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# Variation in Mitochondrial DNA of Vietnamese Pigs: Relationships with Asian Domestic Pigs and Ryukyu Wild Boars

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**ABSTRACT**—Mitochondrial DNA (mtDNA) sequences (574 bp) of 30 Vietnamese pigs (large and small) were examined and compared with those of 61 haplotypes from wild boars and domestic pigs from various locations in Asia. The large Vietnamese pigs had genetic links to Ryukyu wild boars in southern Japan. The small Vietnamese pigs were closely related to other East Asian domestic pigs. These results indicate that Vietnamese pigs are genetically diverse and may be descendents of wild and domestic pigs from other regions of Asia.

**Key words:** genetic variation, mitochondrial DNA, phylogeography, Ryukyu wild boar, *Sus scrofa*

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## INTRODUCTION

Wild boars (*Sus scrofa*) inhabit wide areas of Asia, Europe and North Africa, and include about 27 subspecies (Herre and Rohrs, 1977). Domestic pigs in Asia and Europe have been independently domesticated from different wild boar subspecies (Giuffra *et al.*, 2000; Watanabe *et al.*, 1985). Several wild boar subspecies inhabit East Asia, where domestication of pigs from local populations of wild boars occurred repeatedly from 6000 to 9000 years ago (Xu, 1950). In China and Vietnam, well-known domestic pigs such as Meishan, Jinhua and Mong Cai have been established and used as a genetic source to develop pig breeds (Lan and Shi 1993; Watanabe *et al.*, 1985). Vietnam is thought to be one of the points of origin of Asian domestic pigs. Vietnamese pigs show a remarkable diversity of serum amylase polymorphisms (Kurosawa *et al.*, 1998).

Two subspecies of wild boar now inhabit Japan: the Japanese wild boar (*S. s. leucomystax*), on the Japanese main islands (Honshu, Shikoku and Kyushu); and the Ryukyu wild boar (*S. s. riukiuanus*), found only on several islands of southwestern Japan and the Ryukyu Islands

(Amami-Oshima, Kakeroma, Tokunoshima, Okinawa, Iriomote and Ishigaki Islands). These 2 subspecies are distinguishable by blood groups, protein polymorphisms (Kurosawa *et al.*, 1984; Kurosawa and Tanaka, 1988), and restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA (mtDNA) (Watanabe *et al.*, 1985). Ryukyu wild boars are also distinguished from Japanese wild boars and East Asian domestic and wild pigs by analysis of mtDNA control and cytochrome *b* (*cytb*) regions (Watanobe *et al.*, 1999). Despite the fact that the Ryukyu Islands are located between Taiwan and Kyushu Island, Japan, no wild boar genetically related to Ryukyu wild boars has been identified in Taiwan or the Asian continent (Watanobe *et al.*, 1999, 2001). The origin of the Ryukyu wild boar is still controversial.

We examined pig skeletons stored at 2 Vietnamese research institutes. These pig skeletons were morphologically classified into 2 size groups: large and small. In the present study, to assess the genetic backgrounds of these skeletons, we examined their morphological characters and mtDNA sequence. We found that large and small Vietnamese pigs have genetic links to the Ryukyu wild boar and East Asian domestic pigs, respectively. Here, we describe the phylogenetic relationships among the Ryukyu wild boar, East Asian domestic pigs and Vietnamese pigs.

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## MATERIALS AND METHODS

### Pig samples and morphological measurement

Samples were taken from 30 pig skeletons stored at the Hanoi Agricultural University and at the Anthropology section of the Institute of Archaeology of the Academy of Science in Hanoi. The specimens were designated as large or small based on skeleton sizes. To compare the body size of pigs, the occlusal length and greatest breadth of the mandibular third molar ( $M_3$ ) were measured by digital calipers (Table 1). When  $M_3$  was broken or missing, other mandibular or maxillary molars were measured (samples 19 and 20, AI 2, 3, 7, and 8). Samples for DNA analysis were taken from the rami of the mandibles. The specimens of the Hanoi Agricultural University were purchased on January 7, 1997, in Ba Vi Village, Ba Vi County, Ha Tay Province, near Hanoi, by a group of Japanese and Vietnamese researchers (Yamamoto *et al.*, 1998). Although the exact origin of these bones is not known, they all appear to have been taken from recently hunted or slaughtered animals. Twenty sub-adult or adult animals (older than about 18 months) from this collection were used.

Samples stored at the Anthropology section of the Institute of Archaeology in Hanoi consisted of 10 pig skulls (7 native wild and 3 domestic) collected by one of the present authors (VTL). The wild boars (samples AI 1–5, 9, and 10) were hunted in various localities in northern Vietnam, and the 3 domestic pig skulls (sample AI 6–8) were collected near Hanoi.

Thirteen other pig samples (1 Turkish wild boar, 5 Taiwanese wild boars, and 7 Korean wild boars) were used in constructing the mtDNA database used in this study.

### DNA extraction

DNA was extracted from a total of 43 pig specimens (20 from Hanoi Agricultural University, 10 from the Institute of Archaeology in Hanoi, and 13 from Turkey, Taiwan and Korea). Genomic DNA was isolated from 0.5 to 1.0 g of bone powder, as described elsewhere (Watanobe *et al.*, 2001). Extracted DNA was directly used as polymerase chain reaction (PCR) templates.

### PCR and direct sequencing of mtDNA

The mtDNA control region was amplified by PCR using primer sets A, B and C, designed from the mtDNA control region to amplify 258-, 305- and 229-bp segments, respectively, of the control region (Watanobe *et al.*, 2001): primer set A, mitL76 (5'-AATATGCGACC-CCAAAATTTAACCATT<sup>130</sup>) and mitH62 (5'-CCTGCCAAGCGGG-TTGCTGG<sup>351</sup>); primer set B, mitL119 (5'-CAGTCAACATGCG-TATCACC<sup>301</sup>) and mitH124 (5'-ATGGCTGAGTCCAAGCATCC<sup>567</sup>); primer set C, mitL104 (5'-TGGACTAGTGACTAATCAGCCCAT<sup>518</sup>) and mitH106 (5'-ACGTGTACGCACGTGTACGC<sup>704</sup>). DNA was first activated with AmpliTaq Gold (Applied Biosystems, Foster City, CA): denaturing at 95°C for 10 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min. This was followed by 50 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min. The PCR products were purified using a Centricon 100 micro-concentrator (Millipore, Bedford, MA), and were sequenced on an Applied Biosystems 377 DNA sequencer with BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA). Nucleotide sequences of 574 bp were formed by connecting the 3 DNA fragments amplified by A, B and C primer sets. The DNA sequences were deposited in the DDBJ/EMBL/GenBank database (accession nos. AB05306-AB05322).

### Alignment of DNA sequences and phylogenetic analysis

The 574-bp DNA sequences from 30 Vietnamese pig samples were constructed from fragments A, B and C, and aligned with 61 haplotypes from a total of 304 samples of wild boars and domestic pigs. The 61 haplotypes included 6 haplotypes from Turkish, Tai-

wanese and Korean wild boars sequenced in this study and 55 mtDNA haplotypes from a mtDNA database (Watanobe *et al.*, 2001). The 304 samples included 122 Japanese wild boars, 13 Ryukyu wild boars, 77 East Asian domestic pigs, 73 European domestic pigs, 3 European wild boars, 3 Northeast Asian wild boars, 1 Turkish wild boar, 5 Taiwanese wild boars, and 7 Korean wild boars. All mtDNA sequences were aligned using GENETYX-MAC software Version 10 (Software Development Co., Tokyo, Japan).

Phylogenetic trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) using the PHYLIP program package, version 3.572 (Felsenstein, 1995), and by the maximum parsimony (MP) method using MEGA version 1.0. In the NJ tree, the numbers of nucleotide substitutions per site between haplotypes was estimated using the two-parameter method (Kimura, 1980). The confidence of each branch in the phylogeny was estimated after 1000 bootstrap replications (Felsenstein, 1985). The MP tree was constructed by the branch-and-bound searching method (Kumar *et al.*, 1993), with bootstrap values calculated after 100 replications.

Corrected genetic differences ( $D_A$ : Nei 1987) between pig groups were calculated using the equation  $D_A = D_{XY} - (D_X + D_Y)/2$ , where  $D_{XY}$  is the average pairwise nucleotide difference between pig groups X and Y, and  $D_X$  and  $D_Y$  are average pairwise nucleotide differences within pig groups X and Y, respectively. The significance of differences between pig groups was tested using 1000 permutations in the ARLEQUIN program package, version 2000 (Schneider *et al.*, 2000).

## RESULTS

### Morphological analysis

When the 30 Vietnamese pig skeletons were divided into groups of large and small pigs, the large pigs were found to be similar in size to wild boars found in East Asia. The large pigs had occlusal lengths of mandibular third molars ( $M_3$ ) ranging from approximately 34.6 mm to 44.5 mm (Table 1), which is similar in size to the wild boars of the Middle East (Flannery, 1983; Hongo and Meadow, 1998). The occlusal length of  $M_3$  of the smaller pigs ranged from approximately 23.5 mm to 29.2 mm. The size of  $M_3$  of the smaller Vietnamese pigs was similar to that of male and female Ryukyu wild boars (Table 1). However, the small Vietnamese pigs had narrow, straight frontal bones (Fig. 1). Reduction in body size and shortening of the cranium, especially of the teeth, is a characteristic of domestication (Flannery, 1983), suggesting that the small Vietnamese pigs are either primitive breed of domestic pigs or a small wild boar.

### Genetic relationship of Vietnamese pigs with other wild boars and domestic pigs

DNA analysis of the 574-nucleotide sequences from a total of 43 individuals revealed 17 Vietnamese haplotypes (Viet 1 to 17), and 6 Korean, Taiwanese and Turkish wild boar haplotypes (Nos. 56 to 61). Fig. 2 shows nucleotide sequences of these haplotypes aligned with those of the representative haplotypes of domestic pigs and wild boars from various localities (Watanobe *et al.*, 2001). Most haplotypes were found in 1 or 2 specimens, but haplotypes Viet 5 and Viet 17 were found in 8 and 4 specimens, respec-

**Table 1.** Pig bone samples from Vietnam

| Location or Source                               | Sample No. | M <sub>3</sub> length | M <sub>3</sub> breadth | Size  | Wild (W) or domestic(D) group <sup>1)</sup> | Sex | DNA haplotype <sup>2)</sup> |
|--|------------|-----------------------|------------------------|-------|---|-----|-----------------------------|
| Hanoi Agricultural University (20 pig bones)     |            |                       |                        |       |   |     |                             |
|  | 1          | 23.5                  | 12.2                   | small | D   | M   | Viet 6                      |
|  | 2          | 24.2                  | 12.9                   | small | D   | F   | Viet 9                      |
|  | 3          | 29.2                  | 13.7                   | small | D   | F   | Viet 1                      |
|  | 4          | 27.7                  | 12.8                   | small | D   | F   | Viet 5                      |
|  | 5          | 26.4                  | 13.3                   | small | D   | F   | Viet 5                      |
|  | 6          | 24.7                  | 12.7                   | small | D   | M   | Viet 11                     |
|  | 7          | 24.3                  | 12.1                   | small | D   | M   | Viet 9                      |
|  | 8          | 23.9                  | 14.3                   | small | D   | F   | Viet 5                      |
|  | 9          | 25.3                  | 13.5                   | small | D   | M   | Viet 5                      |
|  | 10         | 25.2                  | 12.7                   | small | D   | M   | Viet 5                      |
|  | 11         | 25.2                  | 12.9                   | small | D   | M   | Viet 3                      |
|  | 12         | 26.0                  | 13.3                   | small | D   | F   | Viet 8                      |
|  | 13         | 23.6                  | 13.0                   | small | D   | M   | Viet 5                      |
|  | 14         | 26.6                  | 13.5                   | small | D   | F   | Viet 10                     |
|  | 15         | 26.7                  | 13.2                   | small | D   | M   | Viet 5                      |
|  | 16         | 37.3                  | 20.2                   | large | W   | F   | Viet 12                     |
|  | 17         | 42.1                  | 17.7                   | large | W   | F   | Viet 17                     |
|  | 18         | ND <sup>5)</sup>      | 21.4                   | large | W   | M   | Viet 17                     |
|  | 19         | 22.2 <sup>3)</sup>    | 16.0 <sup>3)</sup>     | large | ND <sup>5)</sup>                            | F   | Viet 17                     |
|  | 20         | 23.2 <sup>4)</sup>    | 15.9 <sup>4)</sup>     | small | ND <sup>5)</sup>                            | F   | Viet 5                      |
| Institute of Archaeology in Hanoi (10 pig bones) |            |                       |                        |       |   |     |                             |
|  | AI1        | 40.5                  | 18.0                   | large | W   | F   | Viet 15                     |
|  | AI2        | 38.9 <sup>4)</sup>    | 22.7 <sup>4)</sup>     | large | ND <sup>5)</sup>                            | F   | Viet 13                     |
|  | AI3        | 24.5 <sup>6)</sup>    | 21.2 <sup>6)</sup>     | large | ND <sup>5)</sup>                            | M   | Viet 17                     |
|  | AI4        | 44.5                  | 18.5                   | large | W   | M   | Viet 12                     |
|  | AI5        | 43.9                  | 19.6                   | large | W   | M   | Viet 14                     |
|  | AI6        | 24.1                  | 13.1                   | small | D   | M   | Viet 2                      |
|  | AI7        | ND <sup>5)</sup>      | 12.0 <sup>3)</sup>     | small | ND <sup>5)</sup>                            | F?  | Viet 7                      |
|  | AI8        | ND <sup>5)</sup>      | 9.4 <sup>7)</sup>      | small | ND <sup>5)</sup>                            | M?  | Viet 4                      |
|  | AI9        | 34.6                  | 18.6                   | large | W   | F   | Viet 16                     |
|  | AI10       | 40.9                  | 18.2                   | large | W   | M   | Viet 14                     |
| Modern <i>S. s. riukiuanus</i> <sup>8)</sup>     |            | 28.6                  | 13.3                   |       | W   | M   |                             |
| Modern <i>S. s. riukiuanus</i> <sup>8)</sup>     |            | 26.8                  | 13.6                   |       | W   | F   |                             |

<sup>1)</sup> Based on morphological measurements, bone samples were designated as belonging to Wild (W) or domestic pig (D) groups.

<sup>2)</sup> DNA haplotypes shown in Fig. 3.

<sup>3)</sup> Measurements of mandibular M<sub>2</sub>.

<sup>4)</sup> Measurements of maxillary M<sub>3</sub>.

<sup>5)</sup> ND, not determined.

<sup>6)</sup> Measurements of maxillary M<sub>2</sub>.

<sup>7)</sup> Measurements of mandibular M<sub>1</sub>.

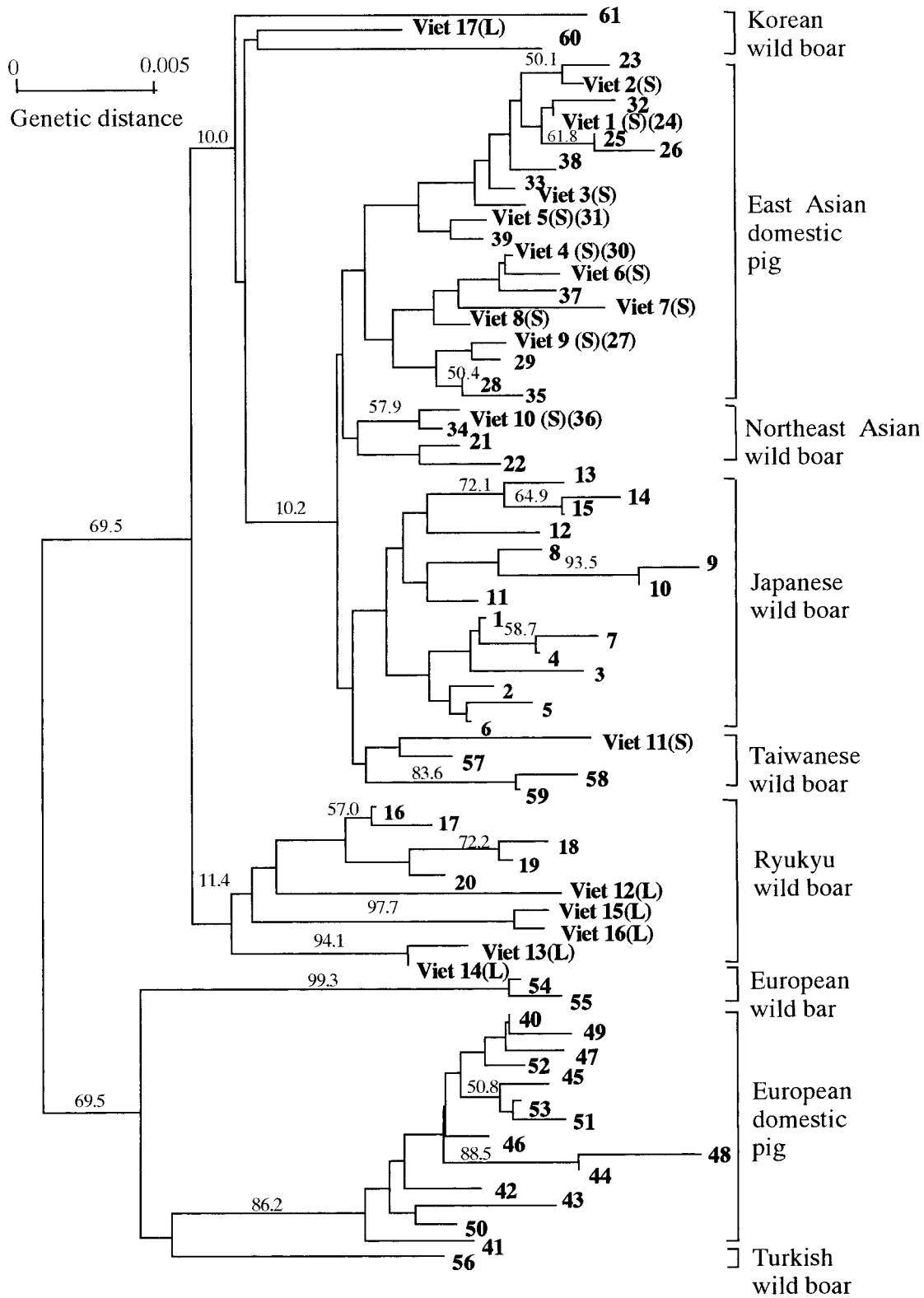
<sup>8)</sup> Measurements of modern Ryukyu wild boars are shown for comparison.

tively.

The NJ relationship among the 17 Vietnamese haplotypes and 61 haplotypes from other parts of the world

showed 2 major clusters: Asian (69.5% bootstrap value) and European (69.5% bootstrap value) (Fig. 3). The Asian cluster was divided into the Ryukyu lineage and East Asian lin-





**Fig. 3.** Phylogenetic tree constructed by the NJ method using 574-bp fragments of the mtDNA control region for 17 Vietnamese pig haplotypes and 61 haplotypes from pig populations in various localities. Haplotype numbers (bold numbers) are the same as in Fig. 2 and a previous study (haplotypes 1 to 55 of Watanobe *et al.*, 2001). The size of the Vietnamese pigs (L, Large; S, Small) is indicated in parentheses following the haplotype number. Bootstrap resampling was performed 1000 times, and resulting bootstrap probabilities greater than 50% are shown on the corresponding branches. Code numbers of the haplotypes are written in parentheses when they fall on the same branch as Vietnamese pig haplotypes.

**Table 2.** Average pairwise genetic differences between pig groups

| Pig group                   | 1            | 2            | 3            | 4            | 5            | 6            | 7            |
|-----------------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 1 Ryukyu wild boar          | <u>2.077</u> | 11.471*      | 9.515*       | 10.386*      | 11.269*      | 10.658*      | 7.201*       |
| 2 Taiwanese wild boar       | 8.933*       | <u>3.000</u> | 6.951*       | 4.720*       | 5.904*       | 6.358*       | 9.800*       |
| 3 East Asian pig            | 6.838*       | 3.813*       | <u>3.277</u> | 5.303*       | 6.855*       | 3.451        | 8.951*       |
| 4 Northeast Asian wild boar | 7.647*       | 1.520*       | 1.964*       | <u>3.400</u> | 4.299        | 4.926*       | 8.855*       |
| 5 Japanese wild boar        | 8.247*       | 2.420*       | 3.232*       | 0.615        | <u>3.968</u> | 6.524*       | 10.102*      |
| 6 Vietnamese pig (S size)   | 8.128*       | 3.367*       | 0.321*       | 1.735*       | 3.049*       | <u>2.982</u> | 9.057*       |
| 7 Vietnamese pig (L size)   | 2.926*       | 5.064*       | 4.076*       | 3.918*       | 4.882*       | 4.330*       | <u>6.473</u> |

Above diagonal : Average number of pairwise differences between pig groups ( $D_{XY}$ )

Diagonal elements : Average number of pairwise differences within pig groups ( $D_X$ )

Below diagonal : Corrected average pairwise difference between pig groups ( $(D_{XY} - (D_X + D_Y))/2$ )

Asterisks on the numbers indicate significant difference ( $P < 0.05$ ).

## DISCUSSION

The large and small skeletons obtained from 2 research Institutes in Hanoi were genetically classified into 2 groups by mtDNA sequence analysis: the large pigs were found to be wild boars related to Ryukyu (Viet 12–16) and Korean wild boars (Viet 17); and the small pigs were mostly related to East Asian domestic pigs, including 2 haplotypes of Northeast Asian wild boar (Viet10) and Taiwanese wild boar (Viet11) (Fig. 3). The large Vietnamese wild boars were genetically distinct from small Vietnamese pigs and East Asian domestic pigs that originated from Chinese domestic pigs and related pigs in our mtDNA haplotype database. In a mtDNA polymorphism study, Lan and Shi (1993) found that the genetic distance between Yunnan native pigs in China and Vietnamese pigs is comparatively large, although southwest China and Vietnam are closely connected geographically. Their results are consistent with the present findings, indicating that Vietnamese pigs, including wild and domestic pigs, possess remarkable genetic diversity.

The present study has demonstrated for the first time that some Vietnamese wild boars are genetically linked to Ryukyu wild boars. The Ryukyu wild boars were once considered to be a feral population of early East Asian domestic pigs that had been brought to the Ryukyu Islands in prehistoric times (Naora, 1937; Semba, 1960; Hayashida, 1960). Based on morphological characters, Imaizumi (1973) reported that Ryukyu wild boars may be a relic of the continental pig population, as are some other endemic species on the Ryukyu Islands. The present results support Imaizumi's hypothesis that Ryukyu wild boars are a unique species established on isolated Ryukyu Islands. The Ryukyu Islands were connected to the Chinese continent several times in the past (Ujiiie and Saito, 1974; Kizaki and Ohshiro, 1980; Ujiiie, 1986). Although the existence of the Pleistocene land bridge is still debated, some members of the continental fauna, including hominids, likely migrated into the Ryukyu Islands from the continent by way of a land bridge. A calculation of the genetic divergence of Ryukyu wild boars from Vietnamese pig populations could be a useful index for eval-

uating the zoogeographical connection between the Ryukyu Islands and the continent over time.

Most haplotypes of the small Vietnamese pigs were closely related to those of the East Asian domestic pig groups (Fig. 3, Table 2). This finding suggests that the small Vietnamese pigs are domestic pigs. If so, there are 2 possibilities: 1) they were domesticated locally in Vietnam; and 2) they were introduced from neighbouring areas such as southwest China. European and East Asian domestic pigs have been clearly shown to be derived from different wild populations (Giuffra *et al.*, 2000). Vietnamese pigs, including wild and domestic pigs, may share a common ancestor with other East Asian pigs (Fig. 3). Further morphological and genetic analyses of Vietnamese pigs will provide important information about the history of domestication of pigs in East Asia.

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