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# Polymorphism of the beta 3-adrenergic receptor gene *(ADRB3*) and its distribution in domestic and wild pigs in China*

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**The beta 3-adrenergic receptor (ADRB3) is a G-protein coupled receptor encoded by *ADRB3* gene and involved in regulation of lipolysis and homeostasis. The objective of this study was to detect the genetic variation existed in this gene in eight domestic and wild pig populations. The polymorphism of partial sequences of *ADRB3* gene in 336 pigs was analysed by single strand conformation polymorphism technique based on PCR (PCR-SSCP). *Fst* statistic and analysis of molecular variance (AMOVA) were used to measure the genetic differentiation. The genetic distances between populations were computed and a phylogenic tree was reconstructed by the unweighted pair group method using the arithmetic mean. Five haplotypes named A, B, C, D and E were detected with the frequencies ranging from 0.060 for E to 0.324 for A. The reliability of all haplotype frequencies reached 0.95. The haplotypes involved four polymorphic sites, of which one located in exon 2 with a thymine insertion, and the remaining three in non-coding regions with G/A and G/T substitutions, respectively. The thymine insertion resulted in the absence of two amino acids in the sequence of receptor protein. The haplotype diversity ranged from 0.579±0.057 in pure wild Liaoning to 0.792±0.024 in Mashen pigs, indicating abundant genetic diversity to exist in the populations. The *Fst* estimates showed that the degree of population differentiation between domestic and wild pigs was moderate, but at a lower level within domestic populations. About 94.38% of the genetic variation originated from within the populations by AMOVA.**

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Beta 3-adrenergic receptor (ADRB3) is a member of G protein-coupled receptors family [Collins and Surwit 2001] and the major mediator of the lipolytic and thermogenic effects of high catecholamine in brown and white adipose tissue [Cannon and Nedergaard 2004]. *ADRB3* gene was predominantly found in adipocytes [Strosberg 1997]. In rodent *ADRB3*-knockout models a marked reduction in lipolysis stimulated by beta-3 agonist, and a decreased lean mass of the body were found [Revelli *et al*. 1997]. Several polymorphisms involving amino acid substitution had been found in the coding region of human *ADRB3*. Reports on the effects of *ADRB3* polymorphisms on obesity-related characteristics in humans had shown inconsistent results. Some of them showed a relationship between the Trp64Arg mutation and obesity [Kurokawa *et al*. 2008, Marvelle *et al*. 2008], but others found no such association [Ramis *et al*. 2004, Ochoa *et al*. 2004, Nagano *et al*. 2005]. Despite this discrepancy, *ADRB3* had been considered a candidate gene for obesity in human populations [Rankinen *et al*. 2006]. Moreover, associations of genetic variation in ovine *ADRB3* gene with birth weight, growth rate, carcass composition, lamb mortality and cold survival have been reported [Forrest *et al*. 2003, 2006].

The coding regions for porcine *ADRB3* have previously been determined by Smith *et al*. [2001]. The structure of *ADRB3* gene included 0.4 kb 5' UTR, 1.2 kb exon 1, 0.7 kb intron, 22 or 28 bp exon 2, and 0.3 kb 3' UTR. So far, two publications were devoted to the genetic polymorphism of *ADRB*3 in pigs [Tanaka *et al*. 2007, Chikun *et al*. 2008], but they did not consider the population genetic analysis.

In this study, we detected polymorphisms of *ADRB*3 gene in domestic (both Chinese and alien) and wild pigs in China, evaluated the distribution of haplotypes of *ADRB*3 and examined differentiation among eight pig populations based upon the genetic variation.

## Material and methods

### Animals

A total of 336 pigs (exotic, domestic and wild as well as some of their crosses) were used as indicated in Table 1. Mashen is a native (domestic) breed from Shanxi province of China. Jinyang White is a newly produced lean-type pig obtained by crossing the Meishan as dam breed with Hampshire and Large White as first and terminal sire breeds, respectively. Pure wild pigs were captured in Liaoning province and kept in Shanxi province. The hybrid wild pigs were obtained by crossing pure wild with domestic pigs, but the domestic breed and the number of crossed generations is not clear. Large White, Landrace, Duroc, and Pietrain pigs were commercial exotic animals imported from abroad and kept in Datong, and Taiyuan farms, respectively. Ear tissue of non-sibling individuals was collected for the experiment.

**Table 1**. Sources and size of pig samples

| Population | Source | Sample size | Code |
|---|---|---|---|
| Large White | Datong, Shanxi, China | 46 | LWH |
| Landrace | Datong, Shanxi, China | 33 | LDR |
| Pietrain | Taiyuan, Shanxi, China | 54 | PIT |
| Duroc | Datong, Shanxi, China | 27 | DUR |
| Jinyang White | Taiyuan, Shanxi, China | 45 | JYW |
| Mashen | Datong, Shanxi, China | 40 | MSH |
| Pure wild pig from Liaoning | Gujiao, Shanxi, China | 26 | LPW |
| Hybrid of Liaoning wild pig | Gujiao, Shanxi, China | 65 | LHW |

**DNA extraction and PCR amplification**

Genomic DNA was extracted from ear tissue by a standard phenol-chloroform method [Sambrook *et al*. 1989]. Briefly, DNA was extracted with an alkaline SDS solution and then purified with phenol-chloroform treatments following ethanol precipitation. The DNA was subsequently detected using the 0.8% agarose gel electrophoresis.

A 313 bp fragment containing partial intron, exon 2, and partial 3'UTR was amplified using primers 5'-GTGAAGACTAGACGGGGTAAGA-3' and 5'-GAATCTCCACAGGGCTCATT-3', designed based on the published porcine *ADRB3* sequence [Tanaka *et al*. 2007, GenBank accession number AB252781]. The primers were synthesized by INVITROGEN.

Polymerase chain reaction (PCR) amplifications were performed in 20 µl reaction mixture containing 1×reaction buffer, 1.5 mM $Mg^{2+}$, 2.5 mM of each dNTP (TIANGEN, China), 10 pM of PCR primers, about 200 ng of porcine genomic DNA, and 2.5 units of Taq polymerase (TIANGEN, China). The amplification reaction consisted of denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 54.8°C for 30 s and 72°C for 30 s, followed by a final extension step at 72°C for 10 min using PTC-200 peltier thermal cycler (APPLIED BIOSYSTEMS, USA).

**Haplotype differentiation by SSCP analysis and sequencing**

PCR-single strand conformation polymorphism (PCR-SSCP) was used to screen polymorphisms. A two µl aliquot of each product was mixed with 8 µl of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured for 10 min at 98°C and plunged into ice to prevent re-annealing of the separated strands, and then applied to a 10% polyacrylamide : bisacrylamide (29:1) gel (16×18 cm, 1.0 mm spacers, 22 well comb) containing 0.01% glycerol. Electrophoresis was performed at 120V and for 12 h in 1×TBE buffer in a 4°C refrigerator. Gels were silver-stained and imaged using Gel-Imaging System (Bio-Rad, USA). The distinct banding patterns represented different haplotypes.

For each *ADRB3* haplotype identified by PCR-SSCP, two homozygous animals were selected for amplification of the gene. The amplified products were gel-purified using DNA purification kit (TIANGEN, China) and cloned into receptive cell TOP 10 using a cloning Kit (TIANGEN, China). Sequences were determined using ABI 3700 DNA sequencer (APPLIED BIOSYSTEMS, USA).

#### Genetic analyses

The number and frequency of haplotypes were computed by the SPSS version 13.0 software package (SPSS Inc., Chicago, IL, USA). Based on pig types, eight populations were divided into four groups. The alien group consisted of Large White, Landrace, Pietrain, and Duroc. The developed and native groups contained only one population each - Jinyang White and Mashen, respectively. The wild group included pure and hybrid wild pigs. Significance of differences in haplotype frequencies between populations was tested by the Mann-Whitney U-test (one-sided test).

We tested the null hypothesis that haplotype distributions were identical between all possible pairs of populations, and across all populations. Unbiased estimates of *P*-values were obtained by log-likelihood based exact tests [Goudet *et al*. 1996] using the software FSTAT Version 2.9.3 [Goudet 2001]. *P*-values were corrected for multiple testing with the strict Bonferroni technique [Rice 1989]. Unbiased haplotype diversity [Nei 1987] was estimated by FSTAT.

The frequency and variance of haplotypes were estimated according to sample structure and sample size of the populations. The reliability ($\beta$) that ensures the estimates not to deviate from the true values more than 0.5 times and the relative deviation ($\eta$) when the reliability reaches 0.9545 were calculated according to Chang *et al*. [2000], so to ensure the data can be applied in the phylogenetic analysis. Non-differentiation exact *P*-values, *Fst* statistics and Reynolds' genetic distance [Reynolds *et al*. 1983] based on haplotype frequencies were calculated by Arlequin version 3.1 [Excoffier *et al*. 2005].

Analysis of molecular variance (AMOVA) was performed using Arlequin version 3.1 to evaluate the genetic differentiations between groups ($\sigma^2_a$, $\phi_{CT}$) within groups, ($\sigma^2_a$, $\phi_{SC}$), and within populations ($\sigma^2_a$, $\phi_{ST}$). The significance of the variance components and F-statistic analogs was tested by 1023 permutations [Excoffier *et al*. 1992].

Nucleotide sequences were aligned using the ClustalW programme contained in MEGA version 4.0 [Tamura *et al*. 2007]. Phylogenetic tree, based on the genetic distances, was constructed with unweighted pair group method with arithmetic mean (UPGMA).

## Results and discussion

#### DNA products and haplotypes

Genomic DNA detected on agarose gels presented a dense, uniform, distinct, and non-trailing banding pattern, demonstrating that its quality was suitable for

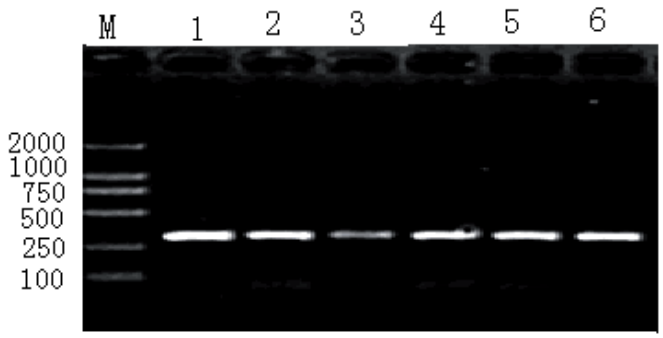


Photo 1. PCR products amplified for *ADRB3* partial sequence in pigs.. M – DNA marker, 1-6 – six PCR products amplified for *ADRB3* partial sequence in pigs.

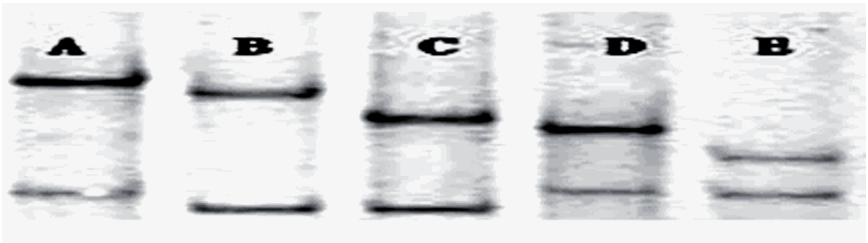


Photo 2. Five haplotypes detected by PCR-SSCP in the porcine *ADRB3* gene.

successive experimentation. Comparison of the PCR products with the DNA size markers suggested that the PCR amplicons accorded with the expected PCR products (Photo 1). The SSCP analysis of PCR amplicons showed five distinct banding patterns representing five *ADRB3* haplotypes (Photo. 2). The haplotype sequences have been submitted to GenBank database (Accession number FJ975133-FJ975138). The base variation occurred at four polymorphic sites (Tab. 2). A thymine insertion at 2338 site in the exon 2 encoded amino acid sequence corresponding to the carboxyl tail end. The remaining variations were two substitutions of G/A and G/T at 2215 and 2221 sites in the intron and one substitution of G/A at 2378 site in 3'UTR.

**Table 2**. Nucleotide variation and haplotypes of porcine *ADRB3*

| Variation | Position | Haplotype | | | | |
|---|---|---|---|---|---|---|
| | | A | B | C | D | E |
| G/A | 2215 | G | A | A | A | G |
| G/T | 2221 | G | T | G | G | T |
| T/- | 2338 | - | T | - | - | T |
| G/A | 2378 | G | G | A | G | G |

**Frequency and diversity of haplotype**

The number and frequency of haplotypes and their diversity estimates for each population are given in Table 3. Haplotypes A, B, and C were distributed in all eight populations. However, the haplotypes D and E were not found in Large White, Landrace, and pure or hybrid wild pigs. In addition, Duroc and Jinyang White did not possess haplotype E. The number of haplotypes detected in each population varied from three to five, and haplotype diversity values from 0.579±0.057 in pure wild to 0.792±0.024 in Mashen pigs.

**Table 3**. Number, distribution and diversity of *ADRB3* haplotypes

| Population code | Haplotype | | | | | Haplotype diversity |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | |
| LWH | 18/0.391 | 10/0.217 | 18/0.391 | | | 0.661±0.026 |
| LDR | 17/0.515 | 12/0.364 | 4/0.121 | | | 0.606±0.049 |
| PIT | 17/0.315 | 14/0.259 | 9/0.167 | 5/0.093 | 9/0.167 | 0.784±0.023 |
| DUR | 10/0.370 | 8/0.296 | 6/0.222 | 3/0.111 | | 0.749±0.034 |
| JYW | 15/0.333 | 11/0.200 | 13/0.244 | 6/0.133 | | 0.744±0.023 |
| MSH | 11/0.275 | 3/0.075 | 8/0.200 | 7/0.175 | 11/0.275 | 0.792±0.024 |
| LPW | 2/0.077 | 10/0.385 | 14/0.539 | | | 0.579±0.057 |
| LHW | 19/0.292 | 26/0.400 | 20/0.308 | | | 0.670±0.016 |

**Table 4**. Estimates of haplotype frequencies and corresponding reliability and precision

| Haplotype | Haplotype frequency ($P$) | Haplotype variance ($V_P$) | Reliability of haplotype frequency (b) | Relative deviation (h) |
|---|---|---|---|---|
| A | 0.324 | $3.271\times10^{-4}$ | 1 | 0.116 |
| B | 0.279 | $3.001\times10^{-4}$ | 1 | 0.124 |
| C | 0.274 | $2.968\times10^{-4}$ | 1 | 0.126 |
| D | 0.063 | $8.745\times10^{-5}$ | 0.999 | 0.299 |
| E | 0.060 | $8.352\times10^{-5}$ | 0.998 | 0.307 |

Table 4 shows the estimates of haplotype frequencies, reliability and precision of the estimates. It can be seen that the reliability estimates of all haplotypes reached 0.998, suggesting that the data were applicable to the phylogenetic analysis.

**Genetic differentiation**

Estimates of pairwise *Fst* values between populations are listed in Table 5. The *Fst* between Landrace and pure wild pig was the highest (0.215) while that between Duroc and hybrid wild pig – the lowest (0.000) *Fst* values were found to be significant between Mashen and the remaining five populations except Duroc and Jinyang White. Generally, differentiation between wild and domestic pigs was higher than that within domestic breeds.

**Table 5**. *Fst* estimates and non-differentiation exact *P*-values based on *ADRB3* haplotype frequency[1]

| Population code | LWH | LDR | PIT | DUR | JYW | MSH | LPW | LHW |
|---|---|---|---|---|---|---|---|---|
| LWH | | 0.006 | 0.022 | 0.014 | 0.024 | 0.114* | 0.115 | 0.005 |
| LDR | 0.446 | | 0.037 | 0.002 | 0.043 | 0.131* | 0.215* | 0.041 |
| PIT | 0.044* | 0.012 | | 0.005 | 0.009 | 0.013 | 0.125* | 0.034* |
| DUR | 0.172 | 0.164 | 0.227 | | 0.017 | 0.055* | 0.106* | 0.000 |
| JYW | 0.006* | 0.011 | 0.631 | 0.593 | | 0.013 | 0.106* | 0.030 |
| MSH | 0.000* | 0.000* | 0.132 | 0.007* | 0.129 | | 0.180* | 0.120* |
| LPW | 0.002* | 0.000* | 0.000* | 0.005* | 0.001* | 0.000* | | 0.048 |
| LHW | 0.451 | 0.047 | 0.000* | 0.050 | 0.001* | 0.000* | 0.039 | |

[1]Above diagonal: *Fst* values (**P*<0.05); below diagonal: non-differentiation exact *P*-values (**P*<0.05).

The significance of genetic differentiation as suggested by the non-differentiation exact *P*-values (Tab. 5) was consistent with that of *Fst* estimates. Significant relations occurred mainly between pure wild pigs and other populations, as well as between Mashen and most of the other populations.

AMOVA analysis showed that most of the genetic differentiation (94.38%) originated from that occurring within populations ($\sigma_c^2$ = 0.354, $\phi_{ST}$ = 0.056). About 3.91% and 1.71% of the total genetic differentiation attributed to among groups ($\sigma_a^2$ = 0.015, $\phi_{CT}$ = 0.039) and among populations within groups ($\sigma_b^2$ = 0.006, $\phi_{SC}$ = 0.017). All three variance components were significant (P<0.05). This result suggests that the within populations variation was larger than between them.

The phylogenetic tree reconstructed according to the genetic distances based on haplotype frequency is shown in Figure 1. The eight populations were divided into two branches. Purebred wild pigs formed one branch, and the remaining seven formed the other. Within the latter branch, Large White, Jinyang White, Duroc and hybrid wild pigs clustered first, and then with Landrace, which formed one sub-branch with the four populations. Pietrain and Mashen pigs grouped into second sub-branch.

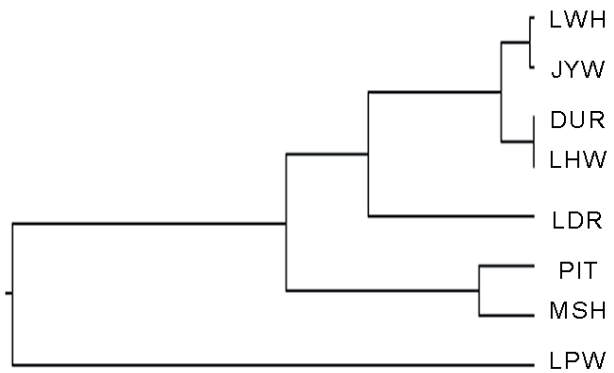


Fig. 1. Dendrogram of eight pig populations by UPGMA method based on the Reynolds' distance.

Evolution and differentiation of species or populations depend on genetic variation of the gene. Five haplotypes involving four polymorphic sites in the partial sequence of *ADRB3* gene were detected by SSCP technique and subsequently proven by sequencing in this study. The polymorphisms resulted from a thymine insertion and three base substitutions. Five haplotypes were also found byTanaka *et al*. [2007], which composed of 47 polymorphic sites in complete *ADRB*3 gene sequence by sequence analysis. Higher frequency of this insertion was observed in this study in Mashen and pure wild pigs. Tanaka *et al*. [2007] reported constantly low frequencies in exotic breeds derived from Western countries and high frequencies in Chinese breeds, Japanese wild boar, and Southeast Asia local pigs. Besides, this insertion was identified in the Chinese native Jinhua and Meishan pigs [Chikun *et al*. 2008]. These results suggest that the insertion in question may originate from Asian breeds and then spread extensively in the domestic pigs of China.

The insertion mutation could result in the absence of two amino acid residues in the carboxyl tail end and thus produce an additional putative β-adrenoceptor kinase (βARK) phosphorylation site with Asn398Asp substitution. As a ligand-binding site, the structural change of this site may affect meat production and quality, because oral administration of some beta-adrenergic agonists increased muscle and decreased fat deposition in cattle and pigs [Mersmann 1998]. Both Chikun *et al*. [2008] and this study didn't evaluate the effect of the polymorphism, because of lacking sufficient variations in *ADRB*3 gene and no access to phenotype records. Further studies must be done on the association of *ADRB3* polymorphism with relevant traits, e.g. birth weight, growth rate or carcass composition.

The haplotype diversity (0.579-0.792) found at *ADRB3* gene *locus* indicates the abundant variations existing in the studied populations. Haplotype diversity of 0.792 in Mashen pigs is comparable with genetic diversity of 0.707-0.864 in 56 Chinese native breeds as reported by Zhang *et al*. [2003] and 0.770 in similar breeds including Mashen, Landrace, Large White and Duroc used in this study (our unpublished results) with microsatellite DNA markers.

The pairwise *Fst* estimates ranged from 0.000 between hybrid wild pigs and Durocs to 0.215 between Landrace and pure wild pig in this study. Based on microsatellite DNA markers, *Fst* was estimated from 0.007 to 0.110 in similar populations (our unpublished results). The mean *Fst* estimates of 0.180 and 0.138 were obtained by Fan *et al*. [2002] in seven Chinese indigenous pig populations and by Thuy *et al*. [2006] in European commercial breeds, respectively. Compared with the results of AMOVA using microsatellite DNA markers by Kim *et al*. [2005] and Sollero *et al*. [2006], lesser genetic variation (5.62%) was found between populations in this study. Besides the breed difference, high *versus* low conservations in genic and microsatellite variations, respectively, could account for the differences.

Dendrogram of eight pig populations by UPGMA method based on Reynolds' distances showed that Large White and Jinyang White are closely related. The latter was a newly developed line, while Large White was used as a terminal sire breed in its

development. So their close relationship is apparent. The relationship between Duroc, Landrace, Large White and Mashen corresponds with the result of Zhang *et al*. [2003] and of Fang *et al*. [2005]. Duroc and the hybrid wild pig occurred to be closely related too. The precise reason is not known, because the hybrids'dams remained unknown. However, it is possible that the unknown parent contained the blood admixture of Duroc due to the extensive use of that breed in pig production in China. The relation of Pietrain and Mashen pig deserves further study.

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