Genetic Analysis of Cattle Cloned From Somatic Cells and Gene Transferred Somatic Cells*

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Abstract Three cloned cattle from the same somatic cell line (TT, QQ, XW) and three transferred cloned cattle from the same gene transferred cell line (JM, LW, 8C2), together with two randomly selected cattle, one LUXI (LX) and one Holstein (HS), were genetically analyzed using 24 fluorescent microsatellite markers. There were 1 ~ 5 alleles at 24 loci, the average being 3.17. Among TT, QQ, XW, JM, LW, 8C2, the somatic cell line and the gene transferred somatic cell line, the probabilities of matching (P_M) between individuals were 1.17×10^{-36} using allele frequencies obtained from public data on internet and 1.90×10^{-23} by defining the allele frequencies at each locus as the reciprocal of the number of alleles at that locus. In addition, their genotypes differed from those of the randomly selected LX and HS at 23 and 20 marker loci respectively.

Key words cloned cattle, genetic analysis, gene transferred somatic cell line

Mammalian cloning by somatic cell nuclear transfer (SCNT) technology has developed rapidly after "Dolly" the sheep was born in 1997. Other animals of the same species such as Nosh and Mouflon, as well as animals from other species including mouse, cattle, goat, pig, cat, rabbit have now been cloned. The detection of genetic materials in the cloned animals was the basis on which the SCNT animals were confirmed to be clones.

Microsatellites (short tandem repeat, STR) are short DNA sequence with a 1 ~ 6 nucleotide core repeated sequence. They are distributed extensively and randomly in the eukaryote genome[1,2]. Their ubiquity in eukaryotic taxa, along with their high degree of polymorphism, codominant inheritance, high degree of conservation between species and ease of genotyping has made them favored makers for the genetic identification of humans and animals, assessing species biodiversity, the construction of genetic linkage map, mapping quantitative trait loci (QTL) and for studying molecular evolution[3~7]. Since the SCNT technology to clone mammals has been established, microsatellites have been used to identify the cloned animals[7~12]. However, reports of using microsatellites to identify cattle cloned with gene transferred somatic cells are scarce.

We used 24 microsatellites, which are distributed evenly on the bovine genome and are recommended internationally for the detection of bovine DNA polymorphisms (9 for parentage identification), to analyse the genetic origin of the 3 cloned cattle and the 3 gene transferred cloned cattle. TT, QQ and XW were cloned from somatic cells, and JM, LW and 8C2 were cloned from gene transferred somatic cells, respectively. The results show the above six cattle, together with the 2 cultured cell lines had the same genetic material, but had no genetic correlation with the randomly selected LX and HS cattle.

1 Materials and methods

1.1 Animals

Somatic cell line (SC1) and gene transferred somatic cell line (GTSC1) were cultured in the State Key Laboratory for Agrobiotechnology. TT, QQ and XW, which were cloned from the same somatic cells, and JM, LW and 8C2, which were cloned from the same gene transferred somatic cells (expressing the transgene GFP), together with randomly selected LX and HS, were selected from Kelong Animal Husbandry Production Ltd. (Shandong province) and from Lutai Dairy Cattle Farm (Hebei province).

A routine phenol/chloroform extraction method was used to extract and purify bovine genomic DNA, and the quality of the DNA was establishing using agarose gel electrophoresis. DNA concentration were estimated using molecular marker and diluted to 20 mg/L.

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1.2 Microsatellite primers

The fluorescent microsatellite primers, which were labeled with HEX, FAM or TAMRA fluorescent dyes on forward or reverse primers, were synthesized by the Shanghai Bioengineer Company. The DNA of the above samples was screened for 24 microsatellites. The sequence of the primers and their distribution on the bovine chromosome are listed (See http://www.projects.roslin.ac.uk/cdiv/).

1.3 Microsatellite genotyping

The total volume of polymerase chain reaction (PCR) was 10 μl, containing 40 ng DNA template, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Triton X-100, 0.01% gelatin, 200 mmol/L dNTP, 5U TaqDNA polymerase and 0.2 pmol forward and reverse primers. The PCR reactions were performed on GeneAmp PCR System 9700 machines(ABI) using the following condition 5 min at 94℃; 30 cycles of each of 30 s at 94℃, annealing 30 s, and 30 s at 72℃; elongation 30 min at 72℃. The products were diluted 5 ~ 20 times with sterilized deionized water and detected on a 6% denaturing polyacrylamide gel on an automatic ABI377 sequence analyzer or POP4 on an ABI3100 sequence analyzer (ABI). Gels on 36 cm gel plates were prepared using Tris, EDTA, acrylamide and Bis (Life Science) (manual of ABI 377 genetic analyzer). The internal size standard (Genescan-350^{ROX}) was obtained from Applied Biosystems USA. Genotypes were analyzed using the software GeneScan3.1, GeneScan 3.7(ABI).

The formula used to calculate P_m (the probability that 2 animals have the same genotype at one locus by chance) at one locus is:

$$P_m = \sum_{i=1}^{n} P_i^2$$

Where n is the number of genotypes present at the marker locus, P_i is the frequency of genotype Ⅰ at the maker locus. If the genetic markers are independent, the probability of 2 animals having the same genotypes at each of k genetic markers (P_M) is:

$$P_M = P_{m1} \times P_{m2} \times P_{m3} \times \ldots \times P_{mk} = \prod_{j=1}^{k} P_j$$

where P_j is the P_m value of the jth marker. As the genotype frequencies of our cattle population are unknown, we used 2 different approaches for determining the P_M:

a. We used the gene frequencies of previously identified alleles available from the internet (www.projects.roslin.ac.uk/cdiv/). In those cases where we identified a novel allele in our animals, the novel genotypes were assigned the genotype of the highest frequency genotype reported at that locus (so as to overestimate the true P_M).

b. We defined the genotype frequencies at each locus as being the reciprocal of the number of possible genotypes at that locus (e.g. if there were 3 alleles, each genotype frequency is 1/9).

2 Results

2.1 Amplified fragment size and the number of alleles

PCR using microsatellite primers at 24 loci yielded specific PCR products that varied in size according to the particular allele present. The number of alleles ranged from 1 ~ 5, the average being 3.17. The cultured somatic cells, the gene transferred somatic cells, and the six cloned cattle exhibited identical genotypes at each of the 24 microsatellite marker loci, and differed from the randomly selected LX and HS at 23 and 20 marker loci, respectively (Table1, Figure 1).

Table 1 The position, PCR condition, fragment, allele and sequences of 24 microsatellite markers

Marker number	Marker	Chromosome	A1	A2	Fragment	Allele	Primer sequences (5′~3′)
1	BM1824 (D1S34)	1	50℃ 10	51℃ 20	181~183	2	GAGCAAGGTGTTTTTCCAATC CATTCTCCAACTGCTTCCTTG
2	BM2113 (D2S26)	2	50℃ 10	51℃ 20	136~152	4	GCTGCCTTCTACCAAATACCC CTTCCTGAGAGAAGCAACACC
3	SPS115 (D15)	15	50℃ 10	51℃ 20	247~255	3	AAAGTGACACAACAGCTTCTCCAG AACGAGTGTCCTAGTTTGGCTGTG
4	ETH3 (D19S2)	19	60℃ 30		116~126	3	GAACCTGCCTCTCCTGCATTGG ACTCTGCCTGTGGCCAAGTAGG
5	ETH10[4] (D5S3)	5	50℃ 10	51℃ 20	213~225	4	GTTCAGGACTGGCCCTGCTAACA CCTCCAGCCCACTTTCTCTTCTC

Continued

Markeri number	Marker	Chromo-some	A1	A2	Fragment	Allele	Primer sequences (5′～3′)
6	ETH225 (D9S1)	9	50℃ 10	51℃ 20	145～151	3	GATCACCTTGCCACTATTTCCT ACATGACAGCCAGCTGCTACT
7	TGLA122 (D21S6)	21	60℃ 30		152～152	1	CCCTCCTCCAGGTAAATCAGC AATCACATGGCAAATAAGTACATAC
8	TGLA126 (D20S1)	20	50℃ 10	51℃ 20	108～126	4	CTAATTTAGAATGAGAGAGGCTTCT TTGGTCTCTATTCTCTGAATATTCC
9	TGLA227 (D18S1)	18	60℃ 30		83～105	4	CGAATTCCAAATCTGTTAATTTGCT ACAGACAGAAACTCAATGAAAGCA
10	INRA063 (D18S5)	18	50℃ 10	51℃ 20	175～177	2	ATTTGCACAAGCTAAATCTAACC AAACCACAGAAATGCTTGGAAG
11	HEL9 (D8S4)	8	50℃ 10	51℃ 20	149～169	5	CCCATTCAGTCTTCAGAGGT CACATCCATGTTCTCACCAC
12	HEL1 (D15S10)	15	50℃ 10	51℃ 20	105～113	3	CAACAGCTATTTAACAAGGA AGGCTACAGTCCATGGGATT
13	INRA035 (D16S11)	16	50℃ 10	51℃ 20	102～104	2	ATCCTTTGCAGCCTCCACATTG TTGTGCTTTATGACACTATCCG
14	ILSTS005 (D10S25)	10	50℃ 10	51℃ 20	184～186	2	GGAAGCAATGAAATCTATAGCC TGTTCTGTGAGTTTGTAAGC
15	INRA023 (D3S10)	3	50℃ 10	51℃ 20	209～217	4	GAGTAGAGCTACAAGATAAACTTC TAACTACAGGGTGTTAGATGAACTC
16	INRA0325 (D11S9)	11	50℃ 10	51℃ 20	180～186	3	AAACTGTATTCTCTAATAGCTAC GCAAGACATATCTCCATTCCTTT
17	MM12 (D9S20)	9	50℃ 10	51℃ 20	107～125	4	CAAGACAGGTGTTTCAATCT ATCGACTCTGGGGATGATGT
18	HAUT24 (D22S26)	22	50℃ 10	51℃ 20	119～131	2	CTCTCTGCCTTTGTCCCTGT AATACACTTTAGGAGAAAAATA
19	HEL135 (D11S15)	11	50℃ 10	51℃ 20	186～194	3	TAAGGACTTGAGATAAGGAG CCATCTACCTCCATCTTAAC
20	ILSTS006 (D7S8)	7	50℃ 10	51℃ 20	327～335	4	TGTCTGTATTTCTGCTGTGG ACACGGAAGCGATCTAAACG
21	HEL51 (D21S15)	21	50℃ 10	51℃ 20	153～167	4	GCAGGATCACTTGTTAGGGA AGACGTTAGTGTACATTAAC
22	INRA037 (D10S12)	11	50℃ 10	51℃ 20	120～132	2	GATCCTGCTTATATTTAACCAC AAAATTCCATGGAGAGAGAAAC
23	INRA005[2] (D12S4)	12	50℃ 10	51℃ 20	139～143	3	CAATCTGCATGAAGTATAAATAT CTTCAGGCATACCCTACACC
24	ETH152 (D5S1)	5	60℃ 30		193～211	5	TACTCGTAGGGCAGGCTGCCTG GAGACCTCAGGGTTGGTGATCAG

Marker 1～9 are recommended in ISAG, toured for parentage testing. A1, A2 is the different annealing temperatures and the cycle numbers of that temperature.

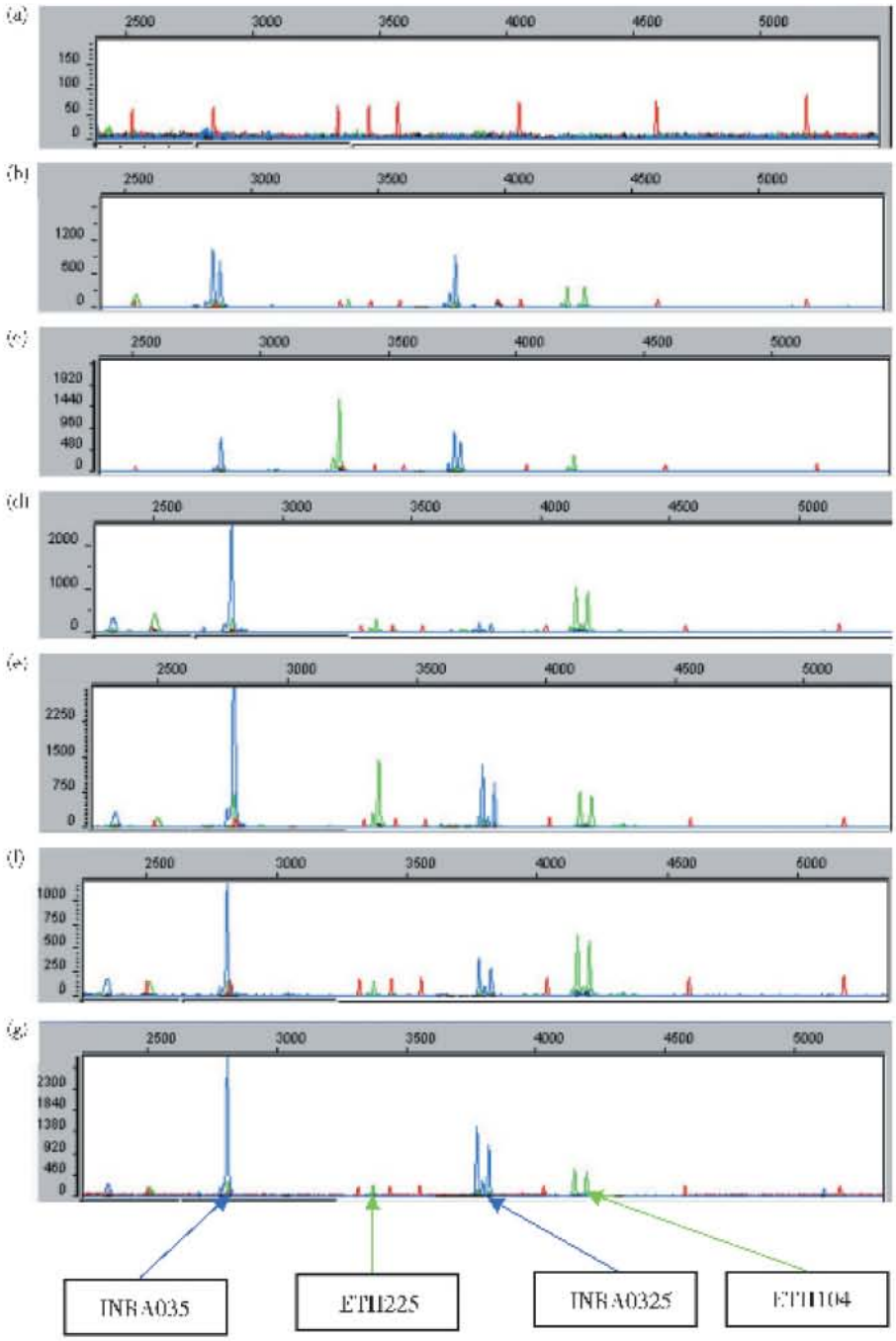

Fig. 1　The genotype of the samples in loci INRA035, ETH225, INRA0325, ETH104

(a) ~ (g) are negative control, HS1, LX, QQ, ST1, LW and TSC1, respectively. Red band is internal size standard, both blue and green band are PCR products.

2.2　P_M (probability of matching)

Based on the statistical method in Judiciary Identification Routine and Technical Damage Regulation, we collected the genotype data at 24 marker loci and computed the P_M values. According to the public data on www. ri. bbsrc. ac. uk/bovmap/bovmap. html, P_M values were calculated in the highest frequencies. The P_M reaches: P_{M1} 1.17×10^{36} between the cultured somatic cell line and cloned cattle TT, QQ and XW from the somatic cells; P_{M1} 1.17×10^{36} between gene transferred somatic cell line and the cloned cattle JM, LW and 8C2 from the gene transferred somatic cells; P_{M1} 1.17×10^{36} between these two cultured cell lines.

Taking the reciprocal of the number of alleles as the allelic frequency, P_M were P_{M2} 1.90×10^{-23} between the cultured somatic cell line and cloned cattle TT, QQ and XW from the somatic cells; P_{M2} 1.90×10^{-23} was between gene transferred somatic cell line and the cloned cattle JM, LW and 8C2 from the gene transferred somatic cells; P_{M2} 1.90×10^{-23} between these two cultured cell lines.

Using data only from the 9 marker loci internationally recommended for parentage identification (www. ri. bbsrc. ac. uk/bovmap/bovmap. html), the probabilities of matching among the two cultured cell lines and the six cloned cattle calculated using two formula above mentioned were P'_{M1} 4.26×10^{-16} and P'_{M2} 5.23×10^{-9}.

3 Discussion

3.1 Genetic identification of the cloned cattle

We observed 1 ~ 5 alleles (the average being 3.17) at 24 microsatellite loci in specific amplified PCR products. According to data on the internet using the highest allele frequency for the purpose of the calculation, the probability of matching among cultured cell lines and the six cloned cattle is P_{M1} 1.17×10^{-36} and P_{M2} 1.90×10^{-23} if computed using our experimental data. The probability of matching based on these two different methods were far smaller than 1×10^{-12}, which is the international standard required for stating that 2 DNA samples have the same genetic origin. Therefore, the cultured cell lines and the six cloned cattle have the same genetic origin, and they differ at 23 and 20 markers respectively from the randomly selected LX and HS cattle. When there exists non-matching at three marker loci, a parentage relationship can be excluded according to the international standard for parentage identification. We can conclude that the cultured cell lines and the six cloned cattle have different genetic origins as compared with the two randomly selected LX and HS cattle.

3.2 Comparison between different methods to calculate P_M

In some individual identification studies, the probability of matching is calculated based on already acquired genotype frequencies. Up to now, no reports on Chinese indigenous scalper and Holstein cattle have been seen. We chose 24 microsatellite loci and found 76 alleles, 6 of which have not been previous reported. So the probabilities of matching were calculated by two different methods, one of which used the highest frequency allele at that locus found in the public data on the Internet, the other used the observed frequency of the allele number in the experiment.

The second method took the reciprocal of the number of alleles as the allelic frequency, so the number of alleles decided the value of the probability of matching. The number of alleles was relatively few in our experiment, so the values of P_{M1} and P'_{M1} were far smaller than that of P_{M2} and P'_{M2}. According to the results from the comparison P_{M1} and P_{M2}, P'_{M1} and P'_{M2}, P'_M calculated by the second method approached P_M calculated by the first method, as the number of loci and alleles increased in a certain range.

3.3 Determination of the number of genetic markers in genetic analysis

The probabilities of matching were P'_{M1} 4.26×10^{-16} and P'_{M2} 5.23×10^{-9} among the two cultured cell lines and the six cloned cattle calculated using the allele numbers of 9 marker loci for parentage identification internationally recommended. It was believed that the origin of the genetic materials could not been judged precisely based only on the nine genetic markers for parentage identification for three reasons. First, the allelic frequency of the population to which the tested sample belonged was unknown; second, 5 alleles in the 9 marker loci have not been reported previously; third, P'_{M2} (5.23×10^{-9}) was higher than the standard for parentage identification internationally (1×10^{-12}). Therefore, if calculated using the average allele number 3.17, at least 12 loci could be required to precisely identify animal relatedness when we had no knowledge about the allelic frequency.

In the process of individual identification and genetic analysis, the number of genetic markers has a considerable impact on experimental cost and labor. When the allelic frequency of the tested population is already known, the number of genetic markers for genetic identification can be estimated on the basis of allelic frequency. However, when that is unknown, estimation can only be performed according to the polymorphism of the genetic markers, the sample size and the genetic structure between samples, etc. If the polymorphism of the genetic markers is large, the sample size is large and a large discrepancy among samples exists, then the number of markers needed is small, and vice versa.

3.4 Identification of cloned cattle from gene transferred somatic cell

Many papers have shown that microsatellite markers can be employed to identify the origin of the genetic materials of somatic cell cloned animals[8~13]. However, this method cannot decide whether or not a foreign gene has been integrated into the genome of the cloned animals. Consequently, the identification of the transferred cloned animals must be performed via Southern blotting, and amplification of specific gene sequence, or Western blotting.

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体细胞克隆牛和转基因体细胞克隆牛的遗传学分析*

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摘要 分析了来自同一细胞系的体细胞克隆牛甜甜、庆庆、浒娃及来源同一培养转基因体细胞系转基因体细胞克隆牛九妹、乐娃和1个妊娠8个月转基因流产胎牛8C2以及随机抽取的1头鲁西黄牛（LX）、1头褐斯坦牛（HS）在24个微卫星位点标记牛的基因型. 结果表明24个多态位点均表现出多态，等位基因数为1～5个，平均为3.17个. 根据网上公布的数据，按其最高频率计算，甜甜、庆庆、浒娃、九妹、乐娃、8C2与培养细胞系、转基因细胞系间匹配概率为1.17×10^{-36}，根据本研究观察到的数据计算，匹配概率为1.90×10^{-23}；而与随机抽取的1头鲁西黄牛及褐斯坦牛的基因型分别在23和20个位点上完全不同.

关键词 克隆牛，遗传学，分析

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