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Full Length Research Paper

Mitochondrial genome of Taiwan pig *(SUS SCROFA)*

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The purpose of this study is to investigate the complete nucleotide sequence of the mitochondrial genome of the Taiwan Lanyu pig *(SUS SCROFA)* and its phylogenetic relationships with other pig breeds. Thirty-four forward and reverse primers were designed. Sequencing was performed in both directions. The results showed that, the complete sequence of the mitochondrial genome of the Taiwan Lanyu *(S. SCROFA)* is 16,747 bp, which was deposited in NCBI GenBank (accession number DQ518915). The complete sequence includes two rRNA (12S and 16S), 22 tRNA and 13 mRNA genes. The length of the D-loop region is 1,314 bp and there are 25 repeat sequences (5'-tacacgtgcg) in the region. It seems that there is a significant difference in the D-loop region between the Lanyu and the European Duroc (10 repeats), the landrace (13 repeats), the large white (6 repeats) or Asian pig breeds such as the Japanese wild boar (1 repeat) and the Ryukyu wild boar (1 repeat). The phylogenetic relationships of the Lanyu by comparing the sequence of the mitochondrial genome and the D-loop region of Asian and European pig breeds were investigated. It revealed that, the genetic distance of the Lanyu is high when compared with both the European and Asian pig breeds; the genetic exchange between the Lanyu and other breeds is not frequent. There is also no evidence of genetic exchange or introgression caused by population migration. Therefore, we conclude that the Lanyu is an independent branch among the breeds.

Key words: Complete genome, mitochondrial DNA, phylogenetic relationships, genetic distance, pig.

INTRODUCTION

Mitochondrial DNA has been widely used for evolution studies, because the evolution is more diversified than nuclear DNA (Brown et al., 1982, 1989; Luikart et al., 2001). For evolution research, a number of studies of the D-loop region sequence mutation in mitochondrial DNA have also been investigated (Watanabe et al., 1985; Lan and Shi, 1993; Huang et al., 1999; Gongora et al., 2004).

The Lanyu pig is an indigenous breed form the Lanyu Islet. The island is located at the southeast of Taiwan (Jiang et al., 2008). Since 1980, the Livestock Research Institute of Council of Agriculture in Taiwan introduced the Lanyu small-ear strain and from then on, the pig group became isolated and did not cross with other breeds. Lanyu pig skin colour and hair is black. The body weight at five months is less than 20 kg; mature pigs weight less than 70 kg. The purpose of this study is to sequence the complete mitochondrial DNA of the unique pig breed in Taiwan. We also studied the phylogenetic relationships of the Lanyu and other breeds from Europe, Japan, Korea and China, in order to investigate the migration of pig populations.

MATERIALS AND METHODS

Blood collection and DNA extraction

10 ml of blood was collected with anticoagulant from jugular vein of the Lanyu boar reared in the Livestock Research Institute, Council of Agriculture (Taitung Animal Propagation Station, LRI- Taitung). DNA extraction was performed according to the user manual of the kit (Puregene Gentra System, Taipei, Taiwan).

Primer design

Primers were designed according to the accession number

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Table 1. Primer pairs for pig complete mitochondrial DNA sequencing.

No.	Forward/reverse sequence	No.	Forward/reverse sequence
1	caaccaaaacaagcattccattcg/ggtttggcaaggcgttataggg	18	aatccaactaacatccaaac/ccagtgaagaacagaacc
2	aatcgcccactctttccc/tgcctgctttcgtagcac	19	cacatagtaaacccaagccc/catctacgaagtgtcagtatcag
3	tcattcaaaccccccttaccc/atgtgaagcaccgccaagtc	20	ccacttcacatccaaccaccac/agcttcgcaggctgcaaac
4	ccacgaaagtgactctaataatcc/cccttacggtactatctctatagc	21	acgaatgaacccaaaaag/tagtataaggggggaggag
5	tgggtactttgaaccaaagc/tgtttgccgagttccttttac	22	tctcacttaatatcttcactac/gtcgttctgtttggtttc
6	aagcctttctcctcgcacac/gaaaccgacctggattgctc	23	attataaccttcaccgccac/tgcaaacactcctaccactc
7	caactcaaccacaaagggataaaac/ggagattgaggatgtgcctg	24	tggcataatgcgaatcactac/cgattagattgatggatgggg
8	agaacagggcacattaggg/gctatgaagaatatggcgaaagg	25	cgtactgctaaaactcggagg/ggtagaatatgtagggctatgagg
9	ctctatcaaccctaatcacaacac/tccgattcagatgagtagtcag	26	acccccatccatcaatctaatc/ggactaggctgagagtgaag
10	agaggttcaaaccctcttatttc/ctctcaataggaggcctg	27	atcctacgccttcactctc/tctgttcgtccgtgtcatc
11	gagggctaaatcaaacccaac/ggatgggaacatagtcagtgag	28	ttcccgtagcactattcgtc/gctttatacagtccgcctatttttc
12	gcatcatcatgccaacactc/cagttaccaaaaccccccaatc	29	aatagtgacaatcggcatcaac/taggcggtgtatgatggagg
13	atgtaattgttacagctc/caactaaatacttttactcc	30	acaacacaacctactacc/ttcttctaagccctctcc
14	tcaccgtaggaatagacgtag/tggaaagggtaagccatagag	31	caataccacaaccaactccac/gcggtaatgatgaatggcag
15	gtttcaagccaacgtcataacc/tgtttctacttcttgggcatcc	32	cacacgattcttcgccttc/gccctccttttctggtttac
16	ccctatatgcctctatggcttac/ttaaaccgagggatgggac	33	gcctccatcttatacttcctaatc/gtacttggcgttttggtttg
17	aacctggagaaatacgac/tgaatgagtgtggtagtag	34	acactaacatgaattggaggac/atgcacgacgtacataggg

AF034253 of NCBI GenBank. A total of 34 primers were used (Table 1).

Fragment amplification by polymerase chain reaction (PCR) and nucleotide sequencing

A total volume of 25 ul of PCR mix consists of 2.5 µl PCR buffer (10 mM Tris-Cl, 50 mM KCl and 1.5 mM MgCl2), 1 ul of dNTPs, 0.5 µl of each forward and reverse primer (10 µM), 0.2 µl Taq (TaKaRa Taq DNA polymerase, 5 U/µl), 100 to 500 ng of DNA and 17.3 ul of 2 dH_2O. The PCR program followed was: pre-denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50 to 58°C based on the primer sequences for 45 s and an extension of 72°C for 45 s; then, a final extension at 72°C for 10 min. PCR products were purified according the manufacturer's instructions using a spin column and gel and a clean extraction kit (BioKit, Hsinchu, Taiwan). The PCR products were sequenced according to dideoxynucleotide chain termination method (Sanger et al., 1977). The sequences were aligned using the program Contigs express in Vector NTI Suite 8 (InforMax, Inc. Wesconsin, USA). Each sequence had overlap of at least 300 bp. Sequences were analyzed in both directions for confirmation.

Phylogenetic analysis

Mitochondrial DNA sequences of the Lanyu pig (DQ 518915) were compared with those of different pig breeds such as the large white (AY574048), Hampshire (AY574046), Berkshire (AY574045), landrace (AF034253), Duroc (AY337045), Italian wild boar (AF30-4201), Yucatan (AB015093), Meishan (D42181), Korean wild boar (AY574047), Jeju native pig (JNP8, DQ334860; JNP10, DQ33-4861), Japanese wild boar (AB015085), Ryukyu wild boar (AB015087) and the Okinawa native pig (AB015092) breeds in NCBI GenBank. Multiple alignments between our sequence and the literature ones were performed using BioEdit software and DAMBE software (Data Analysis in Molecular Biology and Evolution version: 4.5.2) and transfer Fas files were then, converted into a readable format of MEGA3.1 (Molecular Evolutionary Genetics Analysis, Version 3.1) using data analysis in molecular biology and evolution. Neighbor-joining methods and maximum parsimony methods were used to calculate the genetic distances and construct the phylogenetic tree (Saitou and Ne, 1987).

RESULTS

In present studies, the complete mitochondria genome of the Lanyu pig (16,747 bp) was sequenced and deposited in NCBI Genbank (Accession no. DQ 518915). The mitochondrial codons were also investigated. The complete sequence includes two rRNA (12S and 16S), 22 tRNA and 13 mRNA genes (Table 2).

Using neighbor-joining methods, we investigated the phylogenetic relationship of the mitochondrial genome of the Lanyu and other pig breeds (Figure 1). The results showed that the Berkshire, large white and the Korean wild boar belongs to the Asian type. In the Hampshire, landrace and Duroc breeds, the relationship is closer, which were called European type. The Lanyu was classified as a new out-group. As the Lanyu belongs to a breed that is unique in the Taiwan islands, the question as to whether there is a phylogenetic relationship between the Lanyu and other small-ear strains is worthy of study. By searching the sequences of pigs, the Lanyu is found to be independently grouped compared with the Korean wild boar and European breeds. This study has shown that, the Lanyu breed is an independent out-group and is distant to any other breeds. Also using neighbor-joining methods, we analysed the phylogenetic relationship of the mitochondrial D-loop region in the Lanyu and other pig breeds (Figure 2). The Lanyu is an independent group from the two and is a separate out-group. Its phylogenetic relationship with any other pig breeds is far from

Table 2. Location of features in the mitochondrial genome of the Lanyu pig (*S. scrofa*).

Name of gene	Location	Size (bp)		Start codon	Stop codon
D-loop	1–1314	1314			
Repeat region	706–955	250			
tRNA-Phe	1138–1207	70			
12S-rRNA	1208–2163	956			
tRNA-Val	2164–2231	68			
16S-rRNA	2232–3794	1563			
tRNA-Leu(UUR)	3801–3875	75			
NADH1	3878–4837	960		ATG	TAG
tRNA-Ile	4836–4900	65			
tRNA-Gln	4901–4973	73	L[a]		
tRNA-Met	4975–5044	70			
NADH2	5045–6088	1044		ATT	TAG
tRNA-Trp	6087–6154	68			
tRNA-Ala	6161–6227	67	L		
tRNA-Asn	6230–6304	75	L		
Or. L-stand repl.	6301–6347	47			
tRNA-Cys	6337–6402	66	L		
tRNA-Tyr	6402–6467	66	L		
COI	6469–8013	1545		ATG	TAA
tRNA-Ser (UCN)	8017–8087	71	L		
tRNA-Asp	8093–8160	68			
COII	8161–8848	688		ATG	T--[b]
tRNA-Lys	8849–8915	67			
ATPase8	8917–9120	204		ATG	TAA
ATPase6	9078–9758	681		ATG	TAA
COIII	9758–10541	784		ATG	TA-[b]
tRNA-Gly	10542–10610	69			
NADH3	10611–10956	346		ATA	TA-
tRNA-Arg	10958–11026	69			
NADH4L	11027–11323	297		GTG	TAA
NADH4	11317–12694	1378		ATG	T--
tRNA-His	12695–12763	69			
tRNA-Ser (AGY)	12764–12822	59			
tRNA-Leu (CUN)	12823–12892	70			
NADH5	12893–14698	1716		ATA	TAA
NADH6	14698–15225	528	L	ATG	TAA
tRNA-Glu	15226–15294	69	L		
Cyt b	15299–16438	1140		ATG	AGA
tRNA-Thr	16439–16506	68			
tRNA-Pro	16507–16747	64	L		

a: (L), light-strand sense; NADH1–6 and NADH4L, subunits 1 to 6 and 4 L of nicotinamide dinucleotide dehydrogenase; ATPase6 and 8, subunits 6 and 8 of adenosine triphosphatase; COI to COIII, cytochrome c oxidase subunits I to III; cyt b, cytochrome b. b: TA-and T--: TNN indicates the incomplete stop codon, that is, amino acid translation is terminated when the gene forms a stop codon by post-transcriptional polyadenylation.

European type, but its genetic distance is closer to the Asian type. Therefore, it can be counted as a cluster. Pairwise sequence distances were used to analyse the genetic distance of the D-loop region among the pig breeds, which showed that the Lanyu is an independent branch as the genetic distance is far from other pig breeds. In descending order, the Lanyu has the biggest genetic distance with the landrace (0.102), Hampshire (0.098), Duroc (0.097), Mexican Yucatan, Italian wild boar and Jeju native pig (JNP10) (0.095), large white (0.092),

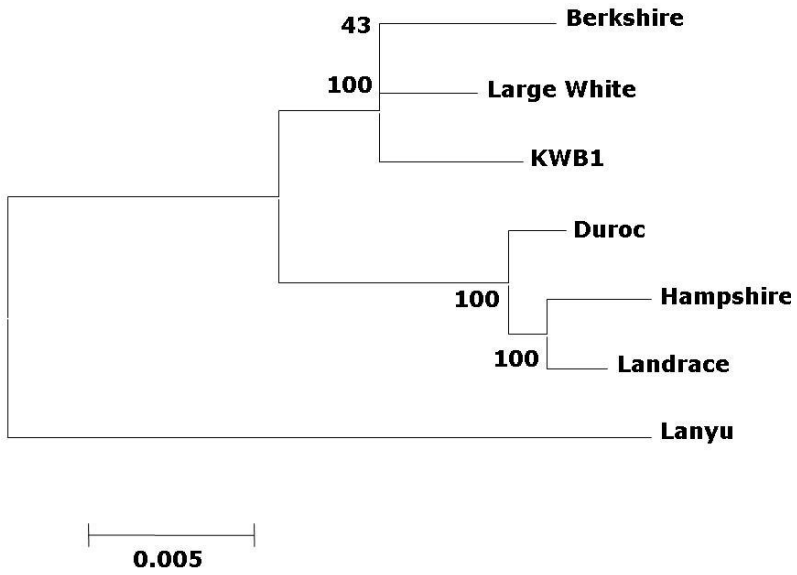

Figure 1. The phylogenetic relationship was analysed using neighbor-joining methods by comparison of the mitochondrial genome sequences of the Lanyu and other breeds. KWBI, Korean wild boar.

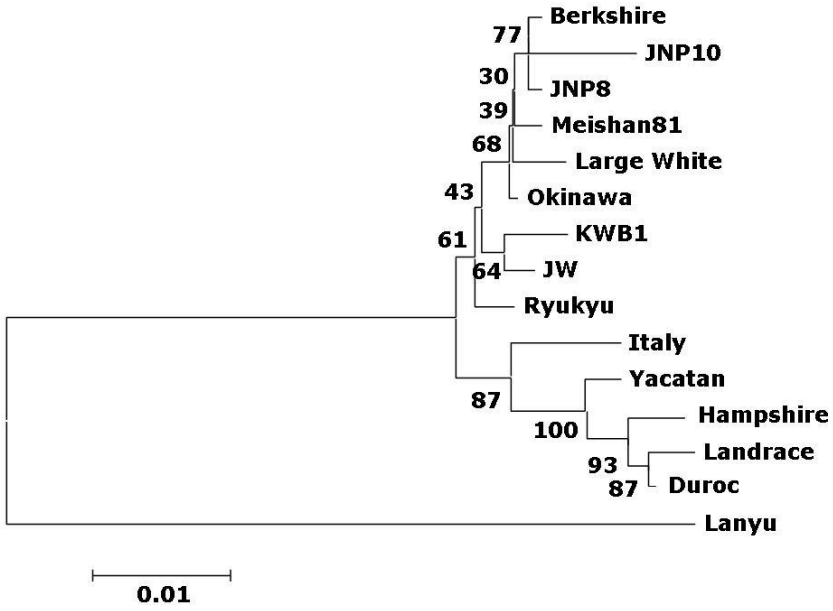

Figure 2. The phylogenetic relationship was analysed using neighbor-joining methods by comparison of the mitochondrial D-loop region of different strains of pig breeds. (1) JNP8, JNP10, Jeju native pig; (2) KWBI, Korean wild boar; (3) JW, Japanese wild boar.

Korean wild boar (0.091), Meishan (0.090), followed by the Berkshire, Jeju native pig (JNP8), Japanese Ryukyu wild boar (0.088). The shortest genetic distance is with the Japanese Okinawa native pig (0.087). Of all the 15 pig breeds, the largest distance from the Lanyu is the landrace (0.012), while the smallest is the Berkshire and Korean Jeju native boar (JNP8) (0.002).

DISCUSSION

The complete sequence of Lanyu pig contains two rRNA (12S and 16S), 22 tRNA and 13 mRNA (Table 2). There are four overlapping regions in the pig mitochondria mRNA and seven base pairs overlapping on NADH4L with NADH4. ATPase6 has 43 and 1 bp overlapping with

ATPase8 and COIII, respectively. As these genes were from the same transcript in the mammals, there is a 1 bp frame shift during transcription. There are 1 bp overlapping in NADH5 and NADH6 and as they are transcribed from different strands of DNA, there is no frameshift. There are 11 mRNA genes starting the amino acid methionine in the Lanyu; these were transcribed from 9 ATG and 2 ATA codons, respectively. The initiating codons of the other two genes were transcribed from ATT (Isoleucine) and GTG (Valine). There are four proteins (CO II, CO III, NADH3 and NADH4) in the stop codons; the stop codon is not TAA and it is different from that in other breeds. The length of the mitochondrial D-loop region of the Lanyu is 1,314 bp. Twenty-five repeat sequences (5'-tacacgtgcg) in this region were found. There are obvious differences between the Lanyu and other breeds, such as the Duroc (10 repeats, AY337045.), landrace (13 repeats, AF034253), large white (6 repeats, AY574048), Japanese wild boar (1 repeat, AB015085) and the Ryukyu wild boar (1 repeat, AB015087). We hypothesized that; these 25 repeat sequences in the D-loop region can easily form a hairpin structure in the com-plementary sequences. Therefore, the copying process may easily introduce errors (Mackay et al., 1993). Similar cases occur in other mammals, such as the rabbit (Dufresne et al., 1996), horse (Xu and Arnason, 1994), seal (Arnason and Johnson 1992), cat (Lopez et al., 1996) and sheep (Zardoya et al., 1995). Many studies have also shown that, the D-loop region has the highest mutation rate in mitochondria. It is thought to be an im-portant region in the analysis of phylogenetic relation-ships and evolution of breeds.

The Lanyu pig is the small ear breed and Lanyu lie in the off-shore island position of Taiwan. The people living there rely mainly on the fact that the aboriginal reaches the Yami of Botel Tobago. Its development and the way Taiwan and other aboriginals of this island build relevantly, have apparent difference. This study analysed the phylogenetic relationship of Lanyu pigs and other pig breeds. Figure 1 showed that, the Berkshire and large white both belong to the Asian type, a similar finding to that of Kim et al. (2002). In Kim's study, the SNPs in the mitochondrial D-loop region were analysed and it was found that, Chinese mainland pig breeds originated from Southeast Asia. In addition, they found that the Berkshire and large white were both of the Asian type. Chinese mainland, Korean and Japanese local breeds have significant differences to the European type. We therefore deduced that, the European breeds may have originated from more than one breed. Sequencing of both the complete mitochondrial genome and the D-loop region has given the same result that the Berkshire and large white belong to the Asian type. Kijas and Andersson (2001) used the phylogenetic relationship to analyse the mitochondria genome of four pig breeds in order to study the origin of domesticated pigs. Using five pig breeds, including the Chinese Meishan, Italian wild boar, Sweden landrace and wild boar, they categorized the origin into three groups, A, E1 and E2. The genetic difference of these three groups is between 0.8 and 1.2%. Branches A (Chinese Meishan) and E1 (European domesticated pigs) were probably separated 900,000 years ago. Long term domestication started from about 9000 years ago. Recent studies have shown that, branch A includes some of the major European breeds, such as the landrace and large white. It is speculated that Asian breeds were introduced into Europe by introgression in the 18 and 19th centuries.

In present studies, both neighbor-joining methods and maximum parsimony methods showed that, the Lanyu is of an independent branch in the phylogenetic analysis of both the mitochondrial complete genome and the D-loop region (Figures 1 and 2). In 1980, the Livestock Research Institute in Taiwan introduced four boars and sixteen sows. They were then isolated and did not introgress with other breeds. In order to preserve the genetic resources of the local pig breeds, the Taiwanese government listed Lanyu pigs as a member of the Taiwan conservation population. This study contributes to the knowledge of phylogenetic relationships of the Lanyu with other pig breeds, which can further be applied to other pig breeds in the world. The Lanyu is of an independent branch but probably still belongs to the Asian type. This result implies that, the frequency of genetic exchange of the Lanyu with other pig breeds is low and they therefore do not have a close relationship. This result also confirms that, after the introduction of the Lanyu into East Taiwan by the Live-stock Research Institute, there was no cross with other pig breeds, the Lanyu therefore becoming an isolated population with a unique genetic combination. Whether the Lanyu has a genetic relationship with the Taiwan wild boar remains to be investigated. From ethnological and archaeological research, anthropologists have found that the Tao people of Lanyu Island migrated from Batan Is-land, of the North Philippines. We hypothesize that, Lanyu pigs were introduced onto Taiwan's Lanyu Island by the early Tao people from the Batan Island of the Philippines.

Kim et al. (2002) used the D-loop region to analyse the Jeju, Chinese, Japanese, European and Mexican Yucatan breeds. They indicated that, the Berkshire and large white were of Asian breeds. The Chinese, Japanese and Korean local breeds were separated in recent years and based on some limiting factors; their classification may differ from the European type. By studying 48 local breeds, China mainland studies have shown that many breeds originated from Southeast Asia (Kim et al., 2002). Paszek et al. (1998) also calculated that, the genetic difference of the Chinese Meishan and European breeds probably originated around 2227 years ago.

Watanabe et al. (2003) collected 180 samples from 10 populations in Shikoku, Kyushu and Honshu in Japan. They used neighbor-joining methods to analyse 574 bp of the mitochondria region and found that, the ancestors of the Japanese wild boar migrated from southeast to northeast Asia in the Pleistocene period. They deduced that,

the Japanese wild boar probably originated in Mongolia. *Sus scrofa* are widely distributed in Asia, Europe and North Africa and their ancestors were processed into at least 16 subspecies. Fossil records show that, wild boars existed in the Pleistocene period in Japan and that the Japanese wild boar existed in Shikoku, Kyushu, Honshu and Ryukyu. Kijas and Andersson (2001) found that, the difference between the Sweden wild boar and Meishan is 1.21 ± 0.09%. This study found that, the phylogenetic relationship of the Ryukyu wild boar and Lanyu is similar to other breeds in the D-loop domain. As Taiwan is close to Ryukyu Island, the appearance of the Ryukyu wild boar is similar to that of the Lanyu pigs. The Ryukyu wild boar exists in the Japanese Amami Islands, Okinawa Main Island, Ishigaki Island and Iriomote Island. The Ryukyu wild boar is small, with a dark brown to black hair colour and a thickset body and is nocturnal and omni-vorous. It belongs to the early Asian wild boar branch.

Luetkemeier et al. (2010) indicated that, Asian domestic populations were derived from multiple Asian ancestral origins whereas the European domestic populations represent a single ancestral European lineage. The complete mitochondrial genome of Taiwan Lanyu pigs was sequenced; it is a reference for the sequence difference and phylogenetic relationship of other small-ear strains in the world. Analysis showed that, the Lanyu could be an independent branch among the other pig breeds. Its far genetic distances with other Asian and European breeds suggest that, the Lanyu did not have a frequent genetic exchange with other breeds. Therefore, there is no genetic exchange and cross between the Lanyu and other breeds.

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