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Phylogeography and Population Structure of the Japanese Wild Boar *Sus scrofa leucomystax*: Mitochondrial DNA Variation

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ABSTRACT—Phylogeographic characteristics and population structure of Japanese wild boar (*Sus scrofa leucomystax*) were investigated using mitochondrial DNA (mtDNA) sequence data. Sixteen Japanese wild boar haplotypes detected from partial sequences of the mtDNA control region (574-bp) from 180 Japanese wild boar specimens from 10 local populations on Honshu, Shikoku, and Kyushu islands and 41 haplotypes from other *S. scrofa* were analyzed using the neighbor-joining method. The Japanese wild boars were more closely related to Northeast Asian wild boars from Mongolia than to the other Asian continental *S. scrofa*. The Japanese and Northeast Asian wild boars were not significantly distinguished by corrected average pairwise difference analysis. The ancestors of Japanese wild boars are suggested to have been part of the continental *S. scrofa* population that spread from Southeast to Northeast Asia during the Middle to Late Pleistocene. The Japanese wild boar mtDNA haplotype cladogram shows 95% parsimoniously plausible branch connections supporting three sympatric clades. Nested clade analysis indicates that these three clades are the result of distinct historical events or gene flow. The present population of Japanese wild boars may have been formed by a few independent migrations of distinct clades from the continent with subsequent mixing on the Japanese Islands.

Key words: Japanese wild boar, migration, phylogeography, genetic population structure, mitochondrial DNA

INTRODUCTION

The wild boar (*Sus scrofa*), which inhabits wide areas of Asia, Europe, and North Africa, is an ancestral species to domestic pigs with at least 16 endemic subspecies (Herre and Rohrs, 1977; Epstein, 1984; Ruvinsky and Rothschild, 1998). Two subspecies inhabiting Japan are the Japanese wild boar (*S. s. leucomystax*) on the Japanese main islands of Honshu, Shikoku, and Kyushu and the Ryukyu wild boar (*S. s. riukiuanus*) on the Ryukyu Islands. Fossil records indicate that *S. scrofa* has probably inhabited the Japanese Islands since the Middle Pleistocene (Fujita *et al*., 2000). General opinion holds that *S. scrofa* migrated to the Japanese Islands across landbridges that repeatedly formed between Japan and the Asian Continent and were subsequently isolated on the islands when the landbridge submerged. However, the identity of the continental population from which the Japanese wild boar was derived and how the present geographical distribution pattern was formed is still unclear.

The genetic characteristics of the Japanese wild boar have been compared with those of the Ryukyu wild boar and other wild and domestic *S. scrofa* at biochemical and molecular levels (Kurosawa *et al*., 1979; Watanabe *et al*., 1986; Kurosawa and Tanaka, 1988; Okumura *et al*., 1996; Watanobe *et al*., 1999). Recent investigations of the phylogenetic relationships between the wild boar and domestic pig have revealed that the domestication of pigs occurred independently from both Asian and European subspecies of the wild boar (Giuffra *et al*., 2000; Kijas and Andersson, 2001). Though the Japanese wild boar was also analyzed as a representative of Asian wild boars in these studies, no information was provided for its origin and history of colonization. Despite the wide distribution of the Japanese wild boar on the Honshu, Shikoku, and Kyushu islands, studies

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of genetic variation and population structure are limited. Only studies of the social structure of local populations (Hirotani and Nakatani, 1987; Nakatani and Ono, 1994), geographical variation of mandible size and shape (Endo *et al*., 2000), and the genetic diversity of mitochondrial DNA (mtDNA) haplotypes from small sample sizes (Okumura *et al*., 1996; Watanobe *et al*., 1999) have been reported.

Analysis of a population's genetic structure for elucidating the evolutionary relationships and assessing extinction risk (Burgman *et al*., 1993; Avise, 2000) can be achieved using recombining nuclear or non-recombining organelle genes (Slatkin, 1987; Pope *et al*., 1996). Mitochondrial DNA (mtDNA) is maternally inherited without recombination and has a higher mutation rate than nuclear DNA, making it highly suitable for studying the genetic structure of populations, both current and historical (Avise, 1994; Patton *et al*., 1996). The sequence divergence of mtDNA has delineated the historical divergence of genetic groups, and haplotype frequencies have revealed recent animal movements among local populations (DeSalle *et al*., 1987; Moritz, 1994). The mtDNA control region has a very high nucleotide substitution rate making it particularly useful for investigating the genetic population structure of closely related animals in restricted areas (Kocher and Wilson, 1991; Vigilant *et al*., 1991; Wilkinson and Fleming, 1996; Hurwood and Hughes, 2001; Sivasundar *et al*., 2001).

Recently, we determined and phylogenetically analyzed a large number of sequences of the mtDNA control region from *S. scrofa* including Japanese wild boar (Okumura *et al*., 2001). The study revealed the independent domestication of Asian and European domestic pigs, followed by secondary genetic introgression of Asian breeds into Euro-American breeds. However, no information on the phylogeography and population structure of Japanese wild boar was provided in that study. In this study, we determined additional sequences from Japanese wild boar, and examined the genetic population structure of the Japanese wild boar using the geographical distribution of mtDNA haplotypes from a large number of Japanese wild boars collected from 16 prefectures in Japan. The population history and phylogeographical characteristics of Japanese wild boars were also assessed using nested clade (Templeton *et al.,* 1995) and ordinary phylogenetic analyses.

MATERIALS AND METHODS

Japanese wild boar samples and sampling sites

In addition to 140 Japanese wild boar mtDNA control region sequences previously reported (Okumura *et al*., 1996; Watanobe *et al*., 1999; Okumura *et al*., 2001; Watanobe *et al*., 2001; accession Nos. D42171-D42178, AB015084-AB015086 and AB041467-AB041473), 40 Japanese wild boars were newly sequenced using the procedure of Okumura *et al*. (1996). A total of 180 individual samples were examined from sampling sites in western Honshu, Shikoku, Kyushu, and Tsushima islands (Fig. 1). Samples were tentatively grouped into local populations (A to J). Samples from populations A and B were collected in Gunma and Shizuoka prefectures, respectively, and were separated by long geographical distances. Though populations C (Gifu prefecture), D (Fukui, Hyogo, and Shiga prefectures), and E (Mie and Nara prefectures) were collected from relatively close geographical locations, these were defined as distinct local populations based on osteometrical analysis of the mandible (Endo *et al*., 2000); a specific skull form distinguishes individuals from Gifu prefecture from individuals from Hyogo or Mie prefectures. Population F was defined as samples collected in Tokushima prefecture. Samples from Shimane and Yamaguchi prefectures were regarded as a single population G despite their relatively wide distribution, as there are no geographical barriers such as high mountain ranges or areas of dense human populations. Samples collected in northern and southern Kyushu were regarded as population H (Kumamoto, Nagasaki, Oita, and Saga) and I (Miyazaki), respectively. These 9 populations (A–I) are thought to have been naturally founded, while population J on Tsushima Island, near northern Kyushu Island, is thought to have been introduced by humans. Three hypothetical regional boundaries (I, II, and III) were defined to assess the regional genetic biases of Japanese wild boars (Fig. 1). Boundary I lies approximately on the Japanese Alps and the boundary III is in straits between Honshu and Kyushu islands dividing eastern and western areas into two regions. Boundary II establishes the longitudinal midpoint of our sampling area. We assumed that the geological separation of Shikoku and Kyushu from Honshu occurred approximately 5,000 years ago (Ohshima, 1990) and had not considerably influenced the genetic distribution of the Japanese wild boars, which first migrated to and then expanded their range on the Japanese Islands in the Middle Pleistocene (Fujita *et al*., 2000).

DNA extraction, PCR and direct sequencing

Total cellular DNA from Japanese wild boars was extracted from frozen muscle or liver tissue using standard Proteinase-K phenol-chloroform protocols (Sambrook *et al*., 1989). Approximately 0.1–0.2 μl of the extracted DNA was used to amplify the mtDNA control region by polymerase chain reaction (PCR). To amplify the most variable fragment in the control region, primers mitL3 (5'-ATATACTGGTCTTGTAAACC-3') in the L strand of threonine tRNA and mitH106 (5'-ACGTGTACGCACGTGTACGC-3') in the H strand of the control region were used. PCR reactions were performed under the following conditions: DNA denaturation and AmpliTaq Gold (Perkin Elmer, Norwalk, CT) activation at 95°C for 10 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min was followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. PCR products were purified using a Centricon 100 micro-concentrator (Amicon, Beverly, MA) and directly sequenced by the dideoxy chain termination method (Sanger *et al*., 1977) on a 373S and 377 DNA Sequencer with Taq DyeDeoxy Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA). Additional primers mitL120 (5'-ACCGCCATTAGATCACGAGC-3') and mitH124 (5'-ATGGCTGAGTCCAAGCATCC-3') were used for DNA sequencing of both L and H strands of the mtDNA control region.

Data Analysis

Multiple sequence alignment was performed using GENETYX-MAC software (Software Development Co., Tokyo, Japan). Forty-one haplotypes of the 574-bp mtDNA control region were used in neighbor-joining analysis (NJ; Saitou and Nei, 1987) made using the PHYLIP program package, version 3.572 (Felsenstein, 1995). Forty haplotypes previously reported by Okumura *et al*. (1996), Watanobe *et al*. (1999), Okumura *et al*. (2001), and Watanobe *et al*. (2001) (accession Nos. D42170-D42185, AB015087-AB015095, AB041464-AB041466 and AB041474-AB041499) were obtained from 167 animals: 14 Ryukyu wild boars (8 from Iriomote Island, 2 from Amami Island, 2 from Kakeruma Island, and 2 from Okinawa main Island in the Ryukyu islands, Japan), 3 Northeast Asian wild boars (1 from near Ulan Bator in northern Mongolia, 1 from near

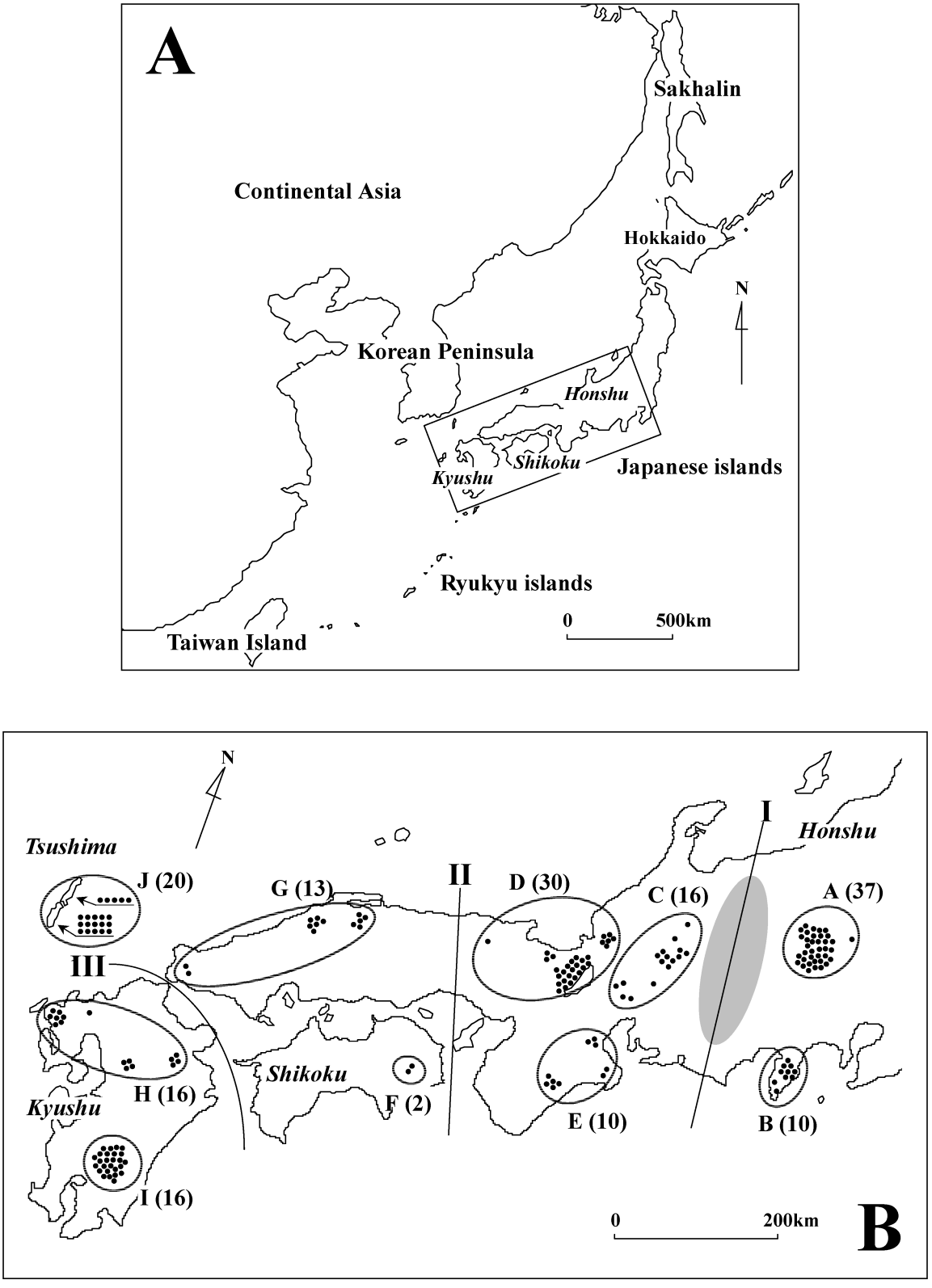

Fig. 1. **(A)** Sampling sites on Honshu, Shikoku, Kyushu, and Tsushima islands of Japan. Relevant locations on continental Asia are also indicated. The boxed area is shown in more detail in **B**. **(B)** The ten local populations defined in this study are shown by circles A to J. Dots indicate the sampling sites and the numbers in parentheses are the numbers of samples used from each local population. Three solid lines (I, II, and III) delineate hypothetical regional boundaries. The approximate location of a high mountainous area, the Japanese Alps, is shown as a shaded ellipse.

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), 119 European domestic pigs (35 Berkshires, 22 Landraces, 20 Large Whites, 20 Durocs, 18 Hampshires, 3 Yucatan miniature pigs and 1 Pietrain), and 3 European wild boars (2 from Italy and 1 from Germany). One haplotype identified in two Northeast Asian wild boars from the Ulan Bator area in northern Mongolia was newly detected in this study and was deposited in the DDBJ/EMBL/GenBank database (accession No. AB066101). The distance matrix for the NJ tree was constructed from distance values using a two-parameter method (Kimura, 1980) with 1000 bootstrap replications.

Corrected genetic differences (D_A; Nei, 1987) between pairs of *S. scrofa* groups were calculated using $D_A=D_{XY}-(D_X+D_Y)/2$, where D_{XY} is the average pairwise nucleotide difference between boar group X and Y, and D_X and D_Y are average pairwise nucleotide differences within boar groups X and Y, respectively. In this analysis, we regarded the European domestic pigs and the East Asian domestic pig haplotypes as belonging to the Asian *S. scrofa* groups because it is well known that introgressions of Asian domestic pig haplotypes into European domestic pigs occurred quite recently (Giuffra *et al.*, 2000; Okumura *et al.*, 2001). The significance of the differences among *S. scrofa* groups was tested using 1,000 permutations in the ARLEQUIN program package, version 2000 (Schneider *et al.*, 2000).

A haplotype cladogram with 95% parsimonious plausible branch connections between haplotypes showing the number of base pair differences between haplotypes was produced by the TCS program (Clement *et al.*, 2000) using the cladogram estimation algorithm described by Templeton *et al.* (1992). The parsimonious haplotype cladogram was used in nested clade analysis to define a series of nested haplotypes or clades. This nesting structure, together with information on the geographical distribution of the haplotypes, was used to estimate two geographical measures for each clade (GeoDis 2.0; Posada *et al.*, 2000): the clade distance (D_c) and the nested clade distance (D_n). D_c is a measure of the geographical extent of a given clade, and D_n is a measure of the average geographical distance of individuals in a clade from those in the next higher-level nesting clade (Templeton, 1998). To contrast geographical dispersion patterns for older (interior, *I*) versus younger (tip, *T*) clades, the mean differences for the both clade and nested clade distances between the interior and tip clades were calculated, and denoted as $(I\text{-}T)_c$ and $(I\text{-}T)_n$, respectively. The distribution of

Table 1. Nucleotide variation and geographical distribution of Japanese wild boar haplotypes

			Nucleotide position[3]																				Local population[4]										
			1	1	2	2	3	3	3	3	4	4	5	5	5	6	6	6	6	6	7													
			5	8	6	8	0	0	8	9	4	5	0	4	6	3	5	6	8	9	0													
Clade[1]		Haplotype[2]	9	6	1	0	3	7	9	1	4	3	2	3	1	8	8	3	4	3	3		A	B	C	D	E	F	G	H	I	J	Total
2-1	1-1	J1 (8)	G	T	C	C	A	T	A	T	A	T	G	A	T	G	T	C	A	A	A		–	–	6	15	1	–	–	–	1	–	23
		J2 (New)	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	G	•	•	–	–	–	–	1	–	–	–	–	–	1
	1-2	J3 (9)	A	•	T	•	•	C	•	•	•	•	A	•	•	•	•	•	•	•	•		24	–	2	4	–	–	–	–	1	–	31
		J4 (10)	A	•	•	•	•	C	•	•	•	•	A	•	•	•	•	•	•	•	•		–	–	–	1	–	–	–	–	–	–	1
2-2	1-3	J5 (12)	•	•	•	•	•	C	•	•	•	C	•	•	C	•	C	•	•	•	•		–	–	–	–	–	–	9	1	–	–	10
	1-4	J6 (13)	•	C	•	T	•	•	•	•	•	•	•	C	•	C	•	•	•	•	•		–	–	2	–	–	–	–	–	–	–	2
	1-5	J7 (14)	•	•	•	T	•	•	•	•	•	•	G	C	•	C	T	•	•	•	•		–	3	–	–	–	–	–	–	6	–	9
		J8 (15)	•	•	•	T	•	•	•	•	•	•	•	C	•	C	T	•	•	•	•		12	5	–	–	–	–	–	–	–	–	17
2-3	1-6	J9 (11)	•	•	•	•	•	•	•	•	•	•	•	•	•	C	•	•	G	•			–	–	–	–	2	–	–	–	–	–	2
	1-7	J10 (1)	•	•	•	•	G	•	•	•	•	•	•	•	•	C	•	•	•	G			–	1	6	10	6	1	3	14	8	20	69
	1-8	J11 (3)	•	•	•	•	G	•	•	•	•	C	•	•	•	A	C	•	•	•	G		–	–	–	–	–	–	–	–	4	–	4
	1-9	J12 (4)	•	•	•	•	G	•	•	•	G	•	•	•	•	C	•	•	•	G			1	1	–	–	–	–	–	1	2	–	5
		J13 (7)	•	•	•	•	G	•	•	C	G	•	•	•	•	C	•	•	•	G			–	–	–	–	–	1	–	–	–	–	1
	1-10	J14 (5)	•	•	•	•	G	•	G	•	•	•	•	C	•	C	•	•	•	G			–	–	–	–	–	–	–	–	1	–	1
		J15 (6)	•	•	•	•	G	•	•	•	•	•	•	C	•	C	•	•	•	G			–	–	–	–	–	–	–	–	3	–	3
		J16 (2)	•	•	•	•	G	•	•	•	•	•	•	C	•	C	•	•	•	•			–	–	–	–	–	–	1	–	–	–	1
			No. of samples																				37	10	16	30	10	2	13	16	26	20	180
			Frequency (%)																			Clade 2-1	64.9	0.0	50.0	66.7	20.0	0.0	0.0	0.0	7.7	0.0	31.1
																						Clade 2-2	32.4	80.0	12.5	0.0	0.0	0.0	69.2	6.2	23.1	0.0	21.1
																						Clade 2-3	2.7	20.0	37.5	33.3	80.0	100.0	30.8	93.8	69.2	100.0	47.8

[1] Clade structure is given in Fig. 3.

[2] Numbers in parentheses indicate previous haplotype identification defined in Watanobe *et al.* (2001).

[3] Nucleotide position 1 corresponds to the first position of the complete DNA sequences of mtDNA control regions described by Okumura et al. (1996). Dots indicate the nucleotide identity with Japanese wild boar haplotype J1.

[4] Localities A to J shown in Fig. 1 are located in the following prefectures: A, Gunma; B, Shizuoka; C, Gifu; D, Fukui, Hyogo and Shiga; E, Mie and Nara; F, Tokushima; G, Shimane and Yamaguchi; H, Kumamoto, Nagasaki, Oita and Saga; I, Miyazaki; and J, Nagasaki (Tsushima Island).

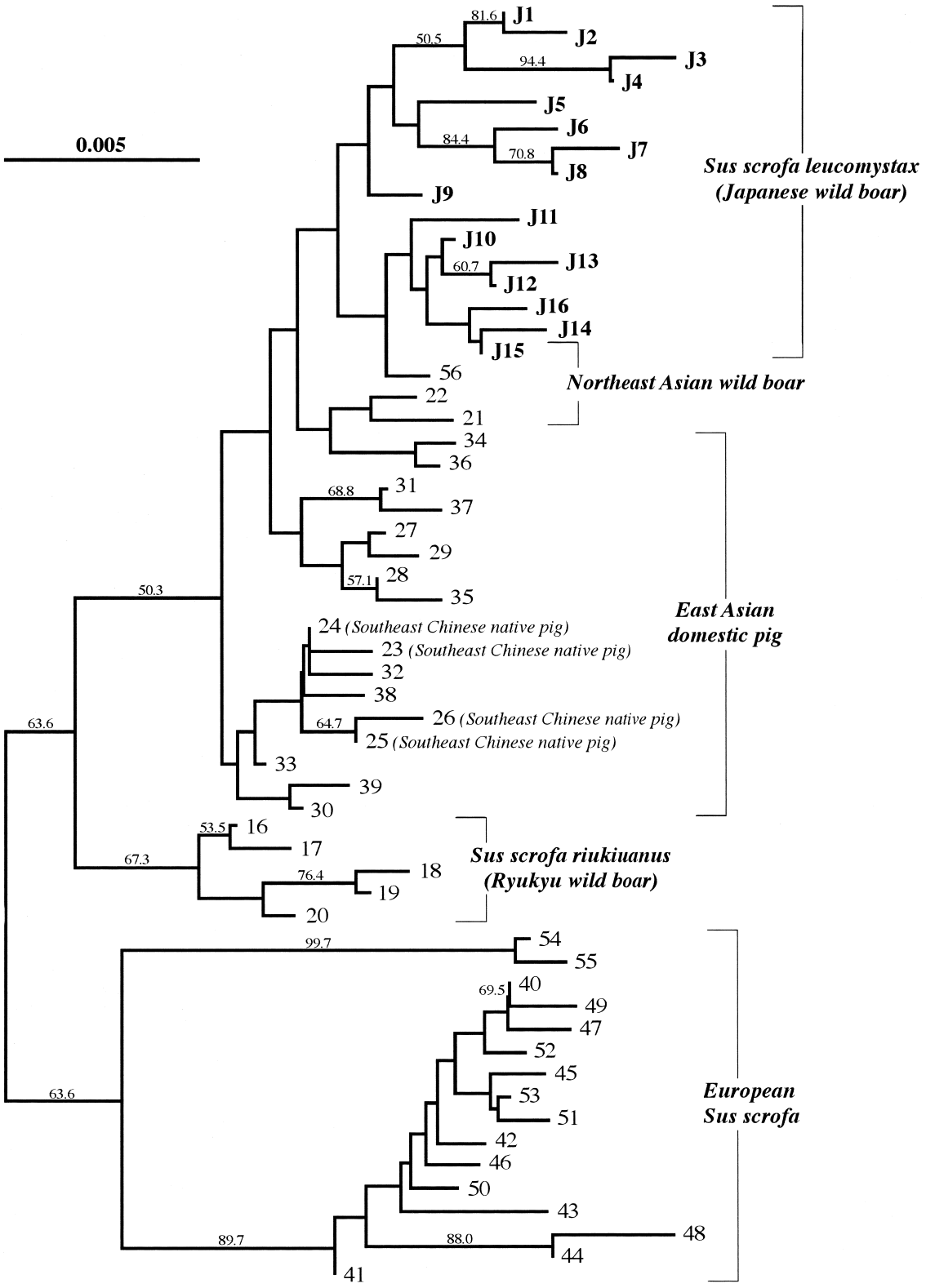

Fig. 2. Neighbor-joining phylogenetic tree was constructed using partial (574-bp) mtDNA control region sequences of 16 Japanese wild boar (J1 to J16) and 41 other *Sus scrofa* haplotypes: Ryukyu wild boar (16 to 20); Northeast Asian wild boar (21, 22, 56, and J15); East Asian domestic pigs (Meishan 23 and 24, Jinhua 25 and 26, Ohmini strain 27, Yontsuan 28, Moncai 29, 34 and 35, Okinawa native pig 33, Berkshire 30 to 32, 36 and 37, and Large White 38 and 39); European *Sus scrofa* (40 to 55). Haplotype designations of Japanese wild boars are given in Table 1 and used in Fig. 3. Bootstrap resampling was done 1,000 times, and the bootstrap probabilities greater than 50% are shown on the corresponding branches.

these distances measured under the null hypothesis of no geographical associations within the nested clade was determined by recalculating distances after each random permutation of the clades against sampling location. This randomization procedure allows us to test for significantly large and small distances for each clade within a nested group of clades with respect to the null hypothesis of no geographical associations within the nested clade. Geographical distances between the local populations were estimated as the mean distance of all pairs of samples included in each population. Distances between sampling sites were estimated as straight lines on a map. The inference key provided by Templeton (1998) was used to interpret the outcome of the geographical association analysis. Local population J was excluded from this analysis because it is thought to be an artificial population, because humans exterminated the original wild boar population in the past (see Discussion).

The genetic structure of regional (defined by boundaries I, II, and III; Fig. 1B) and local populations (designated A to I; Fig. 1B) was quantified by an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) in the ARLEQUIN program using a matrix of nucleotide substitutions between pairs of haplotypes. This procedure calculated the standard variance components at each of the three levels of population subdivision: among geographical regions (R), among local populations within a region (P), and among individuals within a local population (I). The array of haplotype correlation measures is referred to as Φ-statistics, analogous to the standard *F*-statistics of population genetics (Wright, 1965). The significance of the variance components and Φ-statistics was tested using the 1,000 times random permutation procedure, which avoids the parametric assumptions of normality and independence that are not met by molecular distance measures (Excoffier *et al.*, 1992). Φ_{RT} is the difference among regions relative to the total haplotype pool; Φ_{PR} is the amount of variation due to differences among local

Table 2. Corrected average pairwise differences (Nei 1987) between *Sus scrofa* groups and estimated divergence times

Sus scrofa group	Difference	Significance (*P* value)	Divergence time[a]
European vs. Asian	0.01976	+ (0.00000)	500,000 –900,000
Ryukyu vs. other Asian	0.01250	+ (0.00000)	316,000 –569,000
East Asian domestic vs. Northeast Asian and Jpn wild	0.00555	+ (0.00000)	140,000 –253,000
Northeast Asian vs. Jpn wild	0.00108	– (0.06745)	–

[a] The upper and lower divergence times are estimated assuming that the corrected nucleotide difference between European and Asian *Sus scrofa* (0.01976) accumulated for 900,000 years (Kijas and Andersson, 2001) or 500,000 (Giuffra *et al.*, 2000); given as years before present.
'Japanese' is abbreviated as 'Jpn'.

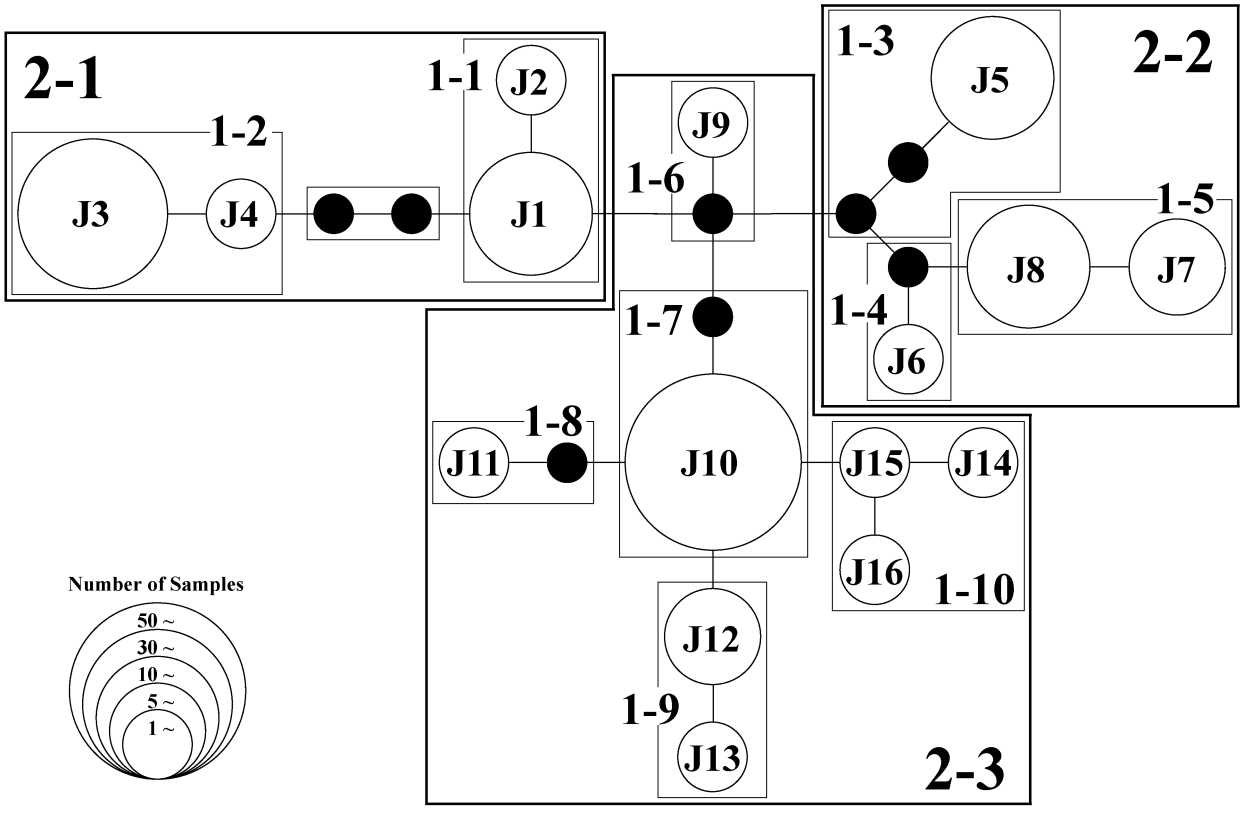

Fig. 3. Cladogram with 95% parsimonious connections for haplotypes of the Japanese wild boar. Open circles represent haplotypes J1 to J16 from Japanese wild boar samples (Table 1). The size of the circle corresponds to number of samples the circle represents (see key). Solid circles indicate haplotypes not detected in the samples. A solid branch between haplotypes indicates a single mutation. Boxes indicate nested clades; 1-x for one-step clades (thin line) and 2-x for two-step clades (heavy line) where x is the number identifying individual clade.

populations within regions; and Φ_{IP} measured the variation due to differences among individual haplotypes within local populations. The artificial population J was excluded from this analysis.

RESULTS

Nucleotide sequence diversity and phylogenetic relationship

A total of 16 haplotypes (J1 to J16) were found among the 180 Japanese wild boars and there were 19 polymorphic sites in the 574-bp sequences (Table 1). One newly detected haplotype (J2) was deposited in the DDBJ/EMBL/GenBank database (accession No. AB055222). The haplotypes differed by between 1 and 9 nucleotide differences. Fifteen of the 16 Japanese wild boar haplotypes (J1 to J14 and J16) were found only in Japanese wild boars, and the haplotype J15 was detected in both Japanese wild boars and Northeast Asian wild boars from northeastern Mongolia.

The neighbor-joining tree of the 16 Japanese wild boar and 41 other *S. scrofa* haplotypes (a total of 57 haplotypes) shows two distinct lineages: Asian (haplotypes J1 to J16, 16 to 39, and 56) and European (haplotypes 40 to 55) lineages (Fig. 2). The Asian lineage is subdivided into two clusters: Ryukyu wild boar cluster (haplotypes 16 to 20) and Japanese wild boar (haplotypes J1 to J16) and Asian continental *S. scrofa* (haplotypes 21 to 39, 56, and J15) cluster. The Asian continental group comprises Northeast Asian wild boars (haplotypes 21, 22, 56 and J15) and East Asian domestic pigs (haplotypes 23 to 39). These East Asian domestic pig haplotypes were derived from Asian native pig breeds in various localities and Asian and European commercial pig breeds such as Ohmini strain, Berkshire, and Large White (see legend of Fig. 2). Moderate bootstrap values support the Asian-European division (63.6%) and the two Asian (67.3% and 50.3%) clusters. Our previous study using the complete sequences of the control region and the cytochrome *b* (cyt *b*) gene showed relatively high bootstrap values for the Asian-European division (94.3%) and the two Asian clusters (93.2% and 99.7%, respectively) (Watanobe *et al.*, 1999).

Japanese wild boar haplotypes were relatively close to East and Northeast Asian *S. scrofa* haplotypes and formed a monophyletic cluster with a Northeast Asian wild boar haplotype 56 detected in two wild boars from northeastern Mongolia. Japanese wild boar haplotype J15 was also found in a northeastern Mongolian wild boar. Haplotypes 21 from southern Mongolian and 22 from the northernmost Chinese wild boars were more closely related to Japanese wild boar haplotypes than to haplotypes from Southeast Chinese native pigs (i.e., haplotypes 23 and 24 from the Meishan pig breed and haplotypes 25 and 26 from the Jinhua pig breed), which were reported to originate from wild boars found in Southeast China (Ozawa, 2000). These phylogenetic relationships seem to provide evidence that the Northeast Asian wild boars are ancestors of Japanese wild boars.

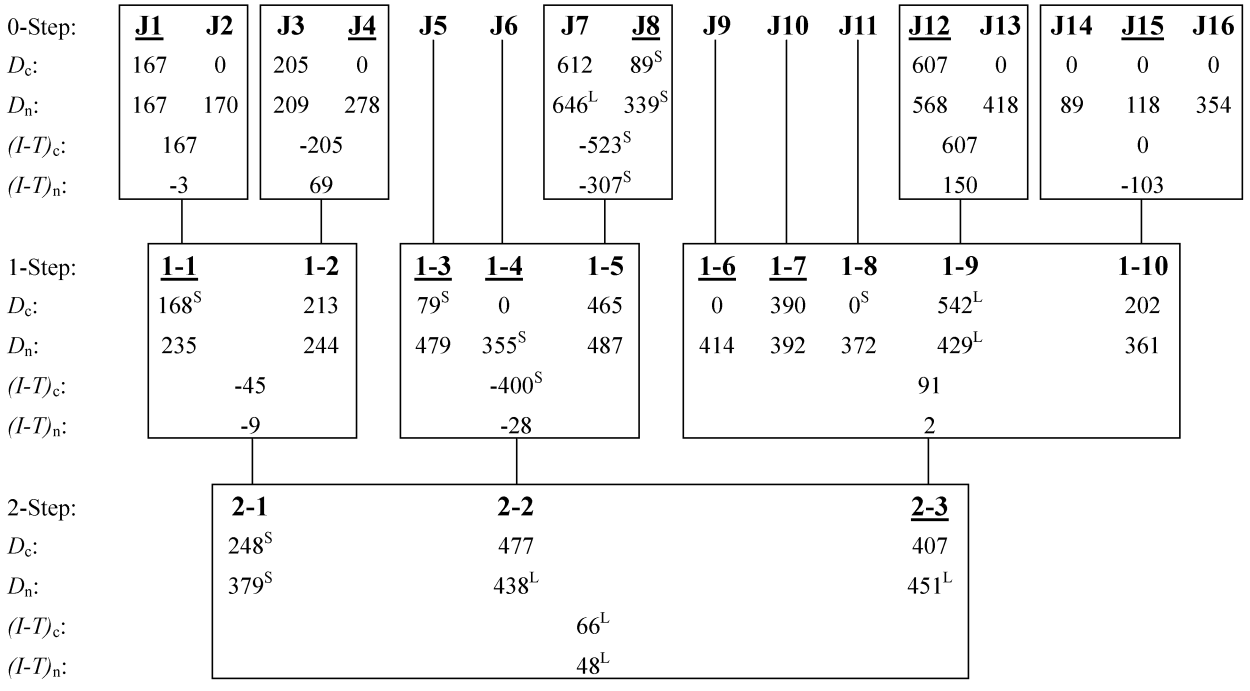

Fig. 4. Results of the nested clade analysis of geographical distances for the mtDNA haplotypes of Japanese wild boar. The haplotype designations are given at the top are grouped in boxes to reflect the one-step nested design given in Fig. 3. Higher-level clade designations are given as one moves down the figure, with boxed grouping indicating the nesting structure. Interior haplotypes or clades within their nested groups are underlined. D_c and D_n indicate average clade and nested clade distances, respectively, whereas $(I\text{-}T)_c$ and $(I\text{-}T)_n$ indicate average differences in clade distances and nested clade distances between interior clades and tip clades within the nesting group, respectively. 'S' and 'L' superscripts identify significantly small and large distances ($P < 0.05$ under the null hypothesis of no geographical association).

Genetic differences and divergence times between *Sus scrofa* groups

Table 2 summarizes the genetic differences between *S. scrofa* groups, the significance of the differences, and the estimated divergence times. The genetic differences between Ryukyu wild boars and other Asian *S. scrofa*, and between East Asian domestic pigs and Northeast Asian and Japanese wild boars were both significant (Table 2). These species were estimated to have diverged approximately 316,000–569,000 and 140,000–253,000 years ago, respectively. These estimated divergence times are relatively conservative because genetic diversity present in the common ancestor of the two *S. scrofa* groups was taken into account when estimating the genetic distances (Nei, 1987). The genetic difference between Northeast Asian and Japanese wild boars was not significant (Table 2). These results also indicate that Northeast Asian wild boars are genetically the closest to Japanese wild boars and that these two *S. scrofa* groups can not be distinguished from each other.

Nested clade analysis

A haplotype cladogram constructed with 95% parsimonious connections shows the genealogical relationship used in the nested clade analysis of mtDNA control region haplo-

Table 3. Chain of inference from nested clade analysis

Clade[1)]	Chain of inference[2)]	Inference
Haplotypes nested in 1-5	1-2-11-12 NO	Contiguous range expansion
Haplotypes nested in other 1-step clades	Fail to reject H_0[3)]	
Clades nested in 2-1	1-2-11-12 NO	Contiguous range expansion
Clades nested in 2-2	1-2-3-4-9 NO	Past fragmentation
Clades nested in 2-3	1-2-3-5-6-7 YES	Restricted gene flow / dispersal but with some long-distance dispersal
Clades nested in entire cladogram	1-2-11-17 NO	Inconclusive outcome

1) Clade structure is given in Fig. 3.
2) The chain uses the key in the APPENDIX on Templeton *et al.* (1998).
3) Null hypothesis.

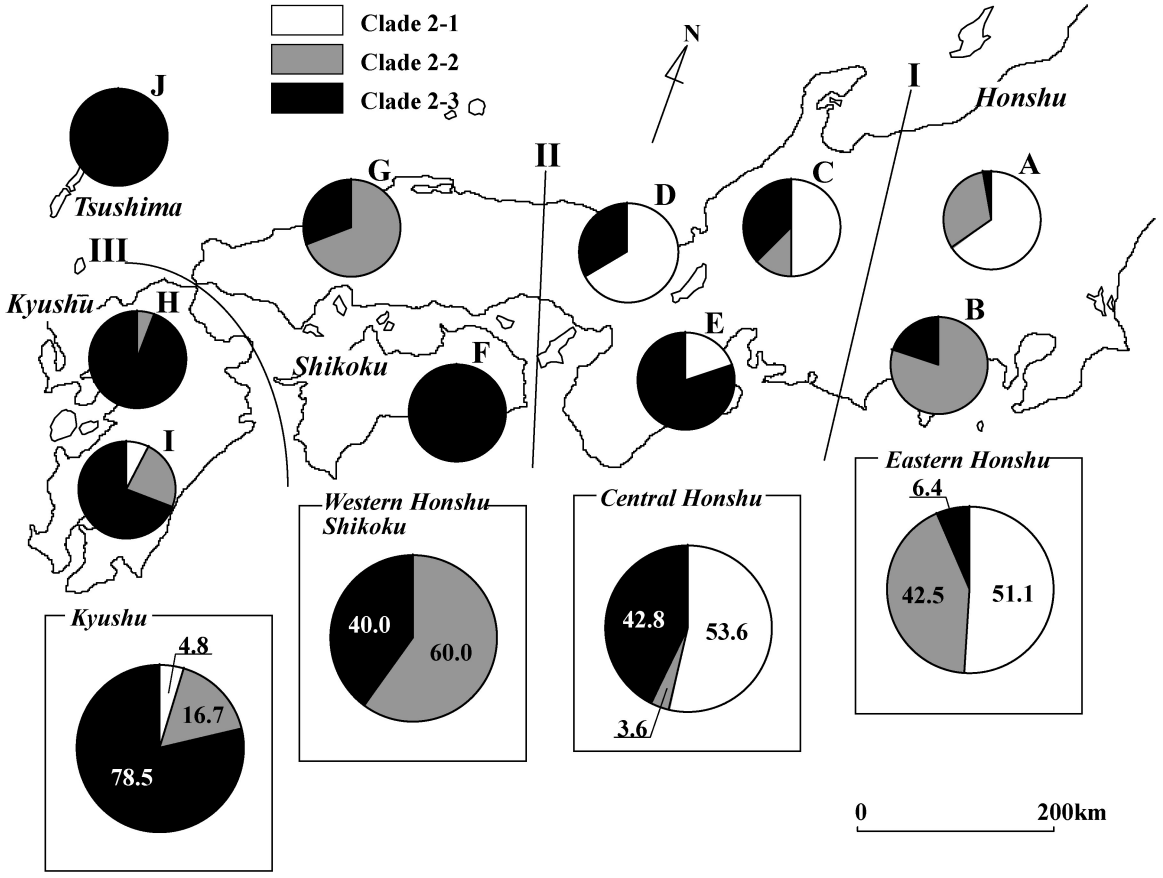

Fig. 5. Geographical distribution of clades identified in Fig. 3 at the 2-step level. White, shaded and black sections of the pie charts show the frequencies of clades 2-1, 2-2, and 2-3, respectively, for each population. Summarized frequencies for each region are given in boxes.

types from Japanese wild boars (Fig. 3). The cladogram requires 23 mutational steps. Ten one-step clades (clade 1-1 to 1-10) and three two-step clades (clade 2-1 to 2-3) were defined in the cladogram (Fig. 3). Among the one-step clades, 1-1, 1-2, 1-5, 1-9 and 1-10 contain both genetic and geographical variation. Clades 1-3 and 1-7 contain only geographical variation, while clades 1-4, 1-6, and 1-8 had neither genetic nor geographic variation (Table 1; Fig. 3). Clade 1-7 with haplotype J10 contains the largest geographical variation; haplotype J10 was detected in nine of ten locations. Among the two-step clades, clades 2-1 and 2-2 placed on the tips, and clade 2-3 placed on the interior within the entire cladogram (Fig. 3). One or two haplotypes with high frequencies were nested in each two-step clade: haplotypes J1 and J3 in clade 2-1, haplotypes J5 and J8 in clade 2-2, and haplotype J10 in clade 2-3.

Fig. 4 shows the results of the nested clade analysis of geographical distance for the mtDNA haplotypes. Values for D_c and D_n were not significant for haplotypes nested in clades 1-1, 1-2, 1-9, and 1-10 of all one-step clades that had both genetic and geographical variation (Fig. 4). The null hypothesis of no association between genealogical relationship and geographical distribution could not be rejected for these haplotypes. However, for haplotypes nested in clade 1-5, one-step clades nested in clades 2-1, 2-2, and 2-3, and two-step clades nested in the entire cladogram, the null hypothesis was rejected, and either historical events or restricted gene flow was suggested to have caused the

Table 4. Hierarchical analysis of molecular variance (AMOVA) among and within regions and localities

Analysis	Variance component	%[1)]	P[2)]	Φ[3)]
4-region analysis	regional boundaries I, II & III			
	among regions (Φ_{RT})	19.74	$P < 0.01$	0.1974
	within regions (Φ_{PR})	18.12	$P < 0.01$	0.2257
	within localities (Φ_{IP})	62.15	$P < 0.01$	0.3785
3-region analysis	regional boundaries I & II			
	among regions (Φ_{RT})	17.76	$P < 0.01$	0.1776
	within regions (Φ_{PR})	20.55	$P < 0.01$	0.2499
	within localities (Φ_{IP})	61.69	$P < 0.01$	0.3831
	regional boundaries I & III			
	among regions (Φ_{RT})	15.95	$P < 0.05$	0.1595
	within regions (Φ_{PR})	22.30	$P < 0.01$	0.2654
	within localities (Φ_{IP})	61.75	$P < 0.01$	0.3825
	regional boundaries II & III			
	among regions (Φ_{RT})	12.12	NS (0.138)	0.1212
	within regions (Φ_{PR})	26.82	$P < 0.01$	0.3053
	within localities (Φ_{IP})	61.05	$P < 0.01$	0.3895
2-region analysis	regional boundary I			
	among regions (Φ_{RT})	20.88	$P < 0.05$	0.2088
	within regions (Φ_{PR})	21.80	$P < 0.01$	0.2755
	within localities (Φ_{IP})	57.32	$P < 0.01$	0.4268
	regional boundary II			
	among regions (Φ_{RT})	11.87	NS (0.088)	0.1187
	within regions (Φ_{PR})	27.46	$P < 0.01$	0.3115
	within localities (Φ_{IP})	60.68	$P < 0.01$	0.3932
	regional boundary III			
	among regions (Φ_{RT})	9.66	NS (0.226)	0.0966
	within regions (Φ_{PR})	29.53	$P < 0.01$	0.3269
	within localities (Φ_{IP})	60.81	$P < 0.01$	0.3919

Separate analyses are given for two, three and four regional divisions using the three boundaries shown in Fig. 1.

[1)] Percentage variance.

[2)] Probability estimated from permutation tests. NS, 'not significant'.

[3)] Φ-statistics given at each hierarchical level. The Φ-statistics are described in detail in the Materials and Methods.

present geographical population structures (Fig. 4; Table 3). In a population range expansion event, it is predicted that some of the older (interior) haplotypes or clades will be confined to the ancestral, pre-expansion area while some of the younger (tip) haplotypes or clades arising in the expanding populations will be located far from their ancestral (interior) haplotypes or clades (Cann *et al*., 1987; Templeton *et al*., 1995). Consequently, both haplotypes nested in clade 1-5 and clades nested in clade 2-1 must have experienced contiguous range expansion events, because D_c (and also D_n in clade 1-5) values for interior clades (haplotypes) were significantly smaller. These estimations were also supported by small $(I\text{-}T)_c$ values (Fig. 4). Clade 2-2, including clade 1-5, is inferred to have contiguously expanded in the range, and consists of three subclades with restricted geographical distributions which were completely separated from each other (Table 1). The significantly small D_n value calculated in the interior subclade 1-4 (Fig. 4) suggests that this clade was fragmented in the past (Table 3). The geographical distribution of the clade 2-3 was the widest, ranging from Kyushu to central Honshu, but was extremely restricted in eastern Honshu (Table 1). A significantly small D_c value was observed in the tip subclade 1-8 within this clade, and the average D_c values increased with increasing clade level in this nested series of clades (Fig. 4). Basically, the older the haplotype, the more widespread it tends to be under the restricted gene flow model (Neigel *et al*., 1991; Neigel and Avise, 1993; Slatkin, 1993). This predicted distance pattern was observed in clade 2-3; the interior (older) subclade 1-7 within clade 2-3 distributed wider than any other tip subclades (Table 1). Thus, clade 2-3 was inferred to have restricted gene flow and dispersal with only some long distance dispersal (Table 3). Consequently, although all two-step clades had overlapping geographical ranges, distinct patterns of historical events and restricted gene flow were inferred for each clade using the nested clade method of analysis.

Hierarchical analysis of molecular variance and geographical distribution of haplotypes

To assess the stability of regional boundaries of genetic characteristics of Japanese wild boars, we separately analyzed the molecular variance of four, three and two regional divisions using three hypothetical boundaries (Figs. 1 and 5). Table 4 summarizes the results of hierarchical analysis of molecular variance (AMOVA) for Japanese wild boar haplotypes. The genetic subdivision among localities within regions (Φ_{PR}) were significantly larger and significantly smaller among individuals within localities (Φ_{IP}) relative to their null distributions as estimated by the permutation test for all analysis categories. In all three categories of the analyses, the regional genetic subdivision of Japanese wild boars (Φ_{RT}) was significant only when regional boundary I was used in the geographical subdivision of their habitat. However, in all cases using regional boundaries II, III, or both, but without I, the genetic subdivision of Japanese wild boars was not significant. These results indicate that Japanese wild boars are regionally subdivided by their genetic characteristics, and that the subdivision imposed by boundary I is stronger than the subdivisions imposed by boundaries II and III (Table 3).

The regional boundary I effectively divides the eastern Honshu region from the rest of Honshu, Shikoku and Kyushu (Fig. 5). A marked difference in the frequency of clade 2-3 was observed between the eastern Honshu region (6.4%) and the rest of the Japan (40.0% to 78.5%), suggesting that the gene flow of clade 2-3 is restricted across regional boundary I. Clade 2-1, predicted by nested clade analysis to have experienced expansion to the contiguous ranges, has a restricted distribution in eastern and central Honshu regions with frequencies greater than 50%. Clade 2-2 was fragmented in the past and has high or moderate frequencies in eastern Honshu, western Honshu and Shikoku and Kyushu regions, and an extremely low frequency (3.6%) in central Honshu.

Within each local population, except for J, two to eight distinct haplotypes were detected, and genetic polymorphism was maintained. However, only haplotype J10 was detected from population J, although a relatively large number of individuals were examined (Table 1). These results suggest that the artificial population J has no or extremely little genetic polymorphism.

DISCUSSION

Previously, we reported that the two endemic *Sus scrofa* subspecies inhabiting Japan, Japanese wild boar *S. s. leucomystax* and Ryukyu wild boar *S. s. riukiuanus*, were highly diverged, and that *S. s. leucomystax* was rather closely related to other East Asian *S. scrofa* (Watanobe *et al*., 1999). The phylogenetic relationship and genetic differences between Japanese wild boars and Asian *S. scrofa* analyzed in this study revealed that Japanese wild boars are particularly closely related to Northeast Asian wild boars within Asian *S. scrofa* (Fig. 2; Table 2). Fossil records of Pleistocene mammalian faunas indicate that the species *S. scrofa* gradually expanded its habitat from southeastern to northeastern Asian Continent in the Middle to Late Pleistocene (Kawamura, 1982). The estimated divergence time between Japanese and Northeast Asian wild boars and the other East Asian domestic pigs (140,000–253,000 years ago; see Table 2) corresponds approximately to the late Middle Pleistocene just when *S. scrofa* was spreading from southeastern to northeastern area of Asian Continent. Thus, the ancestral *S. scrofa* populations of Japanese wild boars were thought to be a part of the population that spread northward in the Middle to Late Pleistocene. The ancestral population of Japanese wild boars probably migrated to Japanese Islands across landbridges repeatedly formed between the Korean Peninsula and Kyushu Island, but not across Sakhalin and Hokkaido Island to the Japanese Islands, because no *S. scrofa* population are found on

Sakhalin and Hokkaido islands today and no *S. scrofa* fossils have been found on Hokkaido Island (Kawamura, 1991).

When the divergence times between northeastern Mongolian wild boars (haplotypes J15 and 56), which are the closest haplotypes to the Japanese wild boars, and three two-step clades defined in this study were estimated using the same methods as in Table 2, clades 2-1, 2-2, and 2-3 were tentatively estimated to have diverged 204,000–367,000, 170,000–307,000, and 12,000–21,000 years ago, respectively. These estimations suggest that clades 2-1 and 2-2 migrated to the Japanese Islands and genetically diverged from their continental ancestor earlier than did clade 2-3. The formation of landbridges between the Korean Peninsula and Kyushu Island has been estimated at around 500,000 and 300,000 years ago based on the fossil records of Japanese land mammal fauna (Dobson and Kawamura, 1998). In a geological study by Ohshima (1990), the landbridge was estimated to have remained in place until around 150,000 years ago but to be absent to the present. Our approximation of the time periods of migration for clades 2-1 and 2-2 corresponds well to the formation of the landbridges. However, in the case of clade 2-3, it is very difficult to explain how it migrated to the Japanese Islands, because no landbridges are assumed to have been present at such a recent time (12,000–21,000 years ago). Although the most recent migration might be explained by the existence of a landbridge in the last Glacial Age or an artificial introduction by humans, these assumptions are not thought to be supported by the current body of evidence. More comprehensive genetic investigation, especially into the continental wild boar populations, is needed to improve the accuracy of estimated divergence times.

The three two-step clades were almost sympatric (Table 1). However, interestingly, they indicated discordant patterns of historical events and gene flows, and a conclusive pattern was not inferred for the entire cladogram (Table 3; Fig. 4). When the founder population was small or modest in size and subsequently expands, the haplotype cladogram should display a star-like form with a common ancestral haplotype at the star's center (Avise, 2000). With the Japanese wild boar, this interpretation can only be applied to clade 2-3 with haplotype J10. Clade 2-3 probably migrated to Kyushu Island first, which is the region that showed the highest frequency of clade 2-3 and maintained the largest number of haplotype components of this clade (Fig. 5; Table 1). Clade 2-3 would have subsequently expanded to western Honshu, Shikoku and central Honshu regions, and would have been restricted in its expansion by regional boundary I in the high mountain range of the Japanese Alps (Table 4; Fig. 5). However, clades 2-1 and 2-2 and the entire cladogram of Japanese wild boar haplotypes have no common central haplotype, and thus have a pattern of migration different from that of clade 2-3 (Fig. 3). This almost sympatric distribution of the three two-step clades and the disagreement between inferred patterns of historical events or gene flow may be explained by differences of the dates of the migration to Japanese Islands and differences in environmental conditions. For example, the *S. scrofa* population had remarkably decreased in the Japanese Islands from the late Middle Pleistocene to early Late Pleistocene (Fujita *et al.*, 2000), when it was assumed that while clades 2-1 and 2-2 had already migrated, but clade 2-3 had not. Though the causes are not explained, the decline of the *S. scrofa* population may have been due to some climatic changes or ecological competition with other large mammals. The inferred historical events, contiguous range expansion of clade 2-1 and past fragmentation of clade 2-2, might be explained by subsequent range expansion and fragmentation as the result of the decrease of the number of individuals. *S. scrofa* have become the dominant large mammal in Japanese Islands after the mammalian extinction event between 20,000 and 10,000 years ago (Kawamura, 1994). It can be assumed that clade 2-3 migrated around this time. Subsequently, admixture of the migrated cladistic populations would have occurred. Nested clade analysis can detect correlations between phylogeny and geology. In this study, historical processes and contemporary gene flow can be inferred even from sympatric distinct clades by analyzing their phylogenetic relationships. This method can assess the strength of geographical and phylogenetic associations through rigorous statistical testing. However, it does not provide a statistical estimate of the certainty or uncertainty of the conclusion obtained through the inference key (Knowles and Maddison, 2002). Our hypothesis for the historical migrations of Japanese wild boars should be tested further using the latest mtDNA data and other genetic markers; for example, microsatellite loci of nuclear DNA can assess the degree of population admixture.

The geographical distribution of mtDNA haplotypes has many implications not only for understanding the recent gene flow among Japanese wild boar populations in distinct localities, but also for the conservation of their populations. A stable genetic structure was indicated at the local level in Japanese wild boars using hierarchical AMOVA (Table 4). Female Japanese wild boars form stronger social bonds within the group compared to juvenile males and consequently do not naturally migrate (Nakatani and Ono, 1994) giving a possible explanation for the limited spread of maternal inherited characters, such as mtDNA, in the absence of artificial movement caused by humans. The stable population structure within each local population provides genetic background data of natural populations that makes it possible to trace animal introductions by humans.

All natural populations of Japanese wild boars have generally maintained polymorphisms within the mtDNA control region with the exception of population J on Tsushima Island (Table 1). The native wild boars on Tsushima Island were exterminated in 1709 A.D. (Watase, 1912), but the wild boar has recently been found and their numbers are gradually increasing. The mtDNA haplotype profile of wild boars from Tsushima Island suggests humans introduced them from population H from northern Kyushu Island. Genetic

monomorphism of boars on Tsushima Island might be due to the expansion of the founder's mtDNA haplotype J10. Recently, artificial introductions of wild boars have frequently been made for hunting in restricted mountain areas and for farming wild boars in Japan. However, unsystematic artificial introduction of wild animals into other localities runs the risk of possibly transforming the genetic population structure of wild animals, and may lead to the extinction of some haplotypes (Avise and Nelson, 1989). Collecting both geographical and genetic characteristics of wild populations, such as for the wild boars in this study, will provide useful background data for conserving and managing wild animals.

Recent phylogeographical research conducted on Japanese land mammals, including Japanese brown bear, sika deer, and sorex shrews (Matsuhashi *et al*., 1999; Nagata *et al*., 1999; Goodman *et al*., 2001; Ohdachi *et al*., 2001), has provided valuable information about ancient migration events and subsequent structuring of their geographic populations. In this study, we newly described the phylogeographical profile of the Japanese wild boar, and also propose a hypothesis of the historical formation of the present population structure of this animal. Future studies of molecular phylogeographical information for many other mammals, in addition to the information from fossil records, will help develop a more comprehensive understanding of the composition of mammalian fauna on the Japanese islands.

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