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Identification of Monozygotic Twins in Pigs Using Optimized Fluorescent Microsatellite Markers

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Abstract: Monozygotic (MZ) twins having the same DNA information have been used for many biomedical studies. Pigs present high similarity with humans and have been used as an ideal mammalian model the use of MZ twins is even more valuable. In the study, researchers present a systematic method for the stepwise exclusion of inconsistently genotyped individuals in a large population by Short Tandem Repeat (STR) genotyping. Five pairs of microsatellite markers with different fluorescent dyes were selected and their reliability was tested. The results demonstrate that the cumulative exclusion rate of all markers was 99.67% in 1,201 pigs; two pairs of MZ twins were initially identified. Researchers have established an effective, easy and cheap way to identify MZ twin pigs.

Key words: Pig, monozygotic twins, paired fluorescent microsatellite marker, STR genotyping, cumulative exclusion rate

INTRODUCTION

Monozygotic (MZ) twins have uniquely identical alleles which have been applied to the study of pathology (Weber and Sebire, 2010), physiology (Beck-Nielsen *et al*., 2003; Ying *et al*., 2006), psychology (Gregory *et al*., 2011) and genetics (Zwijnenburg *et al*., 2010). Mara *et al*. (2005) used twin goats to study Ocular Squamous Cell Carcinoma (OSCC) and identified Papillomavirus-related DNA sequences. Hogg *et al*. (2009) investigated the gene-environment interactions of visual functions by using monozygotic and dizygotic twins of different ages, illustrating the different contributions of genes and the environment to the retina. Kan *et al*. (2010) used monozygotic and dizygotic twins to study the nonlinear epigenetic variance with conventional behavior genetic modeling which appeared unsystematic variance in conventional twin analyses.

Pigs have similar metabolic features, cardiovascular systems and proportionally similar organ sizes to humans (Spurlock and Gabler, 2008) and have been employed as models in the study of human nutrition, pathology and pharmacology (Hughes *et al*., 2003; Schook *et al*., 2005). Considering their anatomy and the fact that pigs are raised widely, monozygotic pig twins are valuable, unique models for the earlier research. However, it is difficult to identify monozygotic pig twins because of their similar appearance and the polyembryony in nature (Grapes *et al*., 2001). Microsatellite markers are a relatively easy way to determine the occurrence of monozygotic twins. Grapes *et al*. (2001) used 180 markers in total with 525 F2 pig individuals and found one pair of female twins by mapping genotype data. The heavy workload and tediousness of this method means it has not been widely applied.

In this study, researchers used the Short Tandem Repeat (STR) Method for MZ twin identification. The five best pairs of twenty candidate microsatellite markers were selected and labeled with two fluorescence dyes for the stepwise exclusion procedure in attempt to establish an effective, economic and easily performed method to identify MZ pig twins.

MATERIALS AND METHODS

Animals and tissue collection: Tail or ear tissues were collected from 1,201 female Yorkshire pigs, ranging from 3 days to 2 months of age. These pigs were raised in intensive piggeries in Jinhua, Zhejiang.

Microsatellite markers: Twenty candidate microsatellite markers were chosen (Schook *et al*., 2005). According to the manufacturer's instructions, paired markers must meet the condition that the interval size was >50 bp and labeled with a different fluorescent dye. Finally, the five best pairs of matched markers were selected their fluorescent labels are given in Table 1.

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Table 1: Microsatellite markers and fluorescent labels

Serial number	Marker ID	Sequences of primers (5'→3')	Average segment size (bp)	Fluorescent dyes
1A	SW886	F*:AATTGGTTTGTCCAGAATTTGG R:GATCATTCCCATTTGTTGAATT	158.0	HEX[a]
1B	SW1989	F:TGATGACTCTCTGGTGGCTG R:TCTTTGCTTAGCCACCCATC	237.0	FAM[b]
2A	SW398	F:AAGTGCCAATGCTTTGTTCC R:CGGAGGAGAAATAAGGGTAGC	179.0	HEX
2B	S0009	F:AAACATACCAAGAAGCCCAG R:TAATCTTTGCCATCCCTTGT	127.5	FAM
3A	S0167	F:AAACTCCAATTTCCATAACATAGG R: CTTCATATATGTGCTAAGACTTCT	213.0	HEX
3B	SW38	F:ACGTCTGTGTCGGTGCCT R:GAGGCTCCTGATAGCAGCC	130.5	FAM
4A	S0289	F:AGGAGCATTTGGCCACGTCTG R:TGTTGACCTTCTGTGATGGGGC	152.0	HEX
4B	SW58	F:TCCTACCAGAAATCCTACCACA R:ATGGGAAGAGAATCTGACAAGG	214.0	FAM
5A	S0071	F:ATTATGCACCCCTACTCCCC R:CCAGAAGCAGGTTTTGAGATGA	184.0	HEX
5B	SW957	F:AGGAAGTGAGCTCAGAAAGTGC R: ATGGACAAGCTTGGTTTTCC	134.0	FAM

1A, 1B etc. means the first pair of markers. *Only forward primers were labeled with fluorescent dyes; [a]HEX: Hexachloro-6-carboxyfluorescein; [b]FAM: 5-(and-6)-Carboxyfluorescein

Extraction and amplification of DNA: DNA was extracted with a DNA extraction kit (BioTeke Corporation DNA Extraction kit, BioTeke Corporation, Shanghai, China), according to the manufacturer's instructions. The Polymerase Chain Reaction assay (PCR) was performed with BioTekePCR mix (2×PowerTaqPCRMasterMix, BioTeke Corporation). The PCR reaction system consisted of 2×MasterMix 10 μL, nuclease-free water 7 μL, each primer 1 μL and 1 μL DNA template, bringing the total reaction volume to 20 μL. The PCR conditions were as follows: 95°C for 3 min followed by 35 cycles of 95°C for 30 sec, 57°C for 30 min, 72°C for 30 sec and a final extension at 72°C for 10 min.

STR genotyping: The first pair of markers was used for amplification in all samples, the size and the quality of the PCR products were scored on 1% agarose gel, the products were then sent to Shanghai OE biotech Cooperation (Shanghai, China) for STR testing with capillary electrophoresis (ABI3730XL, Applied Biosystems, USA) with GeneMapper© Analysis Software 4.0 (Applied Biosystems). Within 1 L if the number and value of sizes were the same, these individuals were prepared for the next paired amplification until they had gone through all the paired markers. Only if the five pairs of markers presented the exact same sizes would researchers preliminarily identify the two pairs of pigs as MZ twins.

Data processing and analysis: All of the data was analyzed and sorted in Microsoft® Excel (Redmond, WA, USA). Pigs from the same litter were rejected if the results of the STR genotyping were not exactly the same. During the analysis and processing of the paired marker data, the collection of the number set was adopted for accuracy. After three pairs of markers were screened, researchers screened the remaining pairs of markers together because of the small number of samples.

RESULTS AND DISCUSSION

STR genotyping results with five paired markers: As shown in Fig. 1, each marker pair was labeled with different fluorescent dyes in the same pig, group A with fluorescent HEX (green) and group B with fluorescent FAM (blue). Within a litter, every two individuals were tested whether they had the same size or not. As earlier verified if the two piglets were the MZ twins, the number of sizes and the value of every size with all the markers were the same (i.e., group 1A) if not the different pigs would have different number or value of sizes in general (i.e., group 4A).

The exclusion rate: Of the twenty candidate microsatellite markers, researchers chose the best five pairs to identify the sizes of each piglet. As shown in Table 1 and Fig. 1, these five pairs were ranked from the highest accuracy and stability to the lowest. The first pair (1A and 1B) was applied to all 1,201 piglets and 842 individuals with the same size as their siblings went on to the second paired test. During the test, weak or dead piglets were eliminated from the analysis. Therefore, researchers excluded 342, 157 and 336 individuals using the following paired markers, respectively in the end researchers excluded 1,197 piglets (Table 2), bringing the total cumulative exclusion rate to 99.67%. Based on the methods described

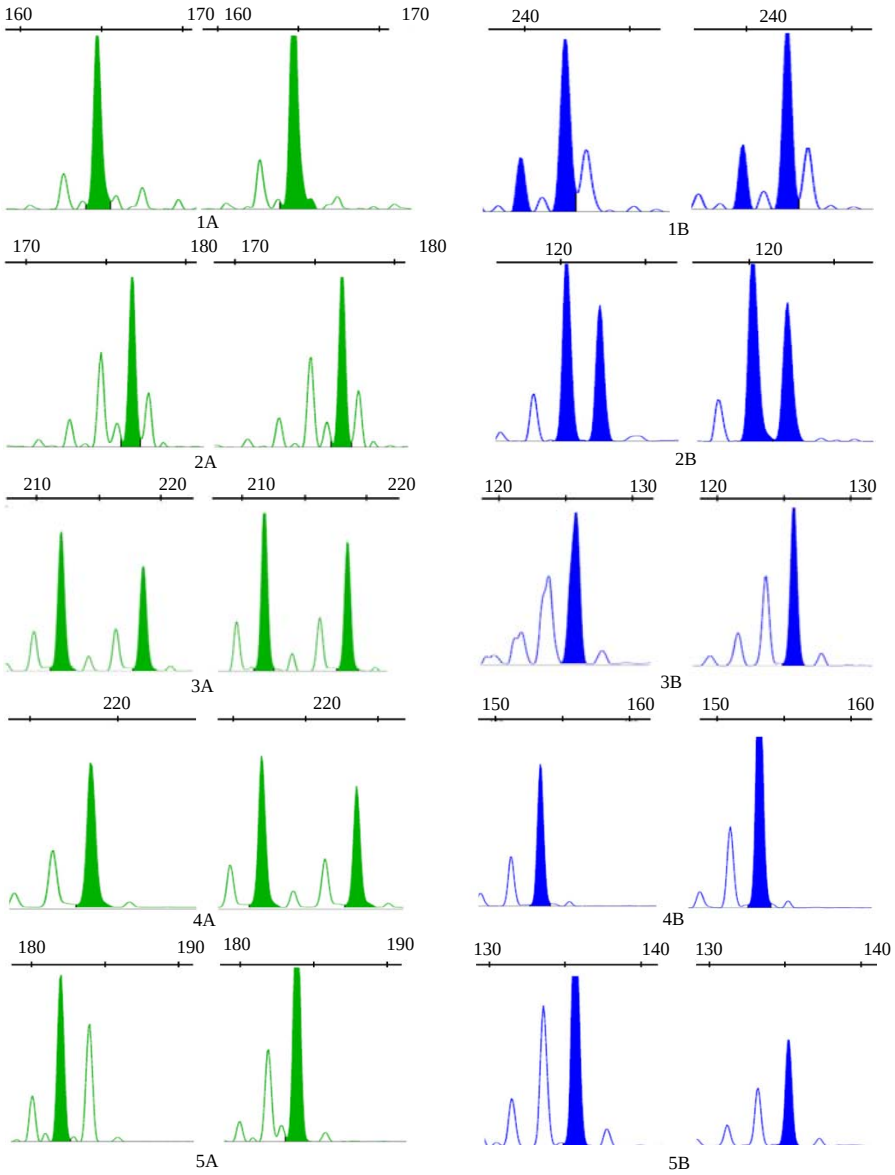

Fig. 1: Sizes of five paired microsatellite markers. Each primer compared the number and value of sizes between two individuals from one litter and the paired markers (like 1A and 1B) showed the same two individuals labeled with different fluorescents

Table 2: Cumulative exclusion rate of the five marker pairs

Microsatellite markers	1st pair	2nd pair	3rd pair	4 and 5th pair
Test number	1,201.00	842.00	501.00	340.00
Number excluded	362.00	342.00	157.00	336.00
Cumulative exclusion number	362.00	704.00	861.00	1197.00
Cumulative exclusion rate (%)	30.13	58.62	71.69	99.67

earlier, two pairs of piglets presented the same number and value of all the markers and were determined as candidate MZ twins (Table 3).

The spontaneous occurrence rate of MZ twins is low in most species. In humans, about 1 in 330 spontaneous live births is a monozygotic birth which means about 1 in 160 babies is a monozygotic twin (Kiely and Kiely, 2001; Bjerre *et al*., 2009). In mice, spontaneous monozygotic twinning conception seems to be very rare with <1 in 1000 twins actually surviving to birth (Murphy and Hey, 1997). In the study, researchers confirmed the ratio of MZ twins in pigs was 0.17% (2/1,201), this ratio is similar to earlier reports of one pair of MZ female twins in 525 F2 individuals of pigs (Grapes *et al*., 2001).

Here, researchers selected five pairs of well-spaced microsatellite markers with each pair being assigned different color fluorescent dyes. Through reliability verification of the markers from the same tissue of the same individual and different tissues from different individuals, the results demonstrate that each marker pair

Table 3: STR genotype data of the two pairs of MZ twins initially identified

Markers		1A		1B		2A		2B		3A		3B		4A		4B		5A		5B	
Litter	ID	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
187	75	165	171	242	/	175	187	122	/	218	/	126	/	133	138	144	174	186	/	110	/
	82	165	171	242	/	175	187	122	/	218	/	126	/	133	138	144	174	186	/	110	/
189	92	165	171	243	/	175	/	122	126	212	/	126	/	139	/	153	170	184	/	109	135
	94	165	171	243	/	175	/	122	126	212	/	126	/	138	/	153	170	184	/	109	135

Litter means the number of the litter; ID means the number of the pig; S1 and S2 mean size of the first and second STR genotypes

can distinguish different individuals. The total cumulative exclusion rate of the five paired markers was 99.67% which was higher than the cumulative rate in Belgian pig populations with seven microsatellite loci (95%) (Van Zeveren *et al*., 1995).

CONCLUSION

Researchers have established a set of effective, easy and cheap methods to identify MZ pig twins which is also available in a large number of samples.

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