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A Genetic Method to Distinguish Crossbred Inobuta from Japanese Wild Boars

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ABSTRACT—To distinguish pig-wild boar crossbred Inobuta from Japanese wild boar populations, a genetic method by using mitochondrial DNA (mtDNA) haplotypes and the nuclear glucosephosphate isomerase-processed pseudogene (*GPIP*) was developed. Sixteen mtDNA haplotypes from 152 wild boars from Kyushu, Shikoku and Honshu islands of Japan were distinct from those from Asian and European domestic pigs. Five alleles of *GPIP* were classified into two groups: 1) alleles *GPIP*1*, *GPIP*3* and *GPIP*3a* from Japanese wild boars, Asian wild boars and domestic pigs; 2) alleles *GPIP*4* and *GPIP*4a* from European wild boars and domestic pigs. An extensive genetic survey was done to distinguish the crossbred Inobuta from 60 wild boars hunted on Tsushima Island, Goto Islands, and Nagasaki and Ooita Prefectures. The mtDNA haplotypes from the 60 samples showed Japanese wild boars, but four wild boar samples from Nagasaki Prefecture had the European *GPIP* allele, *GPIP*4*. These results showed that nuclear DNA polymorphism analysis is useful, in addition to mtDNA haplotype assay, to detect "Inobuta" having the European genotype from Japanese wild boar populations.

Key words: mitochondrial DNA, *GPIP*, Inobuta, Japanese wild boar, PCR-RFLP

INTRODUCTION

The wild boar (*Sus scrofa*) is widely distributed from Eurasia to northeast Africa, and has been classified into at least 16 subspecies (Epstein, 1984; Ruvinsky and Rothschild, 1998). Japan has two subspecies: 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. The Ryukyu wild boar is restricted to the Ryukyu Islands (Amami-Oshima, Kakeroma Islands, Tokuno-Shima, Okinawa Island, Ishigaki Island and Iriomote Island) and their population sizes are very small, while the Japanese wild boar widely inhabits the main islands, except Hokkaido, and its population is larger than the Ryukyu wild boar population. The increase in numbers of Japanese wild boars has been marked in many prefectures in Kyushu and southwest Honshu (Environmental Agency, Japan, 1997–1999). Recently, Japanese wild boars around mountainous villages have caused serious agricultural problems because they damage farms and eat crops. As natural enemies such as the wolf or tiger are extinct in Japan, hunting wild boar could be an efficient means to control wild boar populations. Apart from the increasing numbers of wild boars, breeding wild boars or crossbreeding domestic pigs with wild boars to produce Inobuta have been done as an industry in many prefectures for three decades in Japan. Wild boars or the crossbred Inobuta have escaped from breeding farms accidentally or intentionally (Kodera and Kanzaki, 2001). Although the frequency of escapes from breeding farms has not been estimated exactly, these escapes might influence genetic structures and population sizes of feral wild boars. Artificial migration to, or release of wild boars in, the small islands or mountain areas have been reported (Kodera and Kanzaki, 2001).

Recently we examined the genetic relationship between Japanese wild boar and Ryukyu wild boar populations and Asian and European domestic pigs by comparing their mitochondrial DNA (mtDNA) control region and cytochrome *b* gene sequences (Okumura *et al.,* 1996; 2001; Watanobe *et al.,* 1999). Molecular genetic analyses using mtDNA hapotypes showed Japanese wild boars are different from European and Asian wild boars and domestic pigs, and they have a population structure including many characteristic local populations (Okumura *et al.,*1996; Watanobe *et al.,* 1999). The phylogeographic status of Japanese wild boars

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may provide useful information to trace artificial migrations or the release of feral or breeding wild boars by humans. To distinguish released animals, including the crossbred Inobuta, from wild boar populations, a genetic classification method should be developed.

The mtDNA is a suitable genetic marker to study the genetic variation within a species because of a maternal inheritance without recombination, but it is inadequate to evaluate the paternal genetic influence. In this study, we developed a genetic classification method by using nuclear gene loci in addition to mtDNA to examine wild boar samples from Tsushima Island, Goto Islands and some districts in Nagasaki and Ooita Prefectures, where wild boars have increased suddenly. We here report that nuclear DNA markers combined with mtDNA haplotypes are very useful to distinguish wild boar crossbred with domestic pigs from feral populations.

MATERIALS AND METHODS

Animal samples

To examine the haplotypes of 574bp mtDNA control region, a total of 152 Japanese wild boar samples were collected from 15 prefectures (Table 1). Among them, thirty Japanese wild boars from Shizuoka (10 samples), Shimane (6 samples), Nara (5 samples) and Fukui (9 samples) Prefectures (indicated by italic in Table 1) were newly examined in this study, while the remaining 122 boar samples were from the previous mtDNA database of Watanobe *et al.* (2001).

To examine the polymorphism of glucosephosphate isomerase-processed pseudogene (*GPIP*) (Harbitz *et al.*, 1993), muscle and blood samples were collected from breeds of domestic pigs and wild boars: European lineage (3 samples from Landrace, 2 samples from Large White, 13 samples from Berkshire, 5 samples from Hampshire, 7 samples from Duroc); Asian lineage (2 samples from Meishan, 1 samples from Ryukyu wild boar and 1 sample from Chinese wild boar); and miniature pig (2 samples from Ohmini).

Table 1. Nucleotide variations and distribution of mtDNA haplotypes of Japanese wild boars

		Nucleotide position[c]																																										
		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
		5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	
	Pigs and	5	5	5	5	5	5	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	9	9	9	9	0	0	0	0	0	1	1	1	1	1	
	wild	6	7	7	8	8	9	1	2	4	7	8	9	0	1	1	2	2	3	3	3	4	5	2	2	2	4	4	7	8	9	1	3	7	9	1	4	7	9	9	0	1	2	2	3	
Haplotypes[a]	boars[b]	5	1	8	0	8	3	6	0	9	6	3	5	2	1	4	3	9	5	6	7	1	8	2	3	5	0	1	8	7	6	0	6	7	5	0	5	2	2	7	3	8	4	7	7	Sampling localities[d]
J1(8)	JWB(21)	A	-	-	T	T	G	C	T	T	T	G	C	C	T	C	A	G	C	C	A	T	T	A	A	T	T	T	A	T	G	C	G	A	T	G	T	G	T	C	A	A	T	A	A	Miyazaki,Shiga,Gifu,Mie,*Fukui*(9)
J2(None)	JWB(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	*Nara*(1)
J3(9)	JWB(31)	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	Miyazaki,Shiga,Gifu,Gunma
J4(10)	JWB(1)	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	Shiga
J5(12)	JWB(10)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	C	.	.	.	.	.	.	Kumamoto,Yamaguchi,*Shimane*(3)
J6(13)	JWB(2)	.	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	.	Gifu
J7(14)	JWB(9)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	C	T	.	.	.	.	.	Miyazaki,*Shizuoka*(3)
J8(15)	JWB(17)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	T	.	.	.	.	.	*Shizuoka*(5),Gunma
J9(11)	JWB(2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	G	.	Mie
J10(1)	JWB(43)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	Saga,Kumamoto,Ooita,Miyazaki,*Nara*(4),Hyougo *Shimane*(3),Shiga,Gifu,Tokushima,*Shizuoka*(1)
J11(3)	JWB(4)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	A	C	.	.	.	.	.	G	Miyazaki
J12(4)	JWB(5)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	Miyazaki,Kumamoto,*Shizuoka*(1),Gunma
J13(7)	JWB(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	Tokushima
J14(5)	JWB(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	G	Miyazaki
J15(6)	JWB(3)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	G	Miyazaki
J16(2)	JWB(1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	G	Yamaguchi
J17(New)	JWB	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	Ooita
J18(New)	JWB	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	Ooita
M23(23)	Meishan	.	.	.	.	.	.	.	.	.	C	A	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.	C	.	.	.	.	G	G	
M49(49)	Hampshire	G	C	A	C	C	A	T	.	.	.	.	.	.	.	.	.	A	.	.	.	C	C	.	.	C	.	.	.	C	.	.	A	.	.	A	.	.	C	.	.	.	.	G	.	

a) Numbers in parentheses are haplotype code numbers defined previously in Watanobe *et al.*(2001). Haplotype J2(None) was not included in Watanobe *et al.*,2001. Two representative haplotypes M23 and M49 are shown as reference for Asian domestic pig (Meishan) and European domestic pig (Hampshire), respectively.
b) Numbers in parentheses indicate the sample numbers of Japanese wild boar (JWB) belonging to each haplotype.
c) Nucleotide positions are those of the complete pig mitochondrial DNA reference (Ursing and Arnason, 1998).
d) Italic names indicate the sampling sites obtained Japanese wild boars in this study, while the others are from the previous paper (Watanobe *et al.*, 2001). Numbers in parentheses indicate the sample numbers examined in this study

Muscle samples from 20 Japanese wild boars captured in Gifu, Mie, Shiga, Fukui, Shizuoka and Miyagi Prefectures were also examined the *GPIP* polymorphism.

To examine the genetic characteristics of Japanese wild boars in Kyushu, especially to distinguish crossbred boars, muscle samples were collected from Tsushima Island (20 samples from three districts from 1999 to 2000), Goto Islands (17 samples from three districts from 1999 to 2001), Nagasaki Prefecture (6 samples from two districts in 2000) and Ooita Prefecture (17 samples from eight districts in 2001)(Fig.1), and examined for both the mtDNA haplotype and the *GPIP* polymorphism.

DNA extraction, PCR and direct sequencing

Total DNA was extracted from muscle and blood samples using standard proteinase-K phenol-chloroform methods as described by Watanobe *et al.* (1999). Approximately 0.1~0.2 μg of the total DNA was used to amplify the *GPIP* (507bp) and the mtDNA control region (574bp) by polymerase chain reaction (PCR) (Watanobe *et al.,* 2001).

To amplify the most variable fragment in the mtDNA control region, primers mitL112 (5'-GCGCACAAACATACAAATATGCTG-3') in the L strand and mit H106 (5'-ACGTGTACGCACGTGTACGC-3') in the H strand were used (Watanobe *et al.*, 2001). Primers, mitL11(5'-CCATGCCGCGTGAAACCA-3') and mitH12(5'-ATCGAG-ATGTCTTATTTAAG-3') were used to sequence nucleotide sequence both L and H strands of the mtDNA control region.

To amplify 507bp of the *GPIP,* primers GPIP1(5'-TGCAGT-TGAGAAGGACTTTACTT-3') and GPIP6(5'-GAAGTTACAGGGC-ATCATCTTG-3') were used (Giuffra *et al.,* 2000). The mtDNA and *GPIP* fragments were amplified by using PCR under the following

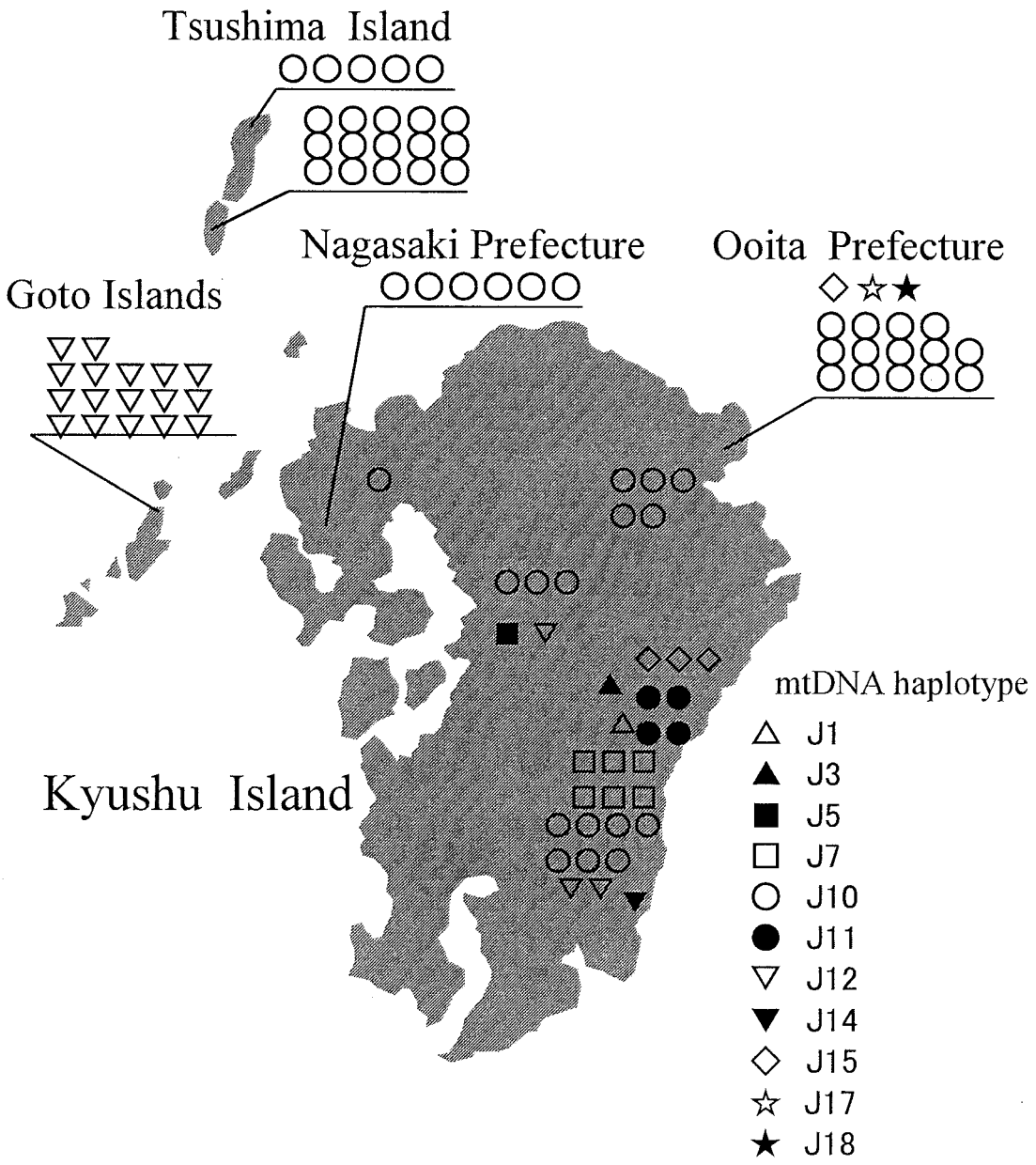

Fig. 1. Distribution of mtDNA haplotypes of Japanese wild boars in Kyushu. The mtDNA haplotypes and their numbers detected are indicated by different symbols. The symbols on the underlines are mtDNA haplotypes detected from the genetic survey in northern Kyushu. The symbols plotted on the map include the mtDNA haplotypes reported previously (Watanobe *et al.*, 2001).

conditions: one cycle of DNA denaturation and activation of AmpliTaq Gold (Perkin Elmer, Norwalk, CT) at 95°C for 9 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, 45 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min (Watanobe *et al.,* 2001). The PCR products were purified by using Centricon 100 micro-concentrators (Amicon, Beverly, MA) and were directly sequenced by using the dideoxy chain termination method with 373S and 310 DNA Sequencers and a Taq Dyedeoxy and BigDye Terminator Cycle sequencing Kits (Applied Biosystems, Foster City, CA). The resulting nucleotide sequences were analyzed by using GENTYX-MAC software (Software Development Co., Tokyo, Japan) for multiple sequence alignment.

DNA cloning

The PCR products of *GPIP* that were amplified by using primers GPIP1 and GPIP6 were phosphorylated with T4 polynucleotide kinase (Takara Shuzo, Kyoto, Japan) and were cloned into the *Sma*I site of the pBluescript SK$^+$ plasmid vector (Stratagene, La Jolla, CA)(Onodera *et al*.,1998). Then the inserted fragments in the plasmid vectors were sequenced by using the dideoxy chain termination method.

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

To confirm the heterozygous DNA sequences, especially at positions 316 and 233 in two European alleles, PCR products using primers GPIP1 and GPIP6 were digested with restriction enzymes *Hha*I and *Bgl*I to identify the genotypes at nucleotide positions 316 and 233, respectively. Then after the bands digested with restriction enzymes were electrophoresed in 2% agarose gel, they were visualized by using ethidium bromide staining and were photographed.

RESULTS

Geographical distribution of mtDNA haplotypes of Japanese wild boars

From a total of 152 individual samples including the 122 Japanese wild boars examined by Watanobe *et al.* (2001), 16 haplotypes of 574bp mtDNA control region were obtained, and they were divided into two groups. Table 1 shows the nucleotide variation of the 16 haplotypes and their distributions. One group (J1 to J9) was widely distributed in Kyushu, Shikoku and Honshu islands, while the other group (J10 to J16) was distributed in southwestern prefectures of Honshu and Kyushu. Nine different haplotypes in Kyushu were plotted on a map (Fig. 1). Haplotype J10 was commonly found in Saga, Ooita, Kumamoto and Miyazaki Prefectures. Eight different haplotypes were detected in Miyazaki Prefecture, suggesting that wild boars with different haplotypes were artificially introduced, or that wild boars from different localities were interbred on breeding farms and the boars escaped from those farms. However, mtDNA haplotypes from Asian and European domestic pigs represented by Meishan or Hampshire were not detected among a total of 152 samples.

Nuclear DNA polymorphism between Japanese wild boars and domestic pigs

To distinguish the genetic lineages between European and Asian pigs, the *GPIP* polymorphisms were examined using total DNAs from 20 Japanese wild boars, a Ryukyu wild boar, a Chinese wild boar, two Meishan pigs, 30 European domestic pigs and two Ohmini miniature pigs (Table 2). From nucleotide substitutions at eight variable sites in the 507bp fragment of *GPIP*, two new alleles, *GPIP*3a* and *GPIP*4a*, and three alleles, *GPIP*1, GPIP*3* and *GPIP*4* that were reported previously (Giuffra *et al*., 2000), were identified in this study (Table 2). *GPIP*1, GPIP*3* and *GPIP*3a* were all from Japanese wild boar, Ryukyu wild boar, Chinese wild boar, Meishan and some European domestic pigs, indicating that these three alleles characterized Asian boars and pigs. Meanwhile, *GPIP*4* and *GPIP*4a* were detected from only European domestic pigs and the Ohmini miniature pig, indicating that they are unique alleles from European boars and pigs (Table 2). Table 3 shows nine *GPIP* genotypes formed from five *GPIP* alleles, and were used as criteria in this study. Heterozygotes at *GPIP* loci between Japanese wild boars and European domestic pigs were confirmed by cloning the PCR products into the vector plasmid and by sequencing their DNA nucleotides (data not shown).

To further check heterozygous polymorphisms at nucleotide positions 233 and 316 in *GPIP*, PCR-RFLP analysis was done (Fig. 2). *Hha*I digested the 507bp PCR product into two fragments (327bp and 180bp) at the nucleotide G

Table 2. Allele frequencies at the *GPIP* locus in domestic pigs and wild boar populations

Type	Allele	Sequence at nucleotide position[1)]								Allele frequencies in domestic pigs and wild boar populations[2)]									
		181	188	225	233	316	388	389	415	JWB (20)	RWB (1)	CWB (1)	Men (2)	Dur (7)	Lan (3)	Ber (13)	Ham (5)	Lw (2)	Ohm (2)
Asian type	*GPIP*1*	G	G	G	C	A	G	A	C	0.82	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25
	*GPIP*3*	A	.	.	.	.	.	T	T	0.03	0.00	1.00	1.00	0.43	0.33	0.19	0.00	0.50	0.50
	*GPIP*3a*	A	A	.	.	.	.	T	T	0.15	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
European type	*GPIP*4*	A	.	A	.	G	C	T	T	0.00	0.00	0.00	0.00	0.57	0.50	0.73	1.00	0.50	0.25
	*GPIP*4a*	A	.	A	T	G	C	T	T	0.00	0.00	0.00	0.00	0.00	0.17	0.08	0.00	0.00	0.00

[1)] Dots mean sequence identities.

[2)] Numbers in parentheses indicate tested numbers of pigs and wild boars. JWB, Japanese wild boar; RWB, Ryukyu wild boar; CWB, Chinese wild boar; Men, Meishan; Dur, Duroc; Lan, Landrace; Ber, Berkshire; Ham, Hampshire; Lw, Large white; Ohm, Ohmini miniature pig

Table 3. Genotype polymorphism of the *GPIP* locus in domestic pigs and wild boars

Genotype	Sequence at nucleotide position								Domestic pigs and wild boars[1)]
	181	188	225	233	316	388	389	415	(No. of samples)
*GPIP*1/GPIP*1*	G	G	G	C	A	G	A	C	JWB(16),
*GPIP*3/GPIP*3*	A	.	.	.	.	.	T	T	CWB(1), Men(2), Dur(1), Ohm(1)
*GPIP*3a/GPIP*3a*	A	A	.	.	.	.	T	T	JWB(3), RWB(1)
*GPIP*1/GPIP*3*	G/A	.	.	.	.	.	A/T	C/T	JWB(1)
*GPIP*4/GPIP*4*	A	.	A	.	G	C	T	T	Dur(2), Ham(5),Ber(6), Lan(1)
*GPIP*3/GPIP*4*	A	.	G/A	.	A/G	G/C	T	T	Dur(4), Ber(5), Lw(2), Lan(1)
*GPIP*4/GPIP*4a*	A	.	A	C/T	G	C	T	T	Ber(2)
*GPIP*3/GPIP*4a*	A	.	G/A	C/T	A/G	G/C	T	T	Lan(1)
*GPIP*1/GPIP*4*	G/A	.	G/A	.	A/G	G/C	A/T	C/T	Ohm(1)

[1)] Same designations as in Table 2.

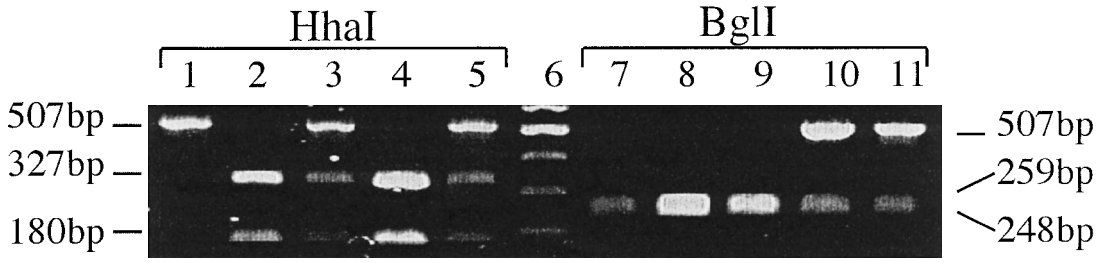

Fig. 2. PCR-RFLP analysis used to distinguish the *GPIP* allele variants. *Hha*I and *Bgl*I RFLPs of PCR products reflect sequence polymorphism at nucleotide positions 316 and 233, respectively. The 507bp PCR product is cleaved to 327bp and 180bp by the *Hha*I (left), and to 259bp and 248bp by the enzyme *Bgl*I (right). Lane 6; 100-bp DNA lader marker. The *GPIP* genotypes of each sample were: lanes 1 and 7, *GPIP*3a/GPIP*3a*; lanes 2 and 8, *GPIP*4/GPIP*4*; lanes 3 and 9, *GPIP*3/GPIP*4*: lanes 4 and 10, *GPIP*4/GPIP*4a*; lanes 5 and 11, *GPIP*3/GPIP4a*.

of the 316 position (Fig. 2, lanes 2, 3, 4 and 5), but not digested at nucleotide A (Fig. 2, lanes 1, 3 and 5). The RFLP with the *Hha*I enzyme could distinguish three alleles *GPIP*1, GPIP*3* and *GPIP*3a* from two alleles *GPIP*4* and *GPIP*4a* at the 316 position (Table 2). *Bgl*I digested the 507bp PCR product into two fragments (259bp and 248bp) at nucleotide C of 233 position in the allele *GPIP*4* (Fig. 2, lanes 7, 8 and 9). However, when nucleotide C was substituted by T in the allele *GPIP*4a*, a non-digested 507bp fragment was observed (Fig. 2, lanes 10 and 11), indicating that RFLP with the *Bgl*I enzyme is the useful method to distinguish the alleles *GPIP*4* from *GPIP*4a* at the 233 position. Nucleotide substitutions at positions 225, 233, 316 and 388 in genotyping of *GPIP* appeared to be critical to distinguish pig-wild boar crossbreds from wild boars (Table 3).

Genetic survey of Japanese wild boars from northern Kyushu

To investigate crossbreeding between pigs and wild boars in Tsushima Island, the Goto Islands, Nagasaki and Ooita Prefectures, mtDNA haplotypes and *GPIP* genotypes of 60 samples were identified (Fig.1, Table 4). The mtDNA haplotypes were all unique for wild boars from Tsushima (haplotype J10) and Goto (J12) Islands. As haplotype J10 was also detected in wild boars from Nagasaki and Ooita Prefectures, it is a common haplotype widely distributed in Kyushu (Fig.1). The mtDNA haplotypes J10 and J15 and new haplotypes J17 and J18 were detected from wild boars from Ooita Prefecture (Tables 1, 4). However, mtDNA haplotypes showing the genetic lineages of Asian and European domestic pigs were not detected in the 60 samples of this study.

Table 4 shows the *GPIP* genotypes of 50 samples examined. Almost all genotypes were *GPIP*1/GPIP*1, GPIP*3/GPIP*3, GPIP*3a/GPIP*3a* and *GPIP*1/GPIP*3* of Japanese wild boars in those samples. European genotypes *GPIP*4/GPIP*4* and *GPIP*3/GPIP*4* were in the four wild boar samples from Nagasaki Prefecture (Table 4). Genotypes *GPIP*4/GPIP*4* and *GPIP*3/GPIP*4* from Nagasaki Prefecture had *Hha*I-digested fragments with homogzygous and heterozygous patterns, respectively (Fig. 2, lanes 2 and 3). The 507bp PCR products from Nagasaki Prefecture boar samples were digested into two fragments by using the *Bgl*I enzyme (Fig. 2, lanes 8 and 9). As allele *GPIP*4* was derived from European domestic pigs and wild boars (Table 2, Giuffra *et al*., 2000), the four wild boars of Nagasaki Prefecture could be Inobuta, a pig crossbred with a wild boar or its descendants. The nucleotide sequences for the new haplotypes J17 and J18 have been submitted to the nucleotide databases of GenBank, EMBL, and DDBJ with the following accession numbers: AB071706 (J17) and AB071707 (J18).

Table 4. Genetic profiles of Japanese wild boars detected from Tsushima Island, Goto Islands, Nagasaki and Ooita Prefectures

Location	No. of	Genetic profiles			
(No. of sampling localities)	samples	mtDNA haplotype	No. of samples	*GPIP* genotype	No. of samples[1)]
Tsushima Island(3)	20	J10	20	*GPIP*1/GPIP*1*	10
				*GPIP*1/GPIP*3*	2
				NT[2)]	8
Goto Islands(3)	17	J12	17	*GPIP*1/GPIP*1*	7
				*GPIP*3/GPIP*3*	1
				*GPIP*3a/GPIP*3*	7
				NT[2)]	2
Nagasaki Prefecture(2)	6	J10	6	*GPIP*3/GPIP*3*	2
				*GPIP*4/GPIP*4*	1
				*GPIP*3/GPIP*4*	3
Ooita Prefecture(8)	17	J10	14	*GPIP*1/GPIP*1*	4
				*GPIP*1/GPIP*3*	4
				*GPIP*3/GPIP*3*	3
				*GPIP*3a/GPIP*3a*	3
		J15	1	*GPIP*3/GPIP*3*	1
		J17(New)	1	*GPIP*1/GPIP*1*	1
		J18(New)	1	*GPIP*3a/GPIP*3a*	1

[1)] Underlines show wild boars with the European allele *GPIP*4*.
[2)] NT, not tested.

DISCUSSION

This study showed that, by using both genetic markers, mtDNA of maternal inheritance and *GPIP* genotypes of biparental inheritance, domestic pigs have crossbred with wild boars in Kyushu. All mtDNA haplotypes identified from 60 wild boar samples were of Japanese wild boar types, whereas the *GPIP* alleles *GPIP*4* and *GPIP*4a* (Table 2, Giuffra *et al*., 2000) specific for European domestic pigs were in four wild boars from Nagasaki Prefecture. In contrast, allele *GPIP*3* obtained from Asian wild boars and domestic pigs (Giuffra *et al*., 2000) was frequently in many European domestic pig breeds (Table 2). This result supports the view that Asian pigs were used to improve European pig breeds during the 18th and early 19th centuries (Jones, 1988). Duroc and Ohmini breeds having *GPIP*3/GPIP*3* are thought to be established through multiple crossbreeding. To distinguish the crossbred Inobuta influenced genetically by European domestic pigs, from Japanese wild boar populations, nuclear *GPIP* genotyping is useful together with using mtDNA haplotypes. The PCR-RFLP method for *GPIP* genotyping developed by us is a simple tool without laborious DNA sequencing and facilitates practical use in the field. In this study, although *GPIP* was used as a nuclear gene to distinguish the genetic difference between Asian and European lineages of pig, any nuclear genes may be found to more clearly distinguish the genetic lineages in the near future.

Populations of Japanese wild boars have increased markedly in southwestern Honshu and Kyushu, like the increasing numbers of sika deer (*Cervus nippon*) in Hokkaido (Environmental Agency, Japan 1997–1999). Increasing numbers of wild boar are marked on small islands such as Tsushima and Goto Islands. Historical records suggest that native wild boars of Tsushima Island inhabiting this island became extinct artificially because of the local government policy Inoshika-Oitsume from 1700 to 1709 (Watase 1912) to reduce damage of agricultural crops by wild boars. Although this policy succeeded in eliminating wild boars from Tsushima Island for about 300 years, wild boars were suddenly discovered at several localities on the island in 1993 and they have increased every year. The total numbers of wild boars captured by hunting were 13 individuals in 1997, 39 in 1998 and 73 in 1999. Two questions have arisen: from where wild boars originated, and how the wild boar populations increased their numbers in limited areas. Two possible explanations are: (1) escape from breeding farms by wild boars or by wild boars crossbred with domestic pigs managed on the island; (2) unsystematic artificial introduction of wild boars to the island for leisure hunting. From our DNA analysis of wild boars, mtDNA haplotype J10 was found widely in Kyushu and Tsushima (Fig. 1), and *GPIP* genotypes of Tsushima wild boars were of typical Japanese wild boars (Table 3). Although whether wild boars on Tsushima Island originated from escapes from breeding farms or from artificial introduction is still unclear, the results of this study show that they originated as introductions from any Kyushu population of typical Japanese wild boars with-

out a crossbreeding history, and that the population has founder effects.

On the Goto Islands, wild boars have also increased markedly in the last decade, although no detailed historical records exist. Only mtDNA haplotype J12 was from the Goto Islands. Also, the Goto population had typical Japanese wild boar *GPIP* alleles. To evaluate the introduction and migration of wild boars in this small islands, a continuous genetic survey of wild boars is needed for a long period.

To understand the situations of farming wild boars and of crossbreeding between wild boars and pigs in Japan, Kodera and Kanzaki (2001) made an extensive survey using both written and verbal questionaires sent to 47 prefectural government offices. They reported that breeding farms of wild boars and crossbreeds existed in 30 (63.8%) and 22 (46.8%) prefectures in Japan, respectively, and that their farms were widely located from Hokkaido to Okinawa. A wild boar market opens only in winter in Miyazaki Prefecture for breeding sources of boars. This stockbreeding of Japanese wild boars could stimulate crossbreeding between wild boars or pigs and wild boars at the industry level. To control populations and to understand the population dynamics for conservation and management, genetic methods are needed to distinguish wild boars from the crossbred Inobuta. Two mtDNA haplotypes characteristic of East Asian and European domestic pigs are in wild boar populations from Miyazaki Prefecture, and introgression from domestic pigs to Japanese wild boars has been frequent (Okumura *et al.* 2001). In this study, the mtDNA and nuclear *GPIP* from hunted wild boars were examined for polymorphism of nucleotide sequences to find out if they are useful genetic markers. The mtDNA haplotypes from 60 samples were all specific to Japanese wild boars, but four samples from Nagasaki Prefecture showed that *GPIP*4* is derived from European boars and pigs (Table 4). These four wild boars from Nagasaki are considered to be descendants of crossbreds with domestic European pigs. The crossbred Inobuta can be generally produced by crossing a male wild boar with a female domestic pig. If the crossbred Inobuta is continuously produced by this crossbreeding system, their genetic characters would be of domestic pig for both mtDNA haplotypes and *GPIP* genotypes. However, the genotypes of wild boars captured by hunting were more complicated, as indicated by the genotypes of wild boars from Nagasaki Prefecture. This evidence suggests more complicated crossbreeding systems between wild boars and domestic pigs in breeding farms or multiple crossbreeding between escaped wild boars and native wild boars are in different districts. In addition to mtDNA haplotyping, *GPIP* genotyping is suitable to detect crossbreeding lineages in wild boar populations.

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REFERENCES

Environmental Agency, Japan (1997–1999) Choujyuu-Kankei-Toukei (in Japanese)

Epstein H (1984) Pig. In “Evolution of Domesticated Animals” Ed by Mason I L, Longman, London, pp145–162

Giuffra E, Kijas M H, Amarger V, Carlborg O, Jeon J-T, Andersson L (2000) The origin of the domestic pigs: Independent domestication and subsequent introgression. Genetics 154: 1785–1791

Harbitz I, Chowdhary B P, Kran S, Davies W (1993) Characterization of a porcine glucosephosphate isomerase-processed pseudogene at chromosome 1q1.6-1.7. Mammal Genom 4: 589–592

Jones G F (1998) Genetic aspects of domestication, common breeds and their origin. In “The Genetics of the pigs” Eds by Ruvinsky A & Rothschild MF, CAB International, Oxon UK, pp 17–50

Kodera Y, Kanzaki N (2001) Present status of wild boar and crossbred pig x wild boar farming and their feral populations in Japan. Widlife conserv Japan 6: 67–78 (in Japanese with English abstract)

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 Genetic 34: 179–189

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. Animal Genet 32: 139–147

Onodera H, Ishiuro N, Horiuchi M, Shinagawa M (1998) Characterization of differentially expressed genes in the bovine T lymphoma cell line. Vet Immunol Immunopathol 62: 209–219

Ruvinsky A, Rothschild M F (1998) Systematics and evolution of the pig. In “The Genetics of the pig” Eds by Ruvinsky A & Rothschild, CAB International, Oxon UK, pp1–16

Ursing B M, Arnason U (1998) The Complete mitochondrial DNA sequence of the pig (*Sus scrofa*). J Mol Evol 47: 302–306

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

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*) analysed by mitochondrial DNA. Mol Ecol 8: 1509–1512

Watase S (1912) Extermination of wild boars in Tsushima Island at the Genroku-Kanei Period. Zool Mag 24: 15–26 (in Japanese)

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