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Maternal Origins of the Jeju Native Pig Inferred from PCR-RFLP Haplotypes and Molecular Phylogeny for Mitochondrial DNA *CYTB* Gene Sequences

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In an effort to gain greater understanding of the maternal lineages of the Jeju native pig (JNP), we analyzed the mitochondrial DNA (mtDNA) *CYTB* gene and compared it with those of other pig breeds. PCR-RFLP analysis was conducted with six pig breeds including JNP, and then the RFLP patterns allowed for the separation of the pig breeds into two distinct haplotypes (*mtCYTB1* and *mtCYTB2*). The JNP *CYTB* sequences were detected in both the European and Asian breed clusters on the phylogenetic tree. The J2 group was sorted with the indigenous cluster of Asian pig lineages and was related closely to Chinese native pig breeds, but a second group, J1, was sorted with the European pig lineages and appeared to be related to Spanish Iberian native pigs, rather than to Asian breeds. These results indicate that the JNP currently raised on Jeju Island have two major maternal origins estimated in Asian and European pigs. We concluded that the JNP that share a common lineage with indigenous Asian pigs were domesticated in the distant past, originating from pigs that were already being raised elsewhere at that time, and that the European pig breeds introduced in the twentieth century have also contributed to the formation of this pig population.

Key words : Maternal lineage, origin, mtDNA, haplotype, Jeju native pig

Introduction

Currently, extant pig species are grouped into wild boars, local native pigs, and domestic pigs, thereby suggesting that domestic pigs and local native pigs originated from independent domestication processes in Europe and Asia from ancestral wild boar stock. The domestication of pigs initially occurred in the Near East from local populations of wild boars approximately 5,000 to 9,000 years before the present (YBP). Because pigs migrated from region to region by land or sea with pre-historic or historic human populations after this initial domestication, the development of pig breeds was influenced by the introgression of genetic materials between European and Asian lineages [7,9,17,25].

As mtDNA is maternally inherited, and the rate of mtDNA evolution is more rapid than that of single-copy nuclear DNA, mtDNA polymorphisms have been analyzed to elucidate the phylogenetic relationships among species and to determine the breeding processes involved in domestication [2,6,26]. Giuffra et al. [7] explained the processes of domestication of wild boars and the introgression of domestic pigs using both mtDNA polymorphisms and nuclear gene sequences. In Asia, Yang et al. [28] evaluated the evolutionary relationship of Chinese pig breeds using nearly-complete mtDNA sequences, and proposed the existence of two clusters indigenous to Asia. Genetic studies of Korean pigs using microsatellite (MS) markers and amplified fragment length polymorphism (AFLP) analysis have shown that native pigs domesticated in the Korean Peninsula evidenced lower genetic diversity and were distinguishable from foreign pig breeds, and have also drawn a picture of a closer relationship between native Korean pigs and Chinese pigs than between the native Korean pigs and other pig breeds [13-15]. Kim et al. [12] utilized mtDNA D-loop sequences to verify the distinct genetic origins of the indigenous Asian pig group, and suggested that they originated from closely-related maternal ancestors. Recently our previous report described that the Korean native pigs had a shared mtDNA D-loop haplotype just with Chinese native pigs and wild boars but did not with other East Asian wild boars including

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Korean wild boars, and suggested that they would be introduced from China after domestication from Chinese wild boars [3].

Pig domestication is estimated to have at least a 2,000-year history on the Korean Peninsula and on Jeju Island. Moreover, several pig breeds (including Duroc, Landrace, Large White, and Berkshire pigs) have been either directly or indirectly introduced into Korea from other countries in the middle to late 20th century [12]. Historical records and legends generally maintain that domestic pigs and wild boars existed in abundance and that pigs have been domesticated there for a very long time. However, the domestication process and the origin of present JNP remain to be clearly elucidated, and the phylogenetic relationship of JNP with other pig populations has yet to be definitively established. In an effort to shed light on these remaining questions, in this study we have assessed the sequences and haplotypes of the mtDNA *CYTB* gene.

Materials and Methods

Total DNA extraction and PCR amplification

Blood samples were obtained from 65 JNP from the Subtropical Animal Experiment Station (SAES), National Institute of Animal Science, Rural Development Administration and Institute for Livestock Promotion (ILP), Jeju-do in South Korea. 62 Berkshire and 10 Hampshire pigs were generously provided from pig farms in Jeju-do and Gyeongsangnam-do. 47 Duroc, 40 Landrace and 76 Yorkshire pigs were collected from SAES, ILP, and farms. Genomic DNA samples were isolated from blood lymphocytes and sera via the sucrose-proteinase K method with slight modification. After isolation, the DNAs were purified by subsequent RNase treatment and ethanol precipitation. The primers utilized for the amplification of the mtDNA *CYTB* fragment were designed from the reported complete mtDNA genome sequences for the Landrace pig (GenBank acc. no. NC_000845). The primer sequences were as follows: CytbF, 5′-TCG TTG TCA TTC AAC TAC AAG AAC-3′ and CytbR, 5′-CCT TCT CTG GTT TAC AAG ACC A-3′. PCR was conducted in 25-μl volumes, each containing 1× reaction buffer, 200 μM of dNTPs, 2.0 units of *Taq* DNA polymerase (Promega, Madison, WI, USA), 10 mM of each primer, and approximately 100 ng of total DNA. Amplification was conducted under the following conditions: an initial 5-min denaturation at 94℃, followed by 30 cycles of 30 sec at 94℃, 60 sec at 53℃, and 100 sec at 72℃. The PCR products were separated on 1% agarose gels containing EtBr, and purified with a QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA, USA).

Cloning and sequencing

Purified DNA was inserted into a plasmid vector using a TOPO^{TM} TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Nucleotide sequencing was conducted using a DYEnamic ET-Dye Terminator Kit (GE Healthcare) and separated on a MegaBace1000 automated DNA sequencer (GE Healthcare). The nucleotide sequences acquired in this study have been submitted to the GenBank database (GenBank accession nos. AY830173- AY830188).

Phylogenetic analysis

In order to extrapolate the phylogenetic relationship of JNPs from the *CYTB* gene sequences, we obtained 88 complete *CYTB* sequences from the GenBank database using a BLAST search of our sequences. However, we did not apply the sequences from the wild boar for the phylogenetic analysis, in order to prevent misunderstandings regarding the genetic source of JNP. We conducted multiple alignments of the sequences using CLUSTAL W software [24]. The identical sequence groups found from the similarity search were symbolized and listed in the footnote below Table 1. After eliminating the identical sequences except for those from the JNPs, we have phylogenetically analyzed the 38 unique *CYTB* sequences with those from *Sus barbatus* (GenBank accession no. AJ314554) for out-group rooting. Phylogenetic analysis was conducted using NEIGHBOR in the PHYLIP program package [5]. The neighbor-joining (NJ) tree was constructed on the basis of pairwise genetic distances calculated via the two-parameter method [16].

PCR-RFLP analysis

CYTB fragments were digested in independent reactions using the different restriction enzymes *Alu* I and *Hae*Ⅲ, respectively. Two-replicate PCR-RFLP tests for all samples were separated on polyacrylamide gel and visualized after EtBr-staining. For the RFLP patterns, digestion pattern *A* was defined as identical with the standard sequence of GenBank accession NC_000845 [18]. *CYTB* haplotypes were defined by combining the enzyme digestion patterns obtained from each individual.

Table 1. Nucleotide substitutions of pig *CYTB* gene sequences

Sample name	Nucleotide positions‡ 111111111111111111111111111111111111111111111111111 555555555555555555555555566666666666666666666666666 344445555556667888889999900000111111222222233344444 700481234580347013890114933349458889111226807913467 708388778748643920569194004829000376457045159156315	Sequence used for phylogenetic analysis	Identical sequences found of databases
Landrace1	AAGTGATGTCTAGTGCAACGACCCATATTCCCTTTTGTGCGCACGCCGAAA	NC_000845	1 of Landrace
JNP3	G.......C.CG.C....T..G....G.....C....CATA...A.T...G	AY830175	1 of JNP
JNP11	........C.CG.C....T......CG.C...C....CATA...A.T...G	AY830182	1 of JNP
CNJN†	........C.CG.C....T.......G.....C....CATA...A.T...G	AY830184	27 of 10 CNP breeds and 4 of JNP
CNP Zang	..A.....C.CG.C....T.......G.....C...ACATA...A.T...G	AF486856	1 of CNP Zang
CNPB3	.......AC.CG.C....T.......G.....C....CATA...A.T...G	AF486868	6 of 3 CNP breeds
JNP2	........C.CG.C....T.C.....G.....C....CATA...A.T...G	AY830174	1 of JNP
JNP9	........C.CGTC....T.......G.....C....CATA...A.T...G	AY830180	1 of JNP
Berkshire	.G...T..C.CG.C...TT.......G.....C....CATAT..A.T...G	AY830185	1 of Berkshire
CNP Meishan	........C.CG.C....T....G..G.....C....CATA...A.TA..G	AY237531	2 of CNP Meishan
Duroc1	........C.CG.C....T.......G.....C....CATA...A.TA...	AF136554	1 of Duroc
JNP8	........C.CG.C..G.T.....G.GG.A.GC.G..CATA...A.T..T.	AY830179	1 of JNP
PTJS†	........CTCG.CA...T.......G.....C....CAT....ATT...G	AY237529	1 of Pietrain and 1 of Japanese Satsuma
Large White3	........C.CG.C....T.......G.....C....CATA...A.T....	AF136552	1 of Large White
Large White2	........CTCG.CA...T.......G.....C....CATA...A.T....	AY237525	1 of Large White
LWSJ†	........CTCG.CA...........G.....C....CAT....ATT...G	AY830188	3 of Large White and 1 of Spanish Jabugo
CNLR†	........C.CG.CAT..T.......G.....C....CATA...A.T...G	AF486867	1 of CNP Wuzhishan and 1 of Landrace
DLIB†	........C.CG.C....T.......G.....C....CAT....A.T...G	AY830186	15 of Duroc, 7 of Landrace, 5 of IB, 2 of Large White, and 1 of Hungarian pig
JNIB†	...................A.....................G........	AY830177	3 of JNP and 12 of IB
JNP12	...................A.....................GT.......	AY830177	1 of JNP
IB20	....A..............A.....................G........	AY237503	4 of IB
JNP4	...................A.............A........G........	AY830176	1 of JNP
IB16	...................A.....................G.....G..	AY237499	3 of IB
Landrace2	......C.....................................G........	AF034253	1 of Landrace
IB15	.....................................G........	AY237498	1 of IB
Hampshire	............................A...........G........	AY830187	1 of Hampshire
IB23	..................................C......G........	AY237506	1 of IB
Duroc2	..................T.....................G........	AB015080	1 of Duroc and 1 of Yucatan miniature pig
JDP	..................T.............C...........A.T....	AJ314557	1 of JDP

The abbreviations JNP, CNP, JDP, and IB indicate Jeju native pig, Chinese native pig, Japanese domestic pig, and Iberian pig, respectively.

†Identical sequences: CNJN (CNP and JNP); CNPB3 (3 CNP breeds); PTJS (Pietrain and Japanese Satsuma); LWSJ (Large White and Spanish spotted black pig); CNLR (CNP and Landrace); DLIB (Duroc, Landrace, Iberian pig, Hungarian pig, and Large White); JNIB (JNP and IB)

‡Number of nucleotide position was followed by the consensus sequence NC_000845.

Results and Discussion

In order to investigate the maternal origin of JNPs, complete sequences of mtDNA *CYTB* gene from JNPs and the other five pig breeds, Berkshire, Duroc, Hampshire, Landrace and Large White, were determined. Additionally, to scan the genetic diversity of each pig population, PCR-RFLP analysis was also conducted.

We noted no differences in the length of newly-determined *CYTB* gene sequences as compared with the previously reported sequences. From our PCR-RFLP analysis of the *CYTB* gene, two types of RFLP patterns were detected in the JNP population, using both restriction enzymes, *Alu I* and *Hae*Ⅲ, respectively (Fig. 1). In other pig breeds, two restriction patterns were also detected, which with the exception of Landrace and Large White pigs, were similar to those from JNP (Table 2). Combining the restriction patterns, two kinds of *CYTB* haplotypes, *mtCYTB1* and *mtCYTB2*, were detected (Table 3). Both haplotypes were found in the four pig breeds-Berkshire, Hampshire, Duroc, and JNP-how-

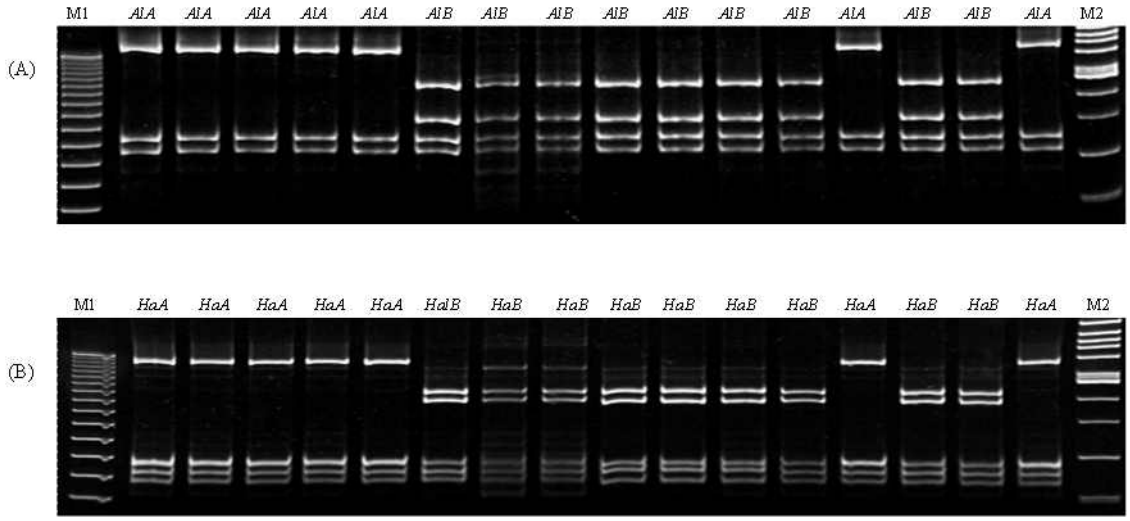

Fig. 1. Cleavage patterns of amplified mtDNA *CYTB* in pigs: restriction digestion by *Alu* I (A) and *Hae*Ⅲ (B). Restriction digestion patterns are indicated by the Italic letters at the top of the lanes. M1 and M2 are a 50-bp step ladder and a 100-bp ladder plus marker, respectively.

Table 2. Restriction digestion patterns and frequencies observed in pig breeds

Digestion pattern†		JNP‡		Berkshire		Duroc		Hampshire		Landrace		Large White	
		n	*f*	n	*f*	n	f	n	*f*	n	*f*	n	*f*
Alu I	*AlA*	19	0.292	5	0.081	37	0.787	4	0.400	40	1.000	0	0.000
	AlB	46	0.708	57	0.919	10	0.213	6	0.600	0	0.000	76	1.000
*Hae*Ⅲ	*HaA*	19	0.292	5	0.081	37	0.787	4	0.400	40	1.000	0	0.000
	HaB	46	0.708	57	0.919	10	0.213	6	0.600	0	0.000	76	1.000

†Digestion patterns were defined in Fig. 1.
‡Jeju native pig.

Table 3. Haplotypes and their frequencies obtained from PCR-RFLP in pig breeds

Haplotype	Digestion pattern	JNP		Berkshire		Duroc		Hampshire		Landrace		Large White	
	Alu I -*Hae*Ⅲ	n	f	n	f	n	f	n	f	n	f	n	f
mtCYTB1	*AlA*-*HaA*	19	0.292	5	0.081	37	0.7878	4	0.400	40	1.000	0	0.000
mtCYTB2	*AlB*-HaB	46	0.708	57	0.919	10	0.213	6	0.600	0	0.000	76	1.000

ever, the Large White pig ha the *mtCYTB2* haplotype, and the Landrace pigs harbor the *mtCYTB1* haplotype. In the JNP, the frequency of *mtCYTB2* is more than two times that of *mtCYTB1* (Table 3). Nucleotide substitutions at fifty-one positions (13 transversions and 38 transition substitutions) were informative in the construction of the phylogenetic tree.

The *CYTB* sequences of JNP are generally divided into two clusters, J1 and J2, corresponding to the haplotypes *mtCYTB1* and *mtCYTB2*, in the NJ tree (Fig. 2A). When constructing the NJ tree with other pig breeds, the J1 and J2 sequences were also discriminated into two distinct clusters. The clustering pattern of the *CYTB* sequences of various pig breeds, including JNP, was similar to those previously documented. The J2 sequences were observed in the Asian indigenous pig lineages, but the J1 sequences were observed in the European pig lineages (Fig. 2B), showing results identical to those of PCR-RFLP. The presence of multiple mtDNA haplotypes and diverse clustering in the phylogenetic tree was suggestive of multiple maternal origins. In our previous report, mtDNA D-loop sequences were divided Korean native pigs into several haplotypes mainly clustered also into two major groups [3], which is similar to the haplotyping result for *CYTB* gene used in this study. Thus we concluded that the PCR-RFLP approach is simpler and more beneficial method to determining maternal lineage of animal pop-

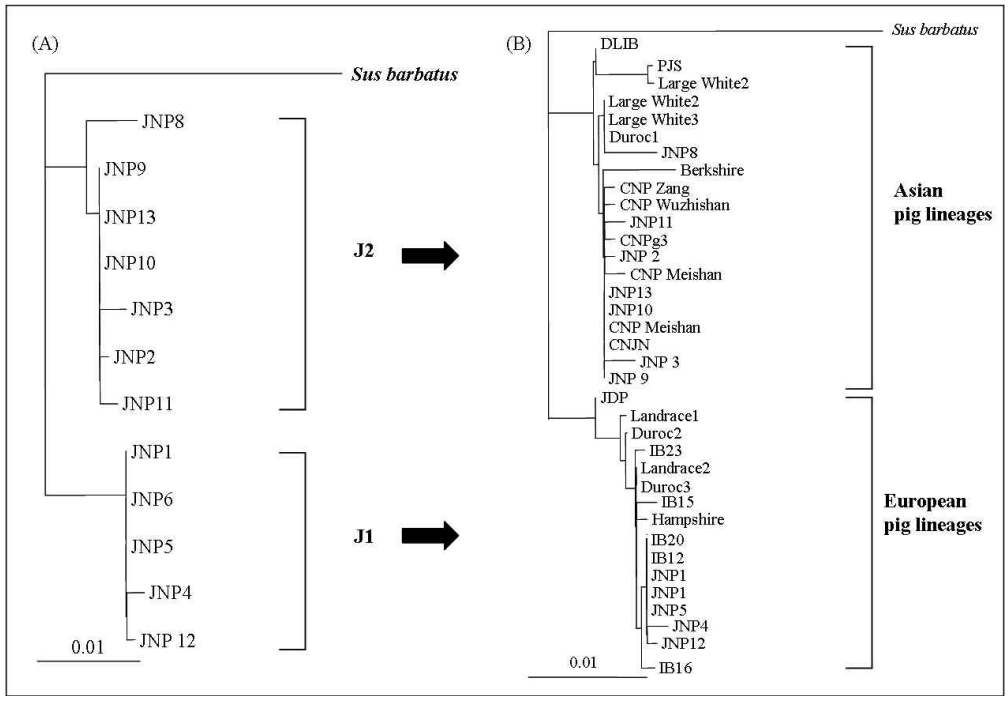

Fig. 2. The NJ trees constructed from the basis on the genetic distances of mtDNA *CYTB* gene sequences: (A) and (B) are NJ trees for *CYTB* sequences of the JNP population and those of JNPs and other pig breeds, respectively. *CYTB* gene sequence of *S. barbutus* (AJ314554) was used for outgroup rooting. The abbreviations used in NJ trees are given in Table 1. The numbers above bar indicate the genetic distances.

ulation than other molecular methods such as DNA sequencing.

These facts were already reported in previous studies concerning mtDNA and nuclear gene phylogenies [1,7,8,19,28], which suggest a maternal introgression between Asian pigs and Europe pigs, and crossbreeding among those. The introgression of Asian pig mtDNA haplotypes into Europe during the 18th and 19th centuries was proven at the molecular level using the mtDNA D-loop and the *CYTB* gene and nuclear genes, such as *MC1R*, *TYR* and *GPIP* [7,10]. Reports on the domestication processes of local pig populations have also used the results of molecular studies. Alves et al. [1] previously reported that the Iberian native pigs (Spain) originated maternally from Spanish wild boars and European domestic pig breeds such as Duroc, Landrace, and Pietrain pigs. Based on the molecular data, Gongora et al. [8] maintained that feral pigs in Australia and New Zealand originated from European pigs and Asian pigs, including Asian wild boars. Fang and Andersson [4] previously reported that native pig breeds from Southeastern China shared the European mitochondrial DNA haplotypes, but those of Northwestern China had Asian-specific haplotypes, and also proposed that the star-like pattern observed with both geographic haplotypes was consistent with a demographic population expansion. These results also support the different origins of the J1 and J2 *CYTB* sequences observed in JNPs. We estimated that the JNP *CYTB* sequences of cluster J1 have been either directly or indirectly introduced because those found in the J1 cluster could not be distinguished from the imported pig breeds and were shared with the European Iberian pigs. However, the J2 group sorted in Asian indigenous lineage with many of Chinese native pig breeds most probably originated from Asian pig ancestors. The studies on the peninsular native pigs of Korea described closer relationship between native Korean pigs and Chinese pigs than between the native Korean pigs and other pig breeds using AFLP and MS analyses [3,13-15]. Especially, Kim et al. [15] has suggested that native pigs distributed throughout the Korean mainland have been experiencing progressive interbreeding with Western pig breeds after originating from a North China pig breed with a black coat color, because they were co-clustered with Western pig breeds and a North China black pig breed, the Min pig, whereas Korean wild boars were co-clustered closely with

central Chinese pig breeds rather than those from North China.

Our present study showed mainly similar to those of mainland native pigs of Korea, but different results were also detected in part, such as appearance of a European mtDNA haplotype. The extant native pig population of Jeju Island is one of local populations of the mainland native pig in South Korea, and they have very similar phenotypic characteristics. Also, scientists believe that they have originated from same genetic background and regard as a same pig breed. But long-term geographical isolation and unique history of insular pig population for breeding and introgression of exotic breeds including European commercial breeds might have led some different study results such as appearance of European pig-haplotype (*mtCYTB1*) suggesting their close relation with European maternal lineages in relatively high frequencies (29.2%) in the JNP population. Based on molecular data we concluded that even if the native pigs of mainland and Jeju Island of South Korea had originated from a same progenitor population maybe from Asian Continent, the present populations had formed by different ways.

Generally, pig domestication in Jeju Island is estimated to have begun sometime prior to approximately 2,000 YBP. After domestication, foreign pig breeds or populations may possibly have been introduced several times into this island from the Korean Peninsula, as well as from other East Asian countries across the South Sea, East China Sea, or Pacific Ocean. In the 20th century, modern European domestic pig breeds were imported both directly and indirectly. The *CYTB* sequences of the J1 group were detected in the European pig lineages in the NJ tree, thus suggesting a certain relationship with those of European pig breeds, especially with Iberian pigs. In particular, the presence of European pig haplotypes observed in JNPs surely reflects the introgression of European pig breeds and shows that the extant native pig population of Jeju Island has a more diverse genetic background, contrary to our previous prediction of a unique evolutionary origin. Recently, Wu et al. [27] has suggested that the pig domestication events in East Asia using wild boars have occurred multiple times, and occurred principally in the Mekong region and Yangtze River, based on the D-loop and the complete mtDNA sequence data of domestic pigs and wild boars. A great deal of archaeological evidence exists to suggest that pigs were present in Jeju Island during this time. Abundant animal remains have been excavated from a great many archaeological sites estimated to be from 1,000 to 2,500 YBP [20-23]. Kim et al. [11] previously addressed the close relationship of several of these pig remains to the extant JNP animals, based on molecular data. Although we previously were not certain as to whether or not ancient insular people used these wild boars for pig domestication or not, it appears that the pigs have preserved their indigenous gene pools inherited from ancient ancestors, according to analyses of the Asian maternal lineage J2, which is shared between Chinese native pig breeds and Asian haplotype-domestic breeds found in the JNP population.

Consequently, we suggest that the extant JNP population originated from a variety of maternal progenitor populations from Europe and Asia. The JNPs fundamentally have their origins in the ancestors of the Asian indigenous pigs, and the introgression of modern European domestic pigs and crossbreeding with those animals must have occurred largely over the last century. However, there is still not sufficient data to adequately evaluate the relationship and evolutionary origins of Korean native pigs, including JNPs. In order to establish clearly the relationship between the domestication histories of native pigs and wild boars in Korea, and to clearly characterize the progenitor population and more accurately elucidate the phylogenetic relationships of the extant wild boars, detailed future studies are recommended, with an expanded sample population of present and ancient pigs from Korea and East Asia.

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초록 : 미토콘드리아 DNA *CYTB* 유전자 서열에 대한 분자 계통과 PCR-RFLP 반수체형에 근거한 제주재래돼지의 모계 기원

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제주재래돼지의 모계 혈통에 대한 보다 명확한 이해를 얻기 위해, 본 연구에서는 제주재래돼지의 미토콘드리아 DNA (mtDNA) *CYTB* 유전자를 분석하고 이를 타 품종들에서 얻은 결과들과 비교하였다. 제주재래돼지를 포함한 돼지 6 품종에서 PCR-RFLP 분석을 수행하였고, RFLP 양상은 돼지 품종들을 뚜렷하게 구분되는 두 가지 반수체형(*mtCYTB1* and *mtCYTB2*)으로 분리시켰다. 제주재래돼지 *CYTB* 서열들은 계통수 상에서 유럽과 아시아 품종 cluster에서 모두 발견되었다. 제주재래돼지 *CYTB*들 중에서 J2 group은 중국재래돼지품종들과 근연이면서 아시아 고유 돼지 계통들과 함께 출현하였으며, 다른 한 group인 J1에 해당하는 서열들은 유럽돼지 계통들과 함께 위치하였고, 아시아 품종들보다는 스페인의 Iberian 재래돼지들과 근연인 것으로 확인되었다. 이 결과들은 현재 제주도에서 사육되고 있는 제주재래돼지 품종의 모계 기원은 크게 아시아계 돼지와 유럽계 돼지인 것으로 추정됨을 보여준다. 따라서 본 연구결과들은 제주재래돼지 집단은 과거에 가축화된 아시아 고유 돼지품종들과 공통 선조를 공유하고, 또한 20세기에 유입된 유럽계 돼지 품종들도 현재의 집단 형성에 기여한 것임을 시사하고 있다.