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The Association of *MC1R* Gene with Coat Color of Banna Mini-Pig Inbred Line (BMI)

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Abstract: In 1980, the Banna Mini-pig Inbred line was established in China. The original ancestors were a sow and her son with the same black color coats. The propagation was conducted by full sibling or parent-offspring mating. With the development of inbreeding, white with black spotting individuals were generated. In the study, a principal coat color encoded candidate gene of MC1R was studied from the typical 8 generation of BMI for revealing the genetic mechanism. It was shown that BMI owned two MC1R alleles named E^{BMI} and E^{bmi}, corresponding to EU604026 and EU604027 in GenBank. Genotypes of E^{BMI}/E^{BMI} and E^{BMI}/E^{bmi} were black color while the white with black spotting phenotype owned the E^{bmi}/E^{bmi} genotype. The E^{BMI} with the length of 963 bp single-coding exon encodes 320 amino acids. Compared with the E^{BMI} sequence, 2 bp was inserted in the 66th nucleotide position of the E^{bmi} encoding regions. The insertion caused a frameshift mutation that introduced a premature stop at codon 55, encoding 54 amino acids. It was indicated that the *MC1R* gene played a key role in the genetic process of the BMI coat color and the dominant of BMI black phenotype to white with black spotting phenotype was confirmed by the combination the pedigree phenotype deduction and genotype testing.

Key words: Banna Mini-pig Inbred line (BMI), coat color, extension, Melanocortin Receptor 1 (MC1R), China

INTRODUCTION

Coat color is an important characteristic of farm animals and has been used as trademarks for the breed during the last 200 years. There are several varieties of coat color phenotypes in pigs. Most of the European breeds such as Large White and Landrace pigs have a white coat color phenotypes however, the black coat is the most common type in the pig breeds of China (Legault, 1998). Most domestic pig breeds have a black color because they carry a recessive nonagouti allele at the Agouti locus and a normal wild-type allele at the Extension locus (Ollivier and Sellier, 1982).

The color variations had been reported due to the distribution of melanocytes. The Melanocortin Receptor 1 (MC1R) plays a central role in regulation of eumelanin and phaeomelanin synthesis within the mammalian melanocyte and is encoded by the classical Extension (E) coat color locus. There are two basic types of melanin (eumelanin and phaeomelanin) in pigs, the former gives black or brown coat color whereas the latter gives yellow or red coat color. Pigment deposition depends on the relative amount of the two types of melanin. MC1R has been confirmed as the extension locus in a number of mammalian species including mouse, cattle, horse, fox, sheep, dog and pig (Robbins *et al*., 1993; Klungland *et al*., 1995; Marklund *et al*., 1996; Vage *et al*., 1997, 1999; Everts *et al*., 2000; Newton *et al*., 2000; Kijas *et al*., 1998). MC1R is a G protein-coupled receptor consisting of seven transmembrane domains (Mountjoy *et al*., 1992).

Binding of a-MSH to its receptor stimulates melanocytes to synthesize cyclic Adenosine Monophosphate (cAMP) by signal transduction via G protein and consequently the melanocytes produce eumelanin (Robbins *et al*., 1993). *MC1R* gene consists of a single-coding exon and the analysis of coat color inheritance within a wild boar/Large White intercross pedigree firmly assigned the MC1R at the distal part of pig chromosome 6p (Mariani *et al*., 1996). Swine are generally considered to be the most ideal biomedical laboratory animals for their anatomical, physiological and metabolic characteristics are similar to human's. Since, 1950s some

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breeds of miniature swine have been developed in several countries such as Yucatan, Hanford, Sinclair, Pitman-Moore, Essex, Minnesota Hormel and Nebraska in the United States, Gottingen in Germany, Oh mini, Clawn and Huei-Jin in Japan, Corsica in France. They have been used in biomedical and some other science fields extensively. The inbred animals are good enough to be used as experimental animals, owing to their clear genetic background, high homozygosity, stable inheritance and so on. Inbred animals can also make less experimental errors using in biological research than noninbred ones (Wright, 1921; Harris, 1997).

In 1980, the Banna Mini-pig Inbred line (BMI) was exploited by Yunnan Agricultural University based on the small-ear pigs at Xishuangbanna, Yunnan province. A pair of progenitors was a sow and her son. Then, the propagation was conducted by means of highly full sibling or parent-offspring inbreeding and each generation underwent the strict selection. As heterozygotic genes were separated and recombined in the process of inbreeding, BMI has already owned six families and eighteen substrains with different phenotypes and genotypes (Zeng and Zeng, 2005). With the development of inbreeding and generating the white with black spots descendants were generated although, the progenitors of BMI were both uniform black phenotype. Namely, BMI were divided into the black and white with black spotting phenotypes in light of the coat color (Fig. 1). According to the coat color difference of the mated individuals, there are three cases.

In the first case, uniform black color or black along with white with black spotting descendants are generated by mating of the two black phenotype pigs. In the second case, the similar results to that of the above come out with mating between the black and white with black spotting pigs. In the 3th case, the descendants own the same coat color as their white with black spotting parents. As it is well known, the coat color genetic process of most mammals is closely related to *MC1R* gene in E locus and the dominant alleles at E locus often lead to the generation of all black individuals (Jackson, 1997) Therefore, it was deduced that the black phenotype was dominant as compared with the white phenotype with black spots. If the E position was supposed to be controlled by a pair of E^{BMI}/E^{bmi} allelic genes in BMI, the black phenotype could possess E^{BMI}/E^{BMI} or E^{BMI}/E^{bmi} genotype and the white with black spotting phenotype should own the E^{bmi}/E^{bmi} genotype. Up to now, it is unclear about the molecular mechanism of domination relation between E^{BMI} and E^{bmi} genes. As the genetic background and pedigree of BMI is well learned, *MC1R* gene was used as the main candidate gene of the research for the coat genetic process and related mechanism was investigated by molecular bio-method, the direct sequencing of PCR products and cloning sequencing of *MC1R* gene with the individuals of E^{BMI}/E^{BMI}, E^{BMI}/E^{bmi} and E^{bmi}/E^{bmi} genotype.

Fig. 1: Illustration of BMI pig coat color phenotypes. (a) E^{BMI}/E^{BMI} black phenotype from left to right, mother No. 624, son No. 725, daughter No. 724 and No. 726. (b) E^{BMI}/E^{bmi} black phenotype and E^{bmi}/E^{bmi} black spotting phenotype, the left most pig is mother No. 600, the second, fourth and fifth are son No. 721, No. 711 and No. 713 and third is the black spotting daughter No. 708 of E^{bmi}/E^{bmi} phenotype

MATERIALS AND METHODS

Samples collection and DNA extraction: According to the entire pedigree of BMI, a typically branch of 8 generation pedigree comprising 85 black coat phenotype pigs and 11 black-spotted phenotype pigs were selected in this study (Fig. 2). The pedigree was drawn with the Cyrillic 2.1 software (Cherwell Scientific Publishing Ltd., Oxford, UK) and the individuals genotypes were deduced by the pedigree. The 96 blood samples in the pedigree of BMI were prepared and the genomic DNA were extracted according to the standard method (Sambrook *et al*., 2002).

PCR amplification and nucleotide sequencing: An MC1R fragment including the entire coding region plus 29 bp of

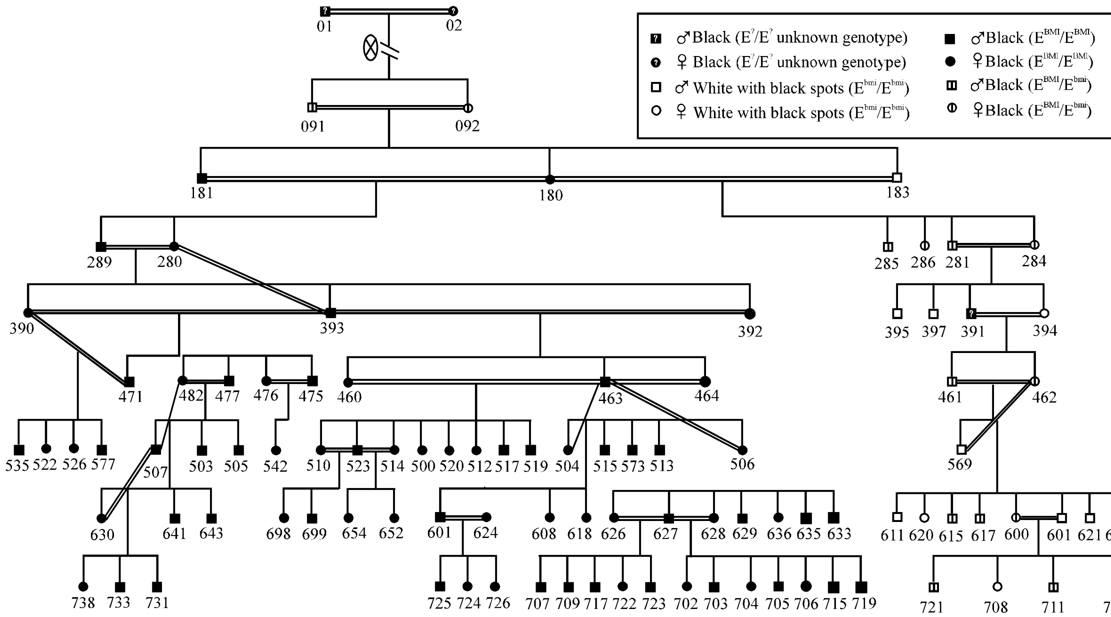

Fig. 2: The pedigree for BMI included in the study

5'-UTR and 132 bp of 3'-UTR was amplified with the forward primer (5'-ACGTGCCCTCCCTGCTCC-3') and reverse primer (5'-CCAGCGTCCATACCTTCAGA-3') designed according to the porcine *MC1R* gene sequence (GenBank accession no. AF326520). The 20 μL reaction system was: 1.5 μL (25 ng μL^{-1}) DNA, 1 μL^{-1} 2.5 mM mixed dNTPs, 2 μL 10×Taq DNA polymerase buffer, 1 μL^{-1} 25 mM $MgCl_2$, 0.4 μL 10 μM forward primer, 0.4 μL 10 μM reverse primer, 0.3 μL 5 U μL^{-1} Taq DNA polymerase and 13.4 μL sterile water. The PCR program initially started with a 94°C denaturation for 3 min, followed by 35 cycles of 94, 62, 72°C/1 min then 72°C extension for 10 min, finally 4°C to terminate the reaction. Amplified DNA fragments were detected by electrophoreseing on 1% agarose gels and then purified.

All purified fragments of 96 samples were sequenced bidirectionally with the commercial fluorometric method, some of those purified products were legated with a pMD18-T vector and transferred into the bacterium DH5α for replication, including No. 183 and its 23 offsprings, 20 black phenotype pigs (offsprings of No. 181 and 180) and. The recombinant plasmid picked out from positive clones was amplified by PCR, digested with EcoR I and Hind III and then sequenced bidirectionally by using universal primer M13. At least 20 independent clones and its plasmid were sequenced for each PCR product.

Sequence analysis: Sequencing data were edited and aligned using the DNASTAR software (DNAStar Inc., Madison, Wisc.). The nucleotide sequence comparison was performed by using the BLAST software at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The ORF prediction was carried out using the ORF finder software at NCBI server.

RESULTS AND DISCUSSION

E^{BMI} and E^{bmi} sequence variation of MC1R in BMI: The complete coding sequences of *MC1R* gene from black pigs and white with black spotting pigs were cloned and sequenced. These nucleotide sequences analysis using the DNASTAR software revealed the only two alleles sequences E^{BMI} and E^{bmi} were detected. The sequence analysis indicated that the genotype of black phenotype pigs was E^{BMI}/E^{BMI} and E^{BMI}/E^{bmi}. However, all of the 11 black spotted phenotype BMI pigs were homozygous for the E^{bmi}/E^{bmi} allele.

The E^{BMI} and E^{bmi} sequences reported in this study was deposited in GenBank with accession numbers corresponding to EU604026 and EU604027. Sequence prediction analysis revealed that the 963 bp fragment from all E^{BMI} genotype contains a ORF encoding 320 amino acids however, the 965 bp fragment from all E^{bmi} genotype contains a ORF encoding 54 amino acids (Fig. 3 and 4). The E^{BMI} and E^{bmi} sequences comparison revealed the presence of six single-base substitutions and one double C insertion mutation in the coding regions of *MC1R* gene (Table 1). The mutation c. 51A>G was a Alanine (A) synonymous mutation. The CC inserted at the position 66 caused a frameshift mutation which did not

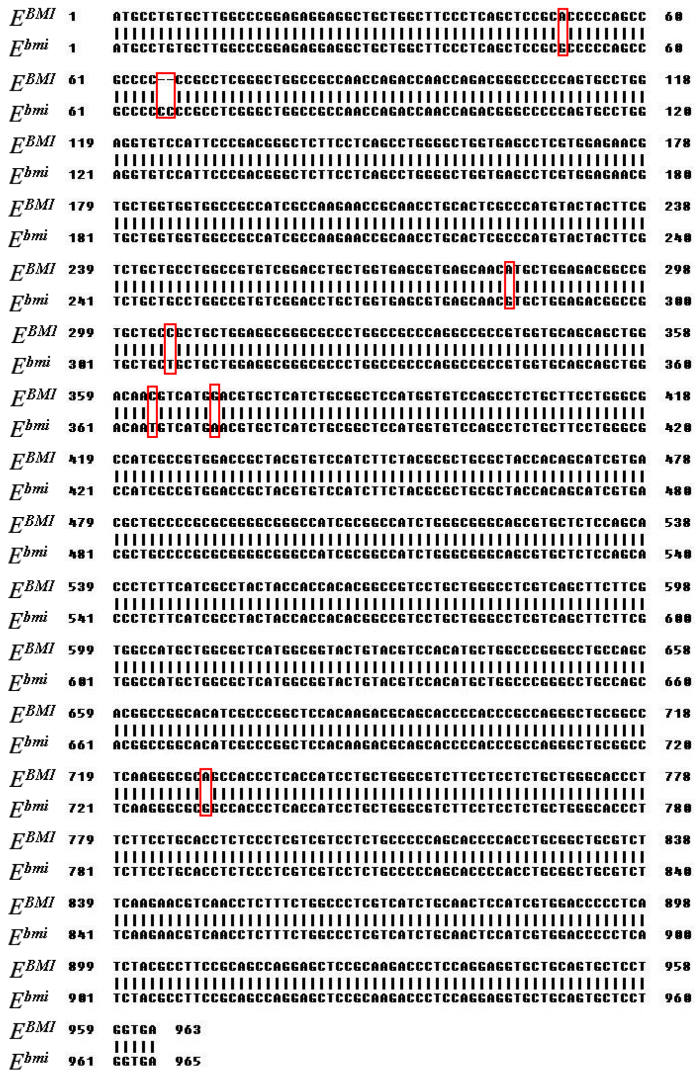

Fig. 3: CDS sequence comparison of E^{BMI} and E^{bmi}; Nucleotides in the pane indicated the mutation

Table 1: Mutation sites of E^{BMI} and E^{bmi} alleles

Allele	51	- -/66, 67	283/285	305/307	363/365	370/372	729/731
E^{BMI}	A	-	A	C	C	G	A
E^{bmi}	G	CC	G	T	T	A	G

only made the amino acids change after 23rd codon but also terminate the translation at the 55th amino acid in advance with a truncated protein product (Fig. 4b). Other mutations sites were c. 283 A>G, c. 305 C>T, c. 363 C>T, c. 370 G>A, c. 729 A>G, respectively between E^{BMI} and E^{bmi}.

MC1R/Extension variants affecting coat color in the BMI: The sequence comparison between E^{BMI} and E^{bmi} sequences of BMI revealed the presence of an insertion of CC nucleotides at codon 23 of E^{bmi}. The insertion of CC occurs in a GC rich region and within a stretch of six Cs that is expanded to a mononucleotide repeat of eight Cs and made change of the translation after codon 23 and translation terminate in advance at codon 55. MC1R is one of the major coat color genes in pigs and classic genetic analyses have established four alleles at the Extension (E) locus in pig (Ollivier and Sellier, 1982; Andersson, 2003). These are E^{+} for wild type, E^{D} for dominant black, E^{P} for black spotting and e for recessive red. About 7 MC1R alleles corresponding to the four phenotypically defined alleles have been reported (Kijas *et al*., 1998, 2001; Giuffra *et al*., 2000; Gustafsson *et al*., 2001). E^{P} is the most interesting MC1R allele in the pig and it contains two causative mutations, a frameshift and a missense mutation. The frameshift is expected to cause a uniform red pigmentation due to the complete loss of MC1R signaling but in fact the phenotype expression of the E^{P}

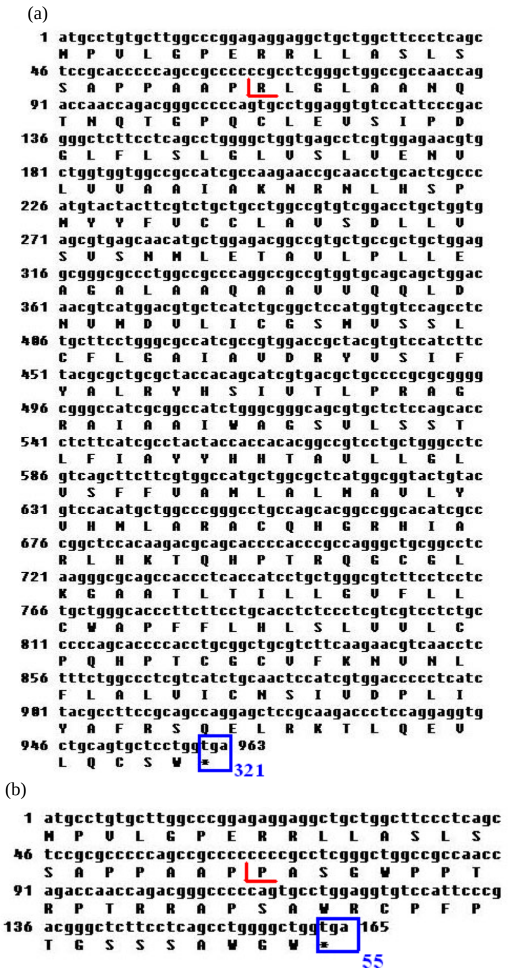

Fig. 4: Translation comparison of *MC1R* gene. a) E^{BMI}, b) E^{bmi}; the red right angle indicated the amino acids difference of E^{BMI} and E^{bmi} after codon 23 and the blue pane indicated the stop codon of E^{BMI} at position 321 and the premature stop codon of E^{bmi} at position 55

allele is highly variable and it is usually associated coat color ranges from red, red with black spots, white with black spots to almost completely solid black. The frameshift mutation is somatically unstable and the black spots reflect somatic reversion events restoring occasionally the reading frame (Kijas *et al*., 2001). White pigs of the Large White and Landrace breeds with CC insert do not show black spots because of epistatic interaction of the Dominant/KIT alleles causing a defect in melanocyte migration (Marklund *et al*., 1998). Previous researches were devoted to explain the change of MC1R alleles by studying the black and black spotting phenotypes, respectively. However, due to the high inbreeding of the BMI pigs, the black individuals with homozygote and heterozygote and the homozygous black spotting phenotype individuals were generated in the same pedigree. That is to say, the mode of MC1R inheritance for phenotypic variation in pig has been established in same family of BMI. Thus, it is of special significance to investigate the variation in MC1R alleles. In the study, the genotypes of all samples could be derived from the pedigree except that of number 391.The direct sequencing of PCR products and cloning sequencing were carried on offsprings of No. 180 and No. 183 including 10 black spotting phenotype pigs and 13 black phenotype pigs and No. 183 itself. The genotype of 72 black phenotype pigs, No. 180 and 181 and their 70 offsprings was considered to be E^{BMI}/E^{BMI} because of all their uniform black color coat phenotype. All 72 black phenotype pigs were directly sequenced by PCR products and 20 of those were selected and sequenced by cloning method. The results indicated that the hypothesis and derived genotype were correct, that is the genotype of No. 181 and 180 and their all offsprings was E^{BMI}/E^{BMI} and in the offsprings of No. 183 and 180 the genotype of all black phenotype pigs was E^{BMI}/E^{bmi} (including No. 391) and the genotype of all black spotting phenotype pigs was E^{bmi}/E^{bmi}.

CONCLUSION

Evidences provided by us in this experiment suggested that the *MC1R* gene played a crucial role in the regulation of BMI coat color trait through phenotype derivation and genotype verification using 8 generations pedigree. From pedigree and the results obtained above researchers can also infer that black coat color phenotype was dominant to the white with black spot phenotype in BMI.

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REFERENCES

Andersson, L., 2003. Melanocortin receptor variants with phenotypic effects in horse, pig and chicken. Ann. N. Y. Acad. Sci., 994: 313-318.

Everts, R.E., J. Rothuizen and B.A. van Oost, 2000. Identification of a premature stop codon in the melanocyte-stimulating hormone receptor gene (MC1R) in labrador and golden retrievers with yellow coat colour. Anim. Genet., 31: 194-199.

Giuffra, E., J.M.H. Kijas, V. Amarger, O. Carlborga and J.T. Jeona and L. Andersson, 2000. The origin of the domestic pig: Independent domestication and subsequent introgression. Genetics, 154: 1785-1791.

Gustafsson, A.C., J.M.H. Kijas, A. Alderborn, M. Uhlen, L. Andersson and J. Lundeberg, 2001. Screening and scanning of single nucleotide polymorphisms in the pig melanocortin 1 receptor gene (*MC1R*) by pyrosequencing. Anim. Biotechnol., 12: 145-153.

Harris, I., 1997. Variables in animal based research: Part 1. Phenotypic variability in experimental animals. Anzccart News, 10: 1-8.

Jackson, I.J., 1997. Homologous pigmentation mutations in human, mouse and other model organisms. Hum. Mol. Genet., 6: 1613-1624.

Kijas, J.M.H., M. Moller, G. Plastow and L. Andersson, 2001. A frameshift mutation in *MC1R* and a high frequency of somatic reversions cause black spotting in pigs. Genetics, 158: 779-785.

Kijas, J.M.H., R. Wales, A. Tornsten, P. Chardon, M. Moller and L. Andersson, 1998. Melanocortin receptor 1 (*MC1R*) mutations and coat color in pigs. Genetics, 150: 1177-1185.

Klungland, H., D.I. Vage, L. Gomez-Raya, S. Adelsteinsson and S. Liens, 1995. The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination. Mammalian Genome, 6: 636-639.

Legault, C., 1998. Genetics of Colour Variation. In: The Genetics of the Pig, Rothschild, M.F. and Ruvinsky, A. (Eds.). CAB International, Wallingford, UK., pp: 51-69.

Mariani, P., M.J. Moller, B. Hoyheim, L. Marklund, W. Davies, H. Ellegren and L. Andersson, 1996. The extension coat color locus and the loci for blood group O and tyrosine aminotransferase are on pig chromosome 6. J. Hered., 87: 272-276.

Marklund, L., M.J. Moller, K. Sandberg and L. Andersson, 1996. A missense mutation in the gene for melanocyte-stimulating hormone receptor (MC1R) is associated with the chestnut coat color in horses. Mammalian Genome, 7: 895-899.

Marklund, S., J. Kijas, H. Rodriguez-Martinez, L. Ronnstrand and K. Funa *et al*., 1998. Molecular basis for the dominant white phenotype in the domestic pig. Genome Res., 8: 826-833.

Mountjoy, K.G., L.S. Robbins, M.T. Mortrud and R.D. Cone, 1992. The cloning of a family of genes that encode the melanocortin receptors. Science, 257: 1248-1251.

Newton, J.M., A.L. Wilkie, L. He, S.A. Jordan and D.L. Metallinoset *et al*., 2000. Melanocortin 1 receptor variation in the domestic dog. Mammalian Genome, 11: 24-30.

Ollivier, L. and P. Sellier, 1982. Pig genetics: A review. Ann. Genet. Sel. Anim., 14: 481-544.

Robbins, L.S., J.H. Nadeau, K.R. Johnson, M.A. Kelly and L. Roselli-Rehfuss *et al*., 1993. Pigmentation phenotypes of variant Extension locus alleles result from point mutations that alter MSH receptor function. Cell, 72: 827-834.

Sambrook, J., E.F. Fritsch, T. Maniatis and P.T. Huang, 2002. Molecular Cloning: A Laboratory Manual. 3rd Edn., Scienc Press, Beijing, China, pp: 16-69.

Vage, D.I., H. Klungland, D. Lu and R.D. Cone, 1999. Molecular and pharmacological characterization of dominant black coat color in sheep. Mammalian Genome, 10: 39-43.

Vage, D.I., D. Lu, H. Klungland, S. Lien, S. Adalsteinsson and R.D. Cone, 1997. A non-epistatic interaction of agouti and extension in the fox, Vulpes vulpes. Nat. Genet., 15: 311-315.

Wright, S., 1921. Systems of mating. I. The biometric relations between parent and offspring. Genetics, 6: 111-123.

Zeng, R. and Y.Z. Zeng, 2005. Molecular cloning and characterization of *SLA-DR* genes in the 133-family of the banna mini-pig inbred line. Anim. Genet., 36: 267-269.