM HEPES (Sigma Co.), 0.05 mM calcium, and 0.1 mM magnesium. Fused couplets were chemically activated by incubation in 5 μM A23187 in CR1-aa for 4 min at 39°C and were cultured for 1 hr in 10 μg/ml cycloheximide (Sigma Co.) and 3 μg/ml cytochalasin D (Sigma Co.) in CR1-aa supplemented with 10% FBS, and then cultured in the same medium without cytochalasin D for 4 hr.

Embryos were cultured in CR1-aa supplemented with 10% FBS at 38.5°C in the humidified 5% CO_2 in air atmosphere for 48 hr. The embryos were co-cultured with mouse fetal fibroblast cells in the same medium, which was changed by half every 48 hr. On days 7, 8, and 9 of in vitro culture, development to morulae and blastocysts was recorded. Morphologically normal blastocysts were nonsurgically transferred into naturally cycling recipient cows 7 days after estrus.

Microsatellite DNA Analysis

DNA samples were compared using microsatellite DNA analysis to determine whether the cloned calves have the same genotype as the donor. Genomic DNA samples were extracted from the ear skin and fibroblast of donor Holstein cow (GN), the ear skin of five live cloned calves, and the ear skin of recipients by 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% SDS, 0.5 mg/ml protein K, and phenol/chloroform. The microsatellite analysis consists of a multiplexed set containing 12 polymorphic bovine loci (ETH3, ETH225, BM1824, BM2113, TGLA126, TGLA53, ETH10, MGTG4B, TGLA227, TGLA122, INRA23, SPS113). The multiplex set contains 12 different PCR primer pairs, amplified in three PCR reactions by GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The forward primers were labeled on the 5′ end with a fluorescent dye (FAM or HEX). The denatured PCR products were loaded into ABI 310 Genetic Analyzer with an internal size standard Genescan 400 Rox (Applied Biosystems) for electrophoretic separation. The fluorescence data collected by GeneScan 3.1 software were exported directly to the Genotyper 2.5 software for automatic genotyping (Applied Biosystems).

TABLE 1. Microsatellite Analysis of 12 Loci

Sample/locus	BM1824	BM2113	ETH225	ETH3	ETH10	MGTG4B	TGLA126	TGLA53	INRA23	SPS113	TGLA122	TGLA227
GN	179.79/188.37	124.47	134.1/145.44	113.45/122.44	214.62	132.95/139.31	116.91/119.08	158.1	204.25	146.36	162.32	82.85/91.71
GN-98120	178.91/187.47	124.41	133.93/145.5	113.49/122.46	213.72	132.84/139.21	116.78/119.08	157.99	204.41	145.43	162.34	83.17/91.84
98120	181.76	128.09	141.6/143.55	122.25	215.49/219.32	139.14	116.78	153.94/155.92	204.4	148.16	141.21/149.48	81.03
GN-99033	178.81/187.47	125.3	133.87/145.41	113.53/122.45	214.59	132.81/139.18	116.78/119.08	157.97	204.31	145.4	162.64	83.12/91.69
99033	187.58	123.35/134.94	134.13/145.42	113.53/118.05	215.48	132.84/137.16	116.79	157.99	200.53/206.12	145.37	149.57/162.36	90/97.39
GN-99065	179.16/187.79	124.45	133.94/145.47	113.41/122.48	213.59	132.75/139.13	116.79/119	157.94	204.37	146.22	162.31	83.13/91.78
99065	176.59	131.12/134.96	134.34/153.82	113.85	209.76/215.53	136.96/151.6	116.77	155.9/157.94	204.25/212.01	145.37	147.51/162.25	90/97.82
GN1-99025	178.91/187.58	125.32	133.82/145.49	113.41/122.36	213.68	132.72/139.04	116.77/119	157.92	204.58	145.44	162.21	82.96/91.77
GN2-99025	178.91/187.47	125.35	133.88/145.49	113.5/122.47	214.51	132.78/139.12	116.75/118.99	157.92	204.47	145.56	162.2	83.29/91.77
99025	180.22	133.06	143.58/145.55	122.36	210.6/214.36	132.67/139.09	116.65/123.27	163.91	205.31/209.26	146.32/150.21	141.03/162.13	89.81

The values indicate the fragment lengths in base pairs. BM1824, BM2113, INRA23, MGTG4B, ETH10, ETH225, SPS113, TGLA53, TGLA227, ETH3, TGLA126, and TGLA122 are primers. GN: donor fibroblast cell; GN-98120, GN-99033, GN-99065, GN1-99025, GN2-99025: live cloned calves; 98120, 99033, 99065, 99025: recipient mother.

354 CATAAACCGTGGGGGTCGCTAT**CC**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

505 CATAAACCGTGGGGGTCGCTAT**CC**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

603 CATAAACCGTGGGGGTCGCTAT**CC**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

644 CATAAACCGTGGGGGTCGCTAT**CC**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

667 CATAAACCGTGGGGGTCGCTAT**CC**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

766 CATAAATCGTGGGGGTCGCTAT**CC**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

843 CATAAACCGTGGGGGTCGCTAT**CC**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

h 1 CATAAACCGTGGGGGTCGCTAT**TT**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

h 2 CATAGACCGTGGGGGTCGCTAT**TT**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

h 3 CATAGACCGTGGGGGTCGCTAT**TT**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

h 4 CATAGACCGTGGGGGTCGCTAT**TT**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

GLV18 CATAAACCGTGGGGGTCGCTAT**TC**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

LM046 CATAAACCGTGGGGGTCGCTAT**CC**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

SM110 CATAAACCGTGGGGGTCGCTAT**CC**AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGG

354 ACCATGCCGCGTGAAACCAGCAACCCGCTA**G**GCAGGG-ATCCCTCTTCTCGCTCCGGGCC

505 ACCATGCCGCGTGAAACCAGCAACCCGCTA**G**GCAGGG-ATCCCTCTTCTCGCTCCGGGCC

603 ACCATGCCGCGTGAAACCAGCAACCCGCTA**G**GCAGGGGATCCCTCTTCTCGCTCCGGGCC

644 ACCATGCCGCGTGAAACCAGCAACCCGCTA**G**GCAGGG-ATCCCTCTTCTCGCTCCGGGCC

667 ACCATGCCGCGTGAAACCAGCAACCCGCTA**G**GCAGGG-ATCCCTCTTCTCGCTCCGGGCC

766 ACCATGCCGCGTGAAACCAGCAACCCGCTA**G**GCAGGG-ATCCCTCTTCTCGCTCCGGGCC

843 ACCATGCCGCGTGAAACCAGCAACCCGCTA**G**GCAGG- - ATCCCTCTTCTCGCTCCGGGCC

h 1 ACCATGCCGCGTGAAACCAGCAACCCGCTA**A**GCAG-GGATCCCTCTTCTCGCTCCGGGCC

h 2 ACCATGCCGCGTGAAACCAGCAACCCGCTA**A**GCAGAGGATCCCTCTTCTCGCTCCGGGCC

h 3 ACCATGCCGCGTGAAACCAGCAACCCGCTA**A**GCAGAGGATCCCTCTTCTCGCTCCGGGCC

h 4 ACCATGCCGCGTGAAACCAGCAACCCGCTA**A**GCAGAGGATCCCTCTTCTCGCTCCGGGCC

GLV18 ACCATGCCGCGTGAAACCAGCAACCCGCTA**G**GCAGGG-ATCCCTCTTCTCGCTCCGGGCC

LM046 ACCATGCCGCGTGAAACCAGCAACCCGCTA**G**GCAAGG-ATCCCTCTTCTCGCTCCGGGCC

SM110 ACCATGCCGCGTGAAACCAGCAACCCGCTA**G**GCAGGG-ATCCCTCTTCTCGCTCCGGGCC

Fig. 1. Blast analysis of D-loop of three kinds of bovine. 354, 505, 603, 644, 667, 766, 843: Holstein cows; h1, h2, h3, h4: Luxi Yellow cows; GLV18, LM046, SM110: Galoway bulls; the bold showed the characteristic loci that are identical in one breed but is or are different in another breed; CC was selected as 3′-end of Holstein breed-specific forward primer N1 (**up**); A was selected as 3′-end of Luxi breed-specific forward primer H1 (**down**).

Sequencing of Mitochondrial D-Loop Region of Holstein Cows, Luxi Yellow Cows, and Galoway Bulls

Since Holstein breed, Luxi breed, and Galoway breed belong to a same species *Bos taurus*, we must find the sequence difference among the three breeds in the hypervariable region (D-loop region) of mtDNA in order to design the breed-specific primers of Holstein and Luxi breeds.

DNA samples from the tissues of ear and ovary were extracted by 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% SDS, 0.5 mg/ml protein K, and phenol/chloroform. DNA samples from the hairs were extracted by 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% SDS, 0.5 mg/ml protein K, 40 mmol/L DTT, and phenol/chloroform. PCR primers corresponding to relatively divergent regions of bovine mtDNA D-loop were used to amplify a 790 bp fragment from DNA samples of the ear tissues of seven Holstein cows, the ovary tissues of four

Fig. 2. Electrophoresis analysis of PCR-amplified D-loop fragments of five cloned calves (primers: N1 and N2). Lines 1, DL-2000 molecular size marker; 2, GN; 3, h2; 4, water; 5, GN-98120; 6, GN-99033; 7, GN-99065; 8, GN1-99025; 9, GN2-99025 (GN: donor fibroblast cell; h2: Luxi Yellow cow; GN-98120, GN-99033, GN-99065, GN1-99025, GN2-99025: live cloned calves).

Luxi Yellow cows, and the hairs of three Galoway bulls, which were designed by aligning the known sequences of *Bos taurus* in GENEBANK. The primers were: forward 5′-AACACAGAATTTGCACCCTAACC-3′(C1); and reverse 5′-ATTGAGTATTGAAAGCGTGAAAAA-3′(C2). PCR amplification was performed by the following cycling program: first 94°C for 5 min, then 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min for 35 cycles, and finally 72°C for 10 min. The 790 bp D-loop fragments were sequenced using Megabase 1000 Sequencer (Amersham Pharmacia Biotech.).

Breed-Specific Primers Design

The sequences were aligned by DNASTAR software and the difference was hunted for among the sequences. The aligned results showed that several different bases in 790 bp D-loop fragments between the Holstein cows and Luxi cows, but these bases were identical in same breeds. The characteristic loci that are identical in one breed but different in another breed were selected as end-base of primer sequence (3′-end) for PCR, and the unmatched end-base will effect the primer extension and lead to amplification failure in another breed. Two pairs of breed-specific primers with the different amplication fragment lengths for Holstein breed (276 bp) and Luxi breed (417 bp) were designed by DNASTAR software, respectively. Based on the sequence of Holstein breed, the forward primer N1 is: 5′-CGTGGGGGTC-GCTATCC-3′ where the CC of 3′-end are two characteristic loci in the sequence of Holstein breed, and the reverse primer N2 is: 5′-CGCTTATCATTATGCTGG-TGC-3′. Based on the sequence of Luxi breed, the forward primer H1 was designed as 5′-GTGAAACCAG-CAACCCGCTAA-3′ where the A of 3′-end is one characteristic locus, and the reverse primer H2 is 5′-TGGTGAATATTTAAGGGGGAGG-3′.

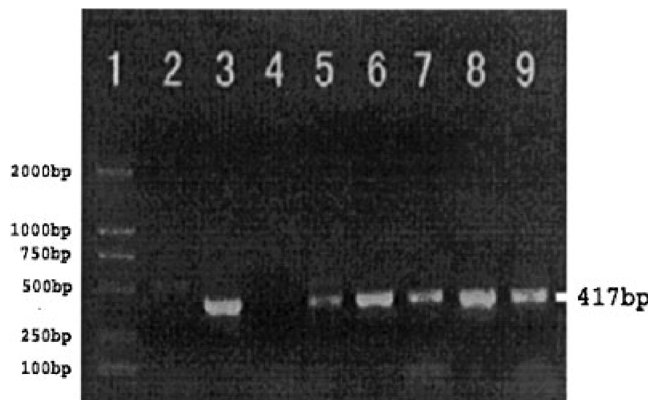

Fig. 3. Electrophoresis analysis of PCR-amplified D-loop fragments of five cloned calves (primers: H1 and H2). Lines 1, DL-2000 molecular size marker; 2, GN; 3, h2; 4, water; 5, GN-98120; 6, GN-99033; 7, GN-99065; 8, GN1-99025; 9, GN2-99025 (GN: donor fibroblast cell; h2: Luxi Yellow cow; GN-98120, GN-99033, GN-99065, GN1-99025, GN2-99025: live cloned calves).

Breed-Specific PCR Amplification and Sequencing of Cloned Bovine

PCR amplification was performed by the following cycling program: first 94°C for 5 min, then 94°C for 1 min, 69°C for Holstein breed-specific primers and 62°C for Luxi breed-specific primers 1 min, 72°C for 1.5 min for 35 cycles, and finally 72°C for 10 min. In order to rule out the possibility of contamination during PCR processing, we used water and DNA samples of Holtein breed as negative controls in Luxi breed-specific primers PCR and used water and DNA samples of Luxi breed as negative controls in Holstein breed-specific primers PCR. The PCR products were separated by agarose electrophoresis and were sequenced by 310 Genetic Analyzer (Applied Biosystems).

RESULTS

As shown in our report (Chen et al., 2003), a total of 13 cloned calves were born from 11 recipients, but eight calves died shortly before or after birth. The five survived clones, two of which are twin, are now 19 months old. The phenotypes of all clones from GN are similar with the donor Holstein cow, especially in the visual coat color pattern on the head and the neck. Microsatellite DNA analysis confirmed that five live cloned calves originated from the donor fibroblast cell of GN and no genetic relationship was shown between cloned calves and recipient mothers (Table 1).

The breed-specific primers were designed by aligning the sequences of 790 bp fragments of seven Holstein cows, four Luxi Yellow cows, and three Galoway bulls (Fig. 1). Before the N1–N2 primers and H1–H2 primers were used to detect the mtDNA of cloned calves, the primers were tested for the breed specificity. The breed-specific primers PCR was conducted by amplifying the 276 and 417 bp fragments of Holstein cows and Luxi Yellow cows. Finally, the best annealing temperature of PCR reactions were selected respectively. The 276 bp PCR products of N1–N2 primers only appeared in Holstein breed, but not in Luxi breed, and the 417 bp PCR products of H1–H2 primers only appeared in the

70

GN: (CGTGGGGGTCGCTATCC) AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGGGCCATCTCATCTAAAAC

GN-98120: (CGTGGGGGTCGCTATCC) AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGGGCCATCTCATCTAAAAC

GN-99033: (CGTGGGGGTCGCTATCC) AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGGGCCATCTCATCTAAAAC

GN-99065: (CGTGGGGGTCGCTATCC) AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGGGCCATCTCATCTAAAAC

GN1-99025: (CGTGGGGGTCGCTATCC) AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGGGCCATCTCATCTAAAAC

GN2-99025: (CGTGGGGGTCGCTATCC) AATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGGGCCATCTCATCTAAAAC

140

GN: GGTCCATTCTTTCCTCTTAAATAAGACATCTCGATGGACTAATGGCTAATCAGCCCATGCTCACACATAA

GN-98120: GGTCCATTCTTTCCTCTTAAATAAGACATCTCGATGGACTAATGGCTAATCAGCCCATGCTCACACATAA

GN-99033: GGTCCATTCTTTCCTCTTAAATAAGACATCTCGATGGACTAATGGCTAATCAGCCCATGCTCACACATAA

GN-99065: GGTCCATTCTTTCCTCTTAAATAAGACATCTCGATGGACTAATGGCTAATCAGCCCATGCTCACACATAA

GN1-99025: GGTCCATTCTTTCCTCTTAAATAAGACATCTCGATGGACTAATGGCTAATCAGCCCATGCTCACACATAA

GN2-99025: GGTCCATTCTTTCCTCTTAAATAAGACATCTCGATGGACTAATGGCTAATCAGCCCATGCTCACACATAA

210

GN: CTGTGCTGTCATACATTTGGTATTTTTTTATTTTGGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGC

GN-98120: CTGTGCTGTCATACATTTGGTATTTTTTTATTTTGGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGC

GN-99033: CTGTGCTGTCATACATTTGGTATTTTTTTATTTTGGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGC

GN-99065: CTGTGCTGTCATACATTTGGTATTTTTTTATTTTGGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGC

GN1-99025: CTGTGCTGTCATACATTTGGTATTTTTTTATTTTGGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGC

GN2-99025: CTGTGCTGTCATACATTTGGTATTTTTTTATTTTGGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGC

276

GN: CCTGACCCGGAGCATCTATTGTAGCTGGACTTAACTGCATCTTGA (GCACCAGCATAATGATAAGCG)

GN-98120: CCTGACCCGGAGCATCTATTGTAGCTGGACTTAACTGCATCTTGA (GCACCAGCATAATGATAAGCG)

GN-99033: CCTGACCCGGAGCATCTATTGTAGCTGGACTTAACTGCATCTTGA (GCACCAGCATAATGATAAGCG)

GN-99065: CCTGACCCGGAGCATCTATTGTAGCTGGACTTAACTGCATCTTGA (GCACCAGCATAATGATAAGCG)

GN1-99025: CCTGACCCGGAGCATCTATTGTAGCTGGACTTAACTGCATCTTGA (GCACCAGCATAATGATAAGCG)

GN2-99025: CCTGACCCGGAGCATCTATTGTAGCTGGACTTAACTGCATCTTGA (GCACCAGCATAATGATAAGCG)

Fig. 4. Sequences of PCR-amplified D-loop 276 bp fragments of cloned calves. GN: donor fibroblast cell; GN-98120, GN-99033, GN-99065, GN1-99025, GN2-99025: live cloned calves.

individuals of Luxi breed, but not in the Holstein breed, thus the N1–N2 primers and the H1–H2 primers were determined as Holstein breed-specific primers and Luxi breed-specific primers, respectively. The breed-specific primers were used to detect the mtDNA of both Holstein and Luxi breeds in the five live cloned calves. The results showed that the PCR products of both Holstein and Luxi breeds presented in all live cloned calves, that is, the mtDNA of the donor cell and recipient ooplasm coexisted in all live cloned calves (Figs. 2 and 3). The sequences of 276 bp PCR products were identical in donor fibroblasts of Holstein cow (GN) and five live cloned calves (GN-98120, GN-99033, GN-99065, GN1-99025, GN2-99025), and the sequences of 417 bp PCR products were identical in the oocytes originated from Luxi breed and five live cloned calves (GN-98120, GN-99033, GN-99065, GN1-99025, GN2-99025) (Figs. 4 and 5).

DISCUSSION

In this study, the cloned embryos were constructed by injection of Holstein cow somatic cells into the perivitelline space of enucleated Luxi Yellow cow oocytes and fused by electrical stimulation. A total of 13 cloned calves were born after transfer the reconstructed embryos into recipient mothers and five of them have survived. Microsatellite DNA analysis were used for detecting the genotype of clones (Zakhartchenko et al., 1999; Betthauser et al., 2000; Kubota et al., 2000; Polejaeva et al., 2000; Zhou et al., 2000). Although we could judge the calves were originated from the Holstein donor cells by the phenotypes, furthermore, we detected their genotypes by microsatellite DNA analysis and confirmed that five live cloned calves were genetically identical to the donor cow and donor fibroblasts of GN and there was no genetic relationship between cloned

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                                                                                        70
GN-98120: (GTGAAACCAGCAACCCGCTAA) GCAGAGGATCCCTCTTCTCGCTCCGGGCCCATAGACCGTGGGGGTCGCT
GN-99033: (GTGAAACCAGCAACCCGCTAA) GCAGAGGATCCCTCTTCTCGCTCCGGGCCCATAGACCGTGGGGGTCGCT
GN-99065: (GTGAAACCAGCAACCCGCTAA) GCAGAGGATCCCTCTTCTCGCTCCGGGCCCATAGACCGTGGGGGTCGCT
GN1-99025:(GTGAAACCAGCAACCCGCTAA) GCAGAGGATCCCTCTTCTCGCTCCGGGCCCATAGACCGTGGGGGTCGCT
GN2-99025:(GTGAAACCAGCAACCCGCTAA) GCAGAGGATCCCTCTTCTCGCTCCGGGCCCATAGACCGTGGGGGTCGCT
                                                                                   140
GN-98120: ATTTAATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGGGCCATCTCATCTAAAGTGGTCCATTCTTTC
GN-99033: ATTTAATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGGGCCATCTCATCTAAAGTGGTCCATTCTTTC
GN-99065: ATTTAATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGGGCCATCTCATCTAAAGTGGTCCATTCTTTC
GN1-99025: ATTTAATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGGGCCATCTCATCTAAAGTGGTCCATTCTTTC
GN2-99025: ATTTAATGAATTTTACCAGGCATCTGGTTCTTTCTTCAGGGCCATCTCATCTAAAGTGGTCCATTCTTTC
                                                                                   210
GN-98120: CTCTTAAATAAGACATCTCGATGGACTAATGACTAATCAGCCCATGCTCACACATAACTGTGCTGTCATA
GN-99033: CTCTTAAATAAGACATCTCGATGGACTAATGACTAATCAGCCCATGCTCACACATAACTGTGCTGTCATA
GN-99065: CTCTTAAATAAGACATCTCGATGGACTAATGACTAATCAGCCCATGCTCACACATAACTGTGCTGTCATA
GN1-99025: CTCTTAAATAAGACATCTCGATGGACTAATGACTAATCAGCCCATGCTCACACATAACTGTGCTGTCATA
GN2-99025: CTCTTAAATAAGACATCTCGATGGACTAATGACTAATCAGCCCATGCTCACACATAACTGTGCTGTCATA
                                                                                   280
GN-98120: CATTTGGTATTTTTTTATTTTGGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGCCCCGACCCGGAGC
GN-99033: CATTTGGTATTTTTTTATTTTGGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGCCCCGACCCGGAGC
GN-99065: CATTTGGTATTTTTTTATTTTGGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGCCCCGACCCGGAGC
GN1-99025: CATTTGGTATTTTTTTATTTTGGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGCCCCGACCCGGAGC
GN2-99025: CATTTGGTATTTTTTTATTTTGGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGCCCCGACCCGGAGC
                                                                                   350
GN-98120: ATCTATTGTAGCTGGACTTAACTGCATCTTGAGCACCAGCATAATGATAGGCATGGGCATTACAGTCAAT
GN-99033: ATCTATTGTAGCTGGACTTAACTGCATCTTGAGCACCAGCATAATGATAGGCATGGGCATTACAGTCAAT
GN-99065: ATCTATTGTAGCTGGACTTAACTGCATCTTGAGCACCAGCATAATGATAGGCATGGGCATTACAGTCAAT
GN1-99025:ATCTATTGTAGCTGGACTTAACTGCATCTTGAGCACCAGCATAATGATAGGCATGGGCATTACAGTCAAT
GN2-99025:ATCTATTGTAGCTGGACTTAACTGCATCTTGAGCACCAGCATAATGATAGGCATGGGCATTACAGTCAAT
                                                                              417
GN-98120: GGTCACAGGACATAAATTACATTATATATCCCCCCTTCATAAAAA (CCTCCCCCTTAAATATTCACCA)
GN-99033: GGTCACAGGACATAAATTACATTATATATCCCCCCTTCATAAAAA (CCTCCCCCTTAAATATTCACCA)
GN-99065: GGTCACAGGACATAAATTACATTATATATCCCCCCTTCATAAAAA (CCTCCCCCTTAAATATTCACCA)
GN1-99025: GGTCACAGGACATAAATTACATTATATATCCCCCCTTCATAAAAA (CCTCCCCCTTAAATATTCACCA)
GN2-99025: GGTCACAGGACATAAATTACATTATATATCCCCCCTTCATAAAAA (CCTCCCCCTTAAATATTCACCA)
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Fig. 5. Sequences of PCR-amplified D-loop 417 bp fragments of cloned calves. GN-98120, GN-99033, GN-99065, GN1-99025, GN2-99025: live cloned calves.

calves and recipients, which is an important basis for the mtDNA analysis of cloned calves.

Since the Holstein breed and Luxi breed belong to a same species *Bos taurus*, we need to detect the D-loop sequences of two breeds and design the breed-specific PCR primers before we studied the mtDNA of cloned calves. Thus, we selected the conservative sequences of two ends as the universal primers for amplifying a 790 bp D-loop fragment of *Bos taurus* by aligning the known D-loop sequences of bovine in GENBANK. In order to ensure we could design the responsible breed-specific primers, we amplified and sequenced the same D-loop region fragments of Galoway bulls which also belongs to *Bos taurus*. Then we further tested the responsibility of breed-specific PCR primers by amplifying the 276 and 417 bp fragments of Holstein cows and Luxi Yellow cows, and selected the best annealing temperature of PCR reactions, respectively.

We designed two pairs of breed-specific primers with 141 bp difference in length of PCR products, which can help us to distinguish them by agarose electrophoresis. In fact we have determined that the mtDNA of Holstein and Luxi breeds co-exist in five live cloned calves after we detected the PCR products by agarose electrophoresis and before we sequenced the PCR products. In order to further confirm the responsibility of the results, we sequenced the purified PCR products and confirmed that the sequences of 276 bp PCR products were identical in donor fibroblasts of the Holstein cow (GN) and in five live cloned calves, and the sequences of 417 bp PCR products were identical in the oocytes originated from Luxi breed and in five live cloned calves.

In previous studies, many methods have been used to analyze the mtDNA of somatic cell cloned animals or reconstructed embryos. Evans et al. (1999) used DNA sequencing of the D-loop region and PCR/RFLP analysis based on polymorphisms found between donor and recipient samples to differentiate the mtDNA genotypes among the nuclear donor somatic cells, the recipient oocytes and the NT-derived cloned sheep. Steinborn et al. (2000) quantified the percentage of donor mtDNA in the cloned animals by allele-specific real-time PCR using single-nucleotide polymorphisms in parental mtDNAs and mismatched allele-specific primers. Do et al. (2002) demonstrated that the mtDNA from donor cells was detected at all developmental stages of NT embryos by AS-PCR. Recently, Takeda et al. (2003) detected the donor mtDNA in NT calves derived from cumulus cells by PCR-SSCP. In the present study, the breed-specific primers PCR was used and PCR products with different length could be detected by agarose electrophoresis before they were sequenced, that is, we can determinate the source of mtDNA without sequencing, which is easier than the previous methods and we have confirmed the responsibility of results by further sequencing.

In addition, the results of this study showed that all the normal and healthy cloned calves are mtDNA heteroplasmy, suggesting that the mtDNA heteroplasmy does not effect the normal development. In previous studies, Evans et al. (1999) and Loi et al. (2001) reported that the mtDNA of cloned sheep and cloned mouflon was derived exclusively from the recipient sheep oocytes in intraspecies and interspecies NT. But most of the bovine NT studies show the mtDNA heteroplasmy has been observed in cloned bovines (Steinborn et al., 2000, 2002; Takeda et al., 2003) except Lanza et al. (2000) reported that the mtDNA of the cloned gaur fetuses was derived from the recipient bovine oocytes in interspecies NT. In this study, we report that the first cases of mtDNA heteroplasmy occurred in all live cloned bovine. Although the reason leading to heteroplasmy in NT remains unknown, our result of mtDNA heteroplasmy in all healthy cloned mammals suggests that mtDNA heteroplasmy could not have been involved in the high death rate. In our previous study, we reported the mitochondria from donor cells of giant panda and those from recipient rabbit oocytes coexisted in embryos before implantation (Chen et al., 2002) and donor cell-derived mitochondria coexisted with oocyte-derived mitochondria during the macaca-rabbit cloned embryos before implantation (Yang et al., 2003). These mtDNA heteroplasmy results of interspecies cloned embryos are consistent with our present study, so the production of healthy heteroplasmy clones in this study is an important basis of interspecies cloning study.

In conclusion, the present study establishes an easier mtDNA detection method and demonstrates that mtDNA heteroplasmy in all live cloned calves for the first time by detecting the sequences of D-loop fragments with breed-specific primers PCR.

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