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Interpopulation and intrapopulation maternal lineage genetics of the Lanyu pig (*Sus scrofa*) by analysis of mitochondrial *cytochrome b* and control region sequences[1]

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ABSTRACT: The Lanyu pig is an indigenous breed from the Lanyu Islet, which is southeast of Taiwan. Two herds of Lanyu pigs were introduced from the Lanyu Islet into Taiwan in 1975 and 1980. The current population of conserved Lanyu pigs consists of only 44 animals with unknown genetic lineage. The Lanyu pig possesses a distinct maternal genetic lineage remote from Asian and European pigs. The present study aimed to understand the phylogenetic relationship among conserved Lanyu, Asian, and European type pigs based on the *cytochrome b* coding gene, to ascertain the maternal lineage and genetic diversity within the conserved Lanyu pigs, and to address whether genetic introgression from exotic or Formosan wild pigs had occurred in the conserved Lanyu pigs. Entire mitochondrial genomes of both types of Lanyu pig comprised 2 ribosomal RNA, 22 transfer RNA, and 13 protein-coding genes. Only 2 haplotypes of the mitochondrial DNA (mtDNA) control region and *cytochrome b* were identified in the conserved Lanyu pig herds. When maximum likelihood trees were constructed, the Type I Lanyu mitochondrial genes formed a unique clade with a large pairwise distance of both *cytochrome b* and the control region from Asian and European type breeds, Formosan wild pigs, and exotic breeds. Significant loss of genetic diversity of mtDNA within the conserved Lanyu pigs was demonstrated by low haplotype and nucleotide diversities, supported by Fu and Li's D* neutrality test (1.44055; $P < 0.05$). The mtDNA control region sequences of extant pigs in the Lanyu Islet, however, showed high haplotype and nucleotide diversity, and clustered with exotic pigs. These results indicate no maternal lineage mtDNA gene introgression from Formosan wild pigs and introduced exotic pigs to conserved Type I Lanyu pigs, and a severe loss of heterozygosity of mtDNA in conserved Lanyu pigs. The remaining extant pigs on the Lanyu Islet have been introgressed with exotic breeds. Strategies for future conservation of native Lanyu pigs are now even more urgent and important.

Key words: control region, *cytochrome b*, genetic diversity, Lanyu pig, mitochondrial DNA, phylogenetic relationship

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INTRODUCTION

The Lanyu pig is an indigenous miniature pig breed with a black coat color inhabiting the Lanyu Islet near southeast Taiwan (Figure 1). Two herds of Lanyu pigs were transferred to Taiwan for conservation purposes before 1980 (Chyr et al., 2001). On the basis of the polymorphism of the mitochondrial DNA (**mtDNA**) control region, only 2 haplotypes of the control region have been identified in all the conserved Lanyu pigs. The Lanyu pig, with one haplotype (Type I), possesses a unique maternal genetic lineage distinct from Asian and European breeds, whereas Lanyu pigs of the other haplotype (Type II) are clustered in the major Asian pig clade (Wu et al., 2007), indicating that the original Lanyu pig possesses a unique genetic background.

The Formosan wild pig (*Sus scrofa taivanus*) is native to Taiwan (Chao and Fang, 1988). Some traits of

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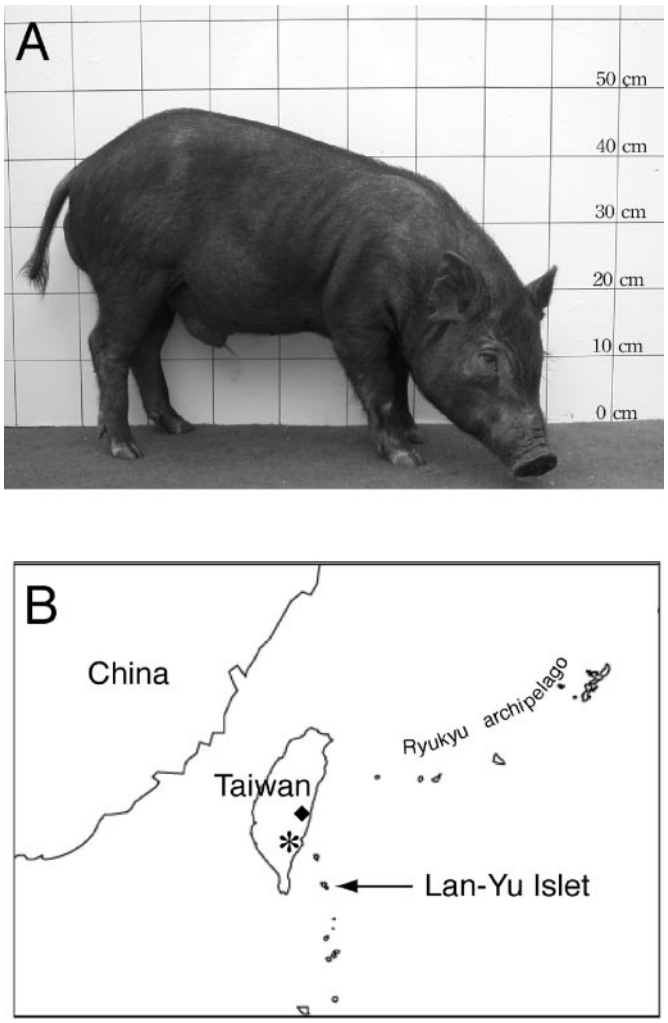

Figure 1. The Lanyu Islet pig breed. (A) A representative 5-mo-old male Lanyu pig. (B) Location of Taiwan and the Lanyu Islet. The diamond (♦) and asterisk (*) represent the location of Hualien and Taitung counties, respectively.

Formosan wild pigs show striking phenotypic differences from Lanyu pigs, but their body conformation and small erect ears are similar. The extent of the genetic relationship between the Lanyu and Formosan wild pig is unknown. In addition, the degree and pattern of introgression of genetic material from the exotic breeds introduced into Lanyu pigs is also currently unknown.

Because the population of conserved Lanyu pigs stood at only 44 animals in 2006 (Wu et al., 2007), this study was undertaken to assist in the future genetic conservation and recovery of this unusual breed. The phylogenetic relationship among conserved Lanyu, Asian, and European pig breeds was determined by analysis of the polymorphism of their *cytochrome b* (***Cytb***) sequences. The genetic diversity within the conserved population, and the presence of any genetic affinity among conserved Lanyu pigs, Formosan wild pigs, and exotic breeds in Taiwan, and among pigs currently extant in the Lanyu Islet were investigated by the diversity of mtDNA. We were also interested in whether any maternal mtDNA genetic introgression had occurred previously between the conserved Lanyu pig and the Formosan wild pig.

MATERIALS AND METHODS

The animal research protocols conformed to those approved by the National Taiwan University Animal Care and Use Committee.

Sample Collection and Preparation of mtDNA

Conserved herd Lanyu pig blood samples were collected from all 39 pigs from the Taitung Animal Propagation Station (**TAPS**) and from all 5 pigs from the National Taiwan University (**NTU**) teaching farm. Blood samples of exotic pigs were obtained from 4 Meishan, 12 Taoyuan, 10 Berkshire, 14 Landrace, 10 Yorkshire, and 5 Duroc pigs from reference stocks at the Taiwan Livestock Research Institute (**TLRI**). Twelve current Lanyu pig blood samples were collected separately from pigs of 6 aboriginal tribes on the Lanyu Islet. Five Formosan wild pig blood samples were obtained from Taitung and Hualien counties (Figure 1). The mtDNA were extracted and purified from platelet-rich plasma by using Qiagen's QIAamp DNA mini kit (Qiagen, Valencia, CA). The quality of purified mtDNA was evaluated via electrophoresis on a 1% agarose gel.

Primer Design and Amplification of mtDNA Fragments by PCR

Entire sequences of the mtDNA control region were amplified by PCR in an MJ thermal cycler (MJ Research, Waltham, MA) using the following primers: L1, 5′-CCAAGACTCAAGGAAGGAGA- 3′ (sequence of position 16,542 to 16,561 of pig mtDNA, GenBank accession number AF034253) and H1, 5′-GGCGCGGATACTTGCATGTG-3′ (position 1,290 to 1,309). The entire sequence of *Cytb* was amplified by using the following primers: CbR1, 5′-GTCCTGCCCTGAGGACAA-3′ (position 15,735 to 15,752); CbL1, 5′-GGTGCTG ATGGCGGAGTT-3′ (position 16,581 to 16,564); CbR2, 5′-CCAAGACTCAAGGAA GGAGA-3′ (position 15,363 to 15,382); and CbL2, 5′-GGCGCGGATACTTGCATGTG-3′ (position 115 to 134). Thermal cycling was conducted in 50-μL volumes using the FastStart High Fidelity PCR system (Roche, Penzberg, Germany), each containing 1 ng of mtDNA, 10-m*M* Tris-HCl (pH 8.3), 1.8-m*M* $MgCl_2$, 0.4-μ*M* each primer, 200-μ*M* each dNTP, and 2 units of FastStart polymerase. Thermal cycling parameters were as follows: 95°C for 5 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 80 s, with a final extension at 72°C for 4 min. The resultant PCR products were then purified by using the PCR-M cleanup system (Viogene, Taipei, Taiwan). The complete control regions were sequenced in both directions by using the following primers: L1; H1; L2, 5′-CCTAT-

GTACGTCGTGCATTA-3′ (position 160 to 179); L3, 5′-TACTTCAGGACC ATCTCACC-3′ (position 434 to 453); H2, 5′-AGTGTAAGTTAGGCTTATTG-3′ (position 963 to 982); and H3, 5′-TTGTGGTAGATTGGCG-TAAA-3′ (position 1,072 to 1,091). The *Cytb* fragments were bidirectionally sequenced by using the following primers: CbR1, CbL1, CbR2, and CbL2.

All sequences were determined by using an Applied Biosystems 3730 DNA sequencer and analyzed with SeqEd software (Perkin-Elmer, Applied Biosystems, Foster City, CA). Full sequences of the control region and *Cytb* were generated by overlapping forward and reverse sequences with SeqEdit software (DNASTAR, Madison, WI; see Hein and Støvlbaek, 1996). The following reference mtDNA control region sequences were obtained from NCBI GenBank: 6 Yorkshire (AM040633 to AM040638), 4 Meishan (I, AY230821; II, AY230827; III, D17739; and IV, AB041474), 6 Taoyuan (I, AM040641 to AM040645; and II, AM040646), 1 Hampshire (AY574046), 1 Berkshire (AM040639), 4 Landrace (AM040613 to AM040616), and 6 Duroc (AM040623 to AM040628) pigs, a Japanese wild pig (AB015085), a Ryukyu wild pig (AB015087), and an Italian wild pig (AB015094). All of the above sequences (except Meishan and Hampshire pigs, and Japanese, Ryukyu, and Italian wild pigs) came from pigs reared in isolation at the TLRI. The *Cytb* sequences were also obtained from the National Center for Biotechnology Information (**NCBI**) GenBank, comprising 5 Ryukyu wild pigs (AB015071 to AB015075), 6 Japanese wild pigs (AB015065 to AB015070), Satsuma (AB015076), Erhualian (AF486861), Tongcheng (AF486862), Wannanhua (AF486873), Taoyuan (DQ534707), Meishan (AB015077), Diannan (AF486869), Large White (AF486874), Berkshire (AY574045), Landrace (AF034253), Duroc (AY337045), Hampshire (AY574046), Yucatan miniature (AB015081), and 2 Italian wild pigs (AB015082 to AB015083; Watanobe et al., 1999; Kim et al., 2002; Yang et al., 2003).

Analysis of the Full-Length mtDNA Genome

The Type I and II mtDNA control regions of Lanyu pigs were identified after their mtDNA was obtained. Twenty pairs of primers were designed according to the Landrace mitochondrial genomic sequence AF034253, and the annealing conditions of PCR were as listed in Table 1. The locations of primers for the full mitochondrial genome sequencing are listed in Supplemental Table 1 (available online at http://jas.fass.org; doi:10.2527/jas.2007-0049). The PCR was performed by using the Long PCR Enzyme Mix (Fermentas, Hanover, MD) in 50-μL volumes with the following parameters: 94°C for 5 min; 32 cycles of 94°C for 30 s, annealing for 30 s, and 68°C for 90 s; with a final extension at 68°C for 10 min. The resultant PCR products were then purified by using the PCR-M cleanup system (Viogene) before direct sequencing was performed. All the amplified sequences were confirmed by bidirectional sequencing.

Table 1. Polymerase chain reaction primers and annealing conditions

Primer pair	Locations in AF034253[1,2]	Annealing temperature, °C
1F/1B	432 to 451/1,398 to 1,381	55
2F/2B	1,313 to 1,332/2,401 to 2,377	55
3F/3B	2,199 to 2,216/3,278 to 3,259	55
4F/4B	3,150 to 3,167/4,112 to 4,095	55
5F/5B	3,983 to 4,002/4,846 to 4,829	55
6F/6B	4,754 to 4,771/5,849 to 5,833	55
7F/7B	5,625 to 5,642/6,893 to 6,876	55
8F/8B	6,579 to 6,597/7,736 to 7,716	55
9F/9B	7,629 to 7,646/8,685 to 8,668	55
10F/10B	8,584 to 8,603/9,568 to 9,587	55
11F/11B	9,437 to 9,457/10,418 to 10,401	62
12F/12B	10,002 to 10,019/10,879 to 10,862	55
13F/13B	10,515 to 10,535/11,584 to 11,571	55
14F/14B	11,530 to 11,547/12,592 to 12,569	55
15F/15B	12,459 to 12,477/13,381 to 13,361	55
16F/16B	13,057 to 13,075/14,156 to 14,138	55
17F/17B	14,089 to 14,106/15,057 to 15,038	55
18F/18B	14,930 to 14,949/15,843 to 15,825	55
19F/19B	15,735 to 15,752/16,581 to 16,564	55
20F/20B	16,485 to 16,504/1,398 to 1,381	65

[1]The first nucleotide of the control region in H-strand is designated as nucleotide position 1.

[2]The nucleotide position numbers of each primer correspond to those in the Landrace pig control region sequence AF034253.

Data Analyses

In the analysis of pairwise distance of the control regions, the tandem repeat motif CGTGCGTACA, with a variable number of repeats in individuals, and the Type I and II Lanyu-specific repeat motifs (ACACAAACC and TAAAACACTTA, respectively) in the mtDNA control region were excluded from the analysis (Wu et al., 2007). Sequence alignment of the control region and *Cytb* was performed by using MegAlign multiple alignment software (DNASTAR; Hein and Støvlbaek, 1996). Haplotype and nucleotide diversities within the conserved Lanyu pigs, exotic pigs, and extant pigs on the Lanyu Islet were obtained with DNA Sequence Polymorphism (DnaSP) software version 4.10.9 (Rozas et al., 2003). The PHYLIP program package (version 3.66) was used to obtain the maximum likelihood (**ML**) phylogenetic tree (Felsenstein, 2006). Tree-Puzzle (version 5.0) software, based on the quartet puzzling method, was used to analyze confidence intervals of the phylogeny and likelihood-mapping analyses (Schmidt et al., 2002). The significance of the difference among pig groups was tested by using 10,000 permutations in the quartet puzzling algorithm (Strimmer and von Haeseler, 1996). For the ML analysis, Modeltest version 3.6 software (Posada and Crandall, 1998) was used to determine the best fit model of the data, including nucleotide composition, substitution matrix among nucleotides, and proportion of invariant sites. Nodal supports of the ML tree were evaluated by bootstrap resampling (1,000 replications) using the fast heuristic search al-

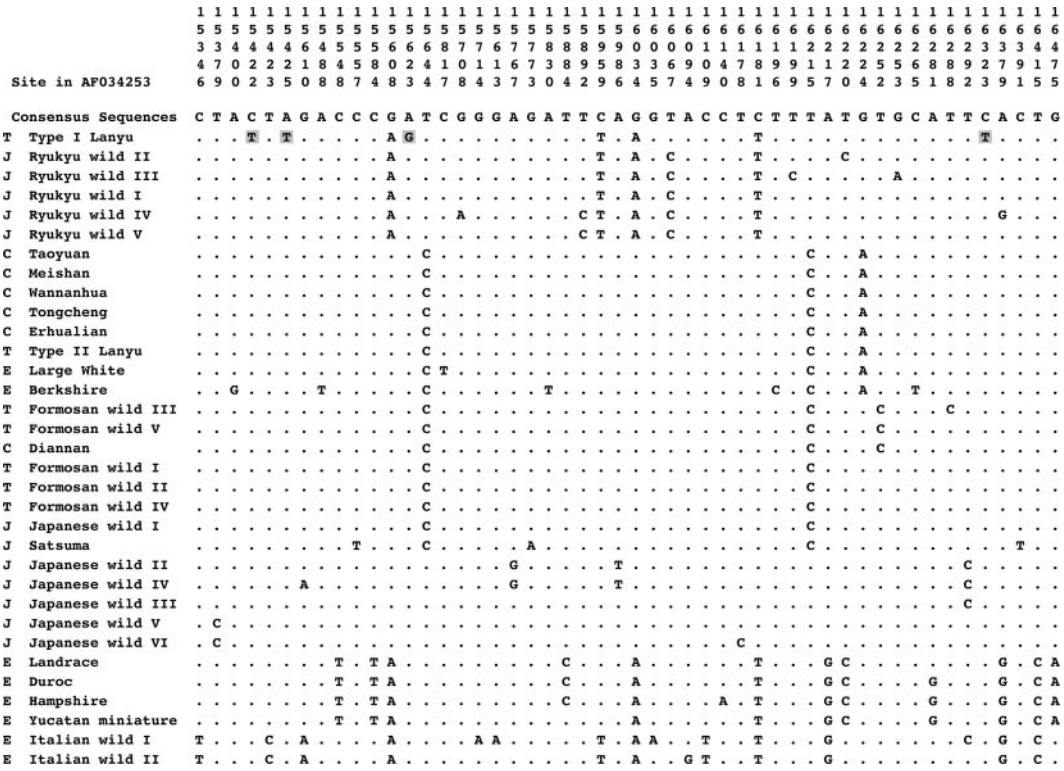

Figure 2. Variable sites of the mitochondrial DNA *cytochrome b* (*Cytb*) sequences of Lanyu, Asian type, and European type pig breeds. The sequence position number (given in the first row) follows those in the *Cytb* sequence of the Landrace breed (accession number, AF034253). Only variable sites in the *Cytb* of these animals, with the sequence positions given above, are shown. Abbreviations in the leftmost column indicate the geographical origins of these animals: T, Taiwan; J, Japan; E, Europe; and C, China. Nucleotides identical to the *Cytb* consensus sequence are denoted by a dot (•). Nucleotides with gray blocks indicate the nucleotide specifically found in the Type I Lanyu haplotype.

gorithm implemented in software PAUP4.0b10 (Swofford, 2002). Fu and Li's D* neutrality test values were determined with DnaSP software.

RESULTS AND DISCUSSION

Variation in mtDNA Control Regions and Cytochrome b Sequences in Conserved Lanyu Pigs

We had previously studied the phylogenetic relationship among conserved Lanyu pigs and 43 Asian and European pig breeds based on the pairwise distance of the mtDNA control region (Wu et al., 2007). Only 2 control region haplotypes were identified in all conserved Lanyu pigs in Taiwan. The Lanyu pigs reared at TAPS had a mixture of Type I (28 head) and Type II (11 head) mtDNA haplotypes, whereas all pigs (5 head) at the NTU teaching farm had the Type I haplotype. Here, to determine whether the sequence polymorphism of the *Cytb* coding gene matched that seen in the noncoding control region, the variation in *Cytb* sequences among the conserved Lanyu pigs was analyzed. Mitochondrial DNA *Cytb* sequences were obtained from all 44 Lanyu pigs kept at TAPS and NTU. Consistent with the control region analyses, all mtDNA *Cytb* sequences of the Lanyu pigs from TAPS and from the teaching farm at NTU could be categorized into 2 types (Figure 2). The phenotypic conformation of both types of Lanyu pigs was indistinguishable. All 5 of the Lanyu pigs at the NTU teaching farm had the same *Cytb* haplotype (Type I), whereas 2 haplotypes were found in the Lanyu herd at TAPS (Figure 2). In the mtDNA control region, 18 polymorphic sites (2 haplotypes), including 17 transitions and 1 transversion, were found by Wu et al. (2007) in the conserved Lanyu pigs. An additional 10 transitions and 1 transversion in *Cytb* were found in the same populations in the present analysis (Figure 2; Supplemental Figure S1, available at http://jas.fass.org). Based on the diversity of *Cytb* sequences, Fu and Li's D* test for neutrality was used to estimate the genetic variation within the population of conserved Lanyu pigs, and the test value was determined to be 1.44055 ($P < 0.05$), indicating significant deviation from neutrality. Among all 44 conserved Lanyu pigs, the low haplotype numbers and nucleotide diversities shown by DnaSP software analysis ($h = 0.384 \pm 0.066$; $\pi = 0.0037 \pm 0.00064$) indicated severe genetic drift. This confirmed that there has indeed been a significant loss of heterozygosity of mtDNA haplotypes in the population of all conserved Lanyu pigs, probably as a result of the small population size and uncontrolled mating in

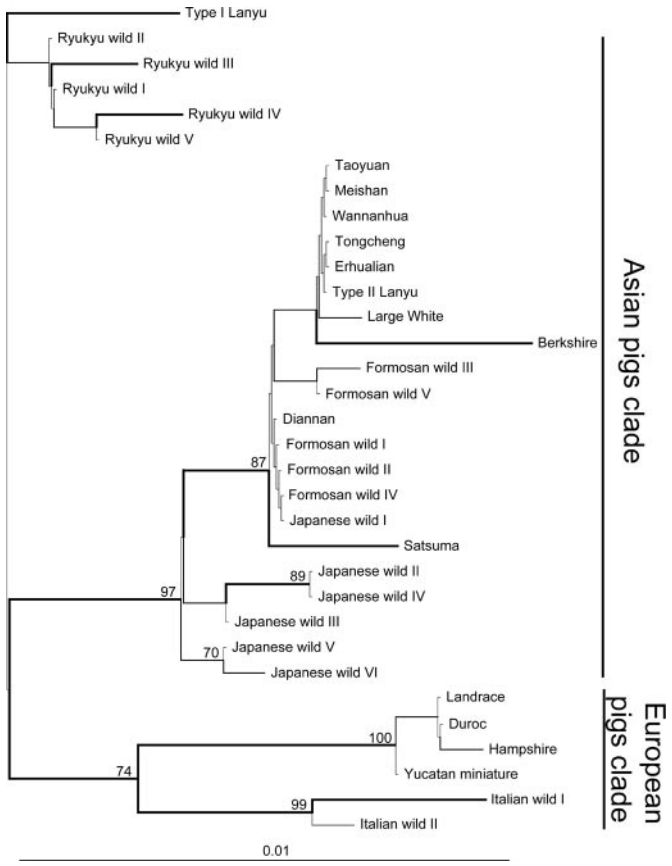

Figure 3. Phylogenetic tree of *cytochrome b* (*Cytb*) of conserved Lanyu, Asian, and European pig breeds. The phylogenetic tree was constructed on the basis of maximum likelihood distances of polymorphism of *Cytb* sequences by using the PHYLIP program package. Those branches with highly significant and significant confidence are shown by bold and midweight lines, respectively. Numbers on the branches are bootstrap values based on bootstrap resampling (1,000 replications).

the past. Additionally, studies of gene frequency and genotypes of 19 microsatellite markers revealed that severe nuclear gene drift has occurred in the conserved Lanyu pig population (Cheng et al., in press).

Lanyu mtDNA Type I Cytb Is Genetically Remote from Asian and European Breeds

We had shown earlier that the Lanyu Type I mtDNA control region sequence was genetically remote from Asian and European breeds (Wu et al., 2007), and we were interested in whether the *Cytb* coding region was similar in distance to the noncoding control region, and whether gene introgression had occurred between Lanyu and Formosan wild pigs. The *Cytb* sequences of Asian and European pig breeds were obtained from the NCBI database (see the Materials and Methods section for details). In addition, the *Cytb* sequences of 5 Formosan wild and 44 conserved Lanyu pigs were determined. Fifty nucleotide substitutions were identified in *Cytb* sequences from the aforementioned pig breeds (Figure 2). The nucleotide substitutions in positions 15,422, 15,425, 15,623, and 16,323 were specifically found in Type I Lanyu *Cytb* sequences. A unique transversion in position 15,425 was also identified in Lanyu Type I (thymine) compared with the consensus sequence (adenine). The pairwise genetic distance among the *Cytb* sequences was determined and an ML phylogenetic tree was constructed. The most appropriate model for this data set was found to be HKY + *I* (−ln = 1,935.7043; K = 5; AIC = 3,881.4087, where AIC refers to Akaike's information criterion). The ML estimates of base frequencies were A: 0.3174; C: 0.2887; G: 0.1292; and T: 0.2647. The estimated proportion of invariable sites was 0.7964. A transition:transversion ratio of 10.4402 was used to obtain the ML tree.

Two major clades (Asian and European clades) were recognized in the ML tree (Figure 3). The treelike topology and phylogenetic signal were obtained by the quartet puzzling method (quartet puzzling support value, 80.2%) supporting the branch assignments in this phylogenetic tree (Supplemental Figure S2, available at http://jas.fass.org). In the Asian clade, Type I Lanyu and Ryukyu wild pig sequences formed 2 separate subclades distinct from the Asian major subclade. Sequences from Japanese and Formosan wild pigs and the Satsuma, Meishan, Taoyuan, Diannan, Wannahua, Tongcheng, Erhualian, Large White, and Berkshire breeds clustered with the Type II Lanyu haplotype in the major Asian subclade.

Characteristics of the Complete Mitochondrial Genome of Different Lanyu Haplotypes

The Lanyu Type I control region and *Cytb* haplotypes were unique sequences. We next compared entire mitochondrial genome sequences to find variations in entire mtDNA genomes of both Lanyu haplotypes. After excluding the tandem repeat motifs (5′-CGTGCGTACA), the length of the Type I Lanyu mitochondrial genome was 16,491 nucleotides (nt; accession number: EF375877), and the length of the Type II Lanyu genome was 16,494 nt (accession number: DQ972936). The total lengths of the 2 types of mtDNA control regions differed because of the varying numbers of ACACAAACC and TAAAACACTTA repeat motifs in their control regions (Wu et al., 2007). With the 5′ end of the control region assigned as the first nucleotide of the Type II sequence, the L-strand replication origin was located at positions 6,223 to 6,269. Both types of Lanyu mitochondrial genome encoded 37 genes, including 2 ribosomal RNA (**rRNA**; 12S and 16S), 22 transfer RNA (**tRNA**), and 13 protein-coding genes, as listed in Table 2. A total of 124 nucleotide substitutions (107 transitions; 17 transversions) were identified between the Lanyu sequences. All protein-coding genes in the mitochondrial genome used identical start and termination codons in both Lanyu sequences.

Table 2. Comparison of the mitochondrial genomes of Lanyu (Type I and Type II) and Landrace pigs

	Position[2]				Start codon			Stop codon		
	Lanyu				Lanyu			Lanyu		
Feature[1]	Type I	Type II	Landrace	Strand[3]	Type I	Type II	Landrace	Type I	Type II	Landrace[4]
D-loop	1 to 1,062	1 to 1,056	1 to 1,175	H						
tRNA-Phe	1,063 to 1,132	1,056 to 1,125	1,176 to 1,245	H						
12S rRNA	1,133 to 2,090	1,126 to 2,087	1,246 to 2,205	H						
tRNA-Val	2,089 to 2,156	2,087 to 2,154	2,206 to 2,273	H						
16S rRNA	2,157 to 3,727	2,155 to 3,726	2,274 to 3,844	H						
tRNA-Leu (UUR)	3,727 to 3,801	3,726 to 3,800	3,845 to 3,919	H						
NADH1	3,803 to 4,762	3,802 to 4,758	3,922 to 4,,878	H	ATG	ATG	ATG	TAG	TAG	TAG
tRNA-Ile	4,761 to 4,829	4,757 to 4,825	4,877 to 4,945	H						
tRNA-Gln	4,828 to 4,898	4,825 to 4,895	4,943 to 5,015	L						
tRNA-Met	4,900 to 4,969	4,897 to 4,966	5,017 to 5,086	H						
NADH2	4,970 to 6,013	4,967 to 6,010	5,087 to 6,130	H	ATA	ATA	ATT	TAG	TAG	TAG
tRNA-Trp	6,012 to 6,079	6,009 to 6,076	6,129 to 6,196	H						
tRNA-Ala	6,086 to 6,153	6,083 to 6,150	6,203 to 6,270	L						
tRNA-Asn	6,155 to 6,229	6,152 to 6,226	6,272 to 6,346	L						
O_L	6,226 to 6,272	6,223 to 6,269	6,346 to 6,382	L						
tRNA-Cys	6,262 to 6,327	6,259 to 6,324	6,379 to 6444	L						
tRNA-Tyr	6,327 to 6,390	6,324 to 6,387	6,444 to 6,509	L						
COI	6,394 to 7,938	6,391 to 7,935	6,511 to 8,055	H	ATG	ATG	ATG	TAA	TAA	TAA
tRNA-Ser (UCN)	7,942 to 8,010	7,939 to 8,007	8,059 to 8,129	L						
tRNA-Asp	8,018 to 8,085	8,015 to 8,082	8,135 to 8,202	H						
COII	8,086 to 8,781	8,083 to 8,778	8,203 to 8,890	H	ATG	ATG	ATG	TCA	TCA	TNN
tRNA-Lys	8,775 to 8,840	8,772 to 8,837	8,891 to 8,957	H						
ATPase8	8,842 to 9,045	8,839 to 9,042	8,959 to 9,162	H	ATG	ATG	ATG	TAA	TAA	TAA
ATPase6	9,003 to 9,683	9,000 to 9,680	9,120 to 9,800	H	ATG	ATG	ATG	TAA	TAA	TAA
COIII	9,683 to 10,466	9,680 to 10,463	9,800 to 10,583	H	ATG	ATG	ATG	TAC	TAC	TNN
tRNA-Gly	10,467 to 10,535	10,464 to 10,532	10,584 to 10,652	H						
NADH3	10,536 to 10,892	10,533 to 10,889	10,653 to 10,998	H	ATA	ATA	ATA	TAT	TAT	TNN
tRNA-Arg	10,883 to 10,951	10,880 to 10,948	11,000 to 11,068	H						
NADH4L	10,952 to 11,248	10,949 to 11,245	11,069 to 11,365	H	GTG	GTG	GTG	TAA	TAA	TAA
NADH4	11,242 to 12,619	11,239 to 12,616	11,359 to 12,736	H	ATG	ATG	ATG	TAC	TAC	TNN
tRNA-His	12,620 to 12,688	12,617 to 12,685	12,737 to 12,805	H						
tRNA-Ser (AGY)	12,689 to 12,747	12,686 to 12,744	12,806 to 12,864	H						
tRNA-Leu (CUN)	12,748 to 12,817	12,745 to 12,814	12,865 to 12,934	H						
NADH5	12,818 to 14,641	12,815 to 14,638	12,935 to 14,755	H	ATA	ATA	ATA	TAA	TAA	TAA
NADH6	14,623 to 15,146	14,622 to 15,149	14,739 to 15,266	L	ATG	ATG	ATG	TAA	TAA	TAA
tRNA-Glu	15,147 to 15,215	15,150 to 15,218	15,267 to 15,335	L						
Cytb	15,220 to 16,359	15,223 to 15,362	15,342 to 16,481	H	ATG	ATG	ATG	AGA	AGA	AGA
tRNA-Thr	16,360 to 16,427	15,363 to 15,430	16,482 to 16,549	H						
tRNA-Pro	16,427 to 16,491	15,430 to 15,494	16,550 to 16,613	L						

[1]NADH1 to NADH6 and NADH4L = subunits 1 to 6 and 4L of *nicotinamide dinucleotide dehydrogenase*; ATPase6 and ATPase8 = subunits 6 and 8 of *adenosine triphosphatase*; COI-COIII = *cytochrome c oxidase subunits I-III*; Cytb = *cytochrome b*; tRNA = transfer RNA; rRNA = ribosomal RNA.

[2]The first nucleotide of the control region in the H strand is designated as nucleotide position 1.

[3]H and L indicate that the gene is transcribed from the H strand or the L strand, respectively.

[4]TNN indicates the incomplete stop codon, where NN is the 5′ end of the adjacent tRNA nucleotide, which formed a stop codon by posttranscriptional polyadenylation.

A triplicate ACACAAACC motif was specifically found in the mtDNA control region of the Type I Lanyu sequence, whereas only 1 ACACAAACC motif was found in Type II sequences and in exotic pigs. Another duplicate TAAAACACTTA motif in domain II of the control region was specifically found in the Type II Lanyu, whereas only 1 TAAAACACTTA motif was seen in the Type I Lanyu and in exotic pigs (except the Satsuma breed; Wu et al., 2007). These 2 repeated motifs may be due to heteroplasmy and may have resulted in different lengths of full mitochondrial genomes in the 2 haplotypes of Lanyu pigs.

A typical animal mitochondrial genome encodes 36 to 37 genes, which are powerful markers for inferring phylogenetic relationships (Saccone et al., 2000; Gerber et al., 2001). The numbers of tRNA (22), rRNA (2), and protein-coding genes (13) in both haplotypes of Lanyu mtDNA were identical to the numbers in domestic pigs

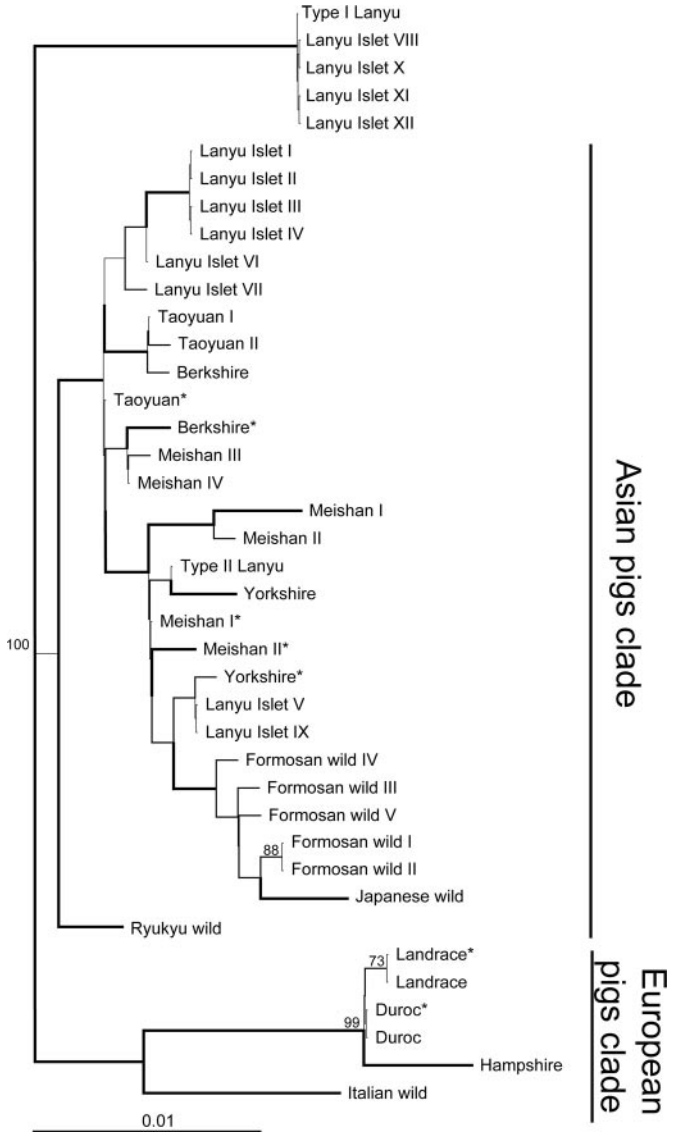

Figure 4. Phylogenetic tree of the mitochondrial DNA (mtDNA) control region of conserved Lanyu pigs, extant domestic Lanyu pigs, Formosan wild pigs, and exotic pigs in Taiwan. Imported exotic pig breeds include the Taoyuan, Meishan, Duroc, Landrace, Yorkshire, Hampshire, and Berkshire breeds. The phylogenetic tree was constructed on maximum likelihood distances of the mtDNA control region polymorphism by using the PHYLIP program package. An asterisk (*) indicates the mtDNA from pigs reared at the Taiwan Livestock Research Institute. Lanyu Islet indicates an extant pig from the Lanyu Islet. Those branches with highly significant and significant confidence are shown by bold and midweight lines, respectively. Numbers on the branches are bootstrap values based on bootstrap resampling (1,000 replications).

(Ursing and Arnason, 1998; Lin et al., 1999). All of the initiation codons (9 ATG, 3 ATA, and 1 GTG) of the 13 protein-coding genes were ATG, except the codons for *NADH dehydrogenase subunit 2* (***NADH2***), *NADH3*, and *NADH5*, which were ATA, and for *NADH4L*, which was the rare GTG. The ATA initiation codon for the *NADH2* gene was found in both types of Lanyu mtDNA, which differed from the rare ATT initiation codon in Landrace and Swedish domestic pigs (Ursing and Arnason, 1998; Lin et al., 1999). Some other slight differences noted were the termination codons of 6 and 2 of 13 protein-coding genes used TAA and TAG, respectively, except those for *NADH3, NADH4, cytochrome c oxidase subunit (**CO**) II*, *COIII*, and *Cytb*, which were TAT, TAC, TCA, TAC, and AGA, respectively (Table 2); and 4 genes (*COII*, *COIII*, *NADH3*, and *NADH4*) terminated with an incomplete TNN stop codon, where NN was the 5′ terminus of the adjacent tRNA gene. The incomplete stop codon (TNN) forms a stop codon by posttranscriptional polyadenylation (Anderson et al., 1981; Ojala et al., 1981; Wolstenholme, 1992).

The vertebrate control region is subdivided into 3 domains. The central domain of the control region, containing the replication origin of the heavy strand, is relatively well conserved. The 2 regions (domains I and II) flanking the central domain are hypervariable in base substitution, insertion, and deletion (Saunders and Edwards, 2000). The complete domestic pig mtDNA has been sequenced and the control region is located between genes encoding tRNA-Pro and tRNA-Phe, containing approximately 1,245 nucleotides (Ursing and Arnason, 1998; Lin et al., 1999).

Phylogenetic Comparison of Exotic, Formosan Wild, and Conserved Lanyu Pigs

Many exotic pig breeds (including Taoyuan, Meishan, Berkshire, Yorkshire, Landrace, and Duroc breeds) were introduced into Taiwan to improve the production performance of local pigs, first by colonizers from 120 to 50 yr ago and then by the Taiwanese government in more recent times (Chyr et al., 2001). To explore whether the genes of exotic breeds and Formosan wild pigs had introgressed into the conserved population of Lanyu pigs, the pairwise distance and ML methods were used to investigate the phylogenetic relationship among the conserved Lanyu pigs, the exotic breeds, and Formosan wild pigs. Some of the mtDNA control region sequences of exotic breeds were obtained from the NCBI Web site, and mtDNA samples of further individuals, including 12 Taoyuan, 4 Meishan, 10 Berkshire, 5 Duroc, 14 Landrace, and 10 Yorkshire breeds, were obtained from the TLRI (Supplemental Figure S3, available at http://jas.fass.org/content/vol86/issue7/). The variable sites of the mtDNA control region of conserved Lanyu, Formosan wild, and exotic pigs are listed in the supplemental data (Supplemental Figure S3, available at http://jas.fass.org/content/vol86/issue7/). Unique nucleotide substitutions, including transitions at nucleotide positions 302, 391, 535, 542, and 657, and a transversion at position 871 (thymine in Type I Lanyu and adenosine in consensus sequence), were found in the control region of Type I Lanyu mtDNA, but not in other sequences used in this study. Pairwise distance analysis

of the mtDNA haplotypes was again performed by using DnaSP. A phylogenetic ML tree was constructed by using the PHYLIP program package (Figure 4), which showed that all pig sequences clustered into 3 major clades. Sequences from many European pig breeds, including the Hampshire, Landrace, Duroc, and Italian wild pig breeds, were categorized as one major clade (referred to here as the European pig clade). Most other sequences, including those from the Formosan wild, Japanese wild, Ryukyu wild, Taoyuan, Meishan, Yorkshire (Large White), and Berkshire breeds, and the Type II Lanyu sequence, were assigned to another major clade (referred to here as the Asian pig clade). The Type I Lanyu sequence clustered as a unique clade distinct from the 2 major clades mentioned above, indicating that the maternal lineage of pigs containing the Type I Lanyu sequence had never crossbred with the Formosan wild and the exotic breeds. The pairwise distance showed that the 2 Lanyu sequences were very different from each other in their maternal lineages.

The treelike topology and phylogenetic signal (quartet puzzling support value, 86.2%) obtained by the quartet puzzling method supported the branches in this phylogenetic tree (Supplemental Figure S4, available at http://jas.fass.org/content/vol86/issue7/). In the ML analysis, the most appropriate model for this data set was found to be TIM + *I*(−ln = 2003.8291; K = 7; AIC = 4021.6582). The ML estimates of base frequencies were A: 0.3639; C: 0.2662; G: 0.1230; and T: 0.2470. Estimated symmetrical substitution rates among these nucleotides were 1.0000 for A/C, 106.7043 for A/G, 47.4006 for T/C, 5.1202 for A/T, 5.1202 for C/G, and 1.0000 for G/T. The estimated proportion of invariable sites was 0.9109. A transition:transversion ratio of 12.5899 was used to obtain the ML tree.

Distinct Genetic Lineage of Lanyu, Taoyuan, and Formosan Wild Pigs

We previously showed that the Taoyuan (accession no. AM040645, AM040646) and Lanyu pigs possess distinct control region haplotypes (Wu et al., 2007). Here, we obtained an identical result after we increased the number of individual Taoyuan pigs in our analysis, indicating no mtDNA gene introgression between Taoyuan and Lanyu pigs (Figure 4). In the present study, we found a remote pairwise distance (0.01882 ± 0.00755 and 0.00936 ± 0.00102) of both the control region and *Cytb* coding sequences between Type I Lanyu and Formosan wild pigs. On the basis of the pairwise distance of *Cytb*, the Formosan wild pigs were clustered together with Japanese wild pigs, with 0.00361 ± 0.00120 pairwise distance, whereas the Type II Lanyu sequence clustered with the major Asian breed subclade, with 0.00328 ± 0.00072 pairwise distance. This result indicates no mtDNA introgression between the Formosan wild pig and Lanyu pigs. The Diannan breed has a phenotype similar to that of the Lanyu pig, but its *Cytb* sequence was identical to the *Cytb* sequence in Formosan and Japanese wild pigs, suggesting that the mtDNA of East Asian wild pigs might have introgressed into the Diannan breed.

Gene Introgression from Exotic Pig Breeds into Pigs Extant on the Lanyu Islet

The Lanyu Islet was isolated by the Taiwanese government during the aboriginal culture protection period. When it was opened to tourism and unrestricted travel after 1960, because most of the Lanyu Islet pigs were bred in free-range piggeries, there was a significantly increased opportunity for the introduction of and crossbreeding with exotic pig breeds from Taiwan. To understand the current diversity of mtDNA in pigs distributed throughout the Lanyu Islet, we obtained mtDNA from 12 individual Lanyu pigs reared by 6 tribes on the Lanyu Islet during February 2005. Their mtDNA control region sequences were subjected to phylogenetic analysis and compared with the sequences of Lanyu pigs conserved in Taiwanese, Formosan wild pigs, and exotic pigs in Taiwan. An ML tree was constructed and sequences from the modern pigs from the Lanyu Islet were clustered into 3 groups (Figure 4). Four extant pigs on the Lanyu Islet had control region sequences identical to the Type I Lanyu haplotype. Two pigs had sequences that clustered together with the Meishan I and II, Yorkshire, Japanese wild pig, Formosan wild pig, and Type II Lanyu sequences, but these 2 sequences had lost one of the repeated TAAAACACTTA motifs present in duplicate in the Type II Lanyu haplotype. The remaining 6 extant Lanyu pigs had mtDNA sequences that were grouped together with Taoyuan, Berkshire, and Meishan III and IV sequences (Figure 4). This confirmed that most extant Lanyu pigs on the Lanyu Islet had hybridized with the Taoyuan, Berkshire, Meishan, or Yorkshire exotic pig breeds.

Most pigs extant on the Lanyu Islet have a dark-pigmented coat color, and some of them present phenotypes of exotic domestic breeds that are now or were previously found in Taiwan, such as the Landrace, Yorkshire, Hampshire, Duroc, Taoyuan, Meishan, and Berkshire breeds. The present study supports the hypothesis of recent introgression of Meishan, Taoyuan, Berkshire, and Yorkshire genes into extant pigs on the Lanyu Islet. Although 4 extant pigs on the Lanyu Islet had mtDNA control region sequences identical to the Type I Lanyu, we observed that those pigs possessed physical characteristics typical of Hampshire, Landrace, and Duroc phenotypes, suggesting that the introgression of exotic pig genes was more serious than could be detected by a simple comparison of the variation in maternal-linked mtDNA. No extant pigs on the Lanyu Islet were found to possess the duplicate TAAAACACTTA motif in their control regions, suggesting that the Type II Lanyu sequence might be becoming extinct on the Lanyu Islet, the original habitat of the Lanyu. One pig from the Lanyu Islet actually possessed 4 ACACAAACC repeat motifs, indicating ei-

ther a recent mutation or that 4 ACACAAACC repeat motifs might have existed in Lanyu pigs previously. These results demonstrated significant genetic introgression from exotic pig breeds into the pigs extant on the Lanyu Islet, and showed that gene drift is currently occurring on the Lanyu Islet, resulting in the loss of the Type II Lanyu sequence.

Evolution of Lanyu Pigs

On the basis of the mtDNA sequences and morphometric data from museum specimens and tissue samples, Lucchini et al. (2005) presented a possible scenario for pig speciation in Southeast Asia (**SEA**): the SEA ancestral pig species (genus *Sus*) might have crossed from Sundaland to the Philippines during the Pliocene (5.3 to 1.8 million years ago). Larson et al. (2005) constructed a consensus tree based on mtDNA obtained from 686 wild and domestic pig specimens from museums worldwide, and showed that basal lineages (origin) of *S. scrofa* occurred on the western island of SEA (**ISEA**) and dispersed into the Indian subcontinent, then dispersed northward into the Asian continent, followed by subsequent westward radiations into mainland Asia, and a final, progressive dispersal across Eurasia into Western Europe. More than 1 domestication event [2 in China (Gansu and Hunan provinces), 1 in India, 1 in Burma and Thailand, and another in Cape York of Northern Australia], followed by rapid radiative expansion in Asia, was identified by median-joining network analysis of Asian domestic and wild pig mtDNA haplotypes (Larson et al., 2005). Later on, Larson et al. (2007) investigated human-mediated *Sus* dispersal in ISEA based on the polymorphism of 781 mtDNA sequences from modern and ancient *Sus* specimens. They concluded that the endemic pigs in Taiwan had a genetic link with mainland East Asian, Micronesian, and Philippine pigs, but not with pigs in Brunei, Sumatra, Oceania, and Polynesia.

Based on variation of the control region, the pairwise distances of the Type I Lanyu haplotype versus Asian and European pigs were 0.01726 ± 0.00275 and 0.02216 ± 0.00889, respectively (Wu et al., 2007). The Type I sequence formed a unique clade distinct from the major Asian clade and the European clade in the constructed ML tree. In addition, the pairwise distances between the Type II Lanyu control region versus the Type I Lanyu control region (0.01744 ± 0.00125), the Type II Lanyu sequence versus the Asian clade (0.00471 ± 0.00054), and the Type II Lanyu versus the European clade (0.01941 ± 0.00356) revealed apparent divergence of Type I and Type II Lanyu mtDNA control region sequences (Wu et al., 2007). In the present study, the pairwise distances of both haplotypes of Lanyu *Cytb* sequences versus Asian and European breeds were consistent with the pairwise distances of the control region variants. Our results, together with those of others, suggest that the Type I and II mtDNA haplotypes had quite different origins, as evidenced by the large calculated genetic distance between the 2 types, and the fact that the 2 types specifically possess different numbers of the repeated ACACAAACC and TAAAACACTTA motifs in their control region. The formation of unique Type I haplotype mtDNA on the Lanyu Islet happened earlier than the formation of the Type II haplotype and the haplotypes of Formosan wild pigs. The ancestor of Type I Lanyu pigs may have crossed through Sundaland into Taiwan and the Lanyu Islet before the last glacial maximum (Meijaard, 2003) and may have evolved in isolation in Taiwan and the Lanyu Islet after the last glacial period ended. To address the origin of the Type II haplotype, the Type II mtDNA haplotype was aligned with 172 *Sus* mtDNA control region sequences published by Larson et al. (2005, 2007). The Type II DNA sequence was identical to the Sarawak (Malaya, DQ779294) specimen and differed by 1 base pair from the Guizhou Xiang (China, AY486118) and Guam D (Northern Mariana Island, AY884677), and differed by 2 base pairs from the Andaman (India, AY884705), Large Black (Australia, AY463075), Kune Kune (New Zealand, AY463076), and Satuma (Japan, AB015091) breeds. Our results revealed that the Type II Lanyu pig in Taiwan shared genetic lineage with pigs distributed in ISEA and Oceania, which was missed in the sampling by Larson et al. (2005, 2007). The short pairwise distance between Type II and Asian breed mtDNA (0.00471 ± 0.00054 in the control region and 0.00328 ± 0.00072 in *Cytb*) shows that the Type II Lanyu mtDNA is genetically almost identical to that of Asian breeds, suggesting that the formation of Type II mtDNA might have occurred in recent times. These results promote 3 hypotheses about the formation of the Type II mtDNA of the Lanyu pig. The first hypothesis is that the Type II mtDNA originated in ISEA and spread northward into the main Asian continent and Japan, and then eastward into Australia and New Zealand through Taiwan and the Lanyu Islet, mediated by human migrations. The second hypothesis is that the Type II Lanyu sequence originated during domestication in mainland Asia and then crossed through Taiwan and the Lanyu Islet and spread into Japan, ISEA, and Oceania. The third hypothesis is that the Type II Lanyu might have originated in Taiwan and the Lanyu Islet and then spread into the Asian mainland, Japan, ISEA, and Oceania. To evaluate the actual origin of the 2 mtDNA haplotypes of Lanyu pigs in Asia, a further combination of genetic, biogeographic, and zooarcheological data is needed.

The conserved Lanyu pigs possesses a distinct maternal lineage to Asian and European type breeds, with no evidence of mtDNA gene introgression from exotic and Formosan wild pigs during recent times. The results of this study further emphasize their unique maternal genetic characterization and the importance of understanding their genetic origin in assessing the trajectories of prehistoric human migration from mainland East Asia into ISEA. The significant loss of mtDNA diversity in the conserved Lanyu pigs may be due to the

small population size and exotic gene introgressions. The discovery that the majority of pigs extant on the Lanyu Islet are hybrids indicates that more effort is needed for the recovery of this native breed. For further conservation or restoration of the Lanyu pig as a distinct breed, the nuclear phylogenetic relationship of the remaining Lanyu pigs, Asian pigs, and other breeds throughout the world will require further analysis. Future population management will also require deeper analysis of global nuclear genetic characteristics within the population of conserved Lanyu pigs by using microsatellite markers or coding genes.

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