

Genetic variation and phylogenetics of Lanyu and exotic pig breeds in Taiwan analyzed by 19 microsatellite markers

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Running head: Microsatellite phylogenetics of Lanyu pig 1 2 Genetic variation and phylogenetics of Lanyu 3 and breeds analyzed exotic pig in Taiwan bv 19 4 microsatellite markers¹ 5 6 W. H. Cheng^{*2}, H. P. Chu^{†2}, Y. N. Jiang^{*}, S. H. Li[‡], Y. Wang[‡], C. H. Chen[§], K. J. Chen[†], 7 C. Y. Lin[#], and Y. T. Ju^{*3} 8 9 *Department of Animal Science and Technology, National Taiwan University, Taipei, 10 Taiwan; [†]Taitung Animal Propagation Station, Taitung, Taiwan; [‡]Department of Life 11 Science, National Taiwan Normal University, Taipei, Taiwan; [§]Livestock Research 12 Institute, Tainan, Taiwan; [#]Hualien Animal Propagation Station, Hualien, Taiwan. 13

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ABSTRACT

15

16 The Lanyu pig is an indigenous miniature pig breed on Lanyu Islet near Taiwan, with a 17 mitochondrial DNA genetic lineage remote from Asian and European pig breeds. The 18 unknown population genetic structure and increased inbreeding among the small 19 population of conserved Lanyu pigs is now of great conservation concern. Additionally, 20 the presence for more than a century of exotic pig breeds in Taiwan has made gene 21 introgression from exotic pig breeds into Lanyu pigs very possible. The present study 22 thus aimed to investigate nuclear genetic variation within the conserved Lanyu pigs, and 23 the phylogenetic relationship and possible genetic introgression between Lanyu and 24 exotic pig breeds by determining the polymorphism of 19 microsatellite loci. In the 25 Neighbor-Joining tree constructed from 7 pig breeds based on Cavalli-Sforza and Edward 26 chord genetic distances, 3 major clades were recognized, where the Asian and European 27 breeds were separately clustered into 2 clades with a 93.0 and 99.9% bootstrap 28 confidence value, respectively. All individuals of the Lanyu breed formed a unique 29 subclade within the Asian clade based on -ln(proportion of shared alleles), suggesting 30 that the Lanyu breed possesses a unique nuclear genetic structure and that no nuclear gene 31 introgression from exotic breeds into the conserved Lanyu pigs has occurred in recent 32 history. Fifteen of 19 microsatellite loci deviated from Hardy-Weinberg equilibrium (by 33 Wright's statistic), suggesting a significant loss of heterozygosity in the conserved 34 population. The valuable nuclear genetic structure and phylogenetic information should 35 assist future conservation and population management of Lanyu pigs.

- 37 Key words: genetic variation, introgression, Lanyu breed, microsatellite, phylogenetics,
- 38 pig, Taiwan

INTRODUCTION

40

41 The Lanyu pig is an indigenous miniature pig breed from Lanyu Islet, off south-east 42 Taiwan, with a characteristic small body, narrow and straight head, small erect ears, and 43 coarse black hair. Its phenotype differs from the domestic Asian and European pig breeds 44 (Cheng, 1986). For the conservation of Lanyu pigs and to develop a laboratory pig stock, 45 2 herds of Lanyu pigs were moved from Lanyu Islet to Taiwan, to the National Taiwan 46 University (NTU) teaching farm in 1975 and to the Taitung Animal Propagation Station 47 (TAPS) in 1980 (Chyr et al., 2001). Both herds were allowed panmixia from 1975 to 2004. 48 Only 2 mitochondrial DNA haplotypes have been identified in all 44 head of conserved 49 Lanyu pigs: the Type I Lanyu mtDNA sequence is distinct from Asian and European 50 breeds, while Type II shows some intermediate features (Wu et al., 2007). The nuclear 51 genetic structure within conserved Lanyu pigs remains unknown.

52 Many exotic pig breeds were introduced into Taiwan to improve the production 53 performance of local pigs, by colonizers from 120 to150 yr ago and by the Taiwan 54 government in more recent times (Chyr et al., 2001). The Taoyuan breed was introduced from southern China into Taiwan around 1877. The Berkshire breed was imported from 55 56 Japan in 1898. The Taiwan Livestock Research Institute (TLRI) introduced the 57 Hampshire and Yorkshire breeds in 1948, while the Landrace and Duroc breeds were 58 imported by the Taiwan Sugar Corporation in 1960. Most recently, the Meishan breed 59 was introduced from Japan by TLRI in 1994. As most of those exotic breeds were 60 introduced before the conserved Lanyu pigs were first reared in isolation, it is possible 61 that some introgression of nuclear genes from exotic breeds into conserved Lanyu pigs 62 has occurred.

63	While the mitochondrial phylogenetics have been recently determined (Jiang et al.,
64	2008), it remained of interest to determine whether the Lanyu breed possesses a unique
65	nuclear genetic structure that matches that seen in the mitochondrial genome, and to
66	identify whether nuclear DNA from exotic breeds had introgressed into the conserved
67	population of Lanyu pigs. A thorough investigation of the phylogenetics of this
68	conserved Lanyu pig population was necessary for their future population management
69	and to avoid further inbreeding.
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71	MATERIALS AND METHODS
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73	Blood Sample Collection and Genomic DNA Purification
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75	Blood samples from all 44 conserved Lanyu pigs were collected (5 from NTU and 39
76	from TAPS). The exotic pig blood samples, from 36 Taoyuan, 35 Meishan, 32 Landrace,
77	31 Yorkshire, 32 Duroc, and 30 Berkshire animals, were obtained from TLRI. Genomic
78	DNA was extracted and purified from collected blood samples with QIAamp DNA Blood
79	Maxi kit (Qiagen, Valencia, CA). For this paper, the term 'European pig breeds' comprises
80	the Berkshire, Duroc, Landrace, and Yorkshire breeds, while the term 'exotic Asian
81	breeds' is used here for the Meishan and Taoyuan breeds.
82	
83	Microsatellite Genotyping

85 Nineteen microsatellite paired primers randomly located on 15 chromosomes, as 86 recommended by the Domestic Animal Diversity Information System of Food and Agriculture Organization (ISAG-FAO) of the United Nations, were chosen for 87 genotyping (FAO, 2004; Table 1). The primers used for genomic DNA amplification 88 89 were fluorescent end-labeled with FAM, TET, or HEX fluorescent dye. The fragments of 90 microsatellite DNAs in the genome were amplified by the polymerase chain reaction 91 (PCR) (PTC-200 Thermal controller, MJ Research), in 15 µL reaction volumes with 50 92 ng of genomic DNA, 1.5 µL of 10× PCR buffer, 0.375 µL of 8 mM dNTP, 4.5 pmole 93 sense and antisense primers, and 0.6 units of DNA Taq polymerase (Amersham 94 Biosciences, Arlington Heights, IL). The thermal cycling conditions were: an initial 95°C 95 for 5 min, then 37 cycles at 95°C for 30 sec, 48 to 62°C for 30 sec (depending on the 96 locus), and 72°C for 45 sec, with a final extension at 72°C for 7 min. The PCR products 97 were resolved by electrophoresis with a MegaBACE 1000 DNA sequencer (Amersham 98 Biosciences). The fluorescent-labeled marker ET-400 (Amersham Biosciences) was used 99 as an internal size standard for length calibration. Allele sizes were determined using 100 Genetic-Profiler software (version 2.2; Amersham Biosciences).

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102 Data Analysis

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104 The Cavalli-Sforza and Edward chord genetic distances (Cavalli-Sforza and Edwards, 105 1967) between 7 breeds were obtained by the PHYLIP program package (version 3.6) 106 (Felsenstein, 2006). The Nei's D_A genetic distance between 7 breeds was obtained by 107 Microsatellite analyzer (MSA; Dieringer and Schlötterer, 2003). The –ln(proportion of

108 shared allele) distance within the 44 conserved Lanyu pigs and 196 individuals of 6 109 exotic breeds were determined by MSA. Allele frequencies at each locus, expected heterozygosity (H_E), and observed heterozygosity (H_o) in conserved Lanyu pigs were 110 111 calculated using CERVUS software (version 2.0; Marshall et al., 1998). The GENEPOP 112 (Genes in Population, version 3.4b) software package (Raymond and Rousset, 1995) was 113 used to calculate Wright's F-statistic (F_{IS}), and to test for deviation from Hardy-Weinberg 114 equilibrium. F_{IS} was used to determine the reduction in heterozygosity in an individual 115 within conserved Lanyu pigs. F_{ST} as determined by MSA was used to determine genetic 116 diversity among the 7 breeds. The effective number of alleles was estimated according to 117 Kimura and Crow's (1964) formula. The Neighbor-Joining tree for the 7 breeds based on 118 Cavalli-Sforza and Edward chord genetic distance was constructed using the PHYLIP 119 (Version 3.66) software package. Bootstrap values of 1,000 replicates were obtained to 120 test the robustness of the tree topology. MEGA3.1 (Molecular Evolutionary Genetics 121 Analysis) software (Kumar et al., 2004) was used to construct another Neighbor-Joining 122 tree based on -ln(proportion of shared allele) distance for 240 individuals from the 7 123 breeds. 124

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RESULTS

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Allele Frequencies, Heterozygosity, and Hardy-Weinberg Equilibrium Test of 127 128 Conserved Lanyu pigs 129

130 To determine the degree of nuclear genetic diversity within conserved Lanyu pigs, the 131 allele frequencies and heterozygosity of 19 microsatellite markers recommended by the

132 FAO and ISAG were examined, and Hardy-Weinberg equilibrium tests performed. The 133 loci of all 19 microsatellite markers in the 44 conserved Lanyu pigs were polymorphic 134 (Table 1). Total alleles per locus varied from 2 (S0005, S0215) to 6 (S0355, S0226), with 135 a mean value at all loci of 3.74. The effective alleles ranged from 1.38 to 3.33 and the 136 mean number of effective alleles was only 2.39 (Table 1). The polymorphic information 137 content (PIC) was calculated to measure the allele diversity at each of the polymorphic 138 loci (Botstein et al., 1980), and allelic PICs ranged from 0.235 to 0.633, with a mean 139 value of 0.485.

140 To describe the genetic diversity and possible deviations from Hardy-Weinberg 141 equilibrium (HWE) within the population, the mean observed heterozygosities (HO), 142 mean expected heterozygosities (HE), and Hardy-Weinberg equilibrium (HWE) test were 143 calculated (Table 1). The observed heterozygosities of all loci (range 0.095 to 0.571) were 144 lower than expected heterozygosities (from 0.276 to 0.697) within conserved Lanyu pigs. 145 Fifteen of 19 loci showed significant deviation from Hardy-Weinberg equilibrium (P <146 0.05) with 12 loci showing severe deviation (P < 0.01). The Fixation indices (F_{IS}) of each 147 locus ranged from 0.020 (SW911) to 0.858 (S0386), and the mean F_{IS} of all loci was 148 0.332. Fourteen private alleles located at 12 loci were identified, including 166 bp 149 (S0155), 163 bp (SW911), 244 bp (S0068), 174 bp (S0002), 118 bp (SW024), 238 bp and 150 258 bp (S0227), 272 and 276 bp (S0355), 174 bp (S0225), 176 bp (S0226), 136 bp 151 (SW951), 188 bp (S0218), and 227 bp (S0228).

Mean Genetic Diversity and Neighbor-Joining Tree of Conserved Lanyu and Exotic
Pig Breeds

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156 To assess the genetic distance among conserved Lanyu, Taoyuan, Meishan, Berkshire, 157 Landrance, Duroc, and Yorkshire breeds, the Cavalli-Sforza and Edwards chord distances 158 (D_C) and Nei's D_A distances based on the allele frequencies of 19 microsatellite loci were 159 obtained (Table 2). The D_C between Duroc and Landrace breeds was 0.1324, similar to 160 the result from Kim et al. (2005). Shorter D_C were found between Lanyu and Berkshire 161 (0.2360), Yorkshire (0.2383), and Meishan (0.2405) breeds than between Lanyu and 162 Taoyuan (0.2633), Duroc (0.2642), and Landrace (0.2675) breeds. The Nei's D_A between 163 Lanyu and exotic breeds were similar to Dc (Table 2). Nei's D_A between Lanyu and 164 Berkshire (0.6056), Yorkshire (0.6115), and Meishan (0.6172) breeds were shorter than 165 against other exotic pigs (including Taoyuan, Duroc, and Landrace breeds). The genetic 166 distances between European breeds (including Landrace, Yorkshire, Duroc, and 167 Berkshire) were shorter than to Asian breeds (including Lanyu, Meishan, and Taoyuan). 168 To understand the phylogenetic relationship between Lanyu and exotic breeds, a 169 Neighbor-Joining tree was constructed based on Dc. All European pig breeds were 170 clustered in one major clade with a 99.9% bootstrap value after 1000 resamplings (Figure 171 1). The Lanyu breed formed a unique clade. The Meishan was clustered with Taoyuan 172 with a bootstrap value of 93.0%. To address whether Lanyu pigs with Type I or II mtDNA 173 haplotypes (Wu et al., 2007; Jiang et al., 2008) have different relatedness to exotic breeds, 174 D_C distances of haplotype Type I and Type II and different exotic breeds were calculated 175 (Table 3). The Dc between mtDNA Type I haplotype and Type II in Lanyu pigs was 176 0.0225. The Dc between Type I Lanyu haplotype and Berkshire breed and between

177 haplotype Type II Lanyu and Berkshire were 0.2408 and 0.2304, respectively; Type II

178 Lanyu sequence is closer to the Berkshire breed than Type I. In addition, the D_C from

179 Type II Lanyu to European pig breeds was closer than Type I (Table 3).

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181 Genetic Variation and Neighbor-Joining Tree of 240 Individuals of Conserved Lanyu 182 and Exotic Pig Breeds

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184 To determine whether the genetic variation of the 44 conserved Lanyu pigs could 185 demarcate them from all exotic breeds, as seen in the polymorphism of 19 microsatellite 186 markers, a Neighbor-Joining dendrogram of 240 individuals from 7 breeds was 187 established based on the distance of -ln(proportion of shared alleles). All of the 188 conserved Lanyu and exotic pig breeds (except one Berkshire and one Yorkshire pig) 189 were classified into their own cluster, indicating the absence of nuclear gene introgression 190 between conserved Lanyu pigs and 99% of exotic breed pigs examined here (Figure 2). 191 Although the NTU Lanyu pigs clustered with TAPS Lanyu pigs to form a unique clade, 192 the NTU Lanyu pigs formed a unique subclade separated from the major Lanyu pig clade. 193 194 195 DISCUSSION 196 197 198 Genetic Diversity Within Conserved Lanyu Pigs 199 In total, 71 alleles were identified in 19 microsatellite loci within the Lanyu pig 200

201 genome. The PIC value is used to evaluate the informativeness of each microsatellite 202 marker under study. Here the mean measured PIC value of 19 microsatellite markers was 203 0.485, ranging from 0.235 to 0.633, indicating that 10 markers were highly informative 204 (PIC > 0.5), 8 markers were reasonably informative (0.5 > PIC > 0.25), and the final 205 marker was only slightly informative (S0215, PIC < 0.25) (Botstein et al., 1980). The 206 range in size of the alleles was 98 to 276 base pairs, consistent with the result of Yang et al. (2003), who used the same microsatellite markers to study genetic variation among 18 207 208 Chinese indigenous pig breeds. The number of effective alleles at the 19 loci ranged from 209 1.38 (S0215) to 3.33 (S0002), based on the Kimura and Crow (1964) formula. All 210 detected allele numbers at the 19 loci were higher than their effective allele number, 211 which suggests that the 19 markers used in the present study were suitable for determination of genetic polymorphisms and diversity within the Lanyu pigs. The 212 213 average number of alleles (3.74) for 19 loci in the conserved Lanyu pigs is lower than the 214 mean of 13.31 alleles present in 26 microsatellite loci of 18 Chinese breeds (Yang et al., 215 2003) and 8.19 alleles present in 20 microsatellite loci of 10 Chinese breeds (Li et al., 2004), suggesting either homogenous selection or gene flow restriction in the Lanyu pig 216 217 population.

The observed heterozygosities (mean 0.375) of all 19 microsatellite loci were lower than expected (mean 0.559). Fifteen of 19 loci deviated significantly from Hardy-Weinberg equilibrium (P < 0.05), including 12 loci with very significant disequilibria (P < 0.01). All F_{IS} values (mean 0.332) of the 19 microsatellite loci in conserved Lanyu pigs were positive, suggesting a severe loss of heterozygosity in individuals due to panmixia within the conserved population. In addition, our previous 224 studies (Wu et al., 2007; Jiang et al., 2008) of the diversity of mitochondrial cytochrome b 225 sequences in conserved Lanyu pigs showed significant deviation from neutrality (Fu and 226 Li's test = 1.44055, P < 0.05), low haplotype number (h = 0.384 ± 0.066), and low 227 nucleotide diversity ($\pi = 0.0037 \pm 0.00064$). Taken together, the lower number of alleles 228 by locus, lower mean observed heterozygosity than expected heterozygosity, and positive 229 F_{IS} values detected in microsatellite DNA, and loss of the diversity of mtDNA haplotype 230 demonstrated a severe loss of heterozygosity both in mitochondrial and nuclear genomes 231 within the population of conserved Lanyu pigs. The current Hardy-Weinberg 232 disequilibria of conserved Lanyu pigs may be a consequence of the original narrow 233 genetic basis (5 males and 19 females were used to found the 2 herds before 1980), and 234 this founder effect may have been exacerbated by the small conserved population and 235 consanguineous mating.

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237 Genetic Distance and Phylogenetic Relationship Among Lanyu and Other Pig Breeds 238

239 The F_{ST} statistic was calculated to estimate the genetic differentiation between Lanyu 240 and 6 exotic pig breed populations (refer to Suppl. Table S1). The F_{ST} value of each 241 microsatellite locus ranged from 0.2171 (SW122) to 0.6052 (S0215), and all 19 242 microsatellite loci deviated significantly from 0 (P < 0.01). The overall F_{ST} value obtained 243 from 19 microsatellite loci from the 7 breeds was 0.3988 (refer to Suppl. Table S1). This mean FST value indicates that about 39.9% of the total genetic variation can be attributed 244 245 to differences between populations and 60.1% to differences within populations, 246 indicating a clear genetic structuring of breeds used in this study. Nevertheless, small 247 differentiation indices (F_{ST} from 0.077 to 0.22) have also been found in studies of 18 and 248 10 Chinese breeds (Yang et al., 2003; Li et al., 2004), with a higher level of genetic 249 differentiation between 11 European breeds (0.27, Laval et al., 2000). A contrary result 250 was obtained in an analysis of the genetic diversity of 52 European and 46 Chinese pig 251 populations by 39 microsatellite markers based on a pooled DNA samples method, 252 indicating the measured differentiation of breeds might be very dependent on the type 253 (including number) of breeds and the methodological approach (Megens et al., 2008). 254 The breeds used in this study have a high level of genetic differentiation and are very 255 distinct from each other. The high differentiation indices detected in the present analyses 256 may be due to the natural genetic variability of the breeds studied.

257 The Cavalli-Sforza and Edward chord (D_C) , and Nei's D_A (Nei et al., 1983) genetic 258 distances are more accurate ways of measuring the genetic distance from microsatellite 259 frequency data (Takezaki and Nei, 1996). Both D_C and Nei's D_A genetic distances 260 between Lanyu and exotic breeds were calculated; both genetic distances showed 261 consistent results (Table 2). The closest genetic distance was determined to be between 262 Lanyu and Berkshire breeds ($D_C 0.2360$ and Nei's $D_A 0.6056$), followed by, in turn, the 263 Yorkshire, Meishan, Taoyuan, Duroc, and Landrace breeds. The genetic distances 264 between European pig breeds (including Berkshire, Duroc, Landrace, and Yorkshire) 265 were much shorter (D_C range 0.1324 to 0.1743) than the distance (D_C range 0.2347 to 266 0.2633) between Asian pig breeds (including Lanyu, Meishan, and Taoyuan breeds). The distances between Lanyu versus Berkshire or Yorkshire (D_C 0.2360 or 0.2383) were 267 268 closer than between Lanyu and Meishan or Taoyuan breed (D_C 0.2405 or 0.2633), 269 suggesting minimal gene flow between Lanyu, Meishan, and Taoyuan breeds in recent times. The relationship of D_C and Nei's D_A genetic distance between Lanyu and the exotic

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271 breeds is not consistent with the time of importation of each exotic breed into Taiwan. 272 The Berkshire and Yorkshire are categorized as Old English pigs. Their original lop-ear appearance was changed to a prick-ear phenotype before the late 19th century. 273 274 Historical records point quite conclusively to genetic introgression of Chinese pigs into 275 the Berkshire and Yorkshire breeds during their development (Jones, 1998; Rothschild 276 and Ruvinsky, 1998), but there is a lack of any genetic evidence to support the historical 277 record. Our previous study revealed a very close pairwise genetic distance of Type II 278 mtDNA to Yorkshire mtDNA (Wu et al., 2007; Jiang et al., 2008), with a difference of 1 279 nucleotide in cytochrome b (1,140 nucleotides in length) and 4 nucleotides in the control 280 region (1,163 nucleotides in length). The cytochrome b and control region of Berkshire 281 mtDNA clustered with Type II mtDNA in a cluster of Asian breeds based on pairwise 282 distances (Wu et al., 2007; Jiang et al., 2008). The close genetic distances in both mtDNA 283 and microsatellite loci between Lanyu versus Berkshire and Yorkshire, suggests a close genetic lineage between Lanyu and the 2 exotic breeds. The development of the 284 Yorkshire and Berkshire breeds in the early 19th century saw a documented continued 285 286 decrease in body size that has been suggested to be the result of introgression of pig 287 breeds from China (Porter, 1993). As a corollary, a synthetic breed referred to as the 288 Lee-sung pig interbred from the Landrace and Lanyu breeds shows prick-ears and white 289 coat color (refer to Suppl. Fig. S1). In consideration of the sum of the short genetic 290 distances, prick ears, and historical records, we hypothesize that the historical 291 development of the Berkshire and Yorkshire breeds might indeed have involved 292 introgression from ancestors of the Lanyu pig. The Yorkshire has mtDNA similar to Type

293 II haplotype mtDNA in Lanyu pig, while the Berkshire may have seen introgression of 294 mitochondria from other breeds from mainland Asia. Nevertheless, although there is no 295 documentary evidence that the Lanyu pig was previously present on the main island of 296 Taiwan, we can not rule out the possibility that the close genetic distance between Lanyu 297 and Berkshire was due to genetic introgression from Berkshire into Lanyu pigs when the 298 Japanese occupied Taiwan. A closer genetic distance was also identified between Lanyu 299 and Meishan than between Lanyu and Taoyuan. The phenotypes of the Taoyuan and 2 300 extinct breeds (Tingshuanghsi and Minung breeds) in Taiwan are similar to those of the 301 Meishan and Lower Changjiang River Basin breeds in Mainland China (Cheng, 1986; 302 Chyr et al., 2001); hence, we could not rule out the possibility that the Meishan and 303 Lower Changjiang River Basin breeds were introduced into Taiwan before the Taoyuan 304 breed.

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306 Interpopulation Structure among Lanyu and Exotic Breeds

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308 The D_C genetic distances for 19 microsatellite loci among 7 breeds were used to 309 construct their Neighbor-Jointing tree (Figure 1). Three major clades were recognized. 310 The Lanyu breed formed a unique clade, the Meishan and Taoyuan breeds formed the 311 second clade, and the European breeds (including Yorkshire, Berkshire, Landrace, and 312 Duroc) formed the remaining clade. The European breeds were clustered and separated 313 from Asian breeds (Lanyu, Meishan, and Taoyuan breeds) with a high bootstrap value 314 (99.9%). In addition, the nucleotides in positions 109, 131, 145, 390, and 1089 of the 315 control region of Lanyu mtDNA sequences were identical to Asian pigs, a feature 316 suggested to be diagnostic between Asian and European pigs (Gongora et al., 2004; Wu 317 et al., 2007). Together, characterization of mtDNA and diversity of microsatellite loci 318 suggest the Lanyu breed should be classified as an Asian breed. Nevertheless, the Lanyu 319 pig and European breeds were separated from Meishan and Taoyuan breeds with a 320 confidence bootstrap value of 93.0%, suggesting that the Lanyu breed possesses a distinct 321 nuclear genetic lineage to Taoyuan and Meishan breeds. Together, these results suggest 322 minimal gene flow between these breeds in recent times, that the Lanyu breed has a 323 unique genetic lineage in its nuclear genome, and that it is remote from Asian and 324 European type breeds.

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326 Genetic Structure of 240 Individual Lanyu and Exotic Pigs

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328 The Neighbor-Joining tree based on -ln(proportion of shared alleles) distance 329 (Bowcock et al., 1994) was constructed from 240 individuals of 7 breeds by the 330 polymorphism of 19 microsatellite loci. Only 2 out of 240 individual pigs clustered with 331 different breeds, indicating that 99.2% of the individuals can be assigned to their own 332 breeds (Figure 2). This suggests that the polymorphism of the 19 microsatellite loci used 333 in the present study is useful for breed demarcation. The 44 Lanyu pigs were clustered 334 into a single clade, and no exotic pig was clustered in the Lanyu pig clade, again 335 suggesting that no interbreeding occurred between Lanyu and exotic breeds in recent 336 times. The 5 Lanyu pigs from the NTU teaching farm formed a subclade within the Lanyu 337 pig clade, which may be due to the small inbred sample from the NTU teaching farm 338 (Suppl. Fig. S2). Nevertheless, we could not exclude the possibility of a different genetic

341	founders in 1980).
340	NTU teaching farm in 1975 to the TAPS Lanyu pigs (originally 4 male and 16 female
339	lineage of the original founders of the Lanyu pig population (1 male and 3 females) at the

343 Genetic Relationship of mtDNA Haplotype and Microsatellite Loci in Conserved
344 Lanyu Pigs

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346 The maternal genetic structure of conserved Lanyu pigs based on the polymorphism of mitochondrial genomic sequences was analyzed in our previous study. Only 2 specific 347 348 mtDNA haplotypes (Type I and II) were identified (Wu et al., 2007; Jiang et al., 2008). In 349 the present study, the nuclear genetic distance and lineage within the conserved Lanyu pig 350 population was determined based on the polymorphyism of 19 microsatellite loci. 351 Controversially, a close D_C genetic distance (0.0225) was identified between pigs 352 possessing Type I and Type II mtDNA haplotypes, and the phylogenetic structure based 353 on -ln(proportion of shared alleles) distance from all 44 conserved Lanyu pigs did not 354 correlate with the phylogenetic structure based on the pairwise distance of their mtDNA sequences (Suppl. Fig. S2). This lack of correlation may reflect differences in the 355 356 mechanisms and rate of mtDNA and nuclear genome replication, evolution, and 357 reassortment (Gamow, 2004).

358

359 Formation of the Unique Genetic Structure and their Speciation

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361 How does the Lanyu pig possess both nuclear and mitochondrial unique genomes?

The tiny Lanyu Islet measures only 45.74 km² overall. Nearly 3,000 aborigines reside on 362 363 this small islet today. When we examined the haplotypes of mtDNA of the extant pigs on 364 the Lanyu Islet in 2005, only Type I and exotic pig mtDNAs were identified (Jiang et al., 365 2008). No additional haplotype of mtDNA was obtained from the Lanyu Islet, leading us 366 to hypothesize that the Lanyu breed might have developed from a small founder 367 population when they were originally domesticated on the Lanyu Islet. After that, a 368 combination of the geographic barrier limiting gene flow and genetic drift led to the 369 speciation of the Lanyu breed. In addition, the founder effect was again enhanced after 370 they were further isolated as conserved herds from a very small founder population (5 371 males and 19 females) after 1975. Here again, our conclusions should be considered 372 relative to the limited sample of breeds examined, and should not preclude conclusions 373 that might be obtained from a more comprehensive set of breeds.

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IMPLICATIONS

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377 The Lanyu pig possesses unique genetic signatures in both nuclear and mitochondrial 378 genomes, and is quite distinct from both Asian and European domestic breeds. The 379 serious loss of nuclear genetic diversity in the conserved Lanyu pig population identified 380 in this study parallels that found in the mitochondrial genomes in our earlier study, 381 suggesting that significant attention must be directed to management of the remaining 382 animals to maintain their genetic diversity. Specific knowledge of each animal's genetic 383 identity presented here should greatly assist such a goal. This miniature pig breed, 384 nonetheless, has much potential for future development as a novel animal model to study

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464 **TABLES**

465 **Table 1.** The genetic variation of 19 microsatellite loci in 44 individuals of conserved 466 Lanyu pigs in Taiwan. Table includes the number of alleles observed (No. of alleles), 467 effective alleles, allele size range, mean observed heterozygosity (HO), expected 468 heterozygosity (HE), fixation indices (F_{IS}), and polymorphism information content 469 (PIC), loci deviating from Hardy-Weinberg equilibrium (HWE test, * P < 0.05, ** P < 470 0.01)

Leave	A 11-1-	Effective	Allele size		HE	DIC	HWE	F _{IS}
Locus	Allele	allele	range	no	HE	PIC	test	
SW857	3	1.90	104-114	0.286	0.480	0.391	**	0.408
IGF1	3	2.38	194-202	0.442	0.587	0.488	*	0.249
S0155	3	2.65	160-166	0.425	0.630	0.542	**	0.328
S0005	2	1.92	206-238	0.400	0.486	0.365	NS	0.179
SW911	3	2.12	157-163	0.524	0.534	0.421	NS	0.020
S0068	4	3.07	244-256	0.571	0.682	0.614	**	0.176
S0002	5	3.33	174-210	0.452	0.694	0.633	**	0.351
S0228	3	2.24	227-243	0.419	0.559	0.459	*	0.254
SW024	3	1.45	98-118	0.295	0.313	0.282	NS	0.058
S0227	4	1.45	238-258	0.182	0.315	0.285	**	0.425
SW72	5	2.48	150-160	0.537	0.603	0.545	**	0.112
S0218	4	3.22	166-188	0.326	0.697	0.632	**	0.529
S0355	6	2.54	246-276	0.455	0.613	0.543	**	0.261
SW122	4	3.06	116-138	0.425	0.682	0.613	**	0.380
S0225	3	2.17	174-194	0.442	0.545	0.467	*	0.239
S0226	6	2.41	176-214	0.341	0.606	0.541	**	0.440
SW951	5	2.75	120-136	0.268	0.644	0.571	**	0.586
S0215	2	1.38	156-164	0.233	0.276	0.235	NS	0.158
S0386	3	2.92	156-172	0.095	0.666	0.584	**	0.858
mean	3.74	2.39		0.375	0.559	0.485		0.332

471

472

474 **Table 2.** Cavalli-Sforza and Edward chord (below the diagonal) and Nei's D_A (above

475 the diagonal) genetic distances between Lanyu, Taoyuan, Meishan, Landrace, Yorkshire,

476	Duroc, and Berkshire	e pig breeds b	y 19 microsatellite	marker polymorphisms
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	Lanyu	Meishan	Taoyuan	Landrace	Yorkshire	Duroc	Berkshire
Lanyu		0.6172	0.6757	0.6863	0.6115	0.6780	06056
Meishan	0.2405		0.6028	0.7515	0.7320	0.7039	0.7391
Taoyuan	0.2633	0.2347		0.7804	0.7524	0.7959	0.8134
Landrace	0.2675	0.2927	0.3042		0.3413	0.3395	0.3747
Yorkshire	0.2383	0.2852	0.2932	0.1331		0.4473	0.4255
Duroc	0.2642	0.2743	0.3101	0.1324	0.1743		0.4165
Berkshire	0.2360	0.2880	0.3170	0.1461	0.1659	0.1623	

- **Table 3**. Cavalli-Sforza and Edward chord genetic distances between Type I and Type II
- 479 haplotype mtDNA of Lanyu pigs, Taoyuan, Meishan, Landrace, Yorkshire, Duroc, and
- 480 Berkshire pig breeds by polymorphisms of 19 microsatellite markers

	Lanyu I	Lanyu II	Meishan	Taoyuan	Landrace	Yorkshire	Duroc
Lanyu II	0.0225						
Meishan	0.2422	0.2455					
Taoyuan	0.2646	0.2660	0.2347				
Landrace	0.2736	0.2625	0.2927	0.3042			
Yorkshire	0.2425	0.2372	0.2852	0.2932	0.1331		
Duroc	0.2713	0.2537	0.2743	0.3101	0.1324	0.1743	
Berkshire	0.2408	0.2304	0.2880	0.3170	0.1461	0.1659	0.1623

491	LEGENDS to FIGURES
492	
493	Figure 1. Neighbor-Joining tree of Lanyu, Taoyuan, Meishan, Landrace, Yorkshire,
494	Duroc, and Berkshire constructed from Cavalli-Sforza and Edward chord genetic
495	distance by 19 microsatellite marker polymorphisms. The numbers at the branch nodes
496	are the percentage of a group occurrence in 1,000 bootstrap replications of re-sampled
497	loci.
498	
499	Figure 2. The Neighbor-Joining tree was constructed based on -ln(proportion of shared
500	alleles) distances among 240 individuals from 7 pig breeds by 19 microsatellite marker
501	polymorphisms.
502	
503	





515 Figure 2.



Supplemental data:

Suppl. Table. S1. The number of alleles observed, the range of allele size, and Fixation indices (F_{IT} , F_{IS} , and F_{ST}) of 19 microsatellite loci among Lanyu, Taoyuan, Meishan, Landrace, Yorkshire, Duroc, and Berkshire breeds.

Locus	allele	Size	F _{IS}	F _{ST}	F _{IT}
SW857	10	94-114	0.0109	0.3586	0.3656
IGF1	10	194-250	0.1573	0.3198	0.4268
S0155	10	148-182	0.1766	0.4786	0.5707
S0005	20	206-278	0.3207	0.3063	0.5288
SW911	7	155-173	0.0154	0.2769	0.2880
S0068	15	190-262	0.0936	0.4357	0.4885
S0002	13	174-220	0.1473	0.3938	0.4830
S0228	10	221-245	0.3613	0.5553	0.7160
SW024	11	96-138	-0.1489	0.3209	0.2198
S0227	14	210-258	0.2722	0.5049	0.6396
SW72	11	142-162	0.1302	0.3157	0.4048
S0218	14	156-188	0.5298	0.3662	0.7020
S0355	12	226-276	0.1108	0.4582	0.5182
SW122	12	114-138	0.1922	0.2171	0.3676
S0225	9	170-194	0.0868	0.5996	0.6344
S0226	13	176-214	0.1463	0.3386	0.4353
SW951	7	120-136	0.0305	0.3394	0.3595
S0215	8	138-170	-0.1958	0.6052	0.5279
S0386	8	154-172	0.4555	0.4184	0.6833
Overall	11.26		0.1559	0.3988	0.4926



Suppl. Figure S1. A 5-weeks-old Lee-Sung pig. A synthetic breed interbred from the Landrance and Lanyu breeds.



Suppl. Figure S2. Neighbor-Joining tree of the 44 conserved Lanyu pigs. The Neighbor-Joining tree was constructed based on $-\ln(\text{proportion of shared alleles})$ distances of 19 microsatellite markers from 44 heads of conserved Lanyu pigs. The red color indicates the individual pig contains Type I haplotype mtDNA sequences; black color indicates Type II haplotype mtDNA. The microsatellite genotypes of conserved Lanyu pigs are subdivided into four populations, and the different haplotypes of mtDNA are randomly distributed within these four sub-populations. NTU and TAPS prefixes refer to the two different conserved herds.