

Ancient DNA Reveals Genetic Lineage of *Sus scrofa* among Archaeological Sites in Japan

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Abstract We analyzed 201 *Sus* bones excavated from one Jomon and nine Yayoi sites in the southwestern Japanese Islands to determine their genetic relatedness using three fragments of the mitochondrial DNA (mtDNA) control region. Three fragments A (194-bp), B (212-bp) and C (166-bp) were independently amplified from 38, 22 and 28 *Sus* bone specimens, respectively. All three fragments were successfully sequenced from ten specimens to construct 574-bp sequences that were combined with 57 modern *Sus scrofa* sequences including wild boars and domestic pigs to make a phylogenetic tree. Four of 10 specimens excavated from three sites located in the western part of Japan, were closely related to modern East Asian domestic pigs. The other six specimens from three sites were closely related to modern Japanese wild boars. However, parsimonious networks constructed for each fragment showed the difficulty of clearly distinguishing between Japanese wild boars and East Asian domestic pigs. These results reveal the possibility that even if *Sus* bones found at Yayoi sites are identified as East Asian domestic pigs, they were brought as exchanges or foods from Continental Asia to Japan through trade, but no evidence showing *Sus* breeding.

Keywords: ancient DNA, mitochondrial DNA, Japanese wild boar, domestic pig, molecular phylogeny

Introduction

Wild boars (*Sus scrofa*) are inhabiting in many countries throughout Europe, Asia and northwestern Africa, and at least 16 different subspecies have been reported (Ruvinsky and Rothschild, 1998). Domestic pigs are thought to have been independently established from native European and Asian wild boar subspecies, and the

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divergence time between these two ancestral forms was estimated at about 900,000 years ago (Giuffra et al., 2000; Kijas and Anderson, 2001). According to fossil records, Japanese wild boar (*S. scrofa leucomystax*) migrated from the Asian continent to the Japanese Islands during the late Pleistocene (Kawamura, 1991). The Japanese wild boar inhabiting the three main islands (Honshu, Kyushu and Shikoku) is distinguishable from the Ryukyu wild boar (*S. s. riukiuanus*) and continental domestic pigs by genetic differences (Watanobe et al., 1999).

In the prehistoric Japanese islands, the Jomon period (10,000 to 250 B.C.) was followed by the Yayoi period (250 B.C.–300 A.D). These two periods are distinguished based on the mode of subsistence because systematic paddy rice cultivation was introduced from Continental Asia in the Yayoi period. We can get information about the prehistoric subsistence pattern by analyzing archaeological remains by various methods. Especially, various kinds of animal bones excavated in association with other archaeological remains. Among the animal bones, *Sus* are one of the most common and indicating their importance as a protein source for the prehistoric human diet. Whether *Sus* specimens excavated from Yayoi sites are Japanese wild boars harvested by hunting or domestic pigs introduced from Continental Asia has been under discussion for a decade. Nishimoto (1991, 1993) reported that Yayoi people imported domestic pigs, called “Yayoi pig” from Continental Asia. His studies suggest that wild boars and domestic pigs can be distinguished by morphological and numerical difference of the atlas, upper third molar, cranial parts and mandible. According to this criterion, some of *Sus* specimens excavated from Yayoi sites were domestic pigs from Continental Asia rather than domesticated Japanese wild boars. On the other hand, Ozawa (2000) reported that it is difficult to distinguish Japanese wild boar and domestic pig specimens excavated from Yayoi sites based on osteometric characters. Introduction of domestic pigs, called “Yayoi pig” from the Asian continent to Japan in the Yayoi period is still controversial.

Advances in DNA analytical techniques during recent decades allow molecular genetic analyses of ancient animal and human populations (Cooper et al., 1992; Demarchi et al., 2001; Hardy et al., 1995; Horai et al., 1991; Oota et al., 1999; Okumura et al., 1999; Pääbo, 1989; Stone and Stoneking, 1993). A recent study of *Sus* specimens from archaeological sites also showed that analysis of ancient DNA made it possible to investigate genetic relationships between prehistoric and modern *S. scrofa* (Watanobe et al., 2001). These molecular studies demonstrate that direct analysis of ancient DNA extracted from archaeological remains is useful for understanding the genetic lineage and the historical transitions of animals.

To investigate the genetic background of *Sus* specimens from Yayoi sites, we extracted ancient DNA from archaeological specimens, and amplified and sequenced the mitochondrial DNA (mtDNA) control region using polymerase chain reaction (PCR) techniques. Sequences obtained from Yayoi sites were compiled with modern

Sus sequences and analyzed for phylogenetic relationships.

Materials and Methods

Archaeological samples and sites

In this study, a total of 201 *Sus* specimens were collected from 10 archaeological sites including two shellmiddens (Fig. 1). A summary of the specimens and the approximate chronological ages of each site are shown in Table 1.

DNA extraction

Ancient DNA was extracted from *Sus* samples. To avoid possible contamination from the surfaces of the archaeological remains, the soil and outer layers of the bone and tooth samples were removed by scraping with a sterile razor blade. Bone powder (0.5 to 1.0 g) was collected by using an electric drill, suspended in 10 ml of 0.5 M ethylenediaminetetraacetate (EDTA), and decalcified by rotating for a few days. There-



Figure 1. Archaeological sampling sites of ancient *Sus* specimens examined in this study.

Table 1. Sampling sites and the number of archaeological specimens examined in this study

Site ^a No.	Archaeological sites (Prefecture or island, Periods)	No. of specimens examined	No. of samples amplified by PCR	PCR results ^b			
				ID	fA	fB	fC
1	Asahi site (Aichi, Middle Yayoi)	31	18	14	+	–	–
				15	+	–	–
				17	+	–	+
				20	+	–	+
				22	+	–	–
				23	+	+	+
				26	+	–	+
				29	+	+	–
				32	+	–	+
				34	+	–	–
				35	+	–	+
				36	+	–	–
				38	+	+	+
				40	+	+	–
				41	+	–	+
				277	+	–	+
				278	+	+	+
				279	+	–	+
2	Karako-kagi site (Nara, middle to late Yayoi)	25	1	95	–	–	+
3	Tsuboi-niji site (Nara, middle to late Yayoi)	4	0				
4	Higashi-nara site (Osaka, middle Yayoi)	4	0				
5	Kadota shellmidden (Okayama, Early Yayoi)	24	0				
6	Agata site (Ehime, Final Jomon to early Yayoi)	23	6	384	–	+	–
				385	+	+	–
				387	+	+	+
				392	+	+	+
				394	–	+	–
				395	+	+	+
7	Miyamaegawa site (Ehime, Late Yayoi to early Kofun)	7	1	414	+	+	+
8	Shimogoori-kuwanae site (Ooita, Early to middle Yayoi)	57	19	3	+	–	+
				4	+	–	–
				8	+	–	–
				13	+	–	–
				71	+	–	–
				280	–	+	–
				283	+	–	–
				284	+	–	–
				285	+	+	+
				288	–	+	–
				357	+	–	+
				358	–	+	–

Site ^a No.	Archaeological sites (Prefecture or island, Periods)	No. of specimens examined	No. of samples amplified by PCR	PCR results ^b			
				ID	fA	fB	fC
				364	–	–	+
				365	+	+	+
				368	–	+	–
				369	–	–	+
				373	–	+	+
				375	–	+	+
				378	–	+	–
9	Harunotsuji site (Iki island, Nagasaki, Late Yayoi)	14	2	116	+	–	–
				120	+	–	–
10	Miyashita shellmidden (Fukuejima island, Nagasaki, Late Jomon)	12	4	424	–	–	+
				425	+	–	+
				427	+	+	+
				428	+	–	+
Total		201	51		38	22	28

^a The site numbers correspond to that in Fig. 1.

^b fA, fB, and fC indicate the DNA fragment amplified by set A, B, and C primers, respectively.

+: amplified, –: not amplified.

after, the sample was centrifuged at 3,000 rpm for 10 min, the supernatant was removed, and the pellet of bone powder was repeatedly decalcified by treatment with 10 ml of 0.5 M EDTA. After decalcification, the bone powder was treated overnight in 5 ml of a mixture of 0.5 M EDTA containing proteinase K (300 µg/ml) and N-lauroyl sarcosine (0.5%) (Hardy et al., 1995, with slight modifications). The sample was centrifuged at 3,000 rpm for 10 min, the supernatant containing the ancient DNA was extracted twice with phenol, once with chloroform: phenol (1 : 1) and once with chloroform to remove the protein. The supernatant was concentrated using a Centri-con 30 micro-concentrator (Amicon, Beverly, MA) and washed with distilled water. At the end of this process, the DNA samples were concentrated to a final volume of 20–100 µl. The extracted ancient DNA samples were directly used as PCR templates. Precautions were taken to prevent contamination from non-ancient DNA as described by Okumura and his colleagues (1999). Blank extractions were carried through the extraction procedure as negative control to monitor possible contamination.

PCR and direct sequencing of mtDNA

Following the present study, 574-bp of the mtDNA control region was amplified by PCR using three primer sets (A, B and C) (Watanobe et al., 2001). We also used semi-nested PCR to amplify the ancient DNA when little or no PCR product was

detected in the first amplification effort. Semi-nested PCR was performed under the same condition as the first PCR with amplification for 40 cycles. To make sure of the reliability of the result, each sample was amplified at least twice independently, and all PCRs were carried out with both blank extractions and negative controls for PCR.

The PCR products were purified by using a Centricon 100 micro-concentrator (Amicon, Beverly, MA) and used for sequencing templates. Nucleotide sequences of both strands were determined by using an Applied Biosystems 377 DNA sequencer (Applied Biosystems, Foster, CA) with BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, Foster, CA). When the nucleotide sequences obtained from two independent PCR products differed, the third PCR amplification and subsequent direct sequencing was carried out to confirm the sequence. The 574-bp nucleotide sequences were constructed by connecting three DNA fragments amplified by primer sets A, B and C.

Data analysis

Phylogenetic analysis was carried out with the sequences obtained in this study and 57 haplotypes of modern wild boars and domestic pigs in previous studies (Okumura et al., 1996; Watanobe et al., 2001) (Table 2). Multiple-sequence alignment was performed using GENETYX-MAC software (Software Development Co., Ltd., Tokyo, Japan). Genetic distance were calculated using the two-parameter method (Kimura, 1980) and tree were constructed by the neighbor-joining method (Saitou and Nei, 1987) using the PHYLIP program, version 3.572 (Felsenstein, 1995) with bootstrap values (Felsenstein, 1985) generated by 1000 replications. Parsimonious network analysis using partial sequences from archaeological specimens and modern *Sus scrofa* haplotypes was conducted by the split decomposition method (Dopazp et al., 1993).

Results

*DNA sequence of *Sus scrofa* from archaeological sites*

The mtDNA control region DNA was successfully obtained from 51 of the 201 archeological specimens examined from 10 sites (Table 1). Ten of the 51 specimens were replete for all three fragments fA, fB, and fC, while the other 41 specimens were partially sequenced as one or two of the fragments could not be amplified by PCR. The long DNA fragment (fB: 212-bp) was more difficult to amplify than the shorter DNA fragments fA (194-bp) and fC (166-bp), indicating that no long fragment might remain in archaeological specimens because of the degradation of autolysis, oxidation and bacterial digestion (Pääbo, 1989).

Table 3 shows the nucleotide polymorphic sites of all ancient sequences compared with 5 representative modern haplotypes (including one gap) in the 574-bp control. Among 51 ancient DNA sequences, 23 polymorphic sites were identified. Eight of

Table 2. Accession numbers of modern *Sus scrofa* using phylogenetic analyses

Haplo-type	Origin	Accession number	Haplo-type	Origin	Accession number
1	Japanese wild boar	D42172	30	Asian domestic pig	D42185
2	Japanese wild boar	AB015084	31	Asian domestic pig	AB015091
3	Japanese wild boar	AB041469	32	Asian domestic pig	D42182
4	Japanese wild boar	AB041471	33	Asian domestic pig	AB015092
5	Japanese wild boar	AB041472	34	Asian domestic pig	AB041480
6	Japanese wild boar	AB041473	35	Asian domestic pig	AB041481
7	Japanese wild boar	AB041467	36	Asian domestic pig	AB041483
8	JaPanese wild boar	D42173	37	Asian domestic pig	AB041487
9	Japanese wild boar	D42174	38	Asian domestic pig	AB041490
10	Japanese wild boar	D42178	39	Asian domestic pig	D42180
11	Japanese wild boar	D4217476	40	European domestic pig	D42179
12	Japanese wild boar	AB015085	41	European domestic pig	AB041496
13	Japanese wild boar	D42171	42	European domestic pig	AB041497
14	Japanese wild boar	AB015086	43	European domestic pig	AB041499
15	Japanese wild boar	AB041468	44	European domestic pig	AB041498
16	Ryukyu wild boar	AB015087	45	European domestic pig	AB041495
17	Ryukyu wild boar	AB015088	46	European domestic pig	D42170
18	Ryukyu wild boar	AB015089	47	European domestic pig	AB041492
19	Ryukyu wild boar	AB015090	48	European domestic pig	AB041491
20	Ryukyu wild boar	D42184	49	European domestic pig	AB041488
21	Eurasian wild boar	AB041465	50	European domestic pig	AB041484
22	Eurasian wild boar	AB041466	51	European domestic pig	AB041485
23	Asian domestic pig	D42181	52	European domestic pig	AB015093
24	Asian domestic pig	AB041474	53	European domestic pig	AB041489
25	Asian domestic pig	AB041475	54	European wild boar	AB015094
26	Asian domestic pig	AB041476	55	European wild boar	AB015095
27	Asian domestic pig	D42183	56	Japanese wild boar	AB055222
28	Asian domestic pig	AB041482	57	Eurasian wild boar	AB066101
29	Asian domestic pig	AB041479			

these 23 sites found only in the 51 ancient DNA sequences were newly identified in this study, and the remaining 15 sites were shared with the modern haplotypes.

Phylogenetic analysis

Using the 41 sequences consisting of only one or two of the three fragments did not adequately resolve the phylogenetic relationship. Therefore, the neighbor-joining tree was constructed using 57 modern haplotypes (Table 2) and the 10 ancient 574-bp nucleotide sequences. Four ancient DNA sequences were identical to modern sequences of Japanese wild boar (Agata 395 and haplotype 4, Shimogoori 365 and haplotype 1, Asahi 38 and haplotype 8) and Asian domestic pig (Miyamaegawa

414 and haplotype 31), and remaining the 6 sequences (DDBJ/EMBL/GenBank Accession Nos. AB077469 to AB077474) were different from modern *Sus scrofa* sequences (Fig. 2).

The neighbor-joining tree showed that the haplotypes can be divided into two distinct lineages, Asian (haplotypes 1 to 39, 56 and 57) and European (haplotypes 40 to 55) (Fig. 2). The Asian lineage was subdivided into two clusters designated A1 and A2, and the European lineage was subdivided into two clusters designated E1 and E2. The cluster A1 comprised Japanese wild boar (haplotypes 1 to 15, and 56), Northeast Asian wild boar (haplotypes 21, 22, and 57) and East Asian domestic pig (haplotypes 23 to 39), and the cluster A2 comprised Ryukyu wild boar (haplotypes 16 to 20). All ancient haplotypes from archaeological specimens belonged to cluster A1 (Fig. 2). Three haplotypes (Asahi 23, 278, and Shimogoori 285) were closely related to modern Japanese wild boars (haplotypes 1 to 15 and 56), and three haplotypes Asahi 38, Agata 395, and Shimogoori 365 were identical to the modern Japanese wild boar haplotypes 8, 4, and 1, respectively. On the contrary, three haplotypes from Agata 387 and 392, Miyamaegawa 414 and Miyashita 427 were closely related to or identical to modern East Asian domestic pigs (haplotypes 23 to 39). Thus some ancient *Sus* specimens are closely related to Japanese wild boars, and others are closely related to East Asian domestic pigs.

Parsimonious networks among modern Asian *Sus scrofa* haplotypes of cluster A1 and ancient sequences were constructed for each fragment A, B, and C (Figs. 3A, 3B, and 3C, respectively). We found that modern haplotypes of each network were resolved into three groups: Japanese wild boar, East Asian domestic pig, and an intermediate group. The intermediate group included sequences derived from both modern Japanese wild boars and East Asian domestic pigs in each network (e.g. sequences from haplotypes 8, 11 and 56 of Japanese wild boars, and haplotypes 28 and 35 of East Asian domestic pigs in Fig. 3A). Therefore, the intermediate groups could not determine the origin of sequence. The fragment sequences obtained from archaeological specimens were also resolved into the three groups determined by the modern sequences, and some fragment sequences belonged to the intermediate group in each network. In such a case, we should suspend the judgment on their origins of sequences.

Discussion

Sus specimens used in this study included bone parts and fragment were neither distinguishable as Japanese wild boar and East Asian domestic pig based on morphological characters described by Nishimoto (1991, 1993), nor precisely dated. Four of the 10 sequences from the archeological specimens are closely related to East Asian domestic pigs, while six of 10 sequences were genetically recognized as Japanese wild boars (Fig. 2). Although we obtained low bootstrap values for clusters A1

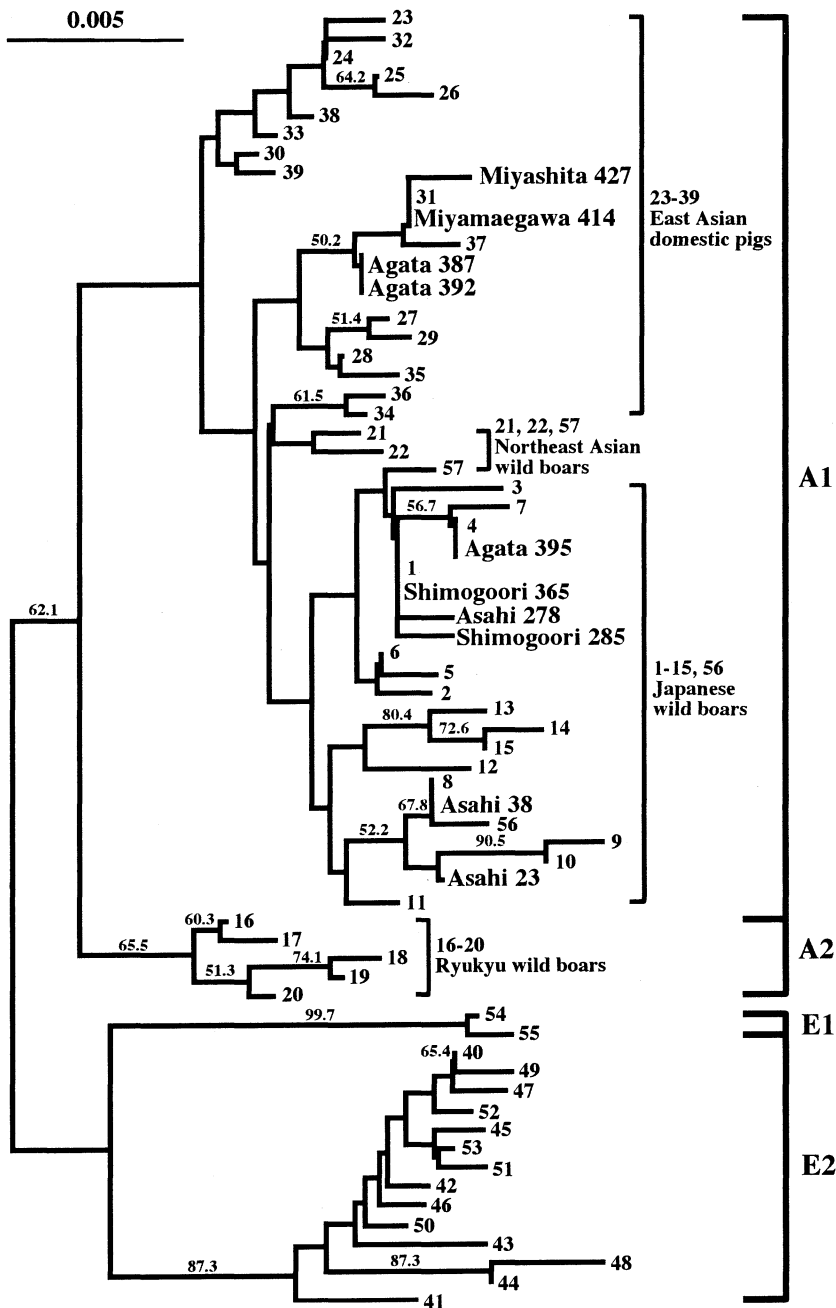
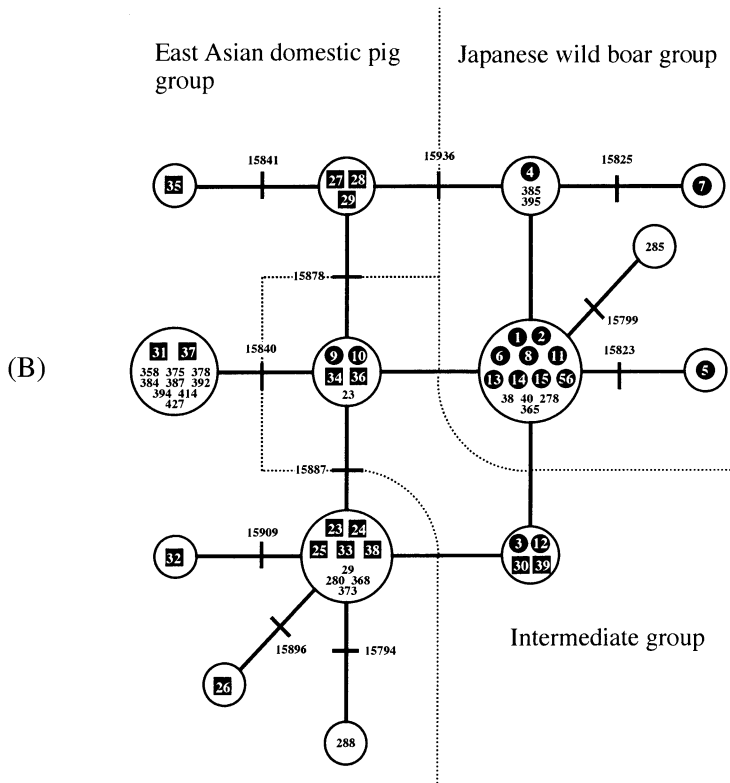
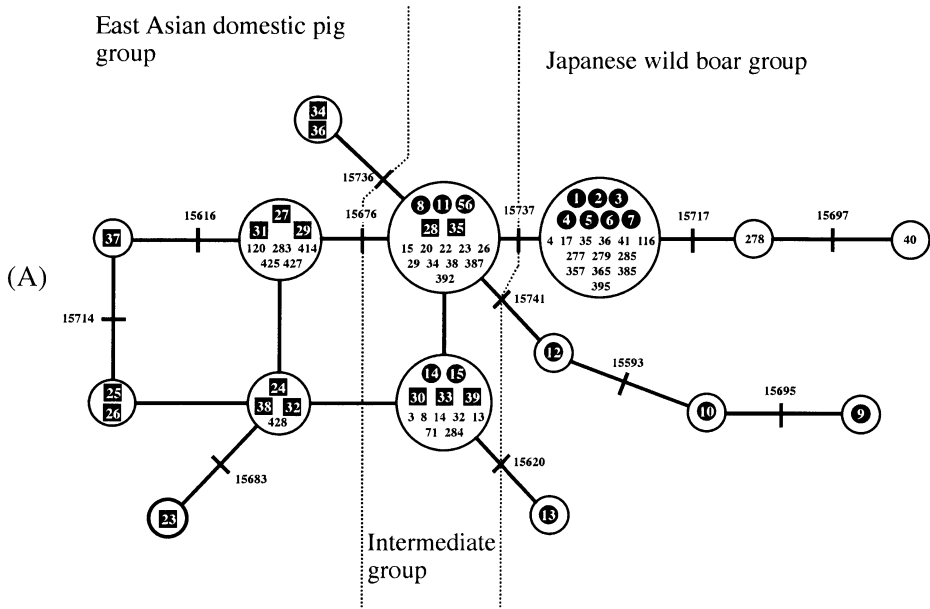


Figure 2. Phylogenetic tree constructed from 57 modern and 10 ancient sequences (574-bp) of the mtDNA control region using the neighbor-joining (NJ) method. The sequences obtained from archaeological specimens in this study are indicated by large print. Bootstrap resampling was done 1,000 times, and probabilities greater than 50% are shown on the corresponding branches.



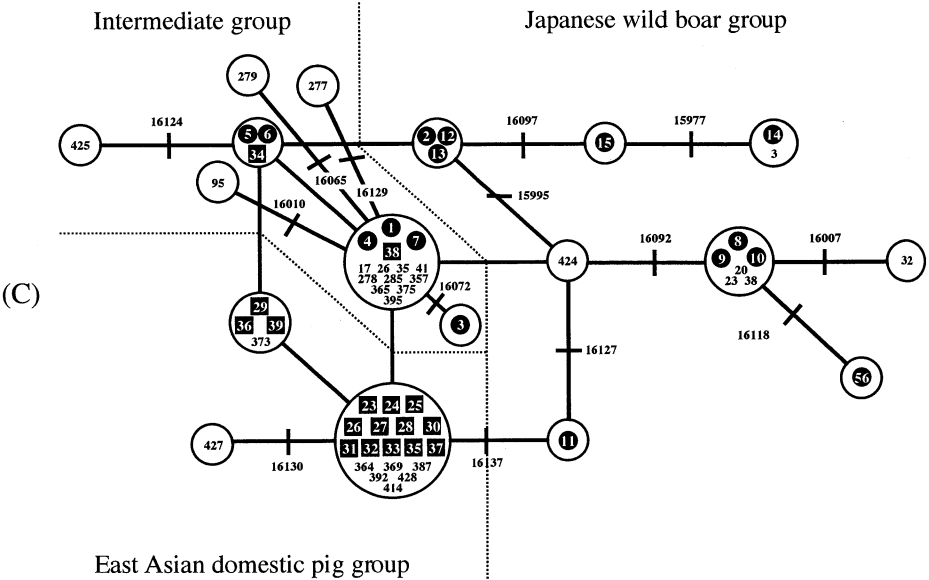


Figure 3. Parsimonious networks constructed using 33 modern Asian *Sus scrofa* haplotypes belonging to cluster A1 (Fig. 2) and ancient sequences based on (A) fragment A (194-bp) with 38 ancient sequences; (B) fragment B (212-bp) with 22 ancient sequences; and (C) fragment C (166-bp) with 28 ancient sequences. In the network, each bar with a nucleotide position number on the branch indicates a nucleotide substitution. Filled squares with white letters indicate the haplotypes from modern domestic pigs, and filled circles with white letters indicate the haplotypes from modern Japanese wild boars. Ancient sequences are shown by ID numbers of archaeological specimens in Table 1. Modern haplotype numbers are the same as in Fig. 2. Three groups (Japanese wild boar, East Asian domestic pig, and combined groups) are defined on the basis of origins of haplotypes.

and A2 in this study (45.7% and 65.5%, respectively), a previous report using the complete sequences of the control region and *cytb* gene showed 99.7% and 93.2% bootstrap values, respectively (Watanobe et al., 1999). Low bootstrap values in this study can be attributed to using a 574-bp of the control region and a large number of haplotypes (Sanderson, 1989).

Another study has used analysis of the mtDNA control region to identify archaeological samples. Ozawa (2000) claimed *Sus* specimens excavated from late Jomon to Yayoi sites were genetically identical to Japanese wild boars based on analysis of partial 255-bp nucleotide sequences of the mtDNA control region. While modern Japanese wild boars could be distinguished from continental Asian *Sus scrofa* by only one nucleotide substitution (nucleotide position number 15,936) in Ozawa's study, we could not make that distinction based on the network of fragment B with one substitution at position 15,936 (Fig. 3B). In a further illustration of this point, the

phylogenetic tree based on the 574-bp mtDNA sequence shows that sequences Agata 387 and 392 are closely related to East Asian domestic pigs (Fig. 2), but we could not make this conclusion using only fragment A (Fig. 3A). The difference between Oza-wa's (2000) and our results may be due not only to the length of the sequences used, but also the total number of modern haplotypes used in the analysis as a sufficient number of reference haplotypes are required to resolve *Sus* specimens from archaeological sites. Alternatively, ancient Japanese wild boars in our study may have possessed the genetic characteristics that classify them with East Asian domestic pigs. We also can not exclude the possibility that ancient, local Japanese wild boar populations shared genetic characteristics with continental *Sus* populations.

Nishimoto (1991, 1993) proposed that "Yayoi pig" was introduced from Continental Asia along with the introduction of new types of stone tools, metal objects, and paddy field rice cultivation techniques through immigration during the Yayoi period. Moreover, Nishimoto (1995) suggested that *Sus* remains excavated from Jomon and Yayoi sites located in southern part of Hokkaido, Sado Island and the Izu Islands provided evidence that human introduced Japanese wild boars to the islands. "Yayoi pig" might have been kept and spread to all parts of Japan during migration to the east and north. It may be difficult to distinguish East Asian domestic pigs from domesticated Japanese wild boars by using morphological characteristics, which are easily influenced by environmental conditions. Our results showed that domestic pigs were probably introduced from Continental Asia to Japan during the Yayoi period based on phylogenetic analysis of the partial 574-bp mtDNA sequences. However, we could not support the spread of "Yayoi pig" using the 574-bp mtDNA sequences. This region of mtDNA could not be amplified from *Sus* specimens of Jomon sites, giving us no opportunity to compare *Sus* specimens from Jomon sites with those from Yayoi sites.

It is significant to note that all 574-bp sequences closely related to East Asian domestic pigs were obtained only from archaeological sites in western Japan, such as Miyashita shellmidden, Agata and Miyamaegawa sites, rather than from eastern sites. Further, all 574-bp sequences derived from *Sus* specimens excavated from the Asahi site in central Japan were closely related to Japanese wild boars, not East Asian domestic pigs. Therefore, our results suggest that *Sus* specimens from southwestern Yayoi sites showing continental genetic lineage were brought from Continental Asia to Japan by trade. Continental Asian domestic pigs were kept in prehistoric Korea during the period corresponding to the Yayoi period in Japan (Ishiguro, unpublished data), and bone and antler artifacts originally from the Korean peninsula were excavated from the Miyamaegawa site on Shikoku Island (Matsui, 2001). Furthermore, the partial sequence obtained from Harunotsuji 120 being closely related to East Asian domestic pigs (Fig. 3A) is consistent with many other remains excavated from the Harunotsuji site showing the exchange with Continental Asia.

However, whether domestic pigs were introduced as livestock or as dressed meat remains unresolved. There might have been very few, if any, domestic pigs introduced as livestock from Continental Asia to Japan giving a potential reason why domestic pigs from Continental Asia could not spread to the eastern Yayoi site.

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