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Prehistoric Introduction of Domestic Pigs onto the Okinawa Islands: Ancient Mitochondrial DNA Evidence

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Abstract. Ancient DNAs of *Sus scrofa* specimens excavated from archaeological sites on the Okinawa islands were examined to clarify the genetic relationships among prehistoric *Sus scrofa,* modern wild boars and domestic pigs inhabiting the Ryukyu archipelago, the Japanese islands, and the Asian continent. We extracted remain DNA from 161 bone specimens excavated from 12 archaeological sites on the Okinawa islands and successfully amplified mitochondrial DNA control region fragments from 33 of 161 specimens. Pairwise difference between prehistoric and modern *S. scrofa* nucleotide sequences showed that haplotypes of the East Asian domestic pig lineage were found from archaeological specimens together with Ryukyu wild boars native to the Ryukyu archipelago. Phylogenetic analysis of 14 ancient sequences (11 haplotypes; 574 bp) indicated that *S. scrofa* specimens from two Yayoi-Heian sites (Kitahara and Ara shellmiddens) and two Recent Times sites (Wakuta Kiln and Kiyuna sites) are grouped with modern East Asian domestic pigs. *Sus scrofa* specimens from Shimizu shellmidden (Yayoi-Heian Period) were very closely related to modern *Sus scrofa riukiuanus* but had a unique nucleotide insertion, indicating that the population is genetically distinct from the lineage of modern Ryukyu wild boars. This genetic evidence suggests that domestic pigs from the Asian continent were introduced to the Okinawa islands in the early Yayoi-Heian period (1700–2000 BP), or earlier.

Key words: Ancient DNA — Mitochondrial DNA — Control region — Molecular phylogeny — *Sus scrofa*

Introduction

The Ryukyu wild boar (*Sus scrofa riukiuanus*) inhabits only the Ryukyu archipelago, which is an island arc southwest of the main Japanese islands (Fig. 1). This subspecies is distinguishable from continental boars (*S. s. ussuricus*), Taiwan wild boars (*S. s. taivanus*), and Japanese wild boars (*S. s. leucomystax*) on body size and other morphological characteristics (Imaizumi 1973; Hayashi et al. 1983). A large number of *Sus scrofa* bones have been excavated from prehistoric sites on the Okinawa islands within the Ryukyu archipelago. Morphologically, most of these prehistoric *S. scrofa* are about the same size as the present-day Ryukyu wild boar. Other bone specimens excavated from early Yayoi-Heian sites [1700–2000 years before present (BP)] are much larger than the modern Ryukyu wild boar (Matsui 1997). Investigation and identification of prehistoric *S. scrofa* including these large specimens are extremely important for estimating the movement of *S. scrofa* as well as the prehistoric cultural exchange or trade systems by humans. However, morphological analysis of these bones is

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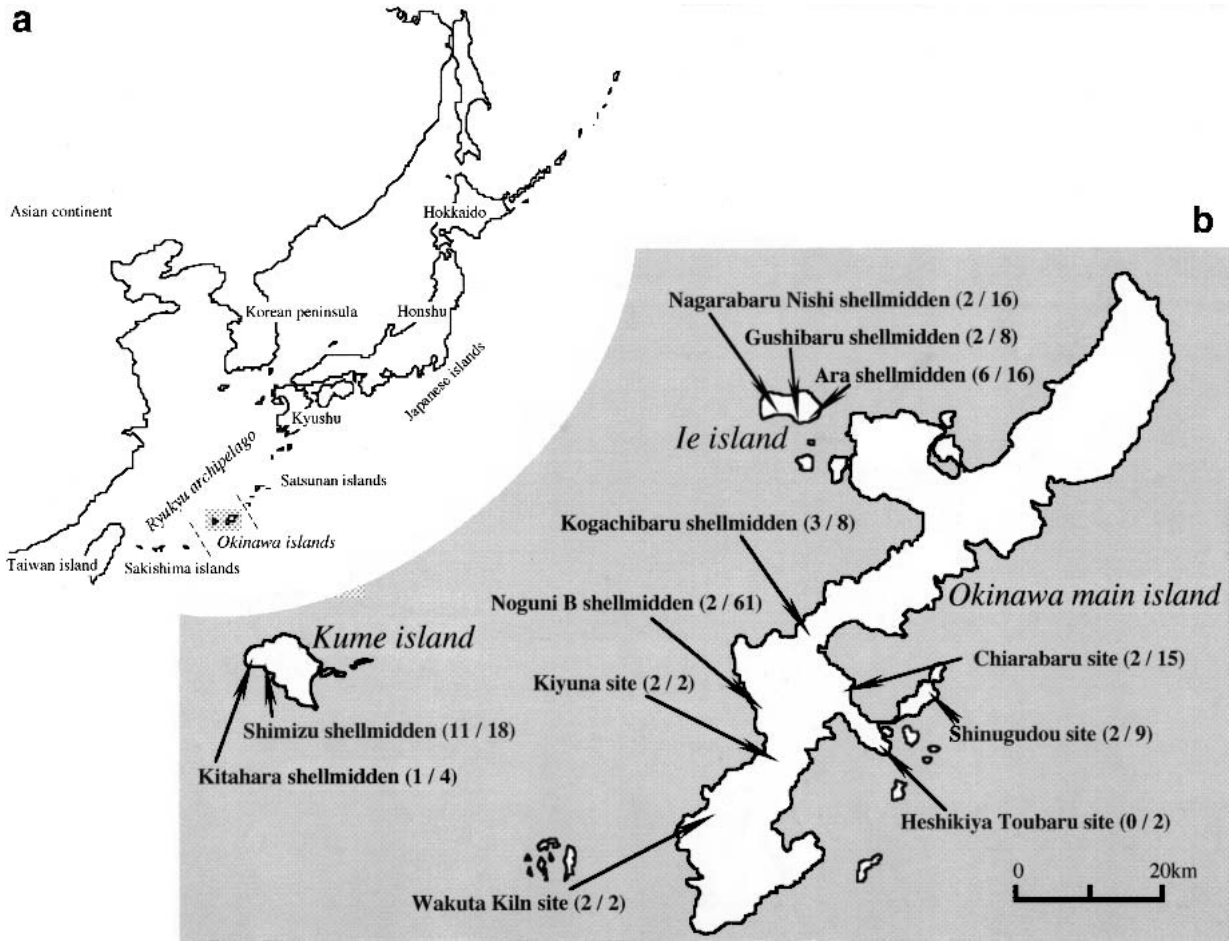

Fig. 1. Maps of the Ryukyu archipelago and Okinawa islands **(a)** and sampling archaeological sites of prehistoric *Sus scrofa* specimens in the Okinawa islands **(b).** Details of the *shaded* area in map a are shown in map b. Chronological periods and approximate dates of the archaeological sites are as follows: early Jomon period—Noguni B shellmidden (6600–7000 BP); late Jomon period—Kogachibaru shellmidden (3500–4000 BP) and Chiarabaru shellmidden (3000–3500 BP); final Jomon period—Shinugudou site (2500–3000 BP); early Yayoi-Heian period (1700–2000 BP, all sites)—Heshikiya Toubaru site, Kitahara shellmidden, Shimizu shellmidden, Nagarabaru Nishi shellmidden, Gushibaru shellmidden, and Ara shellmidden; Recent Times—Wakuta Kiln site (16C AD) and Kiyuna site (18C AD). Numbers in parentheses are the number of samples with DNA data/the number of samples examined in this study.

hampered by poor preservation, making osteometric analysis almost impossible (Matsui 1997).

An alternative approach for identifying the bones is using mitochondrial DNA (mtDNA) analysis of present and prehistoric individuals of *S. scrofa.* We previously examined the phylogenetic relationships among Ryukyu and Japanese wild boars and East Asian domestic pigs based on the mtDNA control region and *cyt b* gene sequences and found that the Ryukyu wild boar is genetically different from other Asian *S. scrofa* and Japanese wild boars (Watanobe et al. 1999). Advances made during recent decades in DNA analytical techniques allow phylogenetic analysis of extinct species (Thomas et al. 1989; Cooper et al. 1992; Krings et al. 1997; Ozawa et al. 1997) and ancient animals and human populations (Horai et al. 1991; Stone and Stoneking 1993; Hardy et al. 1995; Oota et al. 1999). These studies indicate that genetic information retrieved from ancient remains is extremely useful for understanding the transitions of animal populations. Thus, mtDNA analysis can be used to establish genetic relationships between prehistoric *S. scrofa* from the Okinawa islands and modern wild boar and domestic pigs from Okinawa and neighboring regions.

To investigate the genetic background of the prehistoric *S. scrofa* from the Okinawa islands, we extracted ancient DNA from bones and determined the nucleotide sequences of the mtDNA control region using polymerase chain reaction (PCR) techniques. Our results showed complicated phylogenetic relationships among prehistoric *S. scrofa,* modern wild boars, and domestic pigs.

Materials and Methods

Archaeological Sites and Samples

A total of 161 bone specimens (88 mandibles, 38 teeth, 9 ulnae, 8 maxillae, 6 humeri, 6 radii, 3 pelves, 2 femora, and 1 tibiae) was derived from an estimated 73 individuals at minimum collected from

12 archaeological sites (including 7 shellmiddens) from the Okinawa main island, Ie island, and Kume island in the Okinawan islands in the central part of the Ryukyu archipelago (Fig. 1). The estimated minimum number of individuals in each archaeological site was calculated based on the most frequently occurring bone type from each stratum.

Two dating systems are frequently used to describe the prehistory of Okinawa (Takamiya 1996). One system divides the prehistory of Okinawa into the early and late Shellmidden periods. This stresses the uniqueness of the development of prehistoric Okinawa culture. The second system parallels the chronology of mainland Japan, where the early and late Shellmidden periods correspond to the Jomon and Yayoi-Heian periods, respectively. We used the Jomon and Yayoi designations to date each site because most readers are likely to be more familiar with them. The legend to Fig. 1 gives the chronological periods and approximate dates of each site.

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 powders (0.5 to 1.0 g) were collected from each sample using an electric drill. Bone powder samples were suspended in 10 ml of 0.5 *M* ethylenediaminetetraacetate (EDTA), decalcified by rotating for a few days, and then centrifuged at 3000 rpm for 10 min. The supernatant was removed, and pellets of bone powders were repeatedly decalcified with 10 ml of 0.5 *M* EDTA. After decalcification, the bone powders 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 modifications of the method of Hardy et al. (1995). The sample was centrifuged at 3000 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 microconcentrator (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 templates. Precautions were taken to prevent contamination from other nonancient DNA, as described by Okumura et al. (1999). Blank extractions without bone powders 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. Primers mitL76 (5′-AATATGCGACCCCAAAAATTTAACCATT^{130}) and mitH62 (5′-CCTGCCAAGCGGGTTGCTGG^{351}) for set A, primers mitL119 (5′-CAGTCAACATGCGTATCACC^{301}) and mitH124 (5′-ATGGCTGAGTCCAAGCATCC^{567}) for set B, and primers mitL104 (5′-TGGACTAGTGACTAATCAGCCCAT^{518}) and mitH106 (5′-ACGTGTACGCACGTGTACGC^{704}) for set C amplify 258-, 305-, and 229-bp fragments, respectively (Watanobe et al. 2001). The numbers on the upper right of the primers denote the corresponding nucleotide positions of the 3′-terminal nucleotide of the primers (Okumura et al. 1996). L and H designate light and heavy strands, respectively. PCRs were made using 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 s, annealing at 57°C (60°C) for 30 s, and extension at 72°C for 1 min. When few 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, with 1 μl of the first PCR product, 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 microconcentrator (Amicon) for use as sequencing templates. Nucleotide sequences of both strands were determined using an Applied Biosystems 377 DNA sequencer with a 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 the second laboratory. A 574 bp sequence was obtained by connecting the three DNA fragments amplified by primer sets A, B, and C.

Modern Wild Boars and Domestic Pigs

Watanobe et al. (1999) reported nucleotide sequences of the mtDNA control region of 59 Japanese wild boars, 13 Ryukyu wild boars, 12 Asian domestic pigs, 7 European domestic pigs, and 3 European wild boars. In addition, we analyzed 63 Japanese wild boars, 3 Northeast Asian wild boars (1 from near Ulan Bator, northern Mongolia, 1 from near Da-xinganling, southern Mongolia, and 1 from near Xiaoxinganling, China), 65 Asian domestic pigs, and 66 European domestic pigs using the procedure of Okumura et al. (1996) (accession Nos. AB041464–AB041499). In total, 291 modern individual samples were examined. Fifty-five haplotypes designated M1 to M55 obtained for the 574 bp of the mtDNA control region formed a database of modern *S. scrofa* in this study. Five representative haplotypes of the five *S. scrofa* groups, except for the northeast Asian wild boars, are listed in Table 2.

Phylogenetic Analysis

Neighbor-joining analysis (Saitou and Nei 1987) was made using the PHYLIP program package, version 3.572 (Felsenstein 1995). A distance matrix for the neighbor-joining tree was constructed with Kimura's (1980) two-parameter distances. We used 1000 bootstrap replications to determine the confidence intervals of the phylogeny. Parsimony analysis was also performed using the MEGA 1.02 (Kumar et al. 1993).

Results

Preservation and Amplification of Ancient DNA

Archaeological information and success of PCR and sequencing are summarized in Table 1. No negative controls yielded any PCR products. Thirty-three of 161 samples were successfully amplified and sequenced. The hard, less-colored bone samples were successfully amplified corroborating the observations previously made by Hardy et al. (1995) and Okumura et al. (1999). In general, samples from shellmiddens of the early Yayoi-Heian period were well preserved, as most of the shellmiddens in this period were formed on sandy beaches. In contrast, samples from other periods were poorly preserved, as these sites were under cliffs or on gentle hilly areas (Okinawa Prefectural Board of Education 1997) where the soils would be unsuitable for bone preservation.

Table 1. Characteristics of archaeological specimens and their mtDNA amplification

Archaeological site	Bone part	Period	Specimen no.	PCR results[a]			PCR[b]	
				fA	fB	fC	Ordinary	Seminested
Noguni B shellmidden	Radius	Early Jomon	81	+++	−−	+++		fA, fC
	Mandible		130	+−−	−−	++		fA, fC
Kogachibaru shellmidden	Mandible	Late Jomon	143	++	−−	++		fA, fC
	Mandible		144	+++	−−	++		fA, fC
	Mandible		146	++	++	−−		fA, fB
Chiarabaru site	Mandible	Late Jomon	150	++	−−	+−−		fA, fC
	Mandible		151	++	−−	−−		fA
Kitahara shellmidden	Maxilla	Early Yayoi-Heian	87	+++	++	++	fC	fA, fB
Shimizu shellmidden	Mandible	Early Yayoi-Heian	102	+++	−−	+++	fA, fC	
	Mandible		104	+−−	++	++	fA	fB, fC
	Mandible		105	+++	++	++	fA, fC	fB
	Mandible		107	+++	+−−	++	fA	fB, fC
	Mandible		108	+++	−−	+++		fA, fC
	Mandible		109	+++	++	+++	fA, fB, fC	
	Humerus		110	++	+−−	−−	fB	fA
	Femur		111	+++	−−	++	fA, fC	
	Ulna		152	++	++	++		fA, fB, fC
	Ulna		155	++	++	++		fA, fB, fC
	Ulna		156	+++	++	++		fA, fB, fC
Nagarabaru Nishi shellmidden	Teeth	Early Yayoi-Heian	5	++	++	++		fA, fB, fC
	Teeth		13	++	++	++	fA, fB, fC	
Gushibaru shellmidden	Mandible	Early Yayoi-Heian	17	++	++	++	fA, fB, fC	
	Mandible		20	++	++	++	fC	fA, fB
Ara shellmidden	Maxilla	Early Yayoi-Heian	115	++	+++	++	fA, fB	fC
	Mandible		119	+++	−−	−−	fA	
	Mandible		120	+++	++	+++	fA, fB	fC
	Mandible		123	+−−	−−	+++	fA	fC
	Mandible		124	++	−−	+−−	fA, fC	
	Humerus		157	++	−−	++		fA, fC
Wakuta Kiln site	Mandible	Recent Times	125	++	++	+++	fA, fB, fC	
	Mandible		126	+−−	−−	++	fA	fC
Kyuna site	Mandible	Recent Times	127	+++	++	++	fA, fB, fC	
	Mandible		128	++	++	++	fA, fB, fC	

[a] fA, fB, and fC show the DNA fragment amplified by the A, B, and C set primers, respectively. (–) Not amplified; (+) amplified once; (++) amplified twice independently; (+++) amplified three times.
[b] Ordinary or seminested PCR was done to amplify the ancient DNA.

The amplification success of ancient DNA was markedly lower in *S. scrofa* specimens from the Jomon period (7 samples of 93) than from the early Yayoi-Heian period (22/64) and in Recent Times (4/4). The amplification success of ancient DNA appeared to be correlated with the degree of preservation and the age of the archaeological specimens.

Among the 33 successfully amplified samples, fragments A (fA), fB, and fC were amplified from 28, 16, and 27 samples, respectively (Table 1), indicating that the long DNA fragment (fB, 305 bp) is more difficult to amplify than the shorter DNA fragments (fA, 258 bp; and fC, 229 bp). This shows that ancient DNAs have been fragmented by autolysis, oxidation, and/or bacterial digestion (Pääbo 1989).

DNA Sequences of Prehistoric Sus scrofa

Table 2 shows polymorphic sites in the 574 bp control region sequences of all ancient and five representative modern haplotypes. Among the 33 ancient DNA sequences, 29 polymorphic sites were identified. Thirteen of these 29 sites were newly found in this study, and the other 16 sites were shared with the modern haplotypes. Nineteen of the 33 ancient sequences were partial, because one or two DNA fragments (fA, fB, or fC) could not be successfully amplified by PCR. The 574 bp ancient DNA sequences from 14 archaeological specimens were submitted to the DDBJ/EMBL/GenBank database (accession Nos. AB050869–AB050882). Among these 14 ancient sequences, the 11 distinct haplotypes were designated A1 to A11 (Table 2). Haplotypes A1 and A2 were identical to modern haplotypes M33 (from Berkshire and Largewhite) and M31 (from Okinawa native pig and Berkshire), respectively.

Pairwise differences between each ancient sequence and all distinct sequences of the East Asian domestic pig and the Japanese and Ryukyu wild boar are listed in Table 3. Fourteen sequences (specimens 130, 146, 150, 87, 17, 20, 115, 119, 120, 123, 124, 157, 126, and 127)

Table 2. Variability and haplotypes of the mtDNA control region (574 bp) from modern and ancient *Sus scrofa*

Archaeological site	Source/specimen no.	Nucleotide positions[a] fA 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 1 6 8 6 4 9 1 2 6 5 7 2 9 1 9 7 0 9 5 1 2 3 7 4	fB 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4 5 5 2 3 9 9 8 9 1 2 6 7 4 3 2 6 0 2 9 2 1	fC 5 5 5 6 6 6 6 6 6 6 6 7 3 1 6 1 8 0 8 3 9 0 3 3	Haplotype[b]	Cluster[c]
	Meishan pig	A – – TTGCCTTCCACCTTAGCCATT	CCCCAATCTTACGCCCCAC	ATGTGACCATGG	M23	I
	Japanese wild boar	· – – · · · · · C · · TG · · · · · · · · · ·	· · · · · · · · · · · T · · · · · G ·	· C · · · · · · · · AA	M13	I
	Ryukyu wild boar	· – – · · · · T · · · TG · · · · · · · · C ·	· · · · G · · · · · G · · · · · · · ·	· C · · · · · · · · · ·	M16	II
	European wild boar	GC – C · · TT · · · TG · · · · · · · · C ·	· · · · · · C · · · G · · · · · · · ·	AC · G · · · · GCA ·	M54	III
	Landrace	GC – CCA · T · · · TG · · · C · A · · · CC	· · · · · · C · · · · · · · · · · · ·	· · A · · · · · · · ·	M40	IV
Noguni B shellmidden	81	· – – · · · · · · · · TG · T · C · · · · · ·		· C · · · G · · · · · ·	ND	
	130			· C · · · · · · · · · ·	ND	
Kogachibaru shellmidden	143	· – – ·		· C · · · · · · · · · ·	ND	
	144	· – – · · · · T · · TT · · · · · · · · · C ·		· C · · · · · · · · CA ·	ND	
	146	· – – ·	· · · · · · · · · · · · · · · · · · ·		ND	
Chiarabaru site	150	· – – · · · · · · · · T · · · · · · · · · · ·			ND	
	151	· – – · · · · · · · · · · · · · C · · · · · ·			ND	
Kitahara shellmidden	87	· – – · · · · · · · · T · · · · · · · · · · ·	· · · · · · · · · · · · · · · · · · ·	· · · · · · · · · · · ·	A1 (M33)	I
Shimizu shellmidden	102	· – A · · · · · · · · T · · · · · · · · · C ·		· C · · · · · · · · CA ·	ND	
	104	· – A · · · · · · · · T · · · · · · · · · C ·	· · · · G · · · · · G · · · · T · · ·	· C · · · · · · · · CA ·	A7	IIa
	105	· – A · · · · · · · · T · · · · · · · · · C ·	· · · TG · · T · · G · · · · · · · ·	· C · · · · · · · · CA ·	A8	IIa
	107	· – A · · · · · · · · T · · · · · · · · · C ·		· C · · · · · · · · CA ·	ND	
	108	· – A · · · · · · · · T · · · · · · · · · C ·		· C · · · · · · · · CA ·	ND	
	109	· – A · · · · · · · · T · · · · · · · · · C ·	· · T · G · · · · · G · · · · · T · T	· C · · · · · · · · CA ·	A9	IIa
	110	· – A · · · · · · · · T · · · · · · · · · C ·			ND	
	111	· – A · · · TT · · · T · · · · · · · · · C ·		· C · · · · · · · · CA ·	ND	
	152	· – A · · · · · · · · T · · · · · · · · · C ·	· · · · G · · · · · G · · · · · · · ·	· C · · · · · · · · CA ·	A10	IIa
	155	· – A · · · · · · · · T · · · · · · · · · C ·	· · · · G · · · · · G · · · · · · · ·	· C · · · · · · · · CA ·	A10	IIa
	156	· – A · · · · · · · · T · · · · · · · · · C ·	T · · · G · · · · · GT · · T · · · ·	· C · · · · · · · · CA ·	A11	IIa
Nagarabaru Nishi shellmidden	5	· – – · · · · T · · · T · · · · · · · · · · ·	· · · · G · C · · · GT · · · · · · · ·	· C · · · · · · · · CA ·	A5	II
	13	· – – · · · · T · · · T · · · · · · · · · C ·	· T · · G · C · · · GT · · · · · · · ·	· C · · · · · · · · CA ·	A6	II
Gushibaru shellmidden	17			· C · · · · · · · · CA ·	ND	
	20			· C · · · · · · · · CA ·	ND	
Ara shellmidden	115	· – – ·	· · · · · · · · · C · · T · · · · · · ·	· · · · · · · · · · · ·	A2 (M31)	I
	119	· – – ·	· · · · · · · · · C · · T · · · · · · ·		ND	
	120	· – – ·	· · · · · · · · · C · · T · · · · · · ·	· · · · · · · · · · · ·	A2 (M31)	I
	123			· C · · · · · · · · CA ·	ND	
	124	· – – · · · · T · · · T · · · · · · · · · C ·			ND	
	157	· – – · · · · · T · · · T · · · · · · · · · C ·		· C · · · · · · · · CA ·	ND	
Wakuta Kiln site	125	· – – · · · · · · · · · · · · · C · · · · · · ·	· · · · · · · · · · · · T · · · · · · ·	· · · · · · · · · · · ·	A3	I
	126			· · · · · · · · · · · ·	ND	
Kiyuna site	127	· – – · · · · · · · · T · · · · · · · · · · ·	· · · · · · · · · · · · · · · · · · ·	· · · · · · · · · · · ·	A1 (M33)	I
	128	· – – · · · · · · · · T · · · · C · · · T · · ·	· · · · · · · · · · · · · · T · · · · ·	· C · · · · · · · · · ·	A4	I

[a] Nucleotide position 1 corresponds to the first position of the complete sequences of the mtDNA control region described by Okumura et al. (1996). A dot denotes a nucleotide identity with the Meishan pig sequence. A dash denotes a gap site.

[b] ND, not determined.

[c] Clusters are defined by the phylogenetic analysis in Fig. 2.

were identical to sequences from modern animals. The other 19 sequences were unique to the prehistoric specimens examined in this study. Average numbers of pairwise differences within each *Sus scrofa* group (for example, in the case of using sequences of fA + fB + fC, 0.7% within East Asian domestic pig, 0.6% within Japanese wild boar, and 0.5% within Ryukyu wild boar) were tentatively applied as criteria to measure similarity between each ancient sequence and the modern groups. Among seven sequences obtained from the specimens of the Jomon period, three (Nos. 143, 146, and 151) were similar to the sequences of East Asian domestic pigs, one (No. 150) was similar to the sequences detected in both East Asian domestic pigs and Japanese wild boars, one (No. 144) was similar to the sequences detected in Ryukyu wild boars, and the other two (Nos. 81 and 130)

Table 3. Pairwise differences between ancient and modern mtDNA control region sequences (574 bp) of *Sus scrofa*

Archaeological site	Specimen no.	Region[a]	Pairwise nucleotide difference (%)[b] East Asian pigs	Japanese wild boars	Ryukyu wild boars	*Sus scrofa* group[c]
Noguni B shellmidden	81	fA + fC	1.2 ± 0.3	1.6 ± 0.4	2.0 ± 0.4	(Unique)
	130	fC	0.5 ± 0.3	1.5 ± 0.7	0.7 ± 0.7	East Asian pig, Ryukyu wild boar
Kogachibaru shellmidden	143	fA + fC	0.6 ± 0.2	1.5 ± 0.4	1.4 ± 0.4	(Unique)
	144	fA + fC	1.9 ± 0.2	2.0 ± 0.3	0.8 ± 0.3	(Unique)
	146	fA + fB	0.6 ± 0.4	1.2 ± 0.3	1.4 ± 0.2	East Asian pig
Chiarabaru site	150	fA	0.7 ± 0.5	0.9 ± 0.5	1.4 ± 0.4	East Asian pig, Japanese wild boar
	151	fA	0.7 ± 0.4	1.1 ± 0.4	2.5 ± 0.4	(Unique)
Kitahara shellmidden	87	fA + fB + fC	0.5 ± 0.3	1.1 ± 0.2	1.2 ± 0.3	East Asian pig
Shimizu shellmidden	102	fA + fC	1.7 ± 0.3	1.7 ± 0.3	1.1 ± 0.3	(Unique)
	104	fA + fB + fC	1.7 ± 0.2	1.9 ± 0.2	0.9 ± 0.2	(Unique)
	105	fA + fB + fC	1.9 ± 0.2	2.1 ± 0.2	1.1 ± 0.2	(Unique)
	107	fA + fC	1.7 ± 0.3	1.7 ± 0.3	1.1 ± 0.3	(Unique)
	108	fA + fC	1.7 ± 0.3	1.7 ± 0.3	1.1 ± 0.3	(Unique)
	109	fA + fB + fC	2.1 ± 0.2	2.3 ± 0.2	1.2 ± 0.2	(Unique)
	110	fA	1.8 ± 0.5	1.7 ± 0.4	1.7 ± 0.7	(Unique)
	111	fA + fC	2.1 ± 0.5	2.2 ± 0.3	1.1 ± 0.3	(Unique)
	152	fA + fB + fC	1.5 ± 0.2	1.7 ± 0.2	0.7 ± 0.2	(Unique)
	155	fA + fB + fC	1.5 ± 0.2	1.7 ± 0.2	0.7 ± 0.2	(Unique)
	156	fA + fB + fC	1.9 ± 0.3	1.9 ± 0.2	1.2 ± 0.2	(Unique)
Nagarabaru Nishi shellmidden	5	fA + fB + fC	1.5 ± 0.2	1.6 ± 0.2	0.8 ± 0.2	(Unique)
	13	fA + fB + fC	1.8 ± 0.2	1.9 ± 0.2	0.9 ± 0.2	(Unique)
Gushibaru shellmidden	17	fC	1.6 ± 0.4	1.8 ± 0.5	0.5 ± 0.7	Ryukyu wild boar
	20	fC	1.6 ± 0.4	1.8 ± 0.5	0.5 ± 0.7	Ryukyu wild boar
Ara shellmidden	115	fA + fB + fC	0.6 ± 0.3	1.2 ± 0.2	1.9 ± 0.3	East Asian pig
	119	fA + fB	0.8 ± 0.3	1.1 ± 0.2	2.2 ± 0.2	East Asian pig
	120	fA + fB + fC	0.6 ± 0.3	1.2 ± 0.2	1.9 ± 0.3	East Asian pig
	123	fC	1.6 ± 0.4	1.8 ± 0.5	0.5 ± 0.7	Ryukyu wild boar
	124	fA	1.6 ± 0.4	1.7 ± 0.4	0.6 ± 0.7	Ryukyu wild boar
	157	fA + fC	1.6 ± 0.2	1.7 ± 0.3	0.6 ± 0.3	Ryukyu wild boar
Wakuta Kiln site	125	fA + fB + fC	0.5 ± 0.2	1.0 ± 0.2	1.8 ± 0.3	(Unique)
	126	fC	0.2 ± 0.4	1.5 ± 0.7	1.3 ± 0.7	East Asian pig
Kiyuna site	127	fA + fB + fC	0.5 ± 0.3	1.1 ± 0.2	1.2 ± 0.3	East Asian pig
	128	fA + fB + fC	0.9 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	(Unique)

[a] fA, fB, and fC show the DNA fragment amplified by the A, B, and C set primers, respectively.
[b] Mean values ± standard deviations are pairwise nucleotide differences (%) between an ancient and all distinct sequences detected in each *Sus scrofa* group.
[c] *Sus scrofa* group with a sequence identical to each ancient sequence.

were not similar to the sequences from any particular group (Table 3). Among 22 sequences obtained from specimens of the early Yayoi-Heian period, four (Nos. 87, 115, 119, and 120) were similar to the sequences detected in East Asian domestic pigs, and four (Nos. 17, 20, 124, and 157) were similar to the sequences detected in Ryukyu wild boars (Table 3). All DNA sequences from Shimizu shellmidden samples were clearly differentiated from the sequences of Ryukyu wild boars by a specific nucleotide insertion at position 138, although these sequences were closer to Ryukyu wild boars than to East Asian domestic pig or Japanese wild boar (Tables 2 and 3). Three sequences (Nos. 125, 126, and 127) from specimens of Recent Times were similar to the sequences detected in East Asian domestic pigs, and the sequence from No. 128 was not similar to the sequences from any particular group (Table 3). When only one or two regions were available for analysis, there was not enough information to estimate accurately the phylogenetic relationship, which was clarified by phylogenetic analysis using the complete sequences of the control region and the *cyt b* gene (Watanobe et al. 1999). Therefore, a phylogenetic analysis was made using 11 haplotypes from 14 ancient 574 bp sequences constructed from the fA, fB, and fC regions together with 55 modern haplotypes.

Phylogenetic Analysis

The neighbor-joining tree was constructed for 11 haplotypes from 6 archaeological sites (4 sites in the early Yayoi-Heian period and 2 sites in the Recent Times) on the Ryukyu Archipelago along with 55 modern haplotypes. In the tree, haplotypes were clustered into two lineages: Asian (haplotypes M1 to M39) and European

(haplotypes M40 to M55) lineages (Fig. 2). The Asian lineage was subdivided into two clusters, designated I and II, and the European lineage was subdivided into two clusters, designated III and IV (Fig. 2). Cluster I comprised haplotypes of Japanese wild boars, Northeast Asian wild boars, and East Asian domestic pigs (haplotypes M1 to M15 and M21 to M39), and cluster II comprised haplotypes of Ryukyu wild boars (haplotypes M16 to M20). These major topologies were completely identical to trees constructed using parsimonious methods (data not shown). Our previous report (Watanobe et al. 1999) using complete sequences of the control region and the *cyt b* gene also supports these tree topologies. While low bootstrap values for clusters I (30.8%) and II (34.2%) were obtained in this study, our previous study showed higher bootstrap values for both clusters: cluster I, 99.7%; and cluster II, 93.2 (Watanobe et al. 1999).

Ancient haplotypes from the archaeological sites on the Okinawa islands belonged to clusters I and II (Fig. 2). Cluster I included haplotype A1 (=M33) from the Kitahara shellmidden and Kiyuna site, A2 (=M31) from the Ara shellmidden, A3 from the Wakuta Kiln site, and A4 from the Kiyuna site. They were closely related or identical to Okinawa native pig (haplotype M33), Japanese domestic pig breeds (haplotypes M30 to M32), and East Asian domestic pigs. These East Asian pigs consist of Meishan (haplotypes M23 and M24), Jinhua (haplotypes M25 and M26), and Yontsuan (haplotype M28) pigs. Haplotypes A5 and A6 from the Nagarabaru Nishi shellmidden and A7 to A11 from the Shimizu shellmidden belonged to cluster II along with the haplotypes of Ryukyu wild boars (haplotypes M16 to M20). Two haplotypes from Nagarabaru Nishi shellmidden were more closely related to the modern Ryukyu wild boar haplotype from the Okinawa main island (haplotype M20). All haplotypes from Shimizu shellmidden formed a monophyletic group designated subcluster IIa with a 74.9% bootstrap value.

The phylogenetic analysis showed that *S. scrofa* excavated from the Kitahara (haplotype A1) and Ara (haplotype A2) shellmiddens (both date to the early Yayoi-Heian) and the Wakuta Kiln (haplotype A3) and Kiyuna (haplotypes A1, A4) sites (both date to Recent Times) are closely related to modern East Asian domestic pigs. Moreover, the result clarified that the *S. scrofa* from the Shimizu shellmidden belonged to the *S. s. riukiuanus* group (cluster II), but they are a population (cluster IIa) genetically independent from the other Ryukyu wild boars.

Discussion

Prehistoric Introduction of Domestic Pigs

On mainland Japan, pig farming was interrupted from 8C to the late 19C because meat consumption, including pork, was forbidden by a Buddhistic prohibition on hunting and the Shinto belief of the uncleanliness of meat (Hayashida 1971). However, domestic pigs were an essential source of animal nutrients for a long time and domestic pigs called "Okinawa native pigs" continued to be raised on the Ryukyu archipelago until after World War II. Previous molecular studies of modern Okinawa native pigs linked their origins to Chinese domestic pigs (Ozawa 2000). When were these pigs introduced into the Ryukyu archipelago? Based on a morphological study of faunal remains, Kaneko (1990) suggested that domestic pigs were introduced into the Ryukyu archipelago between the late 13th and the 18th centuries. Ozawa (2000) referred to historical documents of the Ryukyu dynasty in his molecular study of Okinawa native pigs and suggested that pigs were introduced into the Ryukyu archipelago at the end of the fourteenth century. In a study of the morphological characteristics of *S. scrofa* bones from the early Yayoi-Heian period sites on Ie island, Matsui (1997) found some bones that were much larger than the average for Ryukyu wild boars. This suggests that the domestic pig may have been on the Okinawa islands as long as 1700 to 2000 years ago.

The phylogenetic analysis showed that the mtDNA haplotypes of the early Yayoi-Heian specimens from the Ara shellmidden on Ie island (haplotype A2 from Nos. 115 and 120) and the Kitahara shellmidden on Kume island (haplotype A1 from No. 87) are more closely related to mtDNA haplotypes of East Asian domestic pigs, including Chinese domestic pigs (Fig. 2). Therefore, our result placed the first introduction of domestic pigs from the Asian continent to the Okinawa islands much earlier than the date reported by Kaneko (1990) and Ozawa (2000) and closer to that of Matsui (1997).

Phylogenetic relationships among continental and Japanese *S. scrofa* (cluster I) and Ryukyu wild boar (cluster II) have low bootstrap values in this study (Fig. 2). However based on previous studies, the phylogenetic relationships between cluster I and cluster II are well supported. The low bootstrap values of this study may be attributed to using a large number of haplotypes (Sanderson 1989) and shorter sequences. In this study, 64 haplotypes of the 574 bp partial sequences of the control region were used in the bootstrap analysis, while only 27 haplotypes from the complete sequences of the control region and the *cyt b* gene (2186 bp) were used in our previous study (Watanobe et al. 1999).

Genetic characteristics of Jomon specimens were not clarified by this study because the DNA regions amplified from the Noguni B and Kogachibaru shellmiddens and the Chiarabaru site were too short to include in the phylogenetic analyses (Table 2). However, some nucleotide sequences of one or two of three regions, fA, fB, and fC, from Jomon specimens were more similar to the nucleotide sequences of East Asian domestic pigs than the Ryukyu and Japanese wild boars (Table 3; Nos. 143

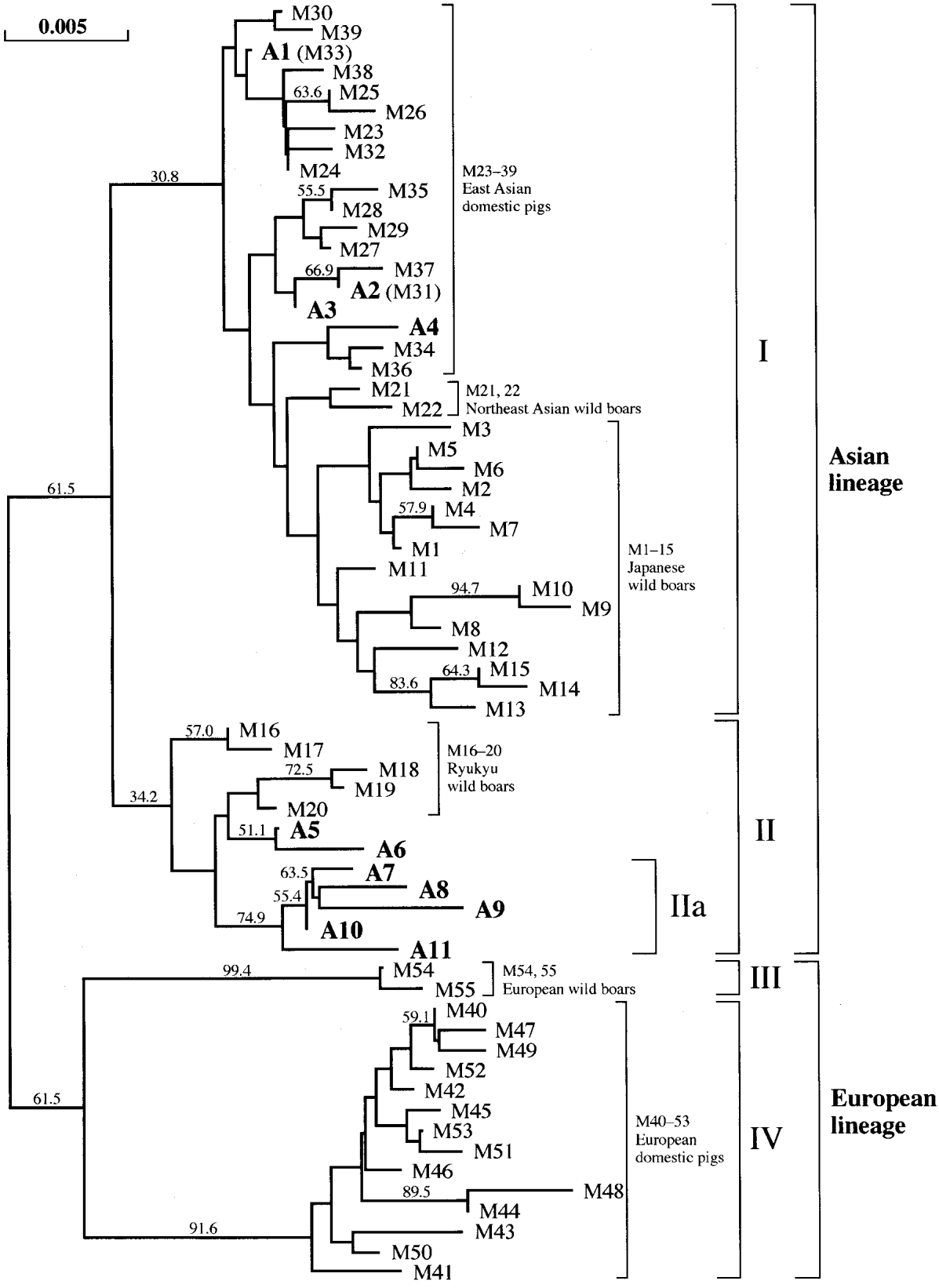

Fig. 2. Neighbor-joining tree constructed from 11 ancient and 55 modern mtDNA control region haplotypes (574 bp). Haplotype numbers are the same as in Table 2. The tree topology has four major distinct clusters (I, II with subcluster IIa, III, and IV). Bootstrap resampling was done 1000 times, and the resulting bootstrap probabilities of the four major clusters and more than 50% at other internal branches are shown.

and 146 from the Kogachibaru shellmidden and No. 151 from the Chiarabaru site). This might place the introduction of domestic pigs as far back as the late Jomon period. *Sus scrofa* bones excavated from the Noguni B shellmidden were 10% smaller than those of modern Ryukyu wild boars (Kawashima and Muraoka 1984), also suggesting that domestic pigs were introduced.

Origin of a Unique S. scrofa *on Prehistoric Kume Island*

We detected a one-nucleotide insertion (A at 138 in Table 2) in all haplotypes (A7 to A11) from Shimizu shellmidden specimens on Kume island. We named this group the Shimizu boar group (cluster IIa). Interestingly, no wild boards inhabit Kume island today, and the following two hypotheses explaining why the Shimizu boar group existed in prehistoric times but are absent today on Kume island are examined. The first hypothesis assumes that this group descended from Ryukyu wild boards that occupied the island more than 10,000 years ago. The Ryukyu wild boar is thought to be a relict from ancient times (Imaizumi 1973), having migrated to the Ryukyu archipelago 1.7–1.2 million years BP or 30,000–10,000 years BP when the archipelago connected to the Asian continent (Kizaki and Oshiro 1980). At that time, the main island of Okinawa and its surrounding satellite islands were probably connected by land bridges. By the end of the Pleistocene, these land bridges submerged and Kume island emerged, isolating this group of Ryukyu wild boars, including the Shimizu boar group. According to this hypothesis, the Shimizu boar would have lived for at least thousands of years and become extinct due to environmental or artificial pressures such as exhaustion of food resources and excessive hunting after the early Yayoi-Heian period. Kume island is too small (59 km^2) to maintain wild boars autogenously for several thousand years, suggesting that this group was probably introduced to Kume island from other areas of the Ryukyu archipelago. However, the unique genetic characteristics of the Shimizu boar group, sharing the nucleotide A insertion and belonging to the independent subcluster IIa, have not been found on other islands of the Ryukyu archipelago (Okumura et al. 1996; Watanobe et al. 1999).

The second hypothesis assumes that the Shimizu boar group was a domestic pig group that was introduced to Kume island from the Asian continent as a source of animal nutrients for prehistoric people. This group may have been obtained as live animals or as meat with bones. In this case, the ancestor of the Shimizu boar group should be found somewhere in the Asian continent. Recently, we sequenced and analyzed the same region of mtDNA from Vietnamese native pigs and Korean wild boars (Hongo et al., unpublished results), and the results indicated that Vietnamese native pigs contained nucleotide variations closely related to the Ryukyu wild boar, including the Shimizu boar group. The insertion of nucleotide A at position 138 in the Shimizu boar group is also found in the Korean wild boar and the domestic Hampshire pig breed (Okumura et al. 2001; Watanobe et al. 2001). These results suggest that the genetic characteristics of the Shimizu boar group may have been derived from ancestors on the Asian continent. Also, many Chinese coins issued in 2C BC and 7C AD have been excavated from Kume island (Kitahara shellmidden, early Yayoi-Heian period) (Okinawa Prefectural Board of Education 1997). Molecular and archaeological evidence suggests that the second hypothesis is more credible. These two hypotheses for the origin of prehistoric *Sus scrofa* on Kume island should be reassessed continuously using the latest information on nucleotide sequences of prehistoric and modern *Sus scrofa* from the Asian continent and the Ryukyu archipelago.

Archaeological Suggestions for Routes of Domestic Pig Introduction

Several possible modes of introduction of continental pig to the Okinawan islands during the early Yayoi-Heian period are considered here. One possibility is that domestic pigs were brought directly from the Asian continent, as many Chinese coins have been excavated from this region. The second possibility is that they were introduced from mainland Japan. In the Okinawan islands, more than 30 early Yayoi-Heian sites have yielded mainland Yayoi pottery shards (Okinawa Prefectural Board of Education 1997). Bracelets and tablets made of shells collected only from the sea around Okinawa and farther south have been found in many Yayoi sites on Kyushu, western Honshu, and as far north as Hokkaido island, indicating that mainland Yayoi and early Yayoi-Heian populations actively engaged in trade.

Items brought to the Okinawan islands are not well enumerated, except for Yayoi pottery. It is possible that the domestic pig was also brought to Okinawa. Nishimoto (1993) analyzed morphological changes of prehistoric *S. scrofa* found on mainland Japan and suggested that domestic pigs were introduced from the Asian continent to Kyushu island during the Yayoi period. These pigs may have been exchanged for valuable shellfish ornaments in the early Yayoi-Heian period. Geographical, archaeological, and genetic data from Ie island support this hypothesis. Ie island is less than 5 km from the Okinawa main island and has wide plains and a natural harbor. Several early Yayoi-Heian sites have been identified on the island. Some of them, such as the Gushibaru shellmidden, may have been port sites for trade between Okinawa main island and Kyushu island. Indeed, a relatively large number of mainland Yayoi pottery shards and shell artifacts used in trade have been excavated from these early Yayoi-Heian period sites (Matsui 1997). While Ie island is very small (23 km^2) for wild boar to survive, a large number of *S. scrofa* remains (from more

than 300 individuals) have been excavated from several early Yayoi-Heian sites (Matsui 1997). Among a large number of prehistoric *S. scrofa* specimens, 40 specimens from Ie island were examined in this study, collected from the Nagarabaru Nishi, Gushibaru, and Ara shellmiddens (Fig. 1). We detected East Asian domestic pig haplotypes (A2, i.e., M31) from two specimens in this study.

In addition to this evidence, the fact that no artifacts identified as being from Taiwan island and the Sakishima islands have been excavated, strongly suggests that domestic pigs were introduced through trade to the Okinawan islands from Kyushu island during the Yayoi period. This hypothesis, together with the direct China–Okinawa hypothesis based on the excavated number of Chinese coins, must be confirmed by studying larger sets of molecular data. mtDNA sequences of prehistoric *S. scrofa* not only from Kyushu, Korea, China, Taiwan, Southeast Asia, and Okinawa islands, but also from the Satsunan islands between Kyushu island and the Okinawa islands should be analyzed, as we have no data from this intermediate region.

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References

Cooper A, Mourer-Chauvire C, Chambers GK, von Haeseler A, Wilson AC, Pääbo S (1992) Independent origins of the New Zealand moas and kiwis. Proc Natl Acad Sci USA 89:8741–8744

Felsenstein J (1995) PHYLIP version 3.572. Executables for PowerMac. University of Washington, Seattle

Hardy C, Callou C, Vigne JD, Casane D, Dennebouy N, Mounolou JC, Monnerot M (1995) Rabbit mitochondrial DNA diversity from prehistoric to modern times. J Mol Evol 40:227–237

Hayashi Y, Nishida T, Hashiguchi T, Shimamora S (1983) Morphological similarities of the mandible between Indonesian native pig and seven types of Asian wild boar. Rep Soc Res Native Livestock 10:140–145 (in Japanese with English abstract)

Hayashida S (1971) The wild boar, domestic pig and Japanese people. Archaeol J 52:11–15 (in Japanese)

Horai S, Kondo R, Murayama K, Hayashi S, Koike H, Nakai N (1991) Phylogenetic affiliation of ancient and contemporary humans inferred from mitochondrial DNA. Philos Trans R Soc Lond Biol 233:409–417

Imaizumi Y (1973) Taxonomic study of the wild boar from the Ryukyu Islands, Japan. Natl Sci Museum Rep 6:113–129 (in Japanese with English summary)

Kaneko H (1990) Natural remains. In: Okinawa Prefectural Board of Education (ed) Report on cultural assets of Okinawa Prefecture, Vol 96. Okinawa Prefectural Board of Education, Naha, pp 123–153 (in Japanese)

Kawashima T, Muraoka M (1984) Animal remains in Noguni shellmiddens, group B site. In: Okinawa Prefectural Board of Education (ed) Report on cultural assets of Okinawa Prefecture, Vol 57. Okinawa Prefectural Board of Education, Naha (in Japanese)

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequence. J Mol Evol 16:111–120

Kizaki K, Oshiro I (1980) The origin of the Ryukyu Islands. In: Kizaki K (ed) Natural history of the Ryukyus. Tsukiji-Shokan, Tokyo, pp 8–37 (in Japanese)

Krings M, Stone A, Schmitz RW, Krainitzki H, Stoneking M, Pääbo S (1997) Neandertal DNA sequences and the origin of modern humans. Cell 90:19–30

Kumar S, Tamura K, Nei M (1993) MEGA: Molecular evolutionary genetics analysis version 1.02. Pennsylvania State University, University Park

Matsui A (1997) Animal remains in Gushibaru shellmidden. In: Okinawa Prefectural Board of Education (ed) Report on cultural assets of Okinawa Prefecture, Vol 130. Okinawa Prefectural Board of Education, Naha, pp 159–187 (in Japanese)

Nishimoto T (1993) The physical character of the pig in the Yayoi Period. Natl Museum Jpn Hist Res Rep 50:49–70 (in Japanese with English summary)

Okinawa Prefectural Board of Education (1997) Summary of archaeological chronology and sites. In: Okinawa Prefectural Board of Education (ed) Cultural properties of Okinawa, Part V: Buried cultural properties. Okinawa Prefectural Board of Education, Naha, pp 18–73 (in Japanese and English)

Okumura N, Ishiguro N, Nakano M, Hirai K, Matsui A, Sahara M (1996) Geographic population structure and sequence divergence in the mitochondrial DNA control region of the Japanese wild boar (*Sus scrofa leucomystax*), with reference to those of domestic pigs. Biochem Genet 34:179–189

Okumura N, Ishiguro N, Nakano M, Matsui A, Shigehara N, Nishimoto T, Sahara M (1999) Variations in mitochondrial DNA of dogs isolated from archaeological sites in Japan and neighbouring islands. Anthropol Sci 107:213–228

Okumura N, Kurosawa Y, Kobayashi E, Watanobe T, Ishiguro N, Yasue H, Mitsuhashi T (2001) Genetic relationship amongst the major non-coding regions of mitochondrial DNAs in wild boars and several breeds of domesticated pigs. Anim Genet 32:139–147

Oota H, Saitou N, Matsushita T, Ueda S (1999) Molecular genetic analysis of a remains of a 2,000-year-old human population in China—and its relevance for the origin of the modern Japanese population. Am J Hum Genet 64:250–258

Ozawa T (2000) Origin of the Ryukyuan native pigs inferred from mitochondrial DNA sequences. Rep Soc Res Native Livestock 18: 209–216

Ozawa T, Hayashi S, Mikhelson VM (1997) Phylogenetic position of mammoth and Steller's sea cow within Tethytheria demonstrated by mitochondrial DNA sequences. J Mol Evol 44:406–413

Pääbo S (1989) Ancient DNA: Extraction, characterization, molecular cloning, and enzymatic amplification. Proc Natl Acad Sci USA 86:1939–1943

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sanderson MJ (1989) Confidence limits on phylogenies: The bootstrap revisited. Cladistics 5:113–129

Stone AC, Stoneking M (1993) Ancient DNA from a pre-Columbian Amerindian population. Am J Phys Anthropol 92:463–471

Takamiya H (1996) Initial colonization, and subsistence adaptation processes in the late prehistory of the island of Okinawa. Indo-Pacific Prehist Assoc Bull 15:143–150

Thomas RH, Schaffner W, Wilson AC, Pääbo S (1989) DNA phylogeny of the extinct marsupial wolf. Nature 340:465–467

Watanobe T, Okumura N, Ishiguro N, Nakano M, Matsui A, Sahara M, Komatsu M (1999) Genetic relationship and distribution of the Japanese wild boar (*Sus scrofa leucomystax*) and Ryukyu wild boar (*Sus scrofa riukiuanus*) analyzed by mitochondrial DNA. Mol Ecol 8:1509–1512

Watanobe T, Ishiguro N, Okumura N, Nakano M, Matsui A, Hongo H, Ushiro H (2001) Ancient mitochondrial DNA reveals the origin of *Sus scrofa* from Rebun Island, Japan. J Mol Evol 52:281–289