

Molecular genetic analysis of the Chinese Erhualian pig breed

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Abstract

The Chinese Erhualian is one of the most prolific pig breeds in the world, but it is in danger of being replaced by other exotic pig breeds because of its slow growth rate and high fat content in the body. To obtain some genetic information for conservation, we analysed the Erhualian pigs by using a PCR-RFLP for the calcium-release-channel (CRC) gene, nine polymorphic microsatellites and the complete mtDNA D-loop sequences, and compared these data with those from other pig breeds from Europe and Asian. The PCR-RFLP analysis of the CRC gene showed that the frequency of the C allele associated with stress resistance was 100% in the Erhualian pigs. Neighbour-Joining trees constructed on the basis of mtDNA D-loop sequences and the microsatellite analysis clearly showed that the Erhualian pigs were located in a separate branch. These data suggest that the Erhualian pigs are different from other breeds. Microsatellite analysis showed that the average allele number (5.3/locus) in the Erhualian pig was intermediate as compared with that (4.8-7.0/locus) in the three European pig breeds. The expected heterozygosity was higher in the Erhualian pig (0.78) than that in these European pig breeds (0.59-0.72), whereas the observed heterozygosity was higher in the European breeds (0.51-0.64) than in the Erhualian pig (0.46). In the Erhualian pig, the fixation index (F_{IS}) was as high as 0.41. These data suggest a high level of inbreeding and/or subpopulation in the Erhualian pigs. For conservation of the germplasm in the Erhualian pigs, it is necessary to take measures to reduce inbreeding and/or subpopulation.

Keywords: Erhualian pig, genetic diversity, conservation, DNA markers

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Introduction

The Erhualian pig located in the Jiangsu and Zhejiang provinces is one of the 48 native Chinese pig breeds (Zhang, 1986). Erhualian pigs can use a high proportion of forage foods in their diets, and offer valuable traits (e.g. early sexual maturity, large litter size, tasty meat and disease resistance) for pig breeders world-wide (Zhang, 1986). The Erhualian is the most prolific pig breed in China, and among the most prolific breeds in the world (Zhang, 1986, Rothschild & Ruvinsky, 1998). The male pigs reach sexual maturity at the average age of 90 days, with a mean body weight of 15.6 kg, whereas the females reach sexual maturity at the age of 64 days, with an average body weight of 15.0 kg. The litter size of 278 sows averaged 15.93 (Yue, 1994). Currently some Chinese native breeds are in danger of being replaced by exotic breeds with very different feed requirements, reproduction rates and meat qualities mainly because of the slow growth rate and high fat content in body in the Chinese breeds (Chang *et al.*, 1999). The Erhualian has been listed under the category of protected breeds by the Chinese Ministry of Agriculture (Chang *et al.*, 1999).

The Erhualian is closely related to the Meishan pig breed (Zhang, 1986), which is now well known in Europe and in the USA (Rothschild & Ruvinsky, 1998). Several Meishan pigs were exported to the USA and Europe in 1980s, and had been used in breeding programs (Ducos *et al.*, 1992) and in pig genome mapping projects (Geldermann *et al.*, 1996; Rohrer *et al.*, 1996). A study on QTL mapping showed that the Meishan pig possessed QTL alleles which could reduce fat content in carcass, despite its fat appearance (Yue *et al.*, 2003). Research efforts in Europe and the USA have been aimed at explaining the mechanisms underlying the high fecundity and lower embryonic mortality of the Meishan pigs (Rothschild & Ruvinsky, 1998). A study carried out in the USA showed that the allelic diversity of the imported Meishan pigs was higher than that of some commercial breeds (Paszek *et al.*, 1998). A PCR-RFLP analysis of calcium-release-channel (CRC) gene showed that the frequency of the T allele associated with stress susceptibility was zero in Meishan pigs (Knorr *et al.*, 1994). Studies on genetic diversity have been carried out for a number of

breeds (e.g. Paszek *et al.*, 1998; Laval *et al.*, 2000; Kim & Choi, 2002). However, molecular studies on the Erhualian pigs are just beginning (Fan *et al.*, 2002; Jiang *et al.*, 2002). There is little detailed information on allele frequencies at the CRC locus, the genetic diversity and genetic relationship of the Erhualian pigs with other pig breeds.

In this study, we analysed the Chinese Erhualian pigs by using three different DNA marker systems, i.e. a PCR-RFLP for the CRC gene, nine polymorphic microsatellites and the complete mtDNA D-loop sequences, and compared the data from the Erhualian pigs with data from other European and Asian pig breeds. The main purpose is to supply some basic information for conservation and application of the prolific Erhualian pigs.

Materials and Methods

Blood samples of 32 unrelated Erhualian pigs and two unrelated Meishan pigs were collected in Jiangsu (22 individuals: 12 females and 10 males) and Zhejiang (10 individuals: 6 females and 4 males) provinces, China. DNA was isolated from whole blood as described (Miller *et al.*, 1988). DNA samples of 30 to 40 individuals from each of the three European breeds (Landrace, Pietrain and Large White) were obtained from University of Hohenheim, Germany (Chen, 1994; Laval *et al.*, 2000).

A PCR-RFLP for the CRC gene was performed only for the Erhualian pigs according to Knorr *et al.* (1994), since genotype data at the CRC locus are available in other breeds studied here (Knorr *et al.*, 1994). Briefly, a 655 bp fragment containing the point mutation (C to T) in the relative position +1843 of the CRC gene (Fujii *et al.*, 1991) was amplified by using a primer pair (5' GTTTGCCACAGGTCCTACCA 3', and 5' ATTCACCGGAGTGGAGTCTC 3'). The amplified fragment was then digested by the use of the *HinP* I (New England Biolab), leading to two fragments: 489 bp and 166 bp for the C/C genotype, and an undigested band of 655 bp for the T/T genotype.

To amplify the complete mtDNA D-loop sequences of pigs, one primer pair (5' CAACCAAACAAGCATTCCATTCGT 3' and 5' CGCCCACAAT-CTGAATATAGCTTATA 3') was designed according to the known pig D-loop sequences (GenBank No. AF276930 and AF276928). PCR was conducted on a PCR-machine PTC-200 (MJ Research) using 50 ng genomic DNA in a 25 µl reaction volume containing 0.4 µM of each primer, 2.00 µM of dNTPs, 1x PCR buffer with 1.5 mM MgCl₂ (Finnzymes) and 0.5 U of DNA-polymerase (Finnzymes). The PCR conditions were: 94 °C for 2 min, and then 35 cycles at 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for one minute followed by an extension at 72 °C for 5 min. The PCR products of about 1044 bp were cloned into a pGEM-T vector (Promega), then sequenced using M13 or M13 reverse primers using BigDye chemicals (ABI/PE) as described (Yue *et al.*, 2000). Forward and reverse sequences were aligned by using Megalign in DNASTAR software package (DNASTAR Inc.). Amplified PCR-products from four Erhualian (Erhualian01-Erhualian04) and two Meishan (Meishan01 and Meishan02) individuals were sequenced, whereas for the three European pig breeds, one individual from each breed was sequenced. The mtDNA D-loop sequences of these five breeds (Erhualian, Meishan, Large White, Landrace and Pietrain) were deposited in the GenBank (accession number: from AY230818 to AY230827). For a phylogenetic analysis, known sequences of the complete mtDNA D-loop of other pig breeds were also used. These breeds included eight Chinese breeds (Nanjang, Wanahua, Gonghei, Dongchang, Jinghua, Putian, Ningxian and Erhualian), a Korean breed (Cheju), two Japanese wild boars (JP-wild01 and JP-wild02) and two European breeds (one UK Walsh and two European wild boars: Eu-wild01 and Eu-wild02). The D-loop sequence of the fifth Erhualian individual (Erhualian05) was derived from GeneBank. Sequences were aligned by using the ClustalX 1.8 (Thompson *et al.*, 1997). Gaps in the aligned sequences were treated as missing. Sequence divergence was calculated using the software MEGA (Kumar *et al.*, 2001). A Neighbour-Joining (NJ) tree was constructed using the MEGA (Kumar *et al.*, 2001). The confidence of branching order was estimated by the use of the bootstrap analysis using 1000 replicates.

Nine microsatellites of 27 microsatellites approved for studying porcine genetic diversity by the FAO-ISAG advisory committee (Laval *et al.*, 2000) were selected for genotyping all the Erhualian, Large White and Pietrain pigs. These nine markers are S0155 (located on chromosome 1: SSC1), Sw240 (SSC2), S0226 (SSC2), S0002 (SSC3), IGF-I (SSC5), Sw951 (SSC10), S0090 (SSC 12), Sw857 (SSC14) and S0026 (SSC16). One primer of each pair was labelled with a fluorescent dye (either Hex or Fam or Tet). Genotyping of microsatellites was carried out using either an automated A.L.F. DNA sequencer (Pharmacia) or an ABI 377 DNA sequencer as described previously (Yue *et al.*, 1999; Yue *et al.*, 2000). Genotype data of the German Landrace were obtained from an earlier study (Laval *et al.*, 2000) and used for comparison.

The number and frequency of alleles at microsatellite loci, Wright's fixation indices (F_{IS} and F_{ST}) and expected (H_E) and observed (H_O) heterozygosity were calculated using the GDA software package (Lewis & Zaykin, 2000). An exact test was carried out to examine the Hardy-Weinberg Equilibrium using the GDA (Lewis & Zaykin, 2000).

The $(\delta\mu)^2$ genetic distance (Goldstein *et al.*, 1995) and Nei's standard genetic distance (Nei, 1978) were computed by using the Microsat program (Minch, 1996). The $(\delta\mu)^2$ genetic distance was then adjusted for small population size (Goldstein & Pollock, 1997). The NJ trees were constructed based on the two genetic distance measures by the use of the MEGA software package (Kumar *et al.*, 2001).

Results

The PCR-RFLP analysis of the CRC gene revealed that all 32 Erhualian pigs had the same genotype C/C, indicating the frequency of the C allele associated with stress resistance was 100% in the Erhualian pigs.

The genetic diversity of the Erhualian pigs is summarised in Tables 1 and 2. At nine microsatellite loci, a total of 48 alleles were detected in the Erhualian pigs. This gives an average number of 5.3 alleles per locus. The allele number of the Erhualian was less than that of the Large white, Pietrain, but higher than that of the Landrace. A total of 15 private alleles was detected in the four breeds. The Large White and Pietrain breeds displayed most private alleles (each had seven private alleles), The Erhualian pigs exhibited one (the allele 120 bp at the locus Sw951), whereas the Landrace showed none. In the Erhualian, disequilibrium of the genotypic frequencies ($P < 0.05$) was found at eight loci except at the locus S0026, whereas in the European pig breeds disequilibrium of genotypes was seen at three to six loci (Table 1).

Table 1 Allelic diversity in different breeds

	Erhualian		Pietrain		Large White		Landrace	
	Size range (bp)	n						
Sw240	94-110	6	94-112	6	94-114*	8	94-108	4
S0155	146-160	6	146-160*	6	146-162	6	146-162*	6
IGF-I	191-203	7	173-203	7	193-207	8	191-207*	7
Sw951	120-134	6	122-130*	3	122-130*	4	122-130	3
S0226	173-201	5	181-203*	8	179-207	11	179-207*	5
S0090	239-247	5	239-245	4	239-251	7	239-245*	4
S0026	96-102*	4	96-104*	4	96-102	4	96-102*	4
Sw857	140-154	5	140-156*	7	140-156*	8	140-154	4
S0002	190-208	4	186-208*	8	190-206	7	186-208*	6
Mean		5.3		5.9		7.0		4.8

n - Numbers of alleles

* Loci showing the Hardy-Weinberg equilibrium

The mean observed heterozygosity (H_O) at all nine microsatellite loci ranged from 0.46 for Erhualian to 0.58 for Pietrain. The expected heterozygosity (H_E) was higher in the Erhualian than in European breeds. The fixation index (F_{IS}) ranged from 0.12 for Pietrain to 0.41 for the Erhualian (Table 2). The average F_{ST} between breeds was 0.11. All F_{ST} calculated by pairs of breeds were different from zero ($P < 0.05$).

Table 2 Comparison of genetic diversity in different breeds

Breed	n	H_O	H_E	F_{IS}
Erhualian	34	0.46	0.78	0.41
Pitrain	32	0.58	0.66	0.12
Large White	30	0.51	0.72	0.28
Landrace	40	0.54	0.59	0.09

n - Number of pigs, H_O - Observed heterozygosity

H_E - Expected heterozygosity; F_{IS} - Average fixation index

A phylogenetic analysis of mtDNA D-loop sequences revealed two distinct clusters, one for Asian breeds (A) and the other for European breeds (B) (Figure 1). Within the cluster of the Asian breeds, the

Erhualian pigs were located on a separate branch, which was near the branch of the two Meishan and the Nanjing pigs. The average pairwise nucleotide distance between the Asian and European breeds was 1.9%.

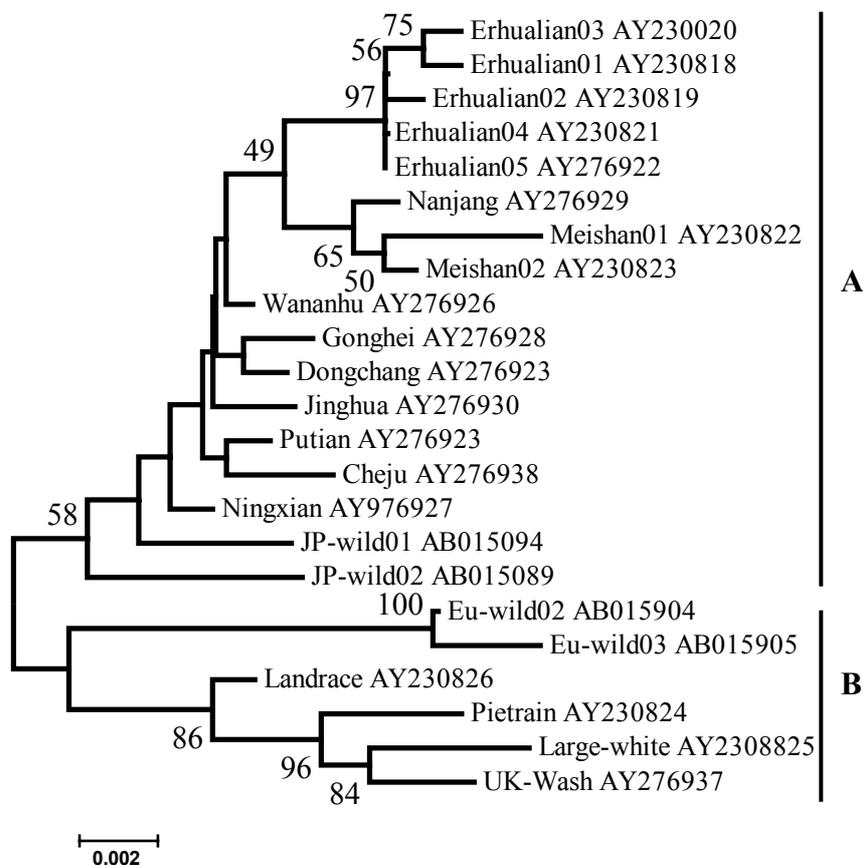


Figure 1 A NJ tree constructed on the basis of the complete mtDNA D-loop sequences. **A:** the cluster of Asian breeds, and **B:** the cluster of European breeds. Bootstrap values ($> 45\%$ after 1000 replicates) are shown on the nodes, whereas scale bar is shown under the tree. The GenBank accession number of each species was listed behind the name of each breed

Based on microsatellite data, the $(\delta\mu)^2$ and Nei's standard genetic distance measures showed the smallest genetic distance was between Large white and Pietrain, and the longest distance between Erhualian and Landrace (data not shown). The genetic distance between the Erhualian and European breeds was much larger than that between different European breeds. Clearly independent cluster of the Erhualian from the European breeds was seen in both trees (Figure 2).



Figure 2 NJ-trees constructed on the basis of the $(\delta\mu)^2$ (left) and Nei's standard (right) genetic distance measures

Discussion

The calcium-release-channel (CRC) gene is an important candidate for pig malignant hyperthermia, which is an inherited skeletal muscle disorder causing major economic losses in the pig industry. A point mutation at the +1843 of the CRC gene (C to T) is the major cause of stress susceptibility (Fujii *et al.*, 1991). DNA tests on the point mutation in several pig breeds clearly demonstrated that the T allele associated with stress susceptibility existed in almost all species tested (Grobet *et al.*; 1992; Houde *et al.*; 1993; Knorr *et al.*, 1994), except the Chinese Meishan pig. In this study we did not find the T allele in the Erhualian pigs, suggesting the Erhualian pigs are free of stress susceptibility. Removal of the T allele from pig populations through traditional selection, even with marker assisted selection is time consuming and costly. Therefore, besides the high reproductive performances, high meat quality and disease resistance, the Erhualian would offer another good trait (i.e. stress resistance) for pig breeders world-wide.

The phylogenetic trees constructed on the basis of complete mtDNA D-loop sequences and the microsatellite analysis showed a marked genetic difference between Asian breeds and European breeds. This result is consistent with those of other studies on the evolutionary relationships between Chinese and Western pig breeds (Li & Enfield, 1989; Chang *et al.*, 1999; Giuffra *et al.*, 2000). Within the cluster of Asian pig breeds, Erhualian pigs were located in a separate branch in the tree constructed by the use of mtDNA D-loop sequences, suggesting the Erhualian breed is genetically different from not only European pig breeds, but also other Chinese native breeds.

The allele number of the Erhualian was less than that of the Large White and Pietrain, but higher than that of German Landrace. As compared with the published data (Laval *et al.*, 2000; Li *et al.*, 2000; Lemus-Flores *et al.*, 2001; Kim & Choi, 2002), the average allele number of the Erhualian was higher than most pig breeds studied. Since the average number of alleles per locus is sensitive to the sample size and microsatellites used, expected and observed heterozygosity should be preferred for comparisons. The Chinese Erhualian showed higher expected heterozygosity than some published data on pigs (Laval *et al.*, 2000; Kim & Choi, 2002), suggesting higher gene diversity in the Erhualian pigs, which corresponds to the results on the Meishan pigs (Paszek *et al.*, 1998). The observed heterozygosity was higher in other pig breeds (Kim & Choi, 2002; Laval *et al.*, 2000) than in the Chinese breed. The fixation index F_{IS} , which is sensitive to heterozygote deficit, was as high as 0.41 in the Chinese breed, suggesting a high level of inbreeding and/or subpopulation in the Erhualian breed and/or appearance of null alleles at microsatellite loci. But the possibility of appearance of null alleles at the nine microsatellites used here can almost be ruled out, because the nine microsatellites were selected from more than 400 microsatellites from linkage maps (Archibald *et al.*, 1995; Rohrer *et al.*, 1996). They have also been used in several genetic diversity studies (Laval *et al.*, 2000; Li *et al.*, 2000), and none has detected null alleles. The deviation from the Hardy-Weinberg Equilibrium at most loci in the Erhualian pigs also suggests subpopulation within this breed. For conservation of Erhualian pigs, measures should be taken to reduce inbreeding and/or subpopulation. Microsatellites could be used to analyse the relatedness of individuals and to arrange the mating pairs properly for reducing inbreeding. It is also useful to exchange some breeding boars from different regions. Genotypes at polymorphic microsatellite loci could help to identify which individuals should be exchanged between different locations.

Conclusion

The Erhualian breed is quite different from other pig breeds, and could offer valuable traits, such as high reproductive performance, disease resistance, stress resistance and high meat quality for pig breeders world-wide. Therefore, its germplasm is worth to be conserved. However, high level of inbreeding and/or subpopulation have been detected in this study. Thus for conservation of this valuable germplasm, measures should be taken to reduce inbreeding and subpopulation.

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