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Prehistoric Sado Island Populations of *Sus scrofa* Distinguished from Contemporary Japanese Wild Boar by Ancient Mitochondrial DNA

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ABSTRACT—Ancient mitochondrial DNA (mtDNA) mainly from Jomon Period *Sus scrofa* bone specimens (6,100–1,700 years old) was examined to clarify the genetic relationships between prehistoric and contemporary *S. scrofa* on Hokkaido, Honshu, Sado, and Izu islands of the Japanese Archipelago. Phylogenetic analysis of the mtDNA control region (574 bp) and analysis of pairwise nucleotide differences between prehistoric and contemporary *S. scrofa* sequences showed the following relationships between these groups: (1) a group genetically similar to contemporary Japanese wild boars was found mainly on Honshu Island, Hokkaido Island, and the Izu Islands, and (2) a monophyletic group distinct from contemporary Japanese wild boars was found on Sado Island. These results suggest that prehistoric people introduced *S. scrofa* from Honshu Island to Hokkaido Island and the Izu Islands. The estimated divergence times between the prehistoric Sado group and the other prehistoric *S. scrofa* is approximately congruent with the geological isolation of Sado Island from Honshu Island. Our results suggest that this extinct *S. scrofa* population was present on Sado Island as recently as around 2,000 years ago.

Key words: ancient DNA, mitochondrial DNA, molecular phylogeny, *Sus scrofa*, Japanese Archipelago

INTRODUCTION

The wild boar (*Sus scrofa*), which inhabits wide areas of Asia, Europe, and North Africa with at least 16 endemic subspecies, is the ancestral species of domestic pigs (Ruvinsky and Rothschild, 1998). One of these subspecies, the Japanese wild boar (*S. s. leucomystax*) inhabits Honshu, Shikoku and Kyushu islands of the Japanese Archipelago (Fig. 1). Fossil records of Pleistocene mammalian fauna indicate that the *S. scrofa* species had expanded its range from southeast to northeast on the Asian Continent in the Early to Middle Pleistocene (Kawamura, 1982), and it was distributed in the mainland of the Japanese Archipelago in the Middle Pleistocene (Fujita *et al*., 2000). Genetic studies of *S. scrofa* showed that the Japanese wild boar was more closely related to the East Asian *S. scrofa*, especially the Northeast Asian wild boar, than to the Ryukyu wild boar (Watanobe *et al*., 1999; Watanobe *et al*., 2001). Moreover, general opinion that *S. scrofa* migrated to the Japanese Archipelago over landbridges repeatedly formed between Japan and the Asian Continent and subsequently were isolated on the islands when the Korea and Tsushima straits formed (Fig. 1) is supported by the phylogeographical analysis of mitochondrial DNA of the Japanese wild boar (Watanobe *et al*., 2003).

On the prehistoric Japanese Archipelago, the Jomon (12,000–2,300 years ago) and Yayoi (2,300–1,700 years ago) periods follow the Paleolithic Period. *S. scrofa* is considered to have been an important source of protein in the prehistoric human diet based on the large numbers of *S. scrofa* bones excavated from many archaeological sites in

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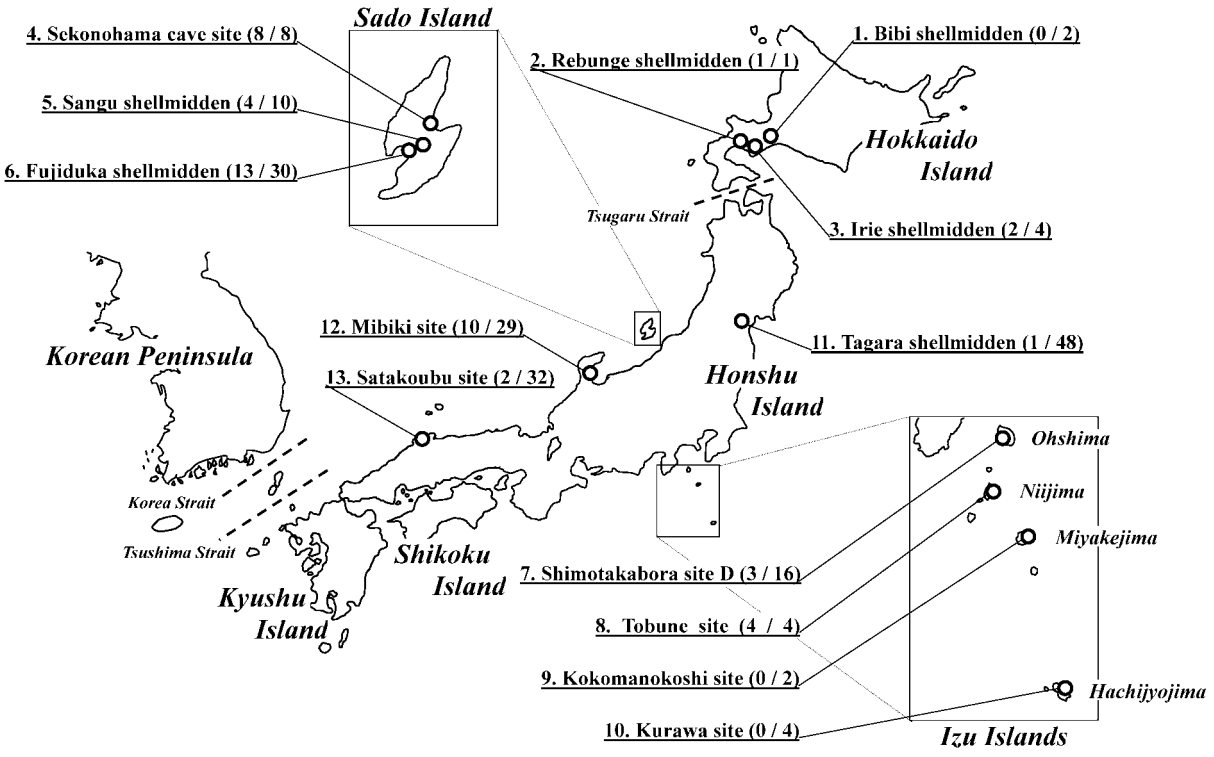

Fig. 1. Archeological sampling sites on the Japanese Archipelago of prehistoric *Sus scrofa* specimens. Sado Island and the Izu Islands are enlarged and shown in insets. Chronological periods and approximate dates (years before present) of archaeological sites are as follows: early Jomon Period–Bibi shellmidden (site 1; 6,000), Mibiki site (12; 6,100–4,700), Satakoubu shellmidden (13; 5,000–3,500); middle Jomon Period–Fujiduka shellmidden (6; 4,700– 4,000); late Jomon Period–Irie shellmidden (3; 4,000– 3,000), Sangu shellmidden (5; 4,000–3,000), Shimotakabora site D (7; 3,000–2,000), Tobune site (8; 4,000–3,000), Kurawa site (10; 5,000–4,000), Tagara shellmidden (11; 3,000–2,500); Yayoi Period (Epi-Jomon Period in Hokkaido)–Rebunge shellmidden (2; 2,300–1,700), Sekonohama cave site (4; 2,000–1,700), Kokomanokoshi site (9; 2,300–1,700). Numbers in parenthesis on the map indicate the number of successfully amplified DNA sequences followed by the number of samples examined in this study.

the Jomon and Yayoi periods. During the Jomon Period, which is characterized by subsistence hunting and gathering, the wild *S. scrofa* is thought to have been a more important food than during the Yayoi Period, which is characterized by systematic rice paddy cultivation introduced from Asian Continent. The natural range of Japanese wild boars is presently limited to three of the four major islands; boars are present on Honshu, Shikoku, and Kyushu islands, but not on Hokkaido Island. However, a large number of *S. scrofa* bones were excavated from archaeological sites not only on the three currently inhabited islands, but also on Hokkaido Island and small neighboring islands such as Sado Island and the Izu Islands (Fig. 1). It remains controversial whether the *S. scrofa* bones excavated from these islands are from native *S. scrofa* or from *S. scrofa* introduced by prehistoric people (Nishimoto, 1995). The origin of prehistoric *S. scrofa* found in archaeological sites of the Jomon Period are of great interest in archaeological, paleontological, and biogeographical contexts.

The DNA of living organisms is an accumulation of the historical record. If the *S. scrofa* bones excavated from these islands are those of native *S. scrofa*, the nucleotide sequences recovered from them should have diverged from the *S. scrofa* on the mainland (i.e. Honshu Island) based on the differences that have accumulated since their isolation after these islands separated from Honshu Island. On the other hand, if the bones excavated from these islands were those of *S. scrofa* introduced by humans at relatively recent times, there would be no or few genetic differences from *S. scrofa* on Honshu Island. Indeed, the currently introduced wild boars on Tsushima Island were shown to have the same mitochondrial DNA (mtDNA) haplotype as those on northern Kyushu Island (Watanobe *et al.*, 2003). Analysis of ancient mtDNA extracted from bones collected at archeological sites has made it possible to investigate the genetic relationships between the prehistoric and contemporary *S. scrofa* (Morii *et al.*, 2002; Watanobe *et al.*, 2001; Watanobe *et al.*, 2002). These studies indicate that genetic information retrieved from archaeological natural remains is extremely useful for understanding the genetic lineage of ancient animals as well as their historical geographic distribution and movements within a given area. Thus, ancient mtDNA analysis can be used to examine genetic relationships between prehistoric *S. scrofa* from Jomon archaeological sites and contemporary *S. scrofa*.

To investigate the genetic background of the prehistoric *S. scrofa* on Hokkaido Island, Sado Island, and the Izu Islands where there are no Japanese wild boars at present,

we extracted ancient DNA from archaeological bone specimens and determined the nucleotide sequences of the mtDNA control region using polymerase chain reaction (PCR) and the sequencing techniques. In this study, we found that the prehistoric *S. scrofa* group on the isolated island was clearly distinct from contemporary Japanese wild boars.

MATERIALS AND METHODS

Archaeological sites and samples

A total of 190 bone specimens (43 mandibles, 24 ulnae, 15 radii, 13 phalanges, 12 calcanei, 11 maxillae, 11 metacarpus, 9 atlantes, 9 humeri, 9 pelves, 9 tibiae, 7 scapulae, 5 femora, 4 crania, 4 tali, 2 teeth, 1 articular, 1 ilium, and 1 pubis) were derived from an estimated minimum of 65 individuals collected from 13 archaeological sites on Hokkaido Island, Sado Island, the Izu Islands, and Honshu Island of the Japanese Archipelago (Fig. 1). The estimated minimum number of individuals at each archaeological site was calculated based on the most frequently occurring bone type from each stratum. The archaeological sites examined in this study mainly dated to the Jomon Period. Only three archaeological sites were not from the Jomon Period: Rebunge shellmidden on Hokkaido Island, Sekonohama cave site on Sado Island, and Kokomanokoshi site on Miyakejima Island in the Izu Islands. Chronological periods and approximate dates of each site are indicated in the legend of Fig. 1. Chronological periods were inferred from the animal remains database (http://lmac152.iwaki-jc.ac.jp/index.htm). Approximate dates were tentatively assigned based on the common chronological table of archaeology.

DNA extraction

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 from each sample using an electric drill. Bone powder samples were suspended in 10 ml of 0.5 M ethylene diamine tetraacetate (EDTA), decalcified by rotating for a few days, and then centrifuged at 3,000 rpm for 10 min. The supernatant was removed, and pellets of bone powder were repeatedly decalcified with 10 ml of 0.5 M EDTA. Following decalcification, the bone powder samples were treated overnight in 5 ml of 0.5 M EDTA with proteinase K (300 μg/ml) and N-lauryl sarcosine (0.5%) with slight modification of the method by Hardy *et al.* (1995). The samples were centrifuged at 3,000 rpm for 10 min, and the supernatant containing the ancient DNA was extracted twice with phenol, once with chloroform:phenol (1:1) and once with chloroform to remove proteins. The supernatant was concentrated using a Centricon 30 micro-concentrator (Amicon) and washed with distilled water. These treatments concentrated the DNA samples to a final volume of 20–100 μl. The extracted ancient DNA was directly used as PCR template. Precautions were taken to prevent contamination from other non-ancient DNA, as described by Okumura *et al.* (1999). Blank extractions without bone powder were used to verify that no contamination occurred during extraction.

PCR and direct sequencing of mtDNA

To amplify the ancient DNA, we used three primer sets A, B, and C designed within the pig mtDNA control region. The primers, with 3' terminal nucleotide position (superscripts) based on the sequence of Okumura *et al.* (1996), are as follows: mitL76 (5'-AATATGCGACCCCAAAAATTTAACCATT^{130}) and mitH62 (5'-CCTGCCAAGCGGGTTGCTGG^{351}) for set A; mitL119 (5'-CAGTCAACATGCGTATCACC^{301}) and mitH124 (5'-ATGGCTGAGTCCAAGCATCC^{567}) for set B; and mitL104 (5'-TGGACTAGTGACTAATCAGCCCAT^{518}) and mitH106 (5'-ACGTGTACGCACGTGTACGC^{704}) for set C. The amplified fragments were 258 bp (including 48 bp of primer sequences) for set A, 305 bp (40 bp of primer sequences) for set B, and 229 bp (44 bp of primer sequences) for set C (Watanobe *et al.*, 2001). L and H designate light and heavy strands, respectively. PCR was carried out under the following conditions: one cycle of DNA denaturation and AmpliTaq Gold (Perkin Elmer) activation at 95°C for 10 min, annealing at 57°C (60°C for primer set A) for 1 min, and extension at 72°C for 1 min was followed by 50 cycles of denaturation at 94°C for 30 sec, annealing at 57°C (60°C) for 30 sec, and extension at 72°C for 1 min. When little or no PCR products were detected, a seminested PCR strategy was used. Seminested PCR amplifications were carried out with primers mitL76 and mitH61 (5'-CTGGTTTCACGCGGCATGG^{336}) for set A, primers mitL120 (5'-ACCGCCATTAGATCACGAGC^{318}) and mitH124 for set B, and primers mitL105 (5'-CCATGCTCACACATAACTGAGGTT^{537}) and mitH106 for set C, using 1 μl of the first PCR product as a template for 30 cycles as described above. To ensure the reliability of the results, we repeated the amplifications at least twice for each sample, and blank extracts were amplified in parallel with samples.

PCR products were purified using a Centricon 100 micro-concentrator (Amicon) and used as sequencing templates. Nucleotide sequences of both strands were determined using an Applied Biosystems 377 DNA sequencer with BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). When nucleotide sequences obtained from two independent PCR products differed from each other, a third PCR amplification and subsequent direct sequencing were done to verify the sequence. Moreover, the nucleotide sequences were independently verified in a second laboratory. A 574-bp sequence was constructed by connecting the three DNA fragments amplified by primer sets A, B, and C.

Contemporary wild boars and domestic pigs

Fifty-five haplotypes from 346 contemporary *S. scrofa* have been previously reported (Morii *et al.*, 2002; Okumura *et al.*, 2001; Watanobe *et al.*, 2002; Watanobe *et al.*, 2003): 180 Japanese wild boars (116 from Honshu Island, 2 from Shikoku Island, 42 from Kyushu Island, and 20 from Tsushima Island), 14 Ryukyu wild boars (8 from Iriomote Island, 2 from Amami Island, 2 from Kakeruma Island, and 2 from the main Okinawa Island in the Ryukyu Islands, Japan), 5 Northeast Asian wild boars (3 from near Ulan Bator in northern Mongolia, 1 from near Da-xinganling in southern Mongolia, and 1 from near Xiao-xinganling, China), 28 East Asian domestic pigs (12 Meishans, 6 Jinhuas, 4 Moncais, 2 Yontsuans, 2 Okinawa native pigs, and 2 Ohmini strains), and 119 European domestic pigs (35 Berkshires, 22 Landraces, 20 Large Whites, 20 Durocs, 18 Hampshires, 3 Yucatan miniature pigs, and 1 Pietrain). Details of the localities and collection information of the 180 Japanese wild boars are described in detail in our previous study (Watanobe *et al.*, 2003). The designation of the 55 contemporary haplotypes (J1 to J16, 16 to 53 and 56), corresponding to those used in our previous study (Watanobe *et al.*, 2003), forms a database for pairwise comparison and phylogenetic analysis. Among these *S. scrofa* haplotypes, J6, 23, 20, and 40 are representative of the Japanese wild boar, the East Asian domestic pig, the Ryukyu wild boar, and the European domestic pig, respectively (Table 1).

Data analysis

Average pairwise nucleotide differences among contemporary *S. scrofa* group sequences and between prehistoric and contemporary *S. scrofa* group sequences were calculated using MEGA, version 2 (Kumar *et al.*, 2001). A neighbor-joining tree (Saitou and Nei, 1987) with 1,000 bootstrap replications was constructed in MEGA 2 and a distance matrix was constructed using two-parameter distances (Kimura, 1980). Net nucleotide differences (d_A; Nei, 1987)

Table 1. Characteristics of archaeological specimen and nucleotide variability of their mtDNA haplotypes

Site No.	Archaeological site (Location)	Source / Specimen No.	Bone type	Nucleotide fA 1111111111111112222222222222233333333 33345566888999124467888889990000022 16864929256123532917023590451237 24
		Japanese wild boar		A−−TTGCCCCCCCCTATGCTTCCGACCGCCATAT
		Meishan pig		.−−.....T.....CA..............
		Ryukyu wild boar		.−−.....T.T.....................C..
		Landrace		GC-CCA..T.T.........C......A...C.C
2	Rebunge shellmidden (Hokkaido)	503	Femur	.−−.....T.....
3	Irie shellmidden (Hokkaido)	291	Talus	.−−.....T.....
		292	Distal phalanx	.−−...T...T.....
4	Sekonohama cave (Sado Island)	442	Mandible	.−−.....T....G..........A....G.
		443	Mandible	.−−.....T....G..........A....G.
		444	Mandible	.−−.....T....G..........A....G.
		445	Atlas vertebra	.−−.....T....G..........A....G.
		446	Articular	.−−.....T....G..........A....G.
		447	Tibia	.−−.....T....G..........A....G.
		448	Metacarpal	.−−.....T....G..........A....G.
		449	Mandible	.−−.....T....G..........A....G.
5	Sangu shellmidden (Sado Island)	434	Cranial	.−−.....T....GC.........A....G.
		435	Mandible	.−−.....T....G....C.....A....G.
		436	Ulna	.−−.....T....GC...C.....A....G.
		438	Teeth	.−−.....T....G.........T.A....G.
6	Fujiduka shellmidden (Sado Island)	321	Pelvis	.−−....T..TT.T.G.....T....A....G.
		323	Calcaneus	.−−.....T....G..........A....G.
		324	Tibia	.−−.....T....G.........TTA....G.
		325	Calcaneus	.−−.....T.T.....
		326	Ulna	.−−.....T....G..........A....G.
		327	Calcaneus	.−−.....T....G.....A...A....C.
		328	Ulna	.−−.....T....G..........A....G.
		329	Tibia	.−−.....T.T..G.....T.....A....G.
		330	Calcaneus	.−−.....T....G..........A....G.
		331	Humerus	.−−.....T....G..........A....G.
		340	Scapula	.−−.....TT.T.G.....
		344	Ulna	.−−.....T....G..........A.....
		345	Tibia	.−−.....T....G..........A....G.
7	Shimotakabora D (Izu Islands)	301	Mandible	.−−.....T.........C.....
		306	Ulna	.−−.....T.........C.....
		314	Talus	.−−.....T.........C.....
8	Tobune (Izu islnads)	295	Femur	.−−.....T.........C.....
		296	Pelvis	.−−.....T.........C.....
		297	Phalanx	.−−.....T.........C.....
		298	Phalanx	.−−.....T.........C.....
11	Tagara shellmidden (Honshu, Miyagi)	194	Mandible	.−−.....T.........C.....
12	Mibiki (Honshu, Ishikawa)	474	Talus	.−−.....T.T.....
		477	Pelvis	.−−.....T.T.....
		478	Pelvis	
		481	Pubis	
		482	Pelvis	
		486	Ulna	.−−.....AT.T.....
		487	Ulna	.−−.....T....G.A.........A....G.
		490	Phalanx	.−−.....T.T.....
		493	Tibia	.−−.....T.....
		500	Atlas vertebra	
13	Satakoubu (Honshu, Shimane)	159	Mandible	.−−.....T.........C.....
		161	Maxilla	.−−.....T.....

[a] Nucleotide position 1 corresponds to the first position of the complete sequences of mtDNA control region described by Okumura *et al.* (1996). fA, fB and fC represent the DNA fragments amplified by primer sets A, B and C, respectively.

[b] ND, not determined.

[c] Clusters correspond to those designated in the phylogenetic tree shown in Fig. 2.

position[a]			
fB	fC	Haplotype[b]	Cluster[c]
333334444444555 588990024567023 089136704325223	5556666667 4673568990 3168834033		
CAATGTTCATGCGCG	ACGGCCATAA	J6	A1
.........C..A..	.T......GG	23	A1
.G......GC..A..	.......C.G	20	A2
...C.....C..A..	.TA.....GG	40	European
....	.....T....	Hokkaido1 (J8)	A1
....	.....T....	Hokkaido1 (J8)	A1
T.....T........	....TT....	Hokkaido2	A1
.........C.....	....	Sado3	A1a
.........C.....	....	Sado3	A1a
.........C.....	....	Sado3	A1a
.........C.....	....	Sado3	A1a
.........C.....	....	Sado3	A1a
.........C.....	....	Sado3	A1a
.........C.....	....	Sado3	A1a
.........C.....	....	Sado3	A1a
.........C..A..	....	Sado4	A1a
.........C..A..	....	Sado5	A1a
.........C..A..	....	Sado6	A1a
.........C..A..	.....T....	Sado7	A1a
.........C..A..	....	Sado8	A1a
.........C.....	....	Sado3	A1a
..........T....	.T...T....	Sado9	A1a
....	.T...T....	Honshu22	A1
.........C....A	....	Sado10	A1a
.....C......A..	....	Sado11	A1a
.........C.....	....	Sado3	A1a
.........C.....	....	Sado12	A1a
.....C......A..	....	Sado13	A1a
....	....	Sado14	A1a
.........C..A..	....	Sado15	A1a
.........C..A..	....	Sado16	A1a
...C.....C..AT.	....	Sado17	A1a
.........C.....	.T...T....	Izu18	A1
.........C..A..	.T...T....	Izu19	A1
.........C.....	.T...T....	Izu18	A1
.........C.....	.T...T....	Izu18	A1
.........C.....	.T...T....	Izu18	A1
.........C.....	.T...T....	Izu18	A1
.........C.....	.T...T....	Izu18	A1
		ND	
............A..	.T...T....	Honshu21	A1
....	.T...T....	Honshu22	A1
	.T......G.	ND	
	....	ND	
	.T........	ND	
		ND	
....A..........	....	Honshu20	A1a
	.T........	ND	
............A..	.....T....	Honshu23	A1
	.T........	ND	
		ND	
		ND	

between contemporary Japanese wild boar haplotypes and prehistoric *S. scrofa* haplotypes groups were calculated and significance was tested using 1,000 permutations in the ARLEQUIN program package, version 2.000 (Schneider *et al*., 2000).

RESULTS

Amplification of ancient DNA

Of the 190 samples from 13 archaeological sites (Table 1), ancient DNA was successfully amplified and sequenced from 48 samples. We could not amplify any ancient DNA fragments from 8 samples of three archaeological sites (Bibi shellmidden, Kokomanokoshi, and Kurawa sites; Fig. 1). The hard and less colored bone samples were successfully amplified, corroborating the observations of Hardy *et al.* (1995) and Okumura *et al.* (1999). Samples from three archaeological sites on Sado Island were especially well preserved, and amplification was successful in 25 out of 48 samples (the amplification frequencies from Sekonohama cave site, Sangu shellmidden, and Fujiduka shellmidden were 100, 40, and 43.3%, respectively; Fig. 1). In contrast, the amplification frequencies (2.1 and 6.3%) from Tagara and Satakoubu shellmiddens were lower than those from the other archaeological sites (Fig. 1). Since the samples from Tagara and Satakoubu shellmiddens were examined three to four years ago, some methodological problems in DNA extractions may have influenced amplification success and the amplification frequencies. Indeed, after we lengthened the decalcification and subsequent proteinase K digestion times in recent extractions for samples from other 11 archaeological sites, the success of amplifying ancient DNA improved.

Among the 48 successfully amplified samples, fragments A (fA), fB, and fC were amplified from 44, 39, and 44 samples, respectively (Table 1). The longest DNA fragment (fB: 305 bp) is more difficult to amplify than the shorter DNA fragments (fA: 258 bp and fC: 229 bp) since ancient DNA is often damaged (Hofreiter *et al*., 2001). Consequently, complete sequences (574 bp) were obtained from only 39 samples.

DNA sequences of prehistoric *Sus scrofa*

A total of 30 polymorphic sites were identified among the 48 ancient DNA sequences; 17 substitutions were newly found in this study and the other 13 were shared with contemporary haplotypes. The 23 ancient and 4 contemporary haplotypes of the 574 bp mtDNA control region are shown in Table 1. The complete sequences (574 bp; fA, fB, and fC) from 39 archaeological specimens were submitted to the DDBJ/EMBL/GenBank database (accession Nos. AB089441-AB089479). Among these 39 prehistoric sequences, 23 distinct haplotypes were detected and designated as Hokkaido1 and Hokkaido2, Sado3 to Sado17, Izu18 and Izu19, and Honshu20 to Honshu23 (Table 1). Prehistoric haplotype Hokkaido1 was found to be identical to contemporary haplotype J8 (from Japanese wild boar) while

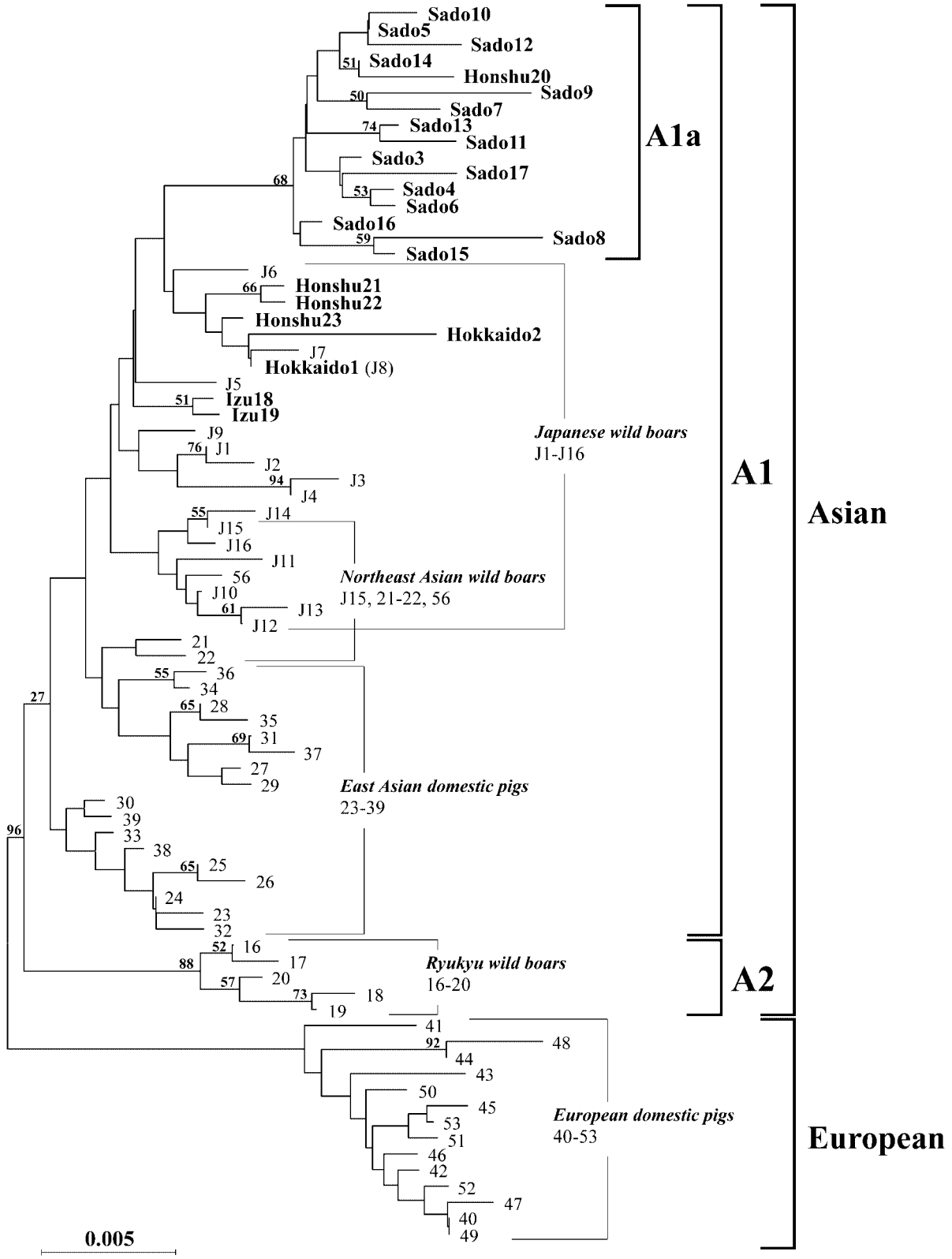

Fig. 2. Phylogenetic tree constructed from 23 prehistoric and 55 contemporary haplotypes of the mtDNA control region by the neighbor-joining method. Haplotype numbers correspond to haplotypes shown in Table 1. The tree topology shows two major lineages (Asian and European) and two Asian clusters (A1 containing subcluster A1a and A2). Bootstrap resampling was done 1,000 times, and the resulting bootstrap probabilities of more than 50% are shown on the corresponding branches.

the remaining 22 prehistoric haplotypes were distinct from contemporary haplotypes. Two prehistoric haplotypes from non-Jomon archaeological sites (haplotype Hokkaido1 from Rebunge shellmidden in the Epi-Jomon Period and haplotype Sado3 from Sekonohama cave site in Yayoi Period) were also detected from neighboring Jomon sites on the same islands (Hokkaido1 from Irie shellmidden and Sado3 from Fujiduka shellmidden, respectively; Fig. 1 and Table 1).

Phylogenetic relationship and geographic distribution of Jomon haplotypes

Haplotypes clustered into two lineages with 96% bootstrap probability in the neighbor-joining tree: Asian (haplotypes J1 to J16, 16 to 39, and 56) and European (haplotypes 40 to 53) lineages (Fig. 2). The Asian lineage was subdivided into two clusters designated A1 and A2. Cluster A1 with a 27% bootstrap value comprised haplotypes of Japanese wild boars, Northeast Asian wild boars, and East Asian domestic pigs (haplotypes J1 to J16, 21 to 39, and 56), and cluster A2 with 88% bootstrap value comprised haplotypes of Ryukyu wild boars (haplotypes 16 to 20). The Asian-European division and clusters A2 and A1a shown in the neighbor-joining tree were also supported by trees constructed using both maximum parsimony and maximum likelihood methods (data not shown). Our previous study using complete sequences of the control region and the cytochrome *b* (cyt *b*) gene showed relatively high bootstrap values for these two Asian clusters (93.2% and 99.7%, respectively) (Watanobe *et al*., 1999).

All 23 prehistoric haplotypes from the archaeological sites in this study grouped in cluster A1 (Fig. 2). Seven prehistoric haplotypes (Hokkaido1 and Hokkaido2, Izu18 and Izu19, and Honshu21 to Honshu23) placed near Japanese wild boars (haplotypes J1 to J16) on the tree and were closely related or identical to haplotypes J5 to J8 (Fig. 2). These seven prehistoric haplotypes were detected in three of three specimens from Hokkaido Island (Nos. 503, 291, and 292), one of 25 specimens from Sado Island (No. 325), seven of seven specimens from the Izu Islands (Nos. 301, 306, 314, 295, 296, 297, and 298), and three of four specimens from Honshu Island (Nos. 474, 477, and 493) (Table 1). The remaining 16 prehistoric haplotypes (Sado3 to Sado17, and Honshu20) formed a monophyletic group designated subcluster A1a (68% bootstrap value) located near haplotypes J5 to J8 of Japanese wild boars (Fig. 2). No contemporary haplotypes were included in this subcluster; prehistoric haplotypes of subcluster A1a were detected in 24 specimens from Sado Island, one specimen from Honshu Island, but none from Hokkaido Island or the Izu Islands (Table 1). As this monophyletic haplotype group is comprised almost exclusively of haplotypes from Sado Island, we designated this group as the Sado group.

Nucleotide differences between contemporary and prehistoric haplotype groups

The distribution of pairwise nucleotide differences between contemporary Japanese wild boar haplotypes (J1 to J16) and prehistoric haplotypes from archaeological specimens is shown in Fig. 3. The range and mode of differences between contemporary Japanese wild boar haplotypes and

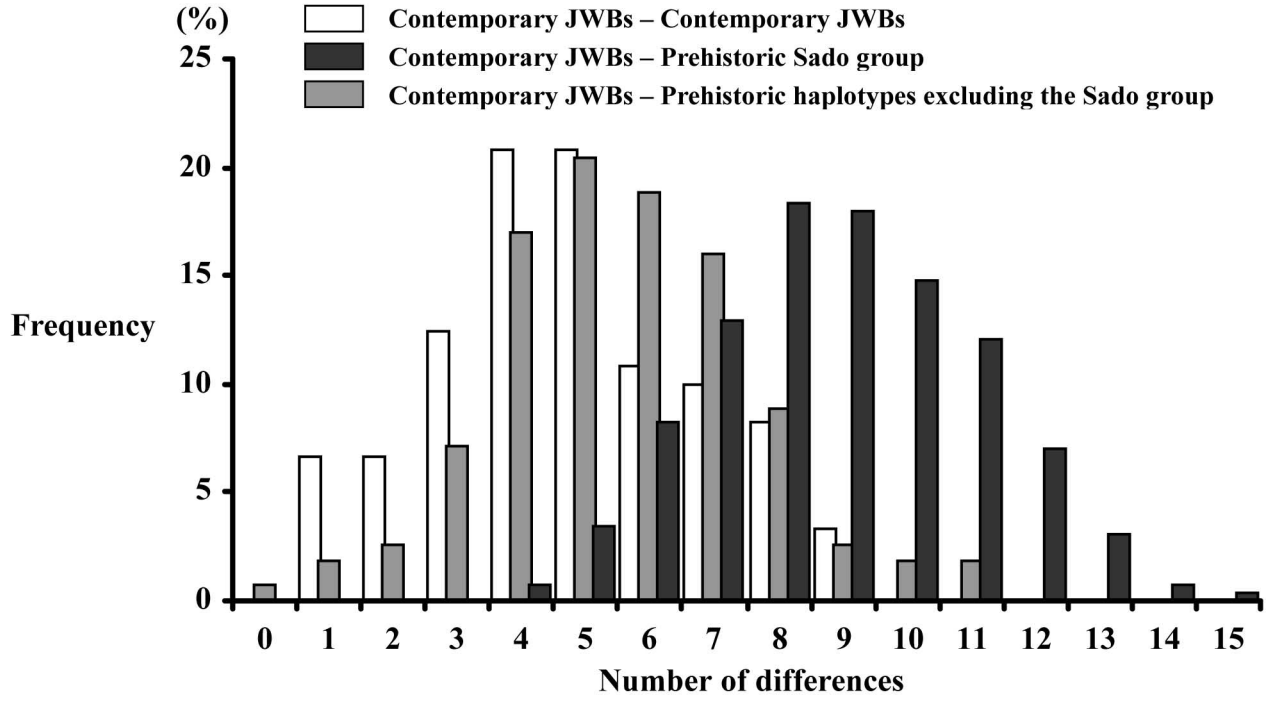

Fig. 3. Frequency of pairwise nucleotide differences among 16 contemporary Japanese wild boars (JWBs), between JWBs and 16 Sado group haplotypes, and JWBs and 7 prehistoric haplotypes excluding Sado group haplotypes. The prehistoric Sado group is the monophyletic group defined as subcluster A1a in Fig. 2.

prehistoric haplotypes excluding the Sado group (haplotypes Hokkaido1 and Hokkaido2, Izu18 and Izu19, and Honshu21 to Honshu23; range=0–11, mode=5) are almost identical to the range and mode of differences among contemporary Japanese wild boar haplotypes (range=1–9, mode=4 and 5; Fig. 3). Furthermore, the net nucleotide differences between these two groups of haplotypes are not significant (0.0024, p=0.107). On the other hand, the range and mode of differences between contemporary Japanese wild boar haplotypes and Sado group haplotypes (haplotypes Sado3 to Sado17, and Honshu20; range=4–15, mode=8) extended beyond the range of differences among contemporary Japanese wild boar haplotypes (Fig. 3). The net nucleotide difference between contemporary haplotypes of Japanese wild boars and Sado group haplotypes was significant (0.0075, p<0.000). These results demonstrated the large genetic difference between the prehistoric Sado group and contemporary Japanese wild boar haplotypes. The high degree of relatedness between the other prehistoric and contemporary Japanese wild boar haplotypes was also confirmed.

The net nucleotide difference between Sado group and the other prehistoric haplotypes was also significant (0.0066, p<0.000). We previously reported the difference between contemporary European and Asian *S. scrofa* used in this study (net nucleotide difference=0.01976; Watanobe *et al*., 2003). This European–Asian divergence is estimated to have occurred 500,000 (Giuffra *et al*., 2000) or 900,000 years ago (Kijas and Andersson, 2001). If we assume that the differences between contemporary European and Asian *S. scrofa* have accumulated for 500,000 or 900,000 years, the level of divergence (0.0066) observed between the Sado group and the other prehistoric haplotypes places the divergence of the Sado group from the other prehistoric haplotypes at 167,000 or 301,000 years ago, respectively.

DISCUSSION

Sado Island

The Sado Island (857 km^2) is the fifth largest isolated island in the Japanese Archipelago after Hokkaido, Honshu, Shikoku, and Kyushu islands. The forest habitat and extent of this island could probably support a *S. scrofa* population, however, none inhabit Sado Island at present. In this study, we found that the prehistoric *S. scrofa* group almost unique to Sado Island was estimated to have diverged from other prehistoric *S. scrofa* on the Japanese Archipelago at 167,000–301,000 years ago (Figs. 2 and 3). This divergence time is within the range of the late Middle to early Late Pleistocene and roughly coincides with the estimated isolation of Sado Island from Honshu Island by the formation of a strait (Ohshima, 1990). This suggests that the prehistoric *S. scrofa* group was a natural population that became isolated. It is well known that endemic populations of *Lepus*, *Mogera*, *Apodemus*, *Microtus*, and *Sorex* also inhabit Sado Island. Ohdachi *et al.* (2001) found that *Sorex shinto* became geologically isolated on Sado Island and was the first population to diverge from *Sorex shinto* on Honshu and Shikoku islands based on mitochondrial cytochrome *b* gene data. This result corroborates our hypothesis of the origin of the prehistoric *S. scrofa* group specific to Sado Island. However, the circumstances of *S. scrofa* distribution are slightly different from those of *Sorex shinto*. While *Sorex shinto* on Sado Island diverged from a monophyletic ancestral *Sorex shinto* population in Japan (Ohdachi *et al*., 2001), the Sado *S. scrofa* group was not the first population to diverge from *S. scrofa* on Honshu, Shikoku and Kyushu islands, but rather seems to have diverged from a part of Japanese wild boar (J6 to J8; Fig. 2) lineage. The difference in divergence patterns between *Sorex shinto* and *S. scrofa* in Japan may be a result of the complex genetic population structure of the Japanese wild boar, which is influenced by the independent and repetitive migration of continental *S. scrofa* (Watanobe *et al*., 2003).

Fossil records indicate that *S. scrofa* on Honshu, Shikoku, and Kyushu islands decreased dramatically or became extinct during the late Middle to early Late Pleistocene (approximately 300,000–50,000 years ago), and revived in the late Late Pleistocene (Fujita *et al*., 2000). An alternative hypothesis suggests the Sado *S. scrofa* population is a remnant of these *S. scrofa* populations, while the other prehistoric *S. scrofa* found on Honshu Island, Hokkaido Island, and the Izu Islands, as well as contemporary Japanese wild boars, are from populations migrating to the Japanese Archipelago in the late Late Pleistocene. In this scenario, the genetic diversity between the Sado group and the other prehistoric *S. scrofa* or contemporary Japanese wild boars would reflect the genetic differences of the ancestral populations on the Asian Continent. Thus, a continental *S. scrofa* population closely related to the Sado group may be identified on the continent, provided that it is not extinct. We previously pointed out that the ancestral population of some contemporary Japanese wild boars is found among the Northeast Asian wild boars (Watanobe *et al*., 2001). However, no continental *S. scrofa* closely related to the Sado group have been found among the Asian continental *S. scrofa* populations yet. Therefore, for the present, the first hypothesis is more plausible than the second to explain the origin of Sado-specific *S. scrofa*. The genetic identity of Asian continental *S. scrofa* is still not sufficiently known, and these two hypotheses should be continuously assessed as more comprehensive genetic data for Asian continental *S. scrofa* populations becomes available.

The Sado *S. scrofa* group was also detected from the Yayoi Period Sekonohama cave site on Sado Island, indicating that this *S. scrofa* had existed as recently as around 2,000 years ago. However, after this time, the *S. scrofa* population became extinct on Sado Island.

Honshu Island

Unfortunately, successful isolation and amplification of ancient mtDNA was limited to a few bone specimens from

three archaeological sites on Honshu Island: Tagara shell-midden, Mibiki site, and Satakoubu site (Fig. 1). However, three haplotypes (Honshu21 to Honshu23) identified from Mibiki site confirm the continuity of the *S. scrofa* population in Japanese Archipelago from the Jomon Period to the present time. Haplotype Honshu20 (included in Sado group) is useful for evaluating the relationship between Honshu Island and Sado Island during the Jomon Period. Honshu20 was detected from only one specimen, suggesting that very few Sado group individuals were present at the Mibiki site, and this can be interpreted as an introduction of the Sado group *S. scrofa* to Honshu sites by humans. Another haplotype (Honshu22) of the non-Sado group was detected from only one of the 25 specimens from Sado Island (Table 1). Together, these results suggest that exchanges between Sado Island and Honshu Island had been conducted at very low frequencies by prehistoric people.

Hokkaido Island and the Izu Islands

At the present, there are no Japanese wild boars on Hokkaido Island. Furthermore, no *S. scrofa* were detected in on Hokkaido Island in the Quaternary mammalian remains (Kawamura, 1991). Ohshima (1990) reported that Hokkaido Island was separated from Honshu Island by the formation of the Tsugaru Strait 150,000–100,000 years ago. Paleontological and geological theory suggests that *S. scrofa* could not naturally colonize Hokkaido Island, however many burned bones of *S. scrofa* have been excavated from Jomon Period archaeological sites on Hokkaido Island. Furthermore, burned bones of *S. scrofa* have also been excavated from Jomon archaeological sites on Honshu Island, providing archaeological evidence to suggest that *S. scrofa* had been introduced to Hokkaido from Honshu together with the animal ritual of the Jomon Period (Nishimoto, 1995). Two haplotypes (Hokkaido1 and Hokkaido2) from prehistoric *S. scrofa* from Hokkaido Island were closely related to or identical to contemporary Japanese wild boar haplotypes (Figs. 1 and 2, Table 1). This is congruent with the archaeological hypothesis and strongly suggests that prehistoric *S. scrofa* of Hokkaido had been introduced from Honshu Island by Jomon people.

The Izu Islands are assumed to have been isolated from Honshu Island during the glacial ages of the Pleistocene, because the strait between them is deeper than the lowest sea level during the glacial ages, thus blocking the natural migration of *S. scrofa.* Therefore, the detection of haplotypes Izu18 and Izu19 closely related to the contemporary Japanese wild boar haplotypes suggests the introduction of Japanese wild boars from Honshu Island to the Izu Islands was carried out by Jomon peoples, as in the case of Hokkaido Island (Table 1 and Fig. 2). The ancient boars excavated from the Izu Islands were of smaller size than those from Honshu Island based on the morphometrical analysis of bone specimens (unpublished data), though it is not clear whether the decrease in size is due to domestication or insular dwarfism.

Genetic and morphological analyses of *S. scrofa* have been used to infer the movements and exchanges of prehistoric humans in the Japanese archipelago. Previously we reported that continental *S. scrofa* haplotypes were detected at the Okhotsk cultural site (1,600–1,300 years ago) in northern Hokkaido. This site was directly influenced by Sakhalin Island and the Northeast Asian Continent, and was distinct from Jomon and Epi-Jomon sites (Watanobe *et al*., 2001). In archaeological sites in southern Kanto of Honshu Island, several significant changes in the size of *S. scrofa* were recorded during the Early Jomon to Medieval Periods, suggesting that distinct *S. scrofa* lines had been introduced to the area (Anezaki, 2003). Based on these results, we can hypothesize that prehistoric *S. scrofa* had been introduced to islands not naturally inhabited by *S. scrofa*, as well as to areas within the natural range of *S. scrofa* through human movements, which were influenced by cultural exchanges.

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