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Mitochondrial DNA Polymorphism and Determination of Effects on Reproductive Trait in Pigs

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Contents

The purposes of this study were to investigate single strand conformation polymorphisms (SSCP) in the D-loop region of pig mitochondrial DNA (mtDNA) and to determinate their association with the reproductive traits of meishan pigs. A total of four types of band patterns, designed SSCP band pattern A, B, C and D, were identified. A type of SSCP band pattern was present in all European–American breeds, but not in East Asian breeds. This result showed the diversified sequence in the D-loop region between European–American and East Asian populations. Two types of band patterns, B and C, were found in Meishan pigs. The average body weight at day 21 of piglets from B type dams was significantly heavier than the body weight of C type ($p < 0.05$). We also tested whether the SSCP patterns would be suitable for paternity testing in a family group and found that bands of all the offspring were derived from their maternal parent. Therefore, we conclude that SSCP may be a marker for identification of maternal ancestors.

Introduction

Mitochondrial DNA (mtDNA) is maternally inherited and has been widely used for phylogenetic studies (Okumura et al. 1996; Jones 1998; Giuffra et al. 2000; Kim et al. 2002), because in mammals its evolution is more diversified than nuclear DNA (Brown et al. 1979, 1982). Restriction fragment length polymorphism (RFLP) analysis of mtDNA has been commonly used in animal breeding for identification and monitoring of animal stocks (Watanabe et al. 1985, 1986; Brown et al. 1989; Hiendleder et al. 1991).

The genetic differentiation in porcine mtDNA were focused on the displacement loop region (D-loop) in mtDNA and showed much diversity within breeds (Horai et al. 1990; Loftus et al. 1994; Pegoraro et al. 1996). The entire sequence of mtDNA D-loops of Landrace has been reported (Ghivizzani et al. 1993) and it has been suggested that porcine D-loop polymorphism and/or sequence diversity has a potential for molecular differentiation in cytoplasmic DNA (Takeda et al. 1995). However, the diversities of sequence combined with polymorphism profiles, such as single strand conformation polymorphism (SSCP) and RFLP analysis, between and within pig breeds have not been fully evaluated.

Genetic variations in the mtDNA D-loop may alter transcription or replication rates of mtDNA. Such D-loop polymorphism may serve as indirect markers for differences elsewhere on the mtDNA genome in the gene coding region directly influencing the phenotypic expression of some traits (Schutz et al. 1994; Mannen et al. 1998). Reports on Holstein cattle have further shown close association of the mtDNA D-loop polymorphism with milk product, reproduction and health (Tess et al. 1987; Brown et al. 1989; Schutz et al. 1992, 1993, 1994). Several factors may be responsible for the maternal influence. This may be due to the cytoplasm of the egg, intra-uterine environment or postnatal environment, e.g. milk production and/or mothering ability (Robison 1972). Few available data are present on the effects of mtDNA for swine economic traits. Toelle et al. (1986) suggested that cytoplasmic effects might be important for birth weight, weaning weight, days to 104 kg and backfat traits in swine.

The SSCP can detect single nucleotide substitution in certain DNA fragment because single-stranded DNA fragments have a unique folded structure (Orita et al. 1989; Hayashi 1991). The purposes of this study were to investigate SSCP of D-loop region in pig mtDNA in eight pig breeds and the association of SSCP band patterns with reproductive traits in Meishan pigs. The SSCP was also applied for identification of maternal ancestor testing.

Materials and Methods

Animals and experimental design

In Taiwan four exotic breeds for hog production are Duroc, Landrace, Hampshire and Yorkshire. Two exotic breeds Meishan and Berkshire were imported for development of genetic resources. Meishan pigs of three gilts and two boars were imported from Japan and raised at Livestock Research Institute (LRI) in July of 1994. Berkshire breed with 12 boars and 55 gilts was imported from USA in 1996. A random mating closed stock herd of Taoyuan pigs (10 boars and 30 sows) were conserved in the Research Farm at LRI. A inbreeding mating closed stock herd of Small-ear pigs were conserved in the Taitung Research Farm at LRI. Landrace (L, $n = 31$), Yorkshire (Y, $n = 17$), Duroc (Du, $n = 59$), Berkshire (Be, $n = 13$), Hampshire (H, $n = 10$), Taoyuan (T, $n = 29$), Small-ear (also name Lanyu; Se, $n = 6$) and Meishan pigs (M, $n = 40$) were used for SSCP analysis of mtDNA D-loop region polymorphism. Except Meishan and Berkshire breeds, DNA samples were collected from unrelated animals (no common grandparents) representing the other six pig breeds. We sampled 13 head offspring from 13 imported Be sows. DNA samples from the imported foundation Meishan parents and their first or second generation offspring were collected. One hundred and forty one litters of M pigs originated from three known

Table 1. Number of sires, dams and litters of tested Meishan pigs

Maternal lineage	No. of litter	No. of sire	No. of dam
Meda	28	10	11
Nehime	113	16	47

maternal ancestors were employed for SSCP investigation. Three foundation females were traced to two maternal ancestries, Meda and Nehime. Number of sires, dams and litters of M pigs for testing are shown in Table 1.

Pellet feeds of different nutrient levels were provided for various stages of M pigs were as following: nursing stage, CP 20%, ME 3135 kcal/kg; growing stage, CP 18.5%, ME 3100 kcal/kg; and for sows and boars, CP 15%, ME 2970 kcal/kg. Pregnant sows were kept in individual pen with solid floor. Their feeds were restricted to 2 kg/day each and water was supplied *ad libitum*. Sows with their piglets were kept in farrowing pen supplied with heating lamp. Piglets were given creep feed from the 10-day of age and were weaned at 5-week of age. Post-weaning piglets were kept in slot-floor pen with feed and water *ad libitum*. Piglets were removed into the growing pen of solid floor (L435 × W145 × H90 cm) at 11 weeks of age. At that time, they were separated by sex; every two to three pigs were kept in one pen with growing stage feed and water supplied *ad libitum*. We bred the M dams at 8-month age. The management of reproduction of all M dams was same as the procedure described by Yen et al. (2003).

DNA extraction

Blood samples were collected with anticoagulant from the jugular veins of pigs. The genomic DNA was isolated from blood by phenol/chloroform extraction and ethanol precipitation (Shiau and Huang 1997).

Polymerase chain reaction

A 392 bp polymerase chain reaction (PCR) product was amplified using the designed oligonucleotide primers SSCP-F (5′-GCACAAACATACAAATATGTGACC-C-3′) and SSCP-R (5′-TATTTAAGGGGAAA GAG TGGGCGA-3′ (Lin et al. 1998). PCR was performed following the revised method described by Horng et al. (2004). In addition to the template DNA, the final 25 μl of the PCR mixture contained mtDNA, 10 pmol each of SSCP-F and -R primers, 0.1 mM dNTPs, 10 × PCR buffer and 0.5 units ProZyme™ DNA polymerase (PROtech Technology Ent. Co. Ltd., Taipei, Taiwan). The first denaturation step at 95°C for 5 min was followed by 30 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The PCR products were detected in 2% agarose gels with ethidium bromide staining.

SSCP analysis and DNA sequencing

The SSCP analysis was performed as the procedure described by Lin et al. (1998) with slight modifications. Two to 6 μl of each PCR product was added to 5 μl of loading buffer (90% formamide, 20 mM NaOH, 0.05% xylene cyanole ff, and 0.05% bromphenol blue). The mixtures were then heated to 95°C for 5 min and put on ice and loaded on a native 8% polyacrylamide gel (39 : 1) (acrylamide : bisacrylamide) containing 5% glycerol in 0.5X TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM ethylenediaminetetraacetic acid, pH 8.0). Electrophoresis was carried out in a Hoefer SE600 unit at 10°C, 200 V in 0.5X TBE buffer for 21 h. The results were visualized by silver staining using a Silver Staining Kit, DNA (Pharmacia Biotech, Uppsala, Sweden). The same primers SSCP-F and SSCP-R were used for DNA sequencing. The sequence method was followed according the manual the BigDye Terminator Cycle Sequencing V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on Model 377 DNA Sequencer (Applied Biosystems).

Statistical analysis

The reproductive performance was assessed by analysis of the total number of piglets at birth (TBN), number of piglets born alive (NBA), average birth weight (ABW), number of live piglets at 21 days (LP21) and average body weight at 21 days (WT21) according to the following model:

$$Y_{ijklmn} = \mu + \mathrm{Inb}_i + \mathrm{Sire}_j + \mathrm{Dcp}_k + P_l + R_m + e_{ijklmn}$$

Where:

Y_{ijklmn} = observed value for the reproductive performance of Meishan dam, μ = overall mean; Inb_i = fixed effect common to the ith of inbreeding coefficient of litter, $i = 0.0$ to 0.3125; Sire_j = fixed effect common to the jth of service sire of dam; Dcp_k = fixed effect common to the kth SSCP band pattern of mtDNA D-loop region for dam of litter, k = B or C; P_l = fixed effect common to the lth parity, $l = 1$ for parity one, $l = 2$ for parity two, $l = 3$ for parity 3–6, $l = 4$ for parity is more than 6; R_m = fixed effect common to the mth farrowing season, m = 1,2,3,4. Seasons here were defined as follows: from March to May as season 1; from June to August as season 2; from September to November as season 3 and from December to February as season 4. And e_{ijklmn} = random residual error, with a mean of zero and a variance of σ_e^2. We used SAS GLM Procedure to analysis these reproductive performances (SAS Institute Inc 1989).

Results

On the basis of PCR-SSCP analysis of eight pig breeds, four different band types (A, B, C and D) were found (Fig. 1). The variation positions of nucleotide substitutions in the D-loop 392 bp regions among five breed pigs showed as Table 2. Fourteen positions (124 c-t; 131 g-a; 145 c-t; 153 c-t; 158 a-g; 181 t-c; 241 t-c; 279 c-t; 294 a-g; 306 c-t; 323 c-t; 390 c-t; 405 t-c; 452 c-t) of nucleotide substitutions were found. Alves et al. (2003) reported 26 positions of nucleotide substitutions in the sequence variation among Iberian pigs and other domestic and wild pig populations in the same mtDNA region and 14 positions were as same as our study. There were none or

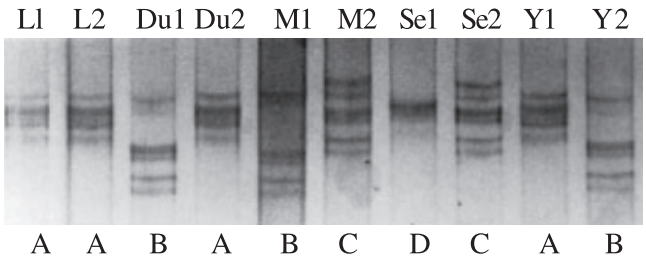

Fig. 1. SSCP analysis of the pig mtDNA D-loop polymorphic region. The PCR amplified 392 bp DNA fragments were denatured by heating and loaded on a native 8% polyacrylamide gel. The results were visualized by silver staining. Four SSCP band patterns (A, B, C and D) were detected. L, M, Du, SE and Y, represent Landrace, Meishan, Duroc, Small-ear pig and Yorkshire. Individuals within a breed were numbered

one nucleotide substitution between A SSCP band pattern and the reference sequence GenBank AF034253. Compare with AF034253, the positions of nucleotide substitution in B SSCP band pattern were 124, 131, 145, 153, 158, 181, 241, 294, 306, 323, 390 and 452, some pigs had 279 or 405 position of nucleotide substitution. Three (241, 405 and 452) positions of nucleotide did not substitute in C and D band patterns. So 241 and 452, two positions of nucleotide substitution were different between B and C band pattern. There was one different position of nucleotide substitution between C SSCP and D SSCP band pattern which was 323 position of nucleotide substitution in D SSCP band pattern and 294 position of nucleotide substitution in C SSCP band pattern. The distributions of four SSCP band patterns of all pigs are summarized in Table 3. SSCP band patterns of L, Y, Du, Be, H, T, Se and M were all A, 6A 6B 5C, 42A 17 B, all A, 9A 1C, all D, 1C 5D and 10B 30C, respectively. The SSCP band types of mtDNA D-loop region for Meda and Nehime were B and C, respectively. Table 4 showed the results of SSCP analysis for these five foundation animals and their randomly selected offspring. SSCP band pattern of dam and maternal ancestries also shown that they were correct following maternally inherited, for example, offspring 12008, 12006 and 12010 showed B SSCP band pattern, SSCP band patterns of its sire (no. 05113) and dam (no. 5008) were C and B respectively (as shown in Fig. 2.). Offspring 12703, 12705 and 12707 showed C SSCP band pattern, SSCP band pattern of its dam (no. 7508) was also C. One hundred forty-one litters of M reproductive data were collected after 4 years breeding in an experimental farm. Table 5 shows the effect of SSCP polymorphism in the mtDNA D-loop region on the reproductive traits of the M dam. TBN, NBA, and LP21 were 11.9 ± 1.1, 10.7 ± 1.2, and 10.6 ± 1.2 for dam with B SSCP band pattern, 12.8 ± 0.8, 11.5 ± 0.8, and 11.0 ± 0.8 for dam with C SSCP band pattern. ABW for dam with B and C SSCP band patterns were 1.00 ± 0.05 and 0.96 ± 0.04, respectively. On WT21 traits, pigs from dam with B SSCP band pattern (4.5 ± 0.3 kg) were heavier than from dam with C SSCP band pattern (3.8 ± 0.2 kg, $p < 0.05$).

Table 3. Distributions of four SSCP band patterns in different breed pigs

Breed	No. of animal	SSCP band pattern			
		A	B	C	D
Landrace	31	31			
Yorkshire	15	6	6	5	
Duroc	59	42	17		
Berkshire	13	13			
Hampshire	10	9		1	
Taoyuan	29				29
Small-ear	6			1	5
Meishan	40		10	30	

Discussion

The T pig is one of the native breeds in Taiwan and is classified as the southern Chinese type (Tai et al. 1988). The result of PCR-SSCP analysis of T mtDNA D-loop region suggested T breed originated from the same maternal ancestries. According to the studies of genetic polymorphism of blood groups, serum proteins and mtDNA, a remarkable genetic difference between East Asian and the European–American pig populations was revealed (Tanaka et al. 1983; Huang 1986; Watanabe et al. 1986). T and other Chinese pigs are of the East Asian populations, while H, L, Du and LW are

Table 2. The variation positions of nucleotide substitutions in the D-loop regions among five breed pigs[a]

Positions of nucleotide substitution[b]	L1	L2	D1	D2	M1	M2	Se1	Se2	Y1	Y2
124 t → a			+		+	+	+	+		+
131 g → a			+		+	+	+	+		+
145 c → t			+		+	+	+	+		+
153 c → t			+		+	+	+	+		+
158 c → g			+		+	+	+	+		+
181 t → c		+	+		+	+	+	+	+	+
241 t → c			+		+					+
279 c → t			+		+	+	+	+		
294 a → g			+		+	+		+		+
306 c → t			+		+	+	+	+		+
323 c → t			+		+		+			+
390 c → t			+		+	+	+	+		+
405 t → c										+
452 c → t			+		+					+

[a]Pigs were as same as those of Fig. 1.
[b]Nucleotide positions are numbered according to the reference sequence GenBank AF034253.

Table 4. Pedigree and SSCP band patterns in the mtDNA D-loop region of tested Meishan pigs

Ear no. of tested pig (SSCP band pattern)	Sex	Ear no. of sire	Ear no. of dam (SSCP band pattern)	Maternal ancestry
00237(C)[a]	Female	Takamori	Nehime	Nehime
00236(C)[a]	Female	Takamori	Nehime	Nehime
00092(B)[a]	Male	Maikeru	Yoshimi	Yoshimi
00227(C)[a]	Male	Takamori	Nehime	Nehime
00071(B)[a]	Female	Maikeru	Meda	Meda
00112(C)	Female	237	236(C)	Nehime
00114(C)	Female	237	236(C)	Nehime
00118(C)	Female	237	236(C)	Nehime
00201(B)	Male	92	71(B)	Meda
00207(B)	Male	92	71(B)	Meda
00209(B)	Male	92	71(B)	Meda
00211(B)	Male	92	71(B)	Meda
00305(C)	Male	92	227(C)	Nehime
00812(C)	Female	92	236(C)	Nehime
00816(C)	Female	92	236(C)	Nehime
05008(B)	Female	237	71(B)	Meda
05113(C)	Male	00205	00106(C)	Nehime
06702(C)	Female	00505	00404(C)	Nehime
06906(C)	Female	00205	00118(C)	Nehime
07508(C)	Female	00505	00604(C)	Nehime
08210(C)	Female	00505	00402(C)	Nehime
10410(C)	Female	92	236(C)	Nehime
10502(C)	Female	00605	3702(C)	Nehime
10604(C)	Female	00205	00402(C)	Nehime
10702(C)	Female	00517	01108(C)	Nehime
12006(B)	Female	05113	05008(B)	Meda
12008(B)	Female	05113	05008(B)	Meda
12010(B)	Female	05113	05008(B)	Meda
12102(C)	Female	04313	05206(C)	Nehime
12108(C)	Female	04313	05206(C)	Nehime
12601(C)	Female	04313	05712(C)	Nehime
12603(C)	Male	04313	05712(C)	Nehime
12609(C)	Male	04313	05712(C)	Nehime
12702(C)	Female	05113	07508(C)	Nehime
12703(C)	Male	05113	07508(C)	Nehime
12704(C)	Female	05113	07508(C)	Nehime
12705(C)	Male	05113	07508(C)	Nehime
12707(C)	Male	05113	07508(C)	Nehime
12708(C)	Female	05113	07508(C)	Nehime

[a]The foundation animals.

relegated to the European–American pig populations (Tanaka et al. 1983; Watanabe et al. 1986). 'A' type of SSCP band pattern presented in all European–American breeds, but not in the Asian breeds, M, T and SE. Our results also supported there are two independent maternal origins between European–American and East Asian populations (Lin et al. 1998; Kim et al. 2002; Yang et al. 2003). Watanabe et al. (1985, 1986) and Mikami et al. (1988) analysed RFLPs of whole porcine mtDNA using four restriction enzymes (BglII, EcoRV, ScaI and StuI), the RFLP type (BglII-EcoRV-ScaI-StuI) of L was A-A-A-A; D was A-A-A-A with a few cases of A-B-A-A; Large White was A-A-A-A or B-A-B-B; H was A-A-A-A and the RFLP types (BglII-EcoRV-ScaI-StuI) of Lanyu (also named Se), T and M pigs are B-A-B-B. PCR-SSCP analysis of mtDNA D-loop region in this experiment also showed that (1) L, Y, H, Be and Du had the same A SSCP band pattern. (2) Some band patterns of Y were as same as M. (3) Se and T had the same D SSCP band pattern. But (1) Y and H had B and C SSCP band patterns, respectively. (2) There were two patterns in M pigs. Takeda et al. (1995) also found two distinct M SSCP patterns in Japan. (3) Se had another C SSCP band pattern. Liu (1991) also reported two RFLP types (BglII-EcoRV-ScaI-StuI) of Se mtDNA in Taiwan.

Two distinct mtDNA haplotypes (Asian and European) of the Large White breed have been reported, suggesting that cross-breeding between European and Asian pigs has occurred during the formation of this breed (Watanabe et al. 1985, 1986; Okumura et al. 1996; Giuffra et al. 2000). This also is expected for the Berkshire breed, as Jones (1998) indicated that the Chinese breeds contributed significantly to the development of Berkshire, Small White and Middle White breeds. Our results showed that the C and D band pattern did not present in Berkshire breed. The reasons were maternal lineage of our imported Be might be not with C and D band pattern and we sampled only 13 head offspring of 13 imported Be sows from 52 imported Be sows, the maternal lineage of sampled pigs with C and D band pattern might be not included. Yen et al. (2003) reported that average of litter size at birth, number born alive, live piglets at 21-day and 56-day ages of M pigs from 160 litters were 11.9, 10.9, 10.7 and 10.2 heads, respectively, and average body weight at birth and at 21-day of age were 0.93 and 4.0 kg, respectively. We applied those M pigs to study the association of

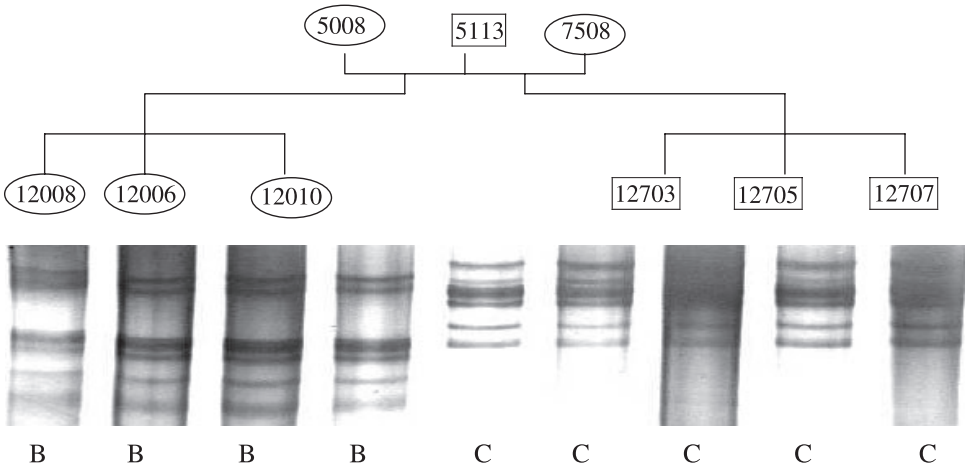

Fig. 2. SSCP analysis of the Meisan family members in the mtDNA D-loop polymorphic region. Leukocyte DNAs from the family members indicated at the top (○: females; : males) were subjected to SSCP analysis. The bands of all offspring were derived from their maternal parent

Table 5. Relationship of SSCP polymorphisms in the mtDNA D-loop region with reproductive traits of Meishan dams

SSCP band type of dam	TBN (head)	NBA (head)	ABW (kg)	LP21 (head)	WT21 (kg)
B	11.9 ± 1.1[a]	10.7 ± 1.2	1.00 ± 0.05	10.6 ± 1.2	4.5 ± 0.3
C	12.8 ± 0.8	11.5 ± 0.8	0.96 ± 0.04	11.0 ± 0.8	3.8 ± 0.2
Significance level	ns	ns	ns	ns	*

*Difference is significant at 5% level, $p < 0.05$.
[a]Least-square mean ± SE.
TBN, total number of piglets at birth; NBA, number of piglets born alive; ABW, average birth weight; LP21, number of live piglets at 21 days; WT21, average body weight at 21 days.

designed SSCP band patterns with their reproductive traits. The average piglet body weight at 21-day of age from SSCP B type dams was significantly heavier than that from C type dams ($p < 0.05$). In another experiment, we collected 381 litters reproductive data (Landrace, 194; Yorkshire, 82, Duroc, 105) which came from 80 head dams, and analysed the relationship between single-strand conformation polymorphisms in the D-loop region of mtDNA and reproductive traits in those dams, the results showed the average body weight at day 21 of piglet from B type dams was also significantly heavier than the body weight of A type ($p < 0.05$) (data not shown). Compare with AF034253, Three positions of nucleotide substitution, 158, 181 and 241 were located at the extended termination associated sequence (ETAS) region of D-loop. The ETAS region is associated with the termination of the nascent H strand during replication and includes two conserved blocks, ETAS1 and ETAS2 (Sbisà et al. 1997), that contain the TAS elements previously described by Doda et al. (1981). The 241 position was located at ETAS2, the others were at ETAS1. Two positions of nucleotide substitution, 241 and 452, were found at B band pattern, not C band pattern. It might be that some transcription factors do not stop to enhance the cytoplasm of intra-uterine environment or postnatal environment, e.g. mothering ability of dams with B band pattern which 241 position nucleotide substitution. It needs to be corroborated by getting more gene expression information about these nucleotide substitutions in relation to the WT21 traits. This result suggested SSCP polymorphisms in the mtDNA D-loop region might be an important source of variation for early postnatal stage of M pigs in Taiwan. This may be related to the cytoplasm of intra-uterine environment or postnatal environment, e.g. mothering ability (Robison 1972). By comparing the mean values of traits among different maternal lineage with differing alleles for a mitochondrial polymorphism can determinate the direct effect of a mitochondrial locus on economic traits. All individuals from a single foundation dam in the maternal lineage should have the same mitochondrial allele, unless a further mutation occurred. Thus, similar to recombinant inbred lines, it is sufficient to genotype only a few individuals of maternal lineage, while records on all individuals can be used to determine the effect of the mitochondrial polymorphism on economic trails (Ron et al. 1992). Our result provides a information for further study of cytoplasmic effects on phenotypic variation in M pigs.

The SSCP analysis of the mtDNA D-loop region in the Meisan family members was performed for paternity testing in a family group, as the mtDNA is maternally inherited; the bands of all offspring were derived from their maternal parent (Fig. 2). Therefore, we conclude that SSCP may be a marker for identification of their maternal ancestors.

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