Phylogenetic relationships of pig breeds from Shandong province of China and their influence by modern commercial breeds by analysis of mitochondrial DNA sequences

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Abstract

Mitochondrial cytochrome b (Cytb) and partial control region (CR) DNA sequence of 165 individuals out of eight pig breeds (Laiwu Black, Dapulian Black, Licha Black, Yimeng Black, Yantai Black, Wulian Black, Luyan White and Changwei White) from Shandong province of China were sequenced to determine phylogenetic relationships and to what extent they were affected by modern commercial breeds (Landrace, Yorkshire and Duroc) in maternal lineage. The eight breeds from Shandong had lower maternal evolutionary divergence than the three commercial breeds. Additionally, they shared the same haplotype (T2) of Cytb. All these results suggest they may have originated from closely related maternal ancestors, if not from a single ancestor. The fact that haplotypes of Dapulian Black and Laiwu Black formed their own subclades, while those of other breeds of Shandong dispersed in the Asian clade of the tree based on CR, indicates that Dapulian Black and Laiwu Black are indigenous breeds with relatively independent maternal lineage, while the others have extensively gene flow with other breeds. Except in Luyan White, no European haplotypes were found in pig breeds from Shandong, demonstrating the maternal introgression from the modern commercial pigs has had no or very little impact on the breeds from Shandong. Comparatively, CR is more suitable to analyze the phylogenetic relationships of closely related groups, whereas the four SNPs of Cytb are useful to determine whether modern commercial breeds (Duroc, Landrace and sometimes Yorkshire) were used as dam in the breeding process of new breeds or synthetic lines in China.

Introduction

Shandong province, with an area of more than 150,000 km² and a diverse terrain, is one of the major districts of pig production in China. Historically, many indigenous pig breeds were developed to meet the demands of the local people. The indigenous pig breeds have special characteristics, such as high reproductive and lactation performance, good meat quality, strong adaptability and disease-resistance. However, for growth performance and lean meat content in pig production, modern commercial breeds were introduced in the early 20th century and hybridized with local pigs (Si et al., 1999). Indigenous pig breeds from Shandong province, as well as other Chinese indigenous pig breeds, have been in direct competition with highly selected modern commercial pig breeds, and many of them are in danger of being replaced, or hybridized with Euro-American originated commercial breeds, primarily Duroc, Yorkshire and Landrace. The extant indigenous pig breeds from Shandong are Laiwu Black (LW), Dapulian Black (DP), Licha Black (LC), Yimeng Black (YM), Yantai Black (YT) and Wulian Black (WL) (Si et al., 1999). Additionally, Changwei White (CW) and Luyan White (LY) are new breeds produced through the hybridization of indigenous breeds from Shandong and modern commercial pig breeds (Si et al., 1999; Guo et al., 2008).

So far, the phylogenetic relationships of Chinese indigenous pig breeds have been evaluated by microsatellite markers (Fan et al., 2002; Yang et al., 2003; Zhang et al., 2003; Li et al., 2004; Fang et al., 2005; Megens et al., 2008) and mitochondrial DNA (mtDNA) sequence (Yang et al., 2003; Fang and Andersson, 2006; Wu et al., 2007; Jiang et al., 2008). However, the vast genetic resources in China are still poorly characterized, except a few very famous breeds. Of the eight pig breeds in Shandong, only LW, YM and LC (Zhang et al., 2003; Fang et al., 2005; Fang and Andersson, 2006; Megens et al., 2008) were included in the previous studies using microsatellite or mtDNA markers. Therefore, a comprehensive assessment of their genetic diversity and relationships with modern commercial breeds represents a major step toward the development of conservation and improvement programs.

Since mitochondrial DNA (mtDNA) is maternally inherited, its evolutionary rate of base substitution is much faster than that of nuclear DNA (Avise, 2000). The analysis of mtDNA sequence has been used to investigate genetic relationships between species and within species. Of the mtDNA genome, control region (CR) and cytochrome b (Cytb) are two molecular markers for investigating the genetic population structure of closely related animals in restricted areas (Ghivizzani et al., 1993; Alves et al., 2003).

The objectives of this study were to determine the phylogenetic relationships among eight pig breeds from Shandong province of China and to what extent they were affected by the modern commercial breeds (Duroc, Yorkshire and Landrace) in maternal lineage by using direct sequencing of Cytb and CR DNA sequence.

Materials and methods

Sample collection and genomic DNA extraction

Ear-punch samples of 165 individuals representing 11 pig breeds were used in the study (geographic location of Shandong province and detailed information of the geo-
graphic regions of sampling are shown in Figure 1 and 2, respectively). Except WL (n=16) and CW (n=14), sample sizes of the other breeds were all 15. Individuals of all breeds except WL were unrelated (without common grandparents), while those of WL had not written pedigree records and were collected randomly. Samples for the eight breeds of Shandong were collected from their conservation farms and those for the three modern commercial breeds were collected from Research Center of Pig Breeding and Reproduction of Shandong province. Total genomic DNA was extracted from ear-punch samples by the standard phenol-chloroform method (Sambrook and Russell, 2001).

Amplification and sequencing of Cytb and CR

Four primer sets were composed according to the reference (Alves et al., 2003) to amplify Cytb and partial CR sequences (Table 1). Polymerase chain reactions (PCRs) were prepared in 50 µL volumes using PTC-2000 DNA Engine (Bio-Rad, USA), each containing 1.5 µL of 100 ng/µL DNA, 5 µL of 10× buffer, 4 µL of 10 mmol/L dNTP, 1.5 µL of each primers (12.5 µmol/L forward and reverse primers), 2 U of PrimeSTAR TM HS DNA Polymerase (TaKaRa, Japan). Thermocycling was performed as follows: a denaturation step at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, and then a final extension at 72°C for 10 min. The PCR products were purified and used as sequencing templates. The nucleotide sequences of both strands were determined using an ABI3700XL DNA sequencer with the Big Dye Terminator v3.1 Cycle Sequencing kit (Life Technologies Corp., Carlsbad, CA, USA). The GenBank accession numbers for sequences determined here are EU660056-EU660130 and EU979216-EU979305 for Cytb and EU660131-EU660205 and EU979126-EU979215 for CR.

Data analyses

The tandem repeat motif of CR was excluded from analysis because the number of repeats was variable within individuals, indicating a high degree of heteroplasmy and thus the repeat itself is not phylogenetically informative (Ghivizzani et al., 1993; Wu et al., 2007). The software of DnaSP 4.10.9 (Rozas et al., 2003) was used to calculate haplotype and nucleotide diversity (Nei, 1987), and average number of nucleotide differences (Tajima, 1983). Genetic distances among groups based on the pairwise analysis were conducted to estimate evolutionary divergence using the maximum composite likelihood method (Tamura et al., 2004) by MEGA4 (Tamura et al., 2007). Neighbor-joining (NJ) phylogenetic trees (Saitou and Nei, 1987) of Cytb and CR were constructed by maximum composite likelihood method (Tamura et al., 2004) and the corresponding sequence of an African Warthog (GenBank number DQ409327) was used as outgroup by MEGA4, too. Bootstrap (Felsenstein, 1985) re-sampling (n=1000) was performed to test the robustness of the tree topologies.

Results

Variation of Cytb and CR mtDNA DNA sequences

The complete Cytb gene (1140 bp) and 1102 bp region of CR (positions 15469 to 16570 according to the reference sequence GenBank...
The genetic diversity within breeds (Table 4) was estimated by measures of haplotype diversity, nucleotide diversity and average number of nucleotide differences. Based on CR, large genetic diversity existed in the breeds studied, with exception of three pig breeds from Shandong, LC, YT and CW, which haplotype diversities were below 0.5000. Averagely, the three commercial breeds had larger genetic diversity than those of the eight breeds from Shandong. However, breeds from Shandong varied widely in genetic diversities. Compared with the three modern commercial breeds, WL had larger genetic diversities; DP, YM, LW and LY had similar ones; whereas LC, YT and CW had smaller ones. Whereas based on Cytb, much smaller genetic diversity was detected than those on CR. With some exception, measures of the genetic diversity on the basis of Cytb were congruent with those based on CR.

Genetic distance by the maximum composite likelihood method (Table 5) was conducted to estimate the evolutionary divergence among breeds. Based on the variation of CR, the genetic distances among pig breeds from Shandong were rather small, ranging from 0.0011 (between LW and LC) to 0.0046 (between DP and LY), while those among the modern commercial pig breeds ranged from 0.0031 (between Duroc and Landrace) to 0.0109 (between Yorkshire and Duroc). Duroc and Landrace had larger genetic distance with breeds from Shandong, ranging from 0.0120 (between Landrace and LY) to 0.0153 (between Duroc and DP), whereas, genetic distance between Yorkshire and the Shandong breeds ranged only from 0.0047 (between Yorkshire and LY) to 0.0065 (between Yorkshire and LY).

Just like genetic diversity within breeds, the genetic distance based on the variation of Cytb was consistent with, but smaller than those on CR in general. Additionally, three breeds from Shandong, LW, LC and DP, had the same Cytb sequence.

Phylogenetic trees constructed on Cytb and CR using the NJ method

To better understand the relationships among eight pig breeds from Shandong and the three modern commercial breeds, several phylogenetic trees based on different methods were conducted on Cytb and CR, but gave similar topologies and bootstrap values. Thus only NJ trees on the basis of maximum composite likelihood method were reported here (Figures 3 and 4).

All pigs analyzed were split into Asian and European clades, with rather high bootstrap values in the phylogenetic trees conducted on Cytb and CR. In the NJ tree on Cytb, all haplotypes of pig breeds from Shandong except one LY haplotype (T4), and two Yorkshire haplotypes (T8 and T9) clustered together into Asian clade, while all haplotypes of Duroc and Landrace, the rest one of Yorkshire and T4 clustered into European clade. In the tree on CR, all pigs had the same classification to the Asian and European clades as in the tree on Cytb except that all LY haplotypes clustered into Asian clade. However, CR had a larger sequence variation and the phylogenetic tree constructed on it showed more informative and detailed relationships of the breeds studied. Two subclades,
named sub1 and sub2, were observed in it. All haplotypes of LW clustered into sub1, with each haplotype of the other six breeds, YT, YM, LY, LC, CW and DP dispersing in it. All the DP haplotypes, excluding the one dispersed in sub1, clustered into sub2. Except those of LW and DP, haplotypes of the other breeds from Shandong distributed dispersedly in the Asian clade. The haplotypes of the Yorkshire varied greatly. Some belonged to the Asian clade and clustered together with breeds from Shandong, while the other belonged to European clade and clustered with Duroc and Landrace.

**Table 3. Haplotypes and polymorphic sites of partial mtDNA CR sequence of eight pig breeds from Shandong and three modern commercial breeds.**

| Haplotypes | Samples | Nucleotide positions |
|------------|---------|---------------------|
| H1         | C2/5-7/12-15 | 15543-15592          |
| H2         | LW1/2-7     | 15543-15592          |
| H3         | LW3/6-8/11-14, DP1/10, LC1/9/12, YM2/9/12/15, YT14/17-7, CW4/8/11 | 15543-15675          |
| H4         | LW5/10/15   | 15543-15570          |
| H5         | DP1        | 15543-15570          |
| H6         | DP2-3-5-7   | 15543-15570          |
| H7         | DP4/15     | 15543-15570          |
| H8         | DP6        | 15543-15570          |
| H9         | DP9/11-14   | 15543-15570          |
| H10        | LC2-8/10-11-13-15, YM1/4/5/10/13, YT1-3-5/7-9/14, LW5/6-15, CW3/6/11 | 15543-15675          |

Nucleotide positions are numbered according to the reference sequence GenBank AJ012119. Nucleotides identical to the first haplotype sequence are denoted by dots (.) and deleted nucleotides are denoted by a dash (–). LW, Laiwu Black; DP, Dapulian Black; LC, Licha Black; YM, Yimeng Black; YT, Yantai Black; WL, Wulian Black; LY, Luyan White; CW, Changwei White; D, Duroc; Y, Yorkshire; C, Landrace.

**Discussion**

**Evolutionary divergence of the eight pig breeds from Shandong and three modern commercial breeds**

Several studies have shown that pigs were independently domesticated in various parts of the world (Giuffra et al., 2000; Larson et al., 2005; Fang and Andersson, 2006; Wu et al., 2007). The facts that all the eight pig breeds from Shandong shared the same haplotypes (T2) of and have very small revolutionary divergence based on both Cytb and CR, suggest the eight breeds are closely related in maternal lineage and may have originated from closely related maternal ancestors, if not from a single one.

The gene introgression of Asian pig breeds into the European pig populations had been documented and supported elsewhere by mitochondrial and nuclear sequence studies (Jones, 1998; Giuffra et al., 2000; Kim et al., 2002; Fang and Andersson, 2006). In our results, haplotypes of Yorkshire varied greatly. Some belonged to the Asian clade and clustered with European clade and clustered with Duroc and Landrace.
ported the gene flow from Asia to Europe and Asian indigenous pig breeds had played a critical role in the development of Yorkshire.

The genetic distances based on CR among pig breeds from Shandong were smaller than those among the modern commercial breeds (0.0031 to 0.0109). Other researchers’ studies based on the mtDNA CR and Cytb sequences. Maximum composite likelihood method was used. The corresponding sequence of an African Warthog (GenBank number D409327) was used as outgroup. Numbers indicated at the nodes are bootstrap values based on 1000 replicates.

The related maternal ancestors of pig breeds from Shandong and the combination of Asian and European maternal origin of modern commercial breeds discussed above may be the main reasons for the smaller maternal evolutionary divergence within Chinese breeds than within European breeds.

Relationships among pig breeds from Shandong province

In the phylogenetic trees constructed on CR, haplotypes of LW and DP formed their own subclades (sub1 and sub2, respectively), while those of other breeds of Shandong dispersed in the Asian clade. In the sub1, besides all the haplotypes of LW, there are each haplotype of the other six breeds. Two possible reasons may explain the dispersal of the haplotypes of six breeds with LW. Firstly, all the six breeds may have played a certain role in the development of LW. Secondly, LW may have contributed to the formation of the six breeds. According to the known data (Si et al., 1999) and characteristics of LW, the second reason is more likely. Except the haplotype H3, which cluster into sub1, all DP haplotypes clustered into sub2.

These results indicate that LW and DP are indigenous pig breeds that have relatively independent maternal lineage while the other six breeds have extensively gene flow with other breeds from Shandong. It was consistent with their conserved condition, too (Si et al., 1999).

Influence of the pig breeds from Shandong by modern commercial breeds

Except in LY, European haplotypes were not found in pig breeds from Shandong, which is consistent with the findings of Fang and Andersson (2006) that no European mtDNA haplotypes were detected in the 21 Chinese indigenous pig breeds, which included 2 breeds analyzed in this study. This result suggests that although modern commercial pig breeds were introduced into Shandong province beginning approximately 100 years...
ago, they were used mainly as sires to improve the growth performance and lean meat content of local pigs, and the maternal introgression from the modern commercial pigs has had no or very little impact on the extant eight pig breeds from Shandong. In addition, the result is in line with the observations that pig breeds from Shandong have characteristics, such as high reproductive performance and good lactation, and are suitable as dams, while modern commercial pig breeds have the characteristics of fast growth rate and high lean meat content and are suitable as sires.

Comparison of Cytb and CR as phylogenetic markers to investigate genetic relationships of pig breeds

Both the mtDNA Cytb and CR DNA sequences are widely used as phylogenetic markers in molecular systematics (Ghivizzani et al., 1993; Alves et al., 2003; Clop et al., 2004; Fang and Andersson, 2006; Wu et al., 2007; Jiang et al., 2008). However, compared with Cytb, CR has a larger sequence variation, and is useful to compare the genetic diversity in the study. In addition, more informative and detailed relationships of the breeds analyzed were obtained from the phylogenetic tree constructed on it. Therefore, CR may be more suitable to analyze the phylogenetic relationships of closely related groups than Cytb. It is consistent with those of Lockhart et al. (1995) and Randi et al. (2001).

However, the major Cytb haplotypes can be easily distinguished on the basis of four single nucleotide polymorphisms (SNPs) located at positions 15036, 15038, 15041 and 15045 bp of the complete sequence of the pig mtDNA (numbering of positions follows the reference sequence GenBank number AJ002189). According to their polymorphisms all pigs were classified into the Asian (A1 = CATA and A2 = CATG) or European (E1 = TGCG and E2 = TGTG) origins (Giuffra et al., 2000; Clop et al., 2004) in maternal lineage. In the European domestic pig breeds, not only European but Asian origin haplotypes as well exist, because of the gene introgression from Asian breeds into European population as mentioned above; whereas in Asian domestic pig breeds only Asian origin haplotypes existed. Therefore, the four SNPs of Cytb as a marker are useful to determine whether modern commercial breeds (Duroc, Landrace and sometimes Yorkshire) were used as dam in the breeding process of new breeds or synthetic lines in China. LY is one example; it is a new breed authenticated by Chinese National Committee of Domestic Animal Resource in 2007. As per the breeding record, YT, used as dam and Landrace, used as sire, crossbred to generate the new breed (Guo et al., 2008). In our study, besides Asian origin haplotypes, one haplotype (T4, 4 SNPs: TGC C G) of European origin was found, too. It demonstrated that in the breeding process of LY, Landrace was used mainly as sire, but in some instances mistakenly used as dam as well.

Except LW and DP, all the other pig breeds from Shandong possess physical characteristics typical of modern commercial breeds, suggesting that the introgression of modern commercial pigs may be more extensive than what can be detected by analysis of maternal-linked mtDNA sequence. An obvious topic for future research will be the use of other genetic markers, such as microsatellites, to further assess the introgression of modern commercial breeds into them in nuclear genome level.

Conclusions

The results indicate that the eight breeds from Shandong may have originated from closely related maternal ancestors, if not from a single one. DP and LW are indigenous pig breeds that have relatively independent maternal lineages while the other six breeds have extensively gene flow with others from Shandong. Except LY, the maternal introgression from the modern commercial pigs has had no or very little impact on the eight pig breeds from Shandong. Comparing the two mtDNA markers, CR is more suitable to analyze the phylogenetic relationships of closely related groups, whereas the four SNPs of Cytb as a marker are useful to determine whether modern commercial breeds (Duroc, Landrace and sometimes Yorkshire) were used as dam in the breeding process of new breeds or synthetic lines in China. Other researches using genetic markers will be needed to further detect introgression of modern commercial breeds into those from Shandong in nuclear genome level.

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