

mtDNA variation in Vietnamese pigs, with particular emphasis on the genetic relationship between wild boars from Vietnam and the Ryukyu Islands

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Abstract. To examine the genetic origin of Ryukyu wild boar, we sequenced mitochondrial DNA (mtDNA: 574-bp) from Vietnamese wild boar and Vietnamese indigenous domestic pigs and compared these sequences with the mtDNA of Ryukyu wild boar by phylogenetic analysis. Numerous Vietnamese wild boars were genetically related to Ryukyu wild boar, suggesting that descendants of the ancestors of Ryukyu wild boar still inhabit Vietnam. The mtDNA sequences of Vietnamese indigenous domestic pigs are extremely diverse and are thought to have contributed to the diversity of mtDNA among East Asian domestic pigs.

Key words: domestic pig, mtDNA, origin, Vietnam, wild boar.

Two subspecies of wild boar inhabit Japan: the Japanese wild boar (*Sus scrofa leucomystax*) on the islands of Honshu, Kyushu, and Shikoku; and the Ryukyu wild boar (*S. s. riukiuanus*) on the Ryukyu Islands (Amami-Oshima, Kakeroma, Tokunoshima, Okinawa, Iriomote, and Ishigaki). These two subspecies are distinguished by morphological features (Endo et al. 1994, 1998, 2002), and by genetic characters such as blood groups and protein polymorphisms (Kurosawa et al. 1984; Kurosawa and Tanaka 1988), restriction fragment length polymorphisms of mitochondrial DNA (mtDNA) (Watanabe et al. 1985), and mtDNA control and cytochrome *b* (*cytb*) regions (Watanabe et al. 1999). Although the Ryukyu Islands are close to Taiwan and mainland China, the Ryukyu wild boar is not closely genetically related to wild boar in Taiwan or mainland China, and there has

therefore been considerable controversy for numerous years concerning the origin of Ryukyu wild boar. Ryukyu wild boars were previously considered to be a feral population of East Asian domestic pigs that had been brought to the Ryukyu Islands by early inhabitants (Naora 1937; Semba 1960). Consequently, based on the morphological characters of Ryukyu wild boars, Imaizumi (1973) reported that the Ryukyu wild boars may therefore be a relic of continental pigs.

Recently, Hongo et al. (2002) reported that mtDNA isolated from ancient Vietnamese pig skeletons by Hanoi Agricultural University and the Institute of Archaeology of the Academy of Science in Hanoi showed a close phylogenetic relationship to mtDNA obtained from Ryukyu wild boars. Interestingly, the skeletons from which they isolated mtDNA were considerably larger than those of

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modern Ryukyu wild boar. Their findings suggest that in prehistoric times, the ancestors of Ryukyu wild boar came to the Ryukyu Islands from the Asian mainland via a land bridge, and that they subsequently became geographically isolated on the Ryukyu Islands where they became uniquely adapted to the local environment. However, it is unclear whether descendants of the ancestors of Ryukyu wild boar still inhabit Vietnam.

In the present study, to estimate the genetic relationships between Ryukyu wild boar and both Vietnamese wild boar and indigenous Vietnamese domestic pigs, we visited Vietnam to collect DNA samples over a three-year period. The present phylogenetic analysis indicates that indigenous Vietnamese domestic pigs belong to the East Asian domestic pig lineage, whereas Vietnamese wild boars are divided into the East Asian domestic pig lineage and the Ryukyu wild boar lineage. Here, we describe the genetic variation observed in mtDNA from indigenous Vietnamese domestic pigs and wild boars, and its relations to the origin of Ryukyu wild boar.

Materials and methods

Samples

A total of 64 tissue samples (1 blood, 38 hair and 25 muscle) were collected from indigenous pig breeds, crossbred pigs and wild boars in Vietnam from 2002 to 2004 (36 samples in 2002, 14 samples in 2003, and 14 samples in 2004) (Table 1). Sample collection sites are shown in Fig. 1. In 2002, we collected 32 samples from indigenous domestic pigs in Lao Cai province, 2 samples from indigenous domestic pigs (Meo breed) in Hanoi, and 2 samples from domestic pigs from Bac Kan. In 2003, we collected 11 samples (1 Lang Hong breed, 4 Mong Cai breed, and 6 cross-bred from Cao Bang province, and 3 samples (1 wild boar, and 2 Mong Cai breed) from Lang Son province. In 2004, we collected 10 samples (7 wild boars, and 3 mini pigs) from Quang Tri province, and 4 samples (wild boar) from Thua Thien Hue province. The distinction between wild boars and domestic pigs can effectively be made using a variety of characteristics, including aural examination, coat color variation, the existence of lean meat, and the incidents of ectoparasites such as ticks and lice on the body.

DNA extraction and mtDNA analysis

Total DNA was extracted from each of the 64 samples. DNA was extracted from hair and muscle samples using the DNeasy Tissue Kit (Qiagen Science, MD,

USA), and from blood using the QIAamp DNA Blood mini kit (Qiagen).

The mtDNA D-loop region (574-bp) was amplified using two primers (mit112 and mit106; Watanobe et al. 2003), and purified using a QIAquick PCR Purification Kit (Qiagen) as described previously (Ishiguro and Nishimura 2005). To determine the sequence of the 574-bp mtDNA, we performed direct sequencing using two primers (mit11 and mit12) and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Novel haplotypes of the 574-bp mtDNA that had not previously been published in pig mtDNA databases were submitted to the nucleotide databases held at GenBank, EMBL and DDBJ.

Alignment of 574-bp mtDNA sequence and phylogenetic analysis

The 574-bp mtDNA sequences were aligned using GENETYX-MAC software Version 10 (Software Development Co., Tokyo, Japan). Haplotypes of the 574-bp mtDNA were determined by comparison with the DNA sequences in a pig mtDNA database (J1–J23, Japanese wild boar; M16–M20, Ryukyu wild boars from Amami Island, Iriomote Island and Okinawa Island; M21–M39, East Asian domestic pig; M40–M55 and E33, European domestic pig and wild boar; M56–M60, Korean and Taiwanese wild boars; Ishiguro and Nishimura 2005). Phylogenetic trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei 1987) using the PHYLIP program (Version 3.572, Felsenstein, University of Washington, Seattle, 1995). The confidence of each branch in the phylogeny was estimated using 1000 bootstrap replications (Felsenstein 1985).

Results

Collection of DNA samples in Vietnam

In 2002, we collected 36 DNA samples from indigenous domestic pigs (in the city of Hanoi, and in the provinces of Lao Cai and Bac Kan), but we did not obtain DNA samples from wild boar (Fig. 1. and Table 1). In 2003, in the provinces of Cao Bang and Lang Son, we collected 14 DNA samples, from 1 wild boar and 13 domestic pigs, including 1 sample of the Lang Hong breed and 6 samples of the Mong Cai breed. In 2004, because we were unable to collect wild boar samples from northern Vietnamese provinces adjacent to China, we went to the provinces of Quang Tri and Thua Thien Hue (in the central Vietnam), where we collected 14

Table 1. Location and mtDNA haplotypes of Vietnamese *Sus scrofa* samples

Sample No.	Year	Domestic or Wild (Breed)	Province or Locaion	Tissue	Haplotype	Accession No. or reference
V1	2002	Domestic (Meo)	Hanoi	Hair	M36	AB041483 ^{a)}
V2	2002	Domestic (Meo)	Hanoi	Hair	M36	
V3	2002	Domestic (Meo)	Lao Cai	Hair	M31	AB015091 ^{a)}
V4	2002	Domestic (Meo)	Lao Cai	Blood	M27	D42183 ^{a)}
V5	2002	Domestic (Meo)	Lao Cai	Hair	M27	
V6	2002	Domestic (Meo)	Lao Cai	Hair	M31	
V7	2002	Domestic (Meo)	Lao Cai	Hair	M31	
V8	2002	Domestic (Meo)	Lao Cai	Hair	M31	
V9	2002	Domestic (Meo)	Lao Cai	Hair	M31	
V10	2002	Domestic (Meo)	Lao Cai	Hair	M31	
V11	2002	Domestic	Lao Cai	Muscle	M31	
V12	2002	Domestic	Lao Cai	Muscle	V12	AB306908
V13	2002	Domestic	Lao Cai	Muscle	M36	
V14	2002	Domestic	Lao Cai	Muscle	M28	AB041482 ^{a)}
V15	2002	Domestic	Lao Cai	Muscle	M36	
V16	2002	Domestic	Lao Cai	Muscle	M28	
V17	2002	Domestic	Lao Cai	Muscle	V17	AB306910
V18	2002	Domestic (Meo)	Lao Cai	Hair	Viet3	AB053614 ^{b)}
V19	2002	Domestic (Meo)	Lao Cai	Hair	Viet3	
V20	2002	Domestic (Meo)	Lao Cai	Hair	V20	AB306911
V21	2002	Domestic (Meo)	Lao Cai	Hair	V21	AB306909
V22	2002	Domestic (Meo)	Lao Cai	Hair	V21	AB306909
V23	2002	Domestic (Meo)	Lao Cai	Hair	V23	AB306912
V24	2002	Domestic (Meo)	Lao Cai	Hair	V24	AB306913
V25	2002	Domestic (Meo)	Lao Cai	Hair	V25	AB306914
V26	2002	Domestic	Lao Cai	Hair	V25	AB306914
V27	2002	Domestic	Lao Cai	Hair	V25	AB306914
V28	2002	Domestic	Lao Cai	Muscle	M36	
V29	2002	Domestic	Lao Cai	Muscle	M36	
V30	2002	Domestic	Lao Cai	Muscle	V30	AB306915
V31	2002	Domestic	Lao Cai	Muscle	M28	
V32	2002	Domestic	Lao Cai	Muscle	M31	
V33	2002	Domestic	Lao Cai	Muscle	M31	
V34	2002	Domestic	Lao Cai	Muscle	M28	
V35	2002	Domestic	Bac Kan	Hair	V31	AB306916
V36	2002	Domestic	Bac Kan	Hair	M28	
V37	2003	Domestic (Lang hong)	Cao Bang	Hair	M33	AB015092 ^{a)}
V38	2003	Domestic (Mong Cai)	Cao Bang	Hair	V38	AB306896
V39	2003	Domestic (Mong Cai)	Cao Bang	Hair	38	AB05960 ^{c)}
V40	2003	Domestic (Mong Cai)	Cao Bang	Hair	M33	
V41	2003	Domestic (Mong Cai)	Cao Bang	Hair	V41	AB306897
V42	2003	Domestic	Cao Bang	Hair	M24	AB041474 ^{a)}
V43	2003	Domestic	Cao Bang	Hair	M24	
V44	2003	Domestic	Cao Bang	Hair	M33	
V45	2003	Domestic	Cao Bang	Hair	38	
V46	2003	Domestic	Cao Bang	Hair	M31	
V47	2003	Domestic	Cao Bang	Hair	V47	AB306898
V48	2003	Wild	Lang Son	Hair	J22	AB302180 ^{d)}
V49	2003	Domestic (Mong Cai)	Lang Son	Hair	M31	
V50	2003	Domestic (Mong Cai)	Lang Son	Hair	V50	AB306899
V51	2004	Wild	Quang Tri	Muscle	M36	
V52	2004	Wild	Quang Tri	Muscle	V52	AB306900
V53	2004	Wild	Quang Tri	Muscle	V53	AB306901
V54	2004	Wild	Quang Tri	Muscle	V54	AB306902
V55	2004	Wild	Quang Tri	Muscle	V55	AB306903
V56	2004	Wild	Quang Tri	Muscle	V56	AB306904
V57	2004	Wild	Quang Tri	Muscle	V57	AB306905
V58	2004	Wild	Thua Thien Hue	Muscle	V56	AB306904
V59	2004	Wild	Thua Thien Hue	Muscle	V59	AB306906
V60	2004	Wild	Thua Thien Hue	Muscle	V57	AB306905
V61	2004	Wild	Thua Thien Hue	Muscle	V61	AB306907
V62	2004	Domestic (Mini pig)	Quang Tri	Hair	Viet8	AB053615 ^{b)}
V63	2004	Domestic (Mini pig)	Quang Tri	Hair	M31	
V64	2004	Domestic (Mini pig)	Quang Tri	Hair	Viet8	

^{a)} Ishiguro and Nishimura (2005). ^{b)} Hongo et al. (2002). ^{c)} Okumura et al. (2001). ^{d)} Ishiguro et al. (2008).

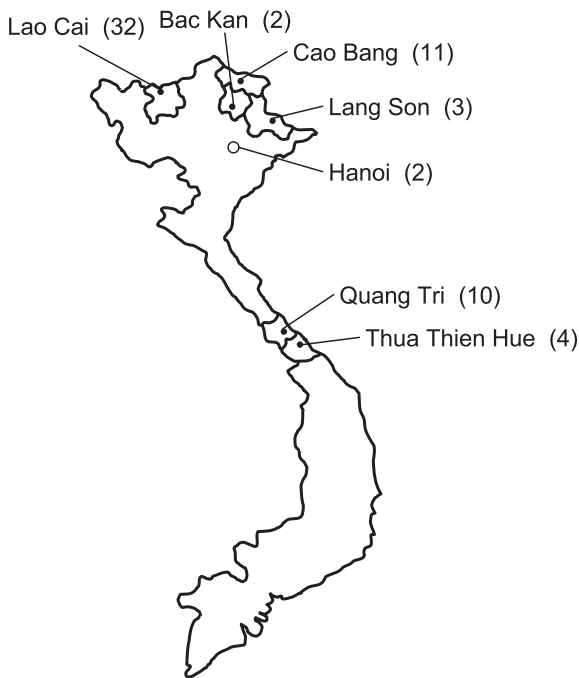


Fig. 1. Sampling sites of 64 domestic and wild pigs in Vietnam. Numbers in parentheses indicate the number of samples per site.

DNA samples from 11 wild boars and 3 mini pigs. This wild boar was a younger than two years old, brown in color, and had numerous ticks on its body.

Nucleotide sequence diversity and phylogenetic relationships

DNA sequences of 574-bp mtDNA from the 64 Vietnamese samples were compared with sequences obtained from a pig mtDNA database, as previously reported (Okumura et al. 2001; Hongo et al. 2002; Watanobe et al. 2003; Ishiguro and Nishimura 2005). Among the 64 Vietnamese samples, we detected 30 different mtDNA haplotypes (21 novel mtDNA haplotypes, and 9 previously reported mtDNA haplotypes) (Table 1). The 9 previously reported mtDNA haplotypes were classified into two groups: an East Asian group (M36, M31, M27, M28, 38, M33, Viet3 and Viet8), and a Japanese wild boar group (J22). Table 2 shows the nucleotide variation observed in the 21 novel mtDNA haplotypes, aligned with the J1 haplotype of Japanese wild boar. Once comparison with the GenBank *Sus scrofa* database revealed that the DNA sequences of the 21 mtDNA haplotypes were novel, the sequences were submitted to the nucleotide databases held at GenBank, EMBL and DDBJ under the accession numbers AB306896 to AB306916 (Table 1).

To clarify the genetic lineage of these novel mtDNA haplotypes, a NJ tree of the 21 mtDNA haplotypes and 69 other *S. scrofa* haplotypes was constructed (Fig. 2). Figure 2 shows the two major lineages formed by the 21 novel mtDNA haplotypes; an Asian cluster (67% bootstrap value) and a European (67% bootstrap value) cluster. The Asian lineage was subdivided into two clusters, a Ryukyu wild boar cluster (35% bootstrap value) and an East Asian cluster (13% bootstrap value), which included the Japanese wild boar, Taiwanese wild boar and Korean wild boar. The 21 novel mtDNA haplotypes were divided into two clusters: Ryukyu wild boar cluster (V54, V55, V57, V59 and V61) and East Asian cluster (the remaining 16 novel mtDNA haplotypes). The five novel mtDNA haplotypes belonging to the Ryukyu wild boar cluster (35% bootstrap value) differ from previously reported mtDNA haplotypes of wild boar from the Ryukyu Islands (M16 and M17 from Amami Island, M18 and M19 from Iriomote Island and M20 from Okinawa Island). The remaining 16 novel mtDNA haplotypes were classified into 3 groups: Korean wild boar group (V52, V53 and V56); East Asian domestic pig and wild boar group (V12, V24, V41, V38, V25, V47, V30, V31, V21, V17, V20 and V50); and Taiwanese wild boar group (V23) (Fig. 2). These results indicate that Vietnamese indigenous domestic pigs and wild boars have made widespread contributions to the genetic diversity of East Asian domestic pig populations. In contrast, we did not find evidence of any genetic contribution from Vietnamese wild boar or indigenous domestic pigs to Ryukyu wild boar lineages.

Discussion

Hongo et al. (2002) demonstrated that the large and small skeletons examined by two research institutes in Hanoi were closely genetically related to Ryukyu and Korean wild boar (large skeletons) and East Asian domestic pigs (small skeletons). However, since the origins and ages of these skeletons are unclear, it remained unknown whether any extant populations in Vietnam are genetically related to Ryukyu wild boar. To determine whether animals belonging to the Ryukyu wild boar lineage exist in Vietnam as wild boar or indigenous domestic pigs, we conducted the present three-year study in Vietnam. Despite searching for Vietnamese wild boar in the mountainous areas of four northern provinces of Vietnam from 2002 to 2003 (Lao Cai, Bac Kan, Cao Bang and Lang Son; Fig. 1), we were unable to obtain

Table 2. Nucleotide variation of Vietnamese wild or domestic pigs detected in this study

Haplotype	Nucleotide positions ^{a)}																				Group								
	182	183	215	242	249	268	277	280	283	302	307	323	324	359	362	388	391	406	431	444		453	502	561	638	658	693	703	
J1	C	T	T	T	G	C	T	C	C	C	T	A	T	A	G	A	T	T	T	A	T	G	T	G	T	A	A	Japanese wild boar	
V23	T	C	G	G	Taiwanese wild boar
V38	T	C	.	.	.	A	.	.	C	G	G	East Asian domestic pig and wild boar	
V41	.	.	.	C	G	C	.	.	.	A	.	.	C	G	G		
V47	.	.	.	C	T	T	A	.	.	C	G	G		
V50	C	.	.	.	A	C	.	C	G	G		
V12	T	C	.	.	C	.	G		
V21	A	.	.	C	G		G
V17	T	.	.	C	A	C	.	C	G		G
V20	.	C	.	.	A	C	.	.	.	A	C	.	C	G	G		
V24	.	.	.	C	.	.	.	T	C	.	.	.	C	A	.	.	C	G		G
V25	.	.	.	C	A	.	.	C	G		.
V30	.	C	C	C	A	.	.	C	G		G
V31	.	C	C	C	.	.	C	G	C	G		G
V52	T	T	.	.	.	C	.	.	G	.	.	.	G	C	A	.	.	C	.	G		Korean wild boar
V53	A	T	.	.	.	C	.	.	G	.	.	.	G	C	A	.	.	C	.	G		
V56	T	T	.	.	T	.	.	.	C	.	.	G	.	.	.	G	C	A	.	.	C	.	G		
V54	T	C	G	C	A	C	.	C	.	G	Ryukyu wild boar	
V55	T	T	C	G	C	A	C	A	C	G	G		
V57	T	T	C	G	C	A	C	.	C	G	G		
V59	T	T	C	G	C	A	C	.	C	.	G		
V61	T	T	C	G	C	A	C	A	C	.	G		

^{a)} Nucleotide position 1 corresponds to the first position of the complete DNA sequences of mtDNA control region described by Okumura et al. (2001).

Dots indicate the nucleotide identity with Japanese wild boar haplotype J1.

samples from wild boars in these areas. The number of wild boars has rapidly decreased in several provinces of Vietnam due to increased hunting pressure and the disappearance of virgin forest. We obtained muscle samples of 12 wild boars from village markets or hunters in three provinces (Lang Son, Quang Tri and Thua Thien Hue) in 2003 and 2004, but we did not obtain any morphometric information of these 12 animals. Morphological characterization of indigenous domestic pigs and wild boars from several Asian countries were described by Groves (1981). Wild boars (*S. Scrofa*) are known to be extensively distributed in Vietnam and the neighboring countries of China, Laos and Cambodia. Recently, Larson et al. (2007) reported the genetic distribution of mtDNA haplotypes of both domestic pigs and wild boars in the South East Asian and Oceania regions. Nonetheless, with the exception of Hongo et al. (2002), relatively little is known about the morphological and genetic characteristics of Vietnamese wild boars.

In the present study, five mtDNA haplotypes (V54, V55, V57, V59 and V61) of the Ryukyu wild boar

lineage were observed in Vietnamese wild boars (Table 1 and Fig. 1), all of which could be distinguished from those of Ryukyu wild boars from Amami Island, Iriomote Island and Okinawa Island (46% bootstrap value) by phylogenetic analysis. The present findings indicate that descendants of the ancestors of Ryukyu wild boar still inhabit Vietnam. They also indicate that the Ryukyu wild boar may be a descendant of the Vietnamese wild boar, and that it developed into a unique species after becoming isolated on the Ryukyu Islands. There have been several opportunities for the ancestor of Ryukyu wild pigs to migrate to the Ryukyu Islands from the Asian continent via a land bridge (Kizaki and Ohshiro 1980; Ujiie 1986). After the Ryukyu Islands were separated from the Asian continent, the wild boars evolved independently on these Islands. The NJ tree shows that the mtDNA haplotypes M16–M19 from Ryukyu Islands in Japan are separated as a small group with 46% bootstrap value from Vietnamese mtDNA haplotypes (V54, V55, V57, V59 and V61) within the Ryukyu wild boar lineage (Fig. 2). This result suggests

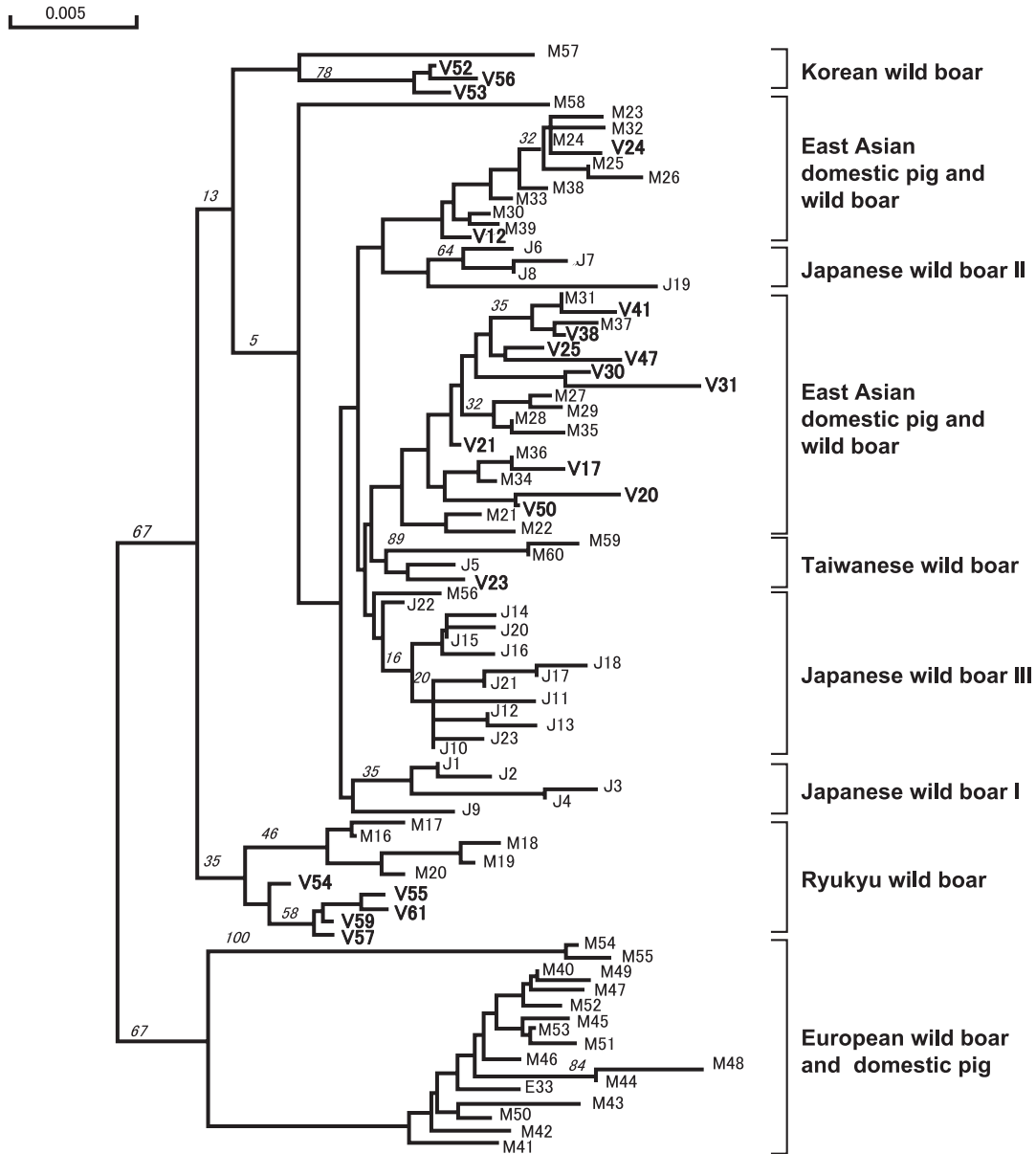


Fig. 2. Neighbor-joining (NJ) phylogenetic tree constructed by the NJ method using the 574-bp mtDNA control region of 21 novel mtDNA haplotypes detected in the present study and 69 previously reported haplotypes (Ishiguro and Nishimura 2005). Bootstrap resampling was performed 1,000 times, with bootstrap probabilities shown in italics on the corresponding branches.

that Ryukyu wild boars on the Ryukyu Islands diverged from the *Sus* population on the mainland in prehistoric times and that they were not introduced into Ryukyu Islands by early human inhabitants. Although it is not clear whether wild boars possessing Ryukyu wild boar genetic characteristics are present in Vietnam and Ryukyu Islands in Japan, no wild boars exhibiting genetic characteristics of the Ryukyu wild boars from the neighboring countries such as Laos, Thailand and Cambodia have been detected to date (Larson et al. 2007).

Eleven of the 12 Vietnamese wild boar mtDNA haplo-

types were classified into wild boar lineages (the exception was V51; haplotype M36 in Table 1), suggesting that they are closely genetically related to East Asian wild boars. However, mtDNA haplotypes belonging to the Ryukyu wild boar lineage have never been observed in Vietnamese indigenous domestic pigs or East Asian domestic pigs. It is unclear why animals from the Ryukyu wild boar lineage are not present among the various domesticated lineages in Vietnam. It may be due to some particular aspect of the historical or biological processes by which *Sus scrofa* was domesticated from wild

boar to domestic pig.

The NJ tree shown in Fig. 2 demonstrates that the 23 mtDNA haplotypes of Japanese wild boars can be classified into three clusters (Fig. 2, I, II and III), which is consistent with the findings of a previous report (Watanobe et al. 2003). The available evidence suggests that the ancestors of Japanese wild boars were part of the Asian *S. scrofa* population that spread from Southeast to Northeast Asia during the Middle to Late Pleistocene (Watanobe et al. 2003). The phylogeographic characterization and population structure of Japanese wild boars indicates that there were at least three independent wild boar migrations, each with distinct genetic characteristics, from the Asian continent to Japan. These three groups of Japanese wild boar ancestors subsequently interbred to form the extant Japanese wild boar population.

In Vietnam, there are several indigenous pig breeds, including Meo, Mong Cai, Lang Bang, Mini pig, I Muongk Huong and Soc (Thuy et al. 2006). In the present study, some Vietnamese indigenous pig breeds (Meo, Lang Hong, Mong Cai and Mini Pig) were identified by their vocalizations, but numerous crossbred pigs were simply classified as “Domestic” (Table 1) because it is difficult to accurately distinguish between several of the indigenous pig breeds using only morphological characteristics. Among the Vietnamese indigenous pig breeds, no domestic pigs were detected in the Ryukyu wild boar lineage. The 21 novel mtDNA haplotypes identified in the present study could be separated into the East Asian domestic pig and wild boar lineages, suggesting that indigenous Vietnamese pigs exhibit a high degree of mtDNA variability, as described previously (Hongo et al. 2002) and demonstrated using microsatellites (Thuy et al. 2006). In addition, like China, Vietnam is considered to be one of the centers of domestication in Asian pigs from native wild boars. Since Vietnamese wild boars and indigenous pigs have been found to be genetically related to domestic pigs and wild boars in numerous other regions of Asia, Vietnam may also be a principal source for Asian feral *Sus scrofa* populations. This genetic diversity in domestic Asian pigs is likely to have contributed to breeding developments in both Asian and European pigs, and the marked genetic diversity observed in indigenous Vietnamese pig breeds is thought to be important for livestock maintenance.

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