

Genetic diversity of mitochondrial DNA D-loop in wild and domestic pigs (*Sus scrofa*) in East Asia

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Chinese domestic pig breeds have abundant genetic resources, but definite information about the geographic specificity and genetic structure is lacking. In this study, we analysed 359 mitochondrial D-loop sequences, including 86 sequences from Chinese domestic pigs and the rest downloaded from GenBank. The haplotype and nucleotide diversity indices ranged from 0.467 ± 0.132 (Duroc) to 1.000 ± 0.500 (Thailand, North Korea and Cambodia) and 0.00067 ± 0.00019 (Duroc) to 0.02000 ± 0.01000 (Thailand), respectively. The neighbour-joining tree revealed two clads in the individual pigs, with a Chinese domestic breed distributed discontinuously among several breeds. The network data indicated that the gene pools of Chinese domestic pigs are likely related to Chinese, Japanese and some other Southeast Asian wild boars.

Key words: mitochondrial DNA, wild boar, domestic pig, genetic diversity, geophylogeny.

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China has a long history of pig breeding (Falkenberg & Hammer 2009; Larson *et al.* 2010), which has not only contributed to the global diversity of pig breeds but also broadened the worldwide genetic pool. In the 20th century, a large number of foreign lean pigs (Yorkshire pig, Duke pig, Landrace pig, etc.) were introduced into China, resulting in a great change in the genetic structure of Chinese pigs. Several of the developed breeds, such as the Harbin White, Shanghai White, Beijing Black and the Xinhui pig, originated from crossing those exotic pigs with local breeds (Sheng *et al.* 2016; Yang *et al.* 2022). Furthermore, breeds such as the Mi, Erhualian, Dongchuan, Hainan, Rongchang and the Min pig have foreign lineages (Ai *et al.* 2013; Tong *et al.*

2022). Chinese pigs are characterised by a high level of genetic diversity, resulting from different ecological environments, migration and fusion, followed by population and selection (China National Commission of Animal Genetic Resources, 2011). There have been several major migrations and integrations throughout the history of China, with the pigs moving along with the population in every migration process (McOrist 2009; China National Commission of Animal Genetic Resources, 2011). A large number of genetic exchanges also took place between the non-local pigs and local pigs, which facilitated the formation of native pig breeds. High fecundity, precocious and easily fat species traits were chosen and fixed during subsequent generations.

Mitochondrial DNA (mtDNA) is the only existing extranuclear genetic material in animals, which has relatively constant biological information, unlike nuclear DNA. MtDNA has a small amount of molecules and a high evolution speed, with a genetically independent, strictly maternal inheritance (Sato & Sato 2013) and almost no recombination occurring (Carelli 2015). Thus, mitochondrial DNA has been widely used to investigate the genetic diversity and evolutionary origins of animals. The mitochondrial displacement-loop region (D-loop) is the control region between tRNA_{Pro} and tRNA_{Phe} in the mtDNA, which is rich in the A-T base and accounts for 6% of the mitochondrial genome. The D-loop is a hypervariable region with a base substitution rate 5~10 times higher than in other regions of the mtDNA molecule.

Porcine mitochondrial DNA research has great significance and broad development prospects for revealing the origins and classifications of pigs throughout the world (Fernández *et al.* 2011). In terms of the principal source of domestic pigs in Europe, multiple centres of domestication have been revealed in Eurasia rather than the Near Eastern region (Larson *et al.* 2005). Okumura *et al.* found that there were extensive introgressions of Asian pig breeds in European breeds (Okumura *et al.* 2001). Europe and Asia were two independent centres of domestication. Moreover, the Asian mtDNA haplotypes presented in European pigs were also found in the breeds from Southeast China. This indicates that the domestic pigs from Southeast China or from other parts of Asia, carrying mtDNA haplotypes belonging to the D2 cluster, resulted in the introgression into Europe (Fang & Andersson 2006). In addition, Jin *et al.* (2012) found that the domestic pigs located in the upper waters of the Yangtze were domesticated in their original locations (Jin *et al.* 2012). The aim of this study is to clarify the genetic diversity and clusters of wild boars in East Asia and to explore the ancestry of Chinese domestic pigs based on the mitochondrial DNA signatures.

Material and Methods

Samples and locations

Ear tissues were collected and stored at -20°C in 75% ethanol before use, representing eight Chinese domestic pig breeds: Harbin White (n = 18, Heilongjiang), Liangshan (n = 10, Sichuan), Qingyu (n = 10, Sichuan), Rongchang (n = 10, Chongqing), Tibetan (n = 10, Tibet), Landrace (n = 10, Sichuan), Duroc (n = 10, Sichuan) and Yorkshire (n = 10, Sichuan) (Table 1). Each individual sample was col-

lected from standardised large-scale farms

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from the ear tissue using a DNA extraction kit (Tiangen, Beijing, China) according to the manufacturer's protocol. A 1274 bp fragment covering the whole mitochondrial control region was amplified by the primer pair (F: 5'-AGCACCCAAAGCTGAAATTC-3', R: 5'-AGCTGTGAGGCTCATCTAGG-3'), which was designed by Primer Premier 5.0 (PREMIER Biosoft Intl., CA, USA). Each amplification reaction was carried out in a 30 µl reaction mixture including 15 µl 2× PCR Master Mix (Thermo Fisher Scientific, Shanghai, China), one µl of DNA (2.5 ng/µl), one µl each of primer (10 pmol/µl) and 12 µl of ddH₂O. The amplification conditions were 95°C for 5 min, 35 cycles at 95°C for 30 sec, 30 sec at 62.5°C and 72°C for 75 sec, then a final extension at 72°C for 5 min in the PTC-200 Programmable Thermal Controller (MJ Research Inc., USA). The amplification products were electrophoresed using a 1.5% agarose gel and sequenced on an ABI 3730XL DNA analyser (Applied Biosystems, USA) using the BigDye® Direct Cycle Sequencing Kit.

Polymorphism detection, diversity, and phylogenetic analysis

The mtDNA D-loop sequences were edited and aligned with the Seqman 5.01 program of DNASTAR Software (DNASTAR Inc. Madison, WI, USA) using the overlapping forward and reverse sequences. To assess the mtDNA diversity, genetic differentiation and phylogeny of the Chinese domestic pigs, another 273 wild boar sequences retrieved from GenBank were jointly analysed based on D-loop sequencing (Supplementary Material, SM.01). The length of all alignments was reduced to 654 bp (Acc. No: KX094894, 1-654) for a further analysis based on the uniform sequence lengths. The population DNA polymorphism was measured by calculating the segregating sites (S), haplotype diversity and nucleotide diversity using the DnaSP6 software package (Rozas *et al.* 2003). Furthermore, the mismatch distribution was drawn with DnaSP6. Phylogenetic trees were constructed using MEGA 6.0 (Tamura *et al.* 2013) with maximum likelihood procedures and 1,000 bootstrap replications. In addition, a phylogenetic network was constructed using Network 4.6.1.0 (www.fluxus-engineering.com/network_terms.htm) based on a median-joining approach (Bandelt *et al.* 1999).

Table 1
Polymorphisms and diversity of Mitochondrial D-loop DNA from different populations

| Origin | N | S | h | Hd (\pm SD) | π (\pm SD) | Reference |
|------------------|-----|----|----|-------------------|-----------------------|---|
| Wild boar | | | | | | |
| South Korea | 26 | 26 | 13 | 0.908 \pm 0.035 | 0.01070 \pm 0.00065 | Cho <i>et al.</i> 2003, 2004, 2005; Larson <i>et al.</i> 2007; Hongo <i>et al.</i> 2002 |
| Russia | 16 | 10 | 8 | 0.875 \pm 0.059 | 0.00605 \pm 0.00062 | Ramayo <i>et al.</i> 2011 |
| Japan | 24 | 18 | 18 | 0.967 \pm 0.024 | 0.00768 \pm 0.00089 | Okumura <i>et al.</i> 1996; Watanobe <i>et al.</i> 1999, 2001 |
| Laos | 8 | 20 | 6 | 0.929 \pm 0.084 | 0.01200 \pm 0.00180 | Wu <i>et al.</i> 2007 |
| India | 6 | 14 | 4 | 0.800 \pm 0.172 | 0.00778 \pm 0.00325 | Larson <i>et al.</i> 2005 |
| Indonesia | 12 | 9 | 10 | 0.970 \pm 0.044 | 0.00288 \pm 0.00038 | Larson <i>et al.</i> 2007 |
| Taiwan | 5 | 7 | 5 | 1.000 \pm 0.126 | 0.00460 \pm 0.00115 | Hongo <i>et al.</i> 2002; Larson <i>et al.</i> 2007 |
| Vietnam | 4 | 8 | 3 | 0.833 \pm 0.222 | 0.00674 \pm 0.00226 | Hongo <i>et al.</i> 2002; Ishiguro <i>et al.</i> 2008; Larson <i>et al.</i> 2007; Wu <i>et al.</i> 2007 |
| Burma | 6 | 11 | 5 | 0.933 \pm 0.122 | 0.00818 \pm 0.00152 | Larson <i>et al.</i> 2005, 2007 |
| Thailand | 2 | 13 | 2 | 1.000 \pm 0.500 | 0.02000 \pm 0.01000 | Larson <i>et al.</i> 2005, 2007 |
| North Korea | 2 | 8 | 2 | 1.000 \pm 0.500 | 0.01227 \pm 0.00613 | Larson <i>et al.</i> 2007 |
| French Polynesia | 6 | 0 | 1 | NA | NA | Larson <i>et al.</i> 2005, 2007 |
| Papua New Guinea | 4 | 2 | 3 | 0.833 \pm 0.222 | 0.00153 \pm 0.00052 | Larson <i>et al.</i> 2005, 2007 |
| Cambodia | 2 | 2 | 2 | 1.000 \pm 0.500 | 0.00394 \pm 0.00197 | Tanaka <i>et al.</i> 2008 |
| China | 149 | 43 | 57 | 0.968 \pm 0.005 | 0.01021 \pm 0.00052 | Larson <i>et al.</i> 2005, 2007; Wu <i>et al.</i> 2007 |
| Domestic pig | | | | | | |
| Harbin white pig | 16 | 21 | 10 | 0.917 \pm 0.049 | 0.00766 \pm 0.00236 | In this study |
| Liangshan pig | 10 | 25 | 9 | 0.978 \pm 0.054 | 0.00958 \pm 0.00317 | In this study |
| Qingyu pig | 10 | 7 | 5 | 0.844 \pm 0.080 | 0.00366 \pm 0.00055 | In this study |
| Rongchang pig | 10 | 5 | 4 | 0.644 \pm 0.152 | 0.00274 \pm 0.00064 | In this study |
| Tibetan pig | 10 | 7 | 4 | 0.800 \pm 0.089 | 0.00458 \pm 0.00056 | In this study |
| Landrace | 10 | 3 | 3 | 0.644 \pm 0.101 | 0.00188 \pm 0.00031 | In this study |
| Duroc | 10 | 1 | 2 | 0.467 \pm 0.132 | 0.00067 \pm 0.00019 | In this study |
| Yorkshire | 10 | 16 | 2 | 0.467 \pm 0.132 | 0.01070 \pm 0.00302 | In this study |

Note: N – sample size; S – polymorphic site number; h – haplotype number; Hd – haplotype diversity; π – nucleotide diversity; SD – standard deviation.

Results and Discussion

There were a total of 85 variable sites, including 28 singleton sites and 57 parsim-informative sites. The information on the sequence numbers, number of polymorphic sites, number of haplotypes and nucleotide diversity of all the pig breeds is listed in Table 1. The haplotype diversity ranged from 0.467 \pm 0.132 (Duroc) to 1.000 \pm 0.500 (wild boars in Thailand, North Korea and Cambodia). The lowest nucleotide diversity was 0.00067 \pm 0.00019 (Duroc). Moreover, the wild boars from Thailand had the highest nucleotide diversity (0.02000 \pm 0.01000). The greatest number of individuals was 149 from the Chinese wild boars, where the nucleotide diversity

was 0.01021 \pm 0.00052. Compared to domestic pigs, the introduced breeds showed a relatively low haplotype diversity, such as 0.467 \pm 0.132 in the Duroc, 0.467 \pm 0.132 in the Yorkshire and 0.644 \pm 0.101 in the Landrace, respectively.

As is shown in the phylogenetic tree (Figure 1), almost all the breeds from different regions could be relatively divided into two clades, with the majority of them showing a concentrated distribution just like their geographical distribution. One clade was mainly made up of the wild boars from Indonesia, Papua New Guinea, Cambodia, India and Laos, which were clustered together and showed a near genetic distance from each other. The other clade was mainly composed of the wild boars from China, Ja-

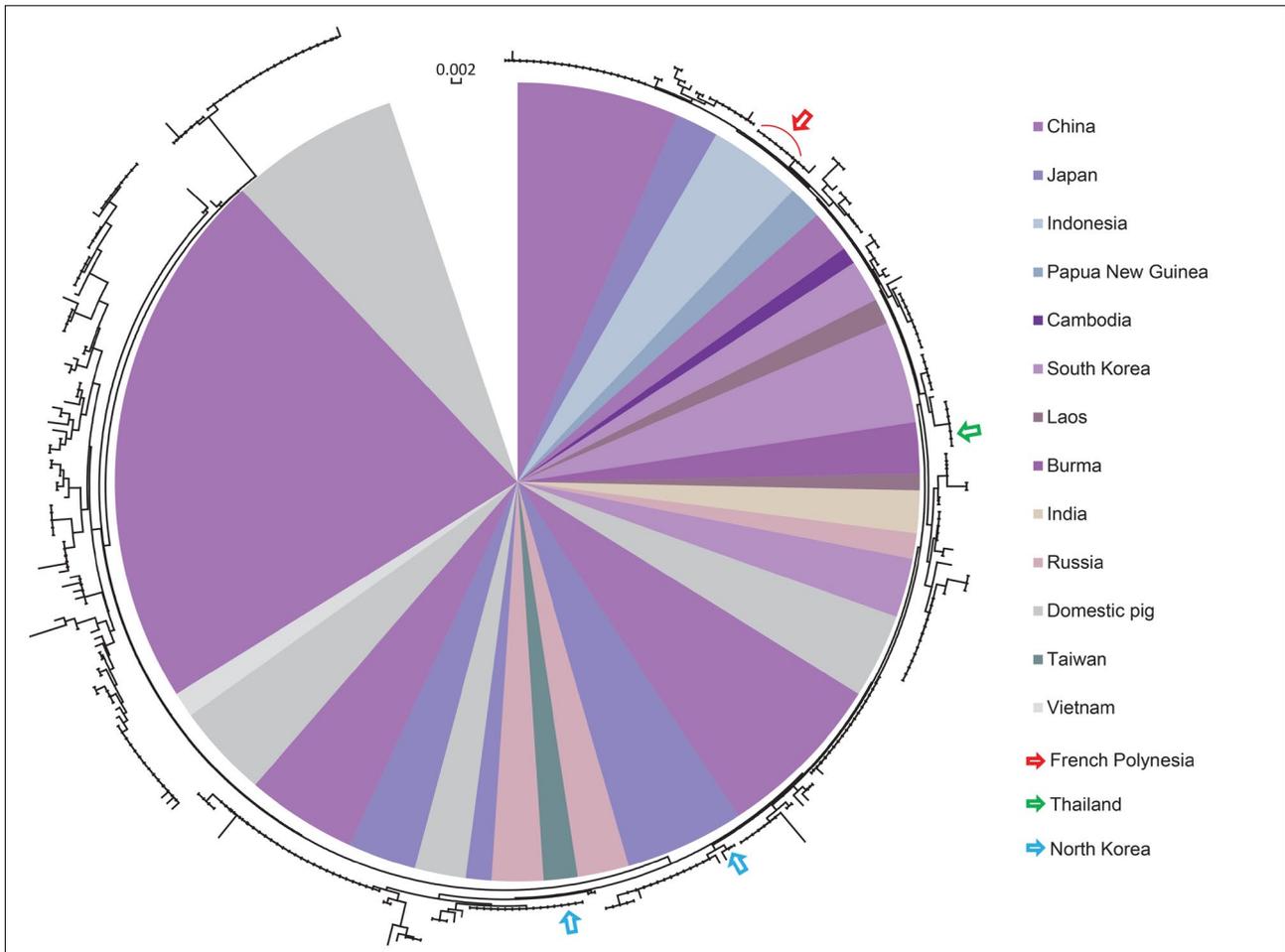


Fig. 1. Phylogenetic tree showing the mtDNA lineages in the studied pigs.

pan and Russia, and the domestic pigs from China. In this case, the distribution was not well separated according to breed, but was decentralised as a small branch. It is worth mentioning at this point that the wild boars from Taiwan were mainly gathered in the Russian breed and showed near a genetic distance to the Russian wild boars. In addition, the wild boars from Vietnam were individually clustered between the Chinese wild boars and Chinese domestic pigs. They had a distant genetic relationship with most of the wild boar breeds from Southeast Asia.

In order to further explore the genetic differentiation of the population, we drew up a median-joining network profile (Figure 2) and found that the results of the network have a correlation with the results of the phylogenetic tree. As with the network, some of the wild boars from Indonesia, Taiwan, Vietnam, Papua New Guinea and French Polynesia have formed a shared haplotype that has diffused to other haplotypes. There was a geographical correlation among the populations around the shared haplotype,

essentially among the wild boars from Southeast Asia. Some of the wild boars from China, Japan and Russia, have also formed a shared haplotype that has been diffused between Japan and Russia. In addition, a large number of the wild boars from China have formed haplotype centres of different sizes. It can be inferred from the haplotype distribution that the formation of Chinese domestic pigs was closely related to the wild boars of China, Japan and some other Southeast Asian countries. Furthermore, the evidence in the mismatch distribution of the investigated samples suggested that one expansion event occurred with four mutational time units (Figure 3). However, the concrete direction of the evolution and migration still needs to be studied further.

The data in this paper, including the haplotype and nucleotide diversity, showed an abundant genetic variation in *Sus scrofa*, which belonged to the European group, Asian group and the Indian group (Groves & Grubb 1993; Maselli *et al.* 2016). In our study, the domestic pigs showed a low genetic di-

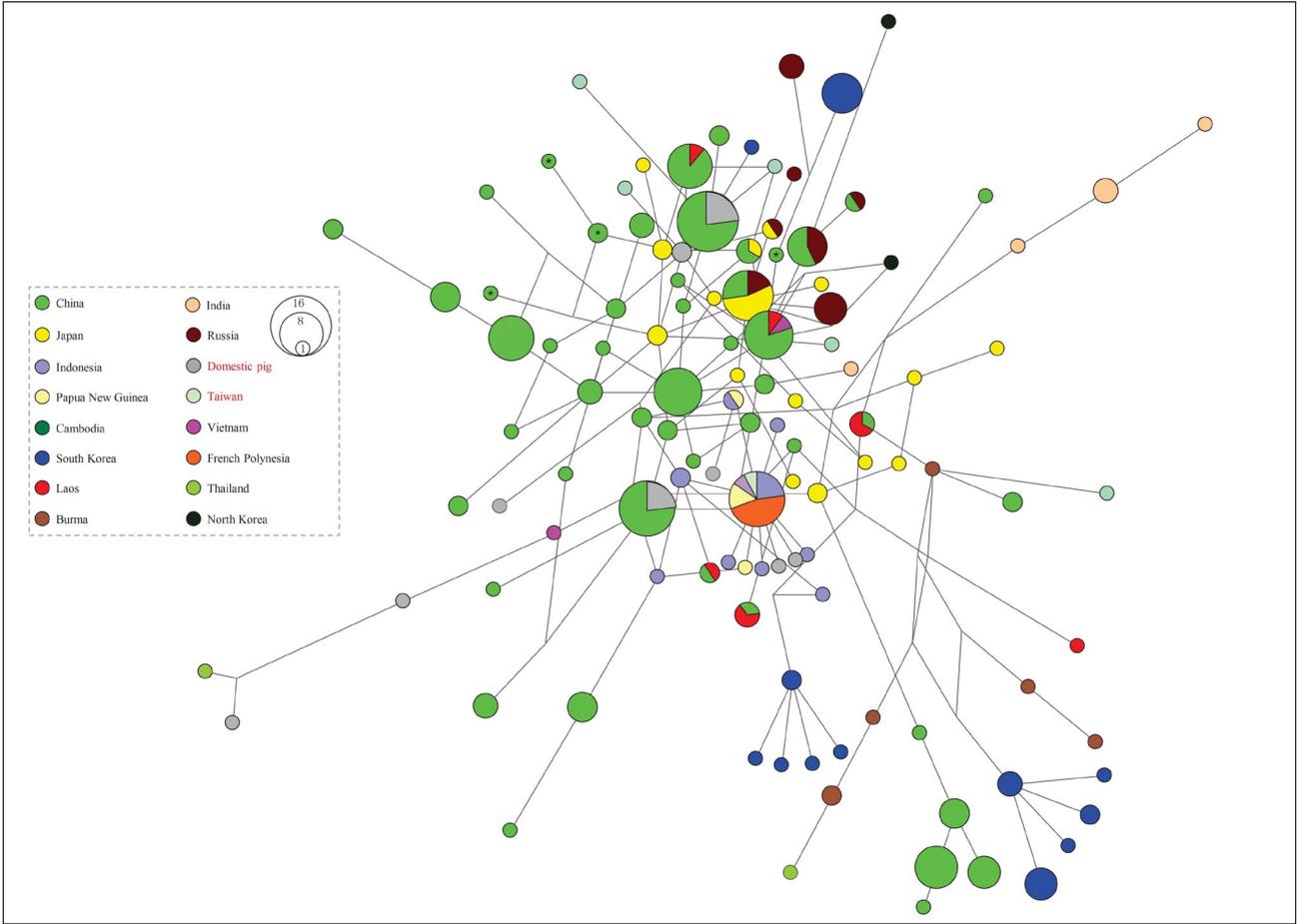


Fig. 2. Network profile of the mtDNA lineages based on mtDNA D-loop sequences. The size of the circle is proportional to haplotype frequency. Circle colors indicate different herds.

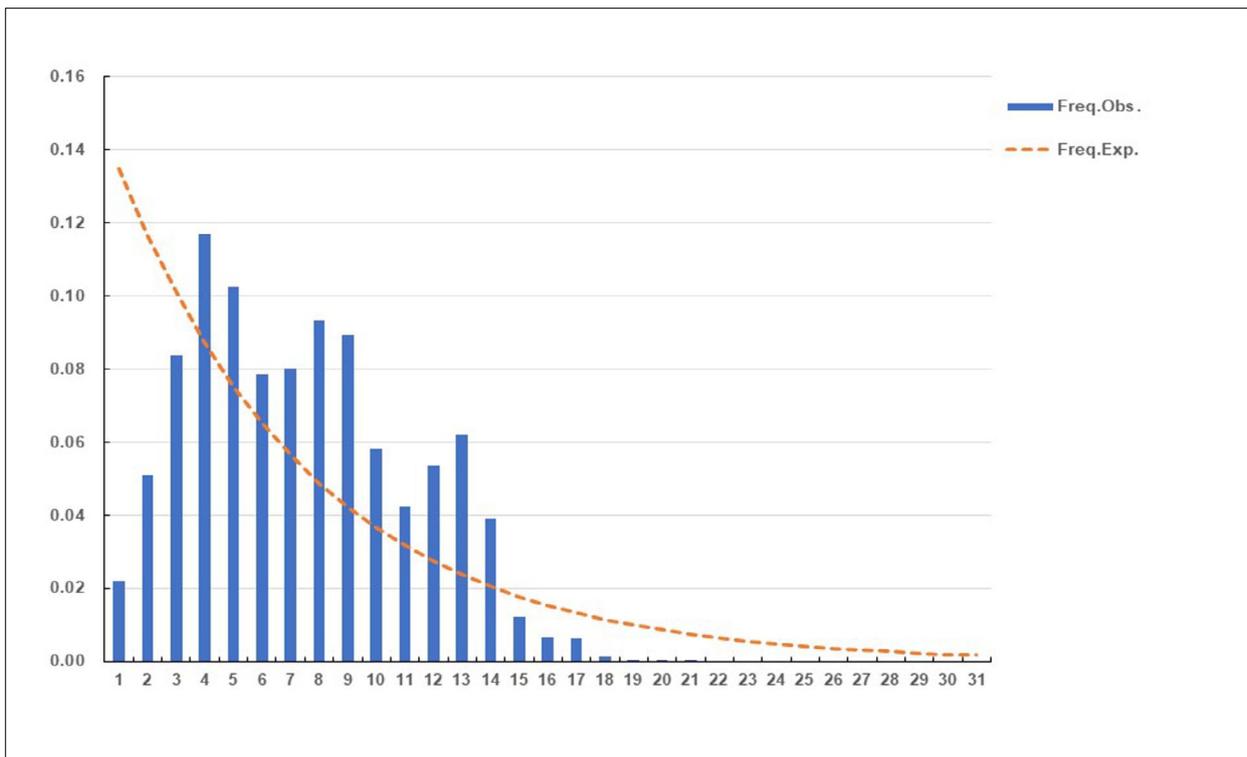


Fig. 3. Mismatch distributions of mtDNA sequences of the investigated pigs.

vergence compared with other breeds from abroad, which is consistent with previous reports analysing mitochondrial control region data from Chinese domestic pigs (Fang & Andersson 2006). As in the phylogenetic tree, we could not distinguish each subclade by a geographic definition, because there might have been a measure of introgression among those breeds during the breeding development. Unlike the distinct separation of domestic pigs and wild boars that was indicated in previous studies (Larson *et al.* 2005; Laval *et al.* 2000), the distribution of the Chinese domestic pig in the phylogenetic tree was dispersed among multiple breeds in the form of small branches. This phenomenon supports the belief that Chinese domestic pigs were derived from multiple Asian ancestral origins (Kim *et al.* 2002; Luetkemeier *et al.* 2010). A relative study suggested that the Chinese, Korean and Japanese breeds were separated in recent years, and that these breeds might have a close genetic distance (Yu *et al.* 2013); however, this issue was not proved with robust evidence, and the Chinese, Korean and Japanese breeds are all separated by other breeds. The Vietnamese wild boars are dispersed among the Chinese domestic breeds and wild boars, which indicates that the Vietnamese wild boars share a common ancestor with other East Asian pigs (s). The Chinese and Japanese wild boars jointly analysed in this paper were distributed almost together with each other, which indicates a close genetic relationship between them. On the other hand, the wild boars from Indonesia and Papua New Guinea formed a small cluster, while another small cluster was formed by the wild boars from Laos, Cambodia, Thailand, Burma and India, which might be caused by geographical isolation.

In this study, we discussed the genetic diversity and evolutionary origins of the wild boars and domestic pigs in East Asia in an effort to establish the main ancestors of Chinese domestic pigs, as well as the genetic relationships between Asian wild boars populations. However, due to the small sample size of some of the pig species, further studies within a larger population are needed to verify our findings in the present study.

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Author Contributions

Research concept and design: T.Z.; Collection and/or assembly of data: J.X.; Data analysis and interpretation: L.N., J.X., K.S.; Writing the article: L.N., T.Z.; Critical revision of the article: T.Z.; Final approval of article: L.N., T.Z.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary Material to this article can be found online at:

<http://www.isez.pan.krakow.pl/en/fovia-biologica.html>

SM.01. Information on the samples used in this paper.

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