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Phylogenomic analysis of mitochondrial DNA in the Huong pig: an indigenous pig of Vietnam

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The Huong pig (*Sus scrofa*) has been included in a project aimed at preserving indigenous Vietnamese pig breeds. This study sequenced the complete mitochondrial (mt) genome of the indigenous Huong pig and determined its phylogenetic relationships with other Asian and European pig breeds. A complete mtDNA sequence of approximately 16,711 base pairs (bp) was obtained and submitted to GenBank (KY964306). In that mtDNA, the content levels of A, C, G, and T were 34.65%, 26.22%, 13.352%, and 25.78%, respectively. The whole mtDNA consisted of 37 genes, including 13 genes coding for proteins, 2 ribosomal RNA genes, 22 genes for tRNA, and a non-control region (D-loop). The length of the D-loop region was 1,315 bp and there were 27 repeat sequences (5'-tacacgtgcg). The phylogenetic relationships, based on both the mtDNA and the D-loop region, indicated the shortest genetic distance was between the Huong and Lantang pig breeds with close relationships to other Asian pig breeds such as South China, Yangtze the River, and Yellow River Region pig groups. In conclusion, the obtained Huong pig mtDNA sequence data can contribute to elucidating the relationships among Vietnamese indigenous pig breeds and support the selection of suitable livestock for pig breeding in the area.

Keywords: Mitochondrial genome, genetic distance, Huong pig, phylogenetic relationship

INTRODUCTION

There are twenty native breeds included in the list of rare livestock gene sources that must be conserved in Vietnam (Dang-Nguyen et al., 2010). Huong pigs (*Sus scrofa*) are one of those breeds and are widely raised in the border districts between Vietnam and China, particularly in Vietnam's Cao Bang province (coordinates: 22°40′ N 106°0′ E). Huong pigs grow slower than other breeds, but maturity occurs earlier. Of particular note, Huong pork has a distinctive aroma. Typical morphological characteristics of Huong pig include white feathery hairs and skin. The top of the head and bottom of the tail are black, and the contiguous areas of white and black hair form a dark streak. Huong pigs have other distinct characteristics from those of domestic pigs in Vietnam including a large head, small and erect ears, straight face, long snout, white streaks running from the middle of forehead to the muzzle, slim abdomen, straight back, and from 8 to 12 breasts (Duy, 2016). This breed lives in rugged high mountainous terrain, thus their trade or sale is very difficult. As the Huong pig population

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only occurs in small groups it can be easy to lose their valuable genetic features. Furthermore, genetic breeding investigations involving Huong pigs have not been undertaken. Thus, such an important indigenous genetic resource should be conserved. Enhanced protection measures for Huong pigs within a country-wide preservation and restoration plan will ensure sustainable use of an important national animal genetic resource.

By applying advanced genetic molecular techniques for the analysis of DNA sequences, evolutionary information can be derived. By comparing DNA sequences within and between groups of animals, evolutionary relationships, levels of variability and geographically-based population substructuring can be determined (Kim et al., 2002). Mitochondrial (mt) DNA polymorphism detection tools have become useful in determining relationships among individuals within a species and between closely related species (Cann et al., 1984). In addition, the D-loop region of mtDNA is reported to include more sequence variation than that in other regions (Cann et al., 1984), and such D-loop variation can reveal past genetic history, even in a contemporary admixture of groups (Huo et al., 2016). A number of studies of D-loop region sequence mutations in mtDNA for application in phylogenetic analyses of closely related groups have been undertaken (Gongora et al., 2004; Lan and Shi, 1993). However, few mtDNA-based studies have indicated genetic differences among Vietnamese indigenous and Asian pig breeds; in particular, the genetic origins of most Vietnamese pig breeds have not been described.

In this study, detailed mtDNA information was analyzed to reveal the phylogenomic and gene organization features of the mt genome of the indigenous Huong pig. The aim was to undertake a comprehensive molecular-based analysis of the origin of the domestic Huong pig breed and its relationship with both Asian and European wild and domestic pigs. The results of this study add to the current knowledge of pig genetics and have important implications for the maintenance and utilization of genetic diversity in a Vietnamese livestock species.

MATERIALS AND METHODS

Sampling and DNA extraction

All animals used for sample isolation were collected in Cao Bang Center for Cultivation, Livestock, and Aquaculture (Cao Bang, Vietnam) under the National Institute of Animal Sciences (Hanoi, Vietnam) care and management guidelines. Five milliliters (5 ml) of blood was collected with anticoagulant from jugular vein of the Huong pigs. Total genomic DNA was extracted according to the user manual of the GeneJET^{TM} Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA), and diluted to a final concentration of 20 ng/ ml.

Primer design and amplification of mtDNA fragments by polymerase chain reaction (PCR)

The complete mt genome of Huong pig was amplified in PCR fragments generated with species specific primer pairs (see below Table 1). The reaction system was carried out in a 50 µl volume, including 5 µl of 10 × buffer (with $Mg2^{+}$), 1 µl of dNTP Mix, 1 µl of 10 mmol/L each primer, 0.5 µl of 5U/ µl Taq DNA polymerase and 2 µl of gemomic DNA. PCR amplification began with an initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at gradients 53 - 55°C for 40 s, and elongation at 95°C for 30 s. The final elongation step was at 72°C for 7 min. All PCR products corrected size as Table 1 were purified with mini spin columns provided in GeneJET™ PCR Purification Kit (Thermo Fisher Scientific, USA).

Sequencing and gene annotation

Sequencing PCR reactions were performed on the automated Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems) at Institute of Genome Research (Hanoi, Vietnam). Briefly, the set up of 10 µl reaction volumes, including 2 µl BigDye Terminator v3.1, 1µl BigDye Sequencing buffer, 1 µl primer (10 µM), 2 µl DNA, and 4 µl water was carried out according to the procedure: denaturation at 96^{o}C for 1 min, and 25 cycles of denaturation at 96^{o}C for 10 s, annealing at 50^{o}C for 5 s, elongation at 60^{o}C for 4 min. Then the samples were purified and incubated with 20 µl Hi-Di, and denatured at 98°C for 2 min. Finally, these samples transfered to plate and sequenced with the sequencer using POP6 and a 36 cm capillary array in 55^{o}C for run temperature.

Phylogenetic analysis

The sequence assemblage was done with DNA Dragon version 1.6.0. The annotation of protein coding, ribosomal RNA genes, and transfer RNA genes were identified by MITOS and DOGMA. Tranfer RNA genes and their putative secondary structures were determined with MITOS software. Mitochondrial circle structure used GenomeVx. Alignment between Huong pig sequencing and other literature ones were performed using MUSCLE algorithm of MEGA 6. Phylogenetic analysis and phylogenetic tree building methods were used Bayesian analysis in BEAST version 1.8.3 and Figure Tree version 1.4.2, respectively.

RESULTS

Genome organization

The mt genome of Huong pig is a circular, double-strand DNA with a length of 16,753 base pairs (bp). Thirty-seven genes were identified in the Huong pig mt genome;

Table 1 Primer pairs and corresponding annealing temperatures used for successful amplification of mitochondrial genome fragments from Huong pig

Primer No.	Primers sequences		Annealing temperature	Size of PCR product (bp)
	Forward (Nucleotide sequence 5'–3')	Reverse (Nucleotide sequence 5'–3')		
D-loop	AGGAGACTAACTCCGCCAT	GCGGATACTTGCATGTGT	54°C	1243
2	ACTAAGTCAATGCCTATTCTG	CAAATGTATGAAACCTCAG	54°C	871
3	CTACACAATAACCTCCCATA	TGGCACGAGATTTACCAACT	54°C	383
4	GCTCATAACGCCTTGCTC	ATTCTTTCATCTTTCCCTT	54°C	1037
5	CACAACCATGCAAGAAGAGACA	ACAACCAGCTATCACCAGGC	54°C	394
6	CCGTAAGGGAAAGATGAAAG	TATGGTTATTTTGACTGGT	54°C	1099
7	CCGTGCAAAGGTAGCATA	CCAACATCGAGGTCGTAA	55°C	417
8	TGGGGTGACCTCGGAGTAC	AATATGGCGAAAGGTCCGG	54°C	1166
9	CGAGCAGTAGCCCAAACA	GGTCGTATCGGAATCGTG	55°C	450
10	GTATCAGGCTTTAACGTAGA	TGGTAATACTGCTGTCATTC	54°C	1128
11	CACAGAAGCAGCCACAAA	ATGGGATAGGGATAAAGT	55°C	540
12	ACATAGGATGAATGACAGC	TGGTGGAAGTAGTCAGAAAC	55°C	1194
13	GCACTGCCTTGAGCCTAC	GTGTTCAGGTTGCGGTCT	55°C	561
14	CCCATTATGATTGGGGGTTT	TGCTGTGTATGCGTCAGGAT	55°C	1134
15	CACTTTGTAATCATATTCGTAG	TAGTTGGAAAGGGTAAGC	53°C	481
16	TTCATCTCACTAACAGCAG	TTGAGTTCGGTTGATTCTG	55°C	1200
17	GCTTCATGCCCATTGTAC	TTATAGCGGAATCCTGTG	55°C	662
18	GCAAGCCCAGAATCAACCG	CGAGGAGGATTGAGGTGTT	55°C	1153
19	ATACCACATAGTAAACCCAA	CCTGTAGCCACAAAGAAA	55°C	584
20	CTAAACACCTCAATCCTCC	TTGGACGTAATCGGTACCG	55°C	1151
21	CCTTGCAGGGTTACTTAT	TTCGGGTTGTGGTTTCTT	53°C	519
22	CGGTACCGATTACGTCCAA	CCGATTAGATTGATGGATG	55°C	1165
23	ACCAGCTCTATCTGCTTA	GAGGCTTTGATGTTGTTA	55°C	472
24	ATGATGACTAATAGCAAGCC	GGGATGTAGTCCGAATTG	55°C	1198
25	CATCGGAGACATTGGATT	AGTTGGCTTGAAGTTGAG	53°C	401
26	CCTACTCCTAGCTGCAGCAG	ATTATGGAGATTACTCGTGG	55°C	1186
27	TCCGCATCATCATTACTA	TTTATGGTGGACTTGGGT	55°C	611
28	TAATTACCACGAGTAATCTC	TTCTACGAGGTCTGTTCCG	55°C	1093
29	GGAGCATCCATATTCTTT	GGTGTAGTTGTCTGGGTCT	53°C	515
30	TCGTAGAATGAATCTGAGG	GGTGATACGCATGTTGACTG	55°C	1099

namely, 2 rRNA genes (*12S* and *16S*), 22 tRNA genes, and 13 protein-coding genes (*COX1* to 3, *ND1* to 6, *ND4L*, *ATP6* and 8, and *Cytb*) (see below Figure 1 and Table 2). There were 59 non-coding base pairs contained in 11 regions dispersed over the entire mt genome, with the largest region comprising 32 bp between tRNA *Cys* and tRNA *Tyr*. The Huong pig mt genome encoded a total of 3,807 amino acids, in which twelve protein-coding genes started with methionine (9 and 3 genes of ATG, ATA, respectively). Only the *ND4L* gene showed a GTG (Valine) start codon. Two genes were observed to have a stop codon with TAG, six with TAA. All incomplete stop codons contained T-- from the alignment of *COX2*, *COX3*, *ND3*, and *ND4* genes. Other stop codons were observed in TAA (6 genes), TAG (2 genes), and AGA (1 gene). There were ten gene overlaps, between *12S rRNA*

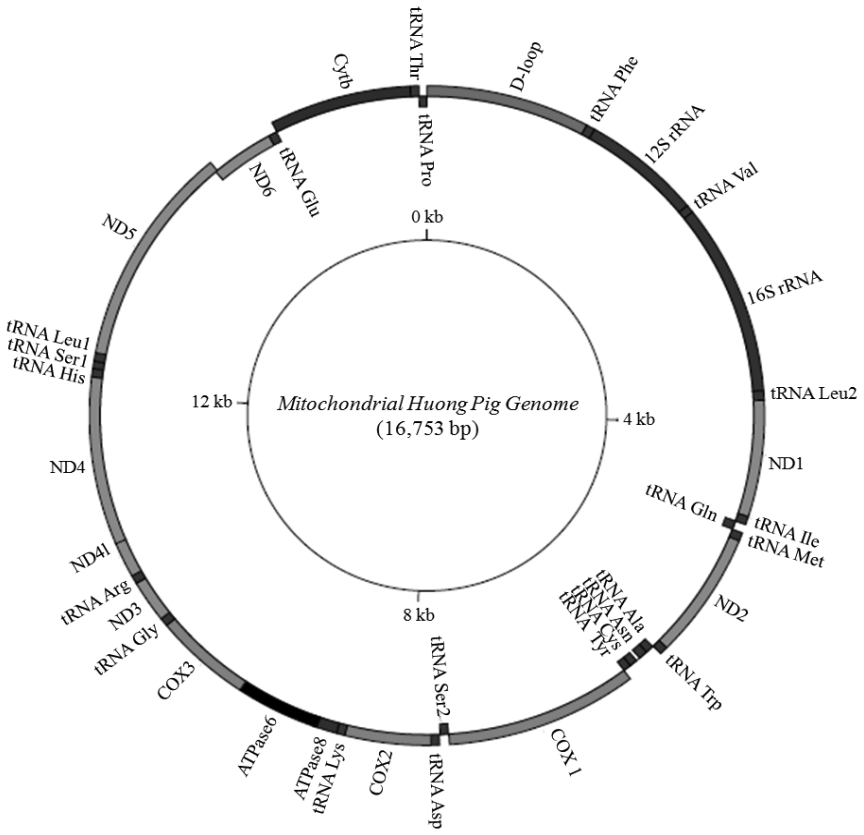

Figure 1 Circular map of the mitochondrial genome of Huong pig obtained by using GenomeVx.

Table 2 Mitochondrial genome organization of Huong pig mtDNA

Gene	Strand	Position		Size (bp)	Codon			Space (+) Overlap (-)
		Start	Stop		Start codon	Stop codon	Anti-codon	
D-loop		1	1315	1315				0
tRNA *Phe*	H	1316	1385	70			GAA	0
12S rRNA	H	1386	2349	964				0
tRNA *Val*	H	2349	2416	68			TAC	-1
16S rRNA	H	2415	3986	1572				-2
tRNA *Leu2*	H	3987	4061	75			TAA	0
ND1	H	4064	5020	957	ATG	TAG		2
tRNA *Ile*	H	5019	5087	69			GAT	-2
tRNA *Gln*	L	5085	5157	73			TTG	-3
tRNA *Met*	H	5159	5228	70			CAT	1
ND2	H	5229	6272	1044	ATA	TAG		0
tRNA *Trp*	H	6271	6338	68			TCA	-2
tRNA *Ala*	L	6345	6412	68			TGC	6
tRNA *Asn*	L	6414	6488	75			GTT	1
tRNA *Cys*	L	6521	6586	66			GCA	32
tRNA *Tyr*	L	6587	6651	65			GTA	0
COX1	H	6653	8197	1545	ATG	TAA		1
tRNA *Ser2*	L	8201	8269	69			TGA	3
tRNA *Asp*	H	8277	8344	68			GTC	7
COX2	H	8345	9032	68	ATG	T--		0
tRNA *Lys*	H	9033	9099	67			TTT	0

Table 2 cont'd

ATPase8	H	9101	9304	204	ATG	TAA		1
ATPase6	H	9262	9942	681	ATG	TAA		-43
COX3	H	9942	10725	784	ATG	T--		-1
tRNA *Gly*	H	10726	10794	69			TCC	0
ND3	H	10795	11140	346	ATA	T--		0
tRNA *Arg*	H	11142	11210	69			TCG	1
ND4L	H	11211	11507	297	GTG	TAA		0
ND4	H	11501	12878	1378	ATG	T--		-7
tRNA *His*	H	12879	12947	69			GTG	0
tRNA *Ser1*	H	12948	13006	59			GCT	0
tRNA L*eu1*	H	13007	13076	70			TAG	0
ND5	H	13077	14897	1821	ATA	TAA		0
ND6	L	14881	15408	528	ATG	TAA		-17
tRNA *Glu*	L	15409	15477	69			TTC	0
Cytb	H	15482	16621	1140	ATG	AGA		4
tRNA *Thr*	H	16622	166689	68			TGT	0
tRNA *Pro*	L	16689	16753	65			TGG	-1

Abbreviations: bp: base pairs; rRNA: ribosomal RNA; *16S rRNA:* large rRNA subunit; *12S rRNA*: small rRNA subunit; tRNA: transfer RNA; *Italic* is replaced by one letter amino acid code; *ND1-6* and *ND4L* genes encoding nicotinamide dinucleotide dehydrogenase subunits 1 to 6 and *4L ATPase6* and *8*: genes encoding adenosine triphosphatase subunits 6 and 8; *COX1* to *3*: genes encoding cytochrome c oxidase subunits I to III; *Cytb*: gene encoding cytochrome b. Intergenic nucleotide refers to the nucleotide distance between pairs of adjacent genes. Start and stop position of ribosomal RNA and non-coding region according to adjacent gene boundaries. T--indicates an incomplete termination codon (the incomplete stop codon; that is, amino acid translation is terminated when the gene forms a stop codon via post-transcriptional polyadenylation).

and tRNA *Val* (1 bp) and *16S rRNA* (2 bp), *ND1* and tRNA *Ile* (2 bp) and tRNA *Gln* (3 bp), *ND2* and tRNA *Trp* (2 bp), *ATPase8* and *ATPase6* (43 bp) and *COX3* (1 bp), *ND4L* and *ND4* (7 bp), *ND5* and *ND6* (17 bp), and tRNA *Thr* and *Pro* (1 bp). Moreover, 28 protein-coding gene sequences were coded on the H strand and 9 other genes in the L strand.

Transfer RNAs and ribosomal RNA genes section

Huong pig mtDNA included 22 tRNA genes, ranging in length from 59 to 75 bp (see below Figure 2). The cloverleaf secondary structures of 21 tRNA genes (excepting tRNA *Ser1*) showed a 4-8 bp DHU arm, a 3-10 bp DHU loop, a 8-10 bp TψC arm, and a 5-9 bp TψC loop. The tRNA *Ser1* was present in the TψC arm and loop but lacked the DHU arm and loop, thus it was only 59 bp long.

Phylogenetic analysis of mitochondrial sequences

The phylogenetic relationships between the Huong pig and 15 indigenous and wild pig breeds (see below Table 3) were determined by analysis of the polymorphisms in the D-loop region and in the complete coding region within the entire mtDNA sequence. Phylogenetic analysis of both the whole mt genome and the D-loop regions (see below Figures 3 and Figure 4) revealed a close relationship between the Huong pig and the Lantang pig breed from the South China region. In addition, the Huong pig genome was clustered into the Asian clade, which includes a pig breeds in the South China, Yangtze River, and Yellow River regions. The mtDNA and D-loop region genetic distance analyses of the various pig breeds included in this study showed that the Huong pig had the greatest genetic distances from European pig breeds (0.0120 and 0.0144, respectively) and the shortest genetic distances from the Lantang pig (0.0002 and 0.0007, respectively).

DISCUSSION

The Huong pig gene content and arrangement were consistent with those of *Sus scrofa*. The mtDNA genome was typically small (16,753 bp) and circular molecular mapping showed the mtDNA consisted of 37 genes and one non-coding (control or AT-rich) region. In addition, other Huong pig data such as the base composition ratio, codon usage, and amino acid frequencies were similar to those of other Asian pigs (Chai et al., 2016; Ran et al., 2016; Wang et al., 2016; Xu et al., 2016). The mt genome of Huong pig has 16 intergenic regions ranging from 1 to 43 bp in length, and the structures of the genes in the Huong mt genome are similar to those of other vertebrates.

The D-loop region of an mt genome is important in phylogeographic studies and in examining the evolution of breeds (Cann et al., 1984). The D-loop region of the Huong pig is 1,315 bp in size. It acts as a promoter for both the heavy and light strands of mtDNA and contains essential transcription and replication elements (Levin et al., 1999). There were 27 repeat sequences (5'-

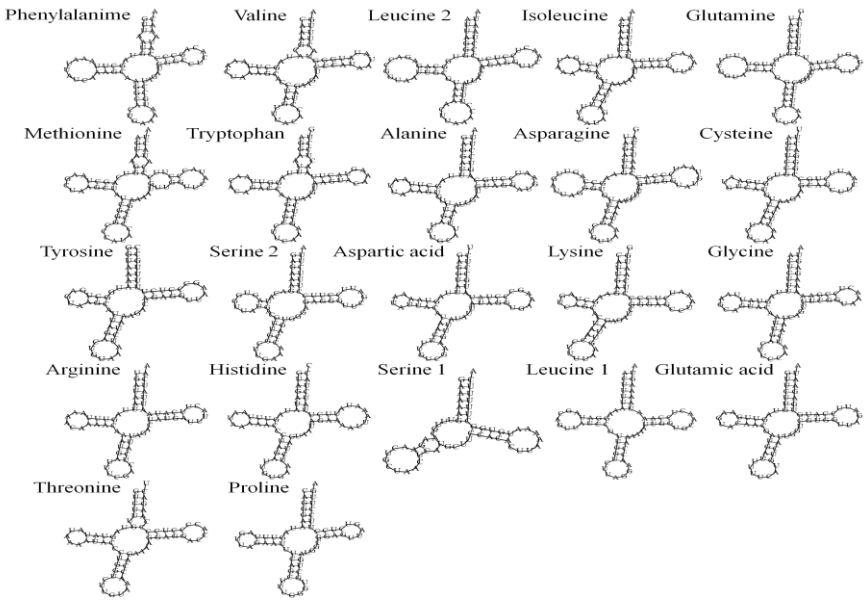

Figure 2 The cloverleaf secondary structures of 22 tRNAs predicted in the mitochondrial genome of Huong pig obtained by using MITOS and DOGMA.

Table 3 Pig breeds and their geographic definitions of regions

Geographic location	Pig breeds	Accession number in NCBI GenBank
Mekong Region	Wild boar (WB) -Vietnam	EF545584.1
	Banna mini	GQ220328.1
South China	WB-Fujian	EF545569.1
	Lantang	KC250274
Yangtze River Region	Aba	EF545578.1
	WB- Jiangxi	EF545579.1
	Jinhua	KC469586
	Xiangg	KC250273
	Bihu	EF545591.1
	Wei	EF545577.1
Yellow River Valley	Bamei	EF545583.1
	Huzu	EF545588.1
European Countries	Duroc	AY337045.1
	Hampshire	AY574046.1
	Large-white	KC250275

tacacgtgcg) in the Huong pig D-loop region, and there were significant differences between the D-loop regions of Huong and European pigs, including Duroc (10 repeats), Landrace (13 repeats), and Large-white (6 repeats), as well as between Huong and other Asian pig breeds such as the Japanese wild boar (WB) (1 repeat), and the Ryukyu WB (1 repeat). This study observed similar AT-rich features in the D-loop sequence of Huong pig (60.5%) and other Asian pig breeds such as Wuzhishan (60.46%), Visayan warty (60.8%), Qianshao spotted (60.50%), Daweizi (60.48%), Ningxiang (60.52%), and Shaziling (60.51%) pigs (Chai et al., 2016; Liu et al., 2015), indicating that many Asian pig breeds have been close relationships and most likely have originated from a common ancestor (Yang et al., 2003). Phylogenetic trees, based on the polymorphic sequences of the D-loop region, were constructed to analyze the genetic lineage between Huong pig and Asian and European type pig breeds. The results showed a close relationship with the Lantang pig breed from the Guangdong Province in South China, whereas there were significant differences between the Huong pig and the European pig groups. Morphological characteristics, including body conformation and color, of Huong and

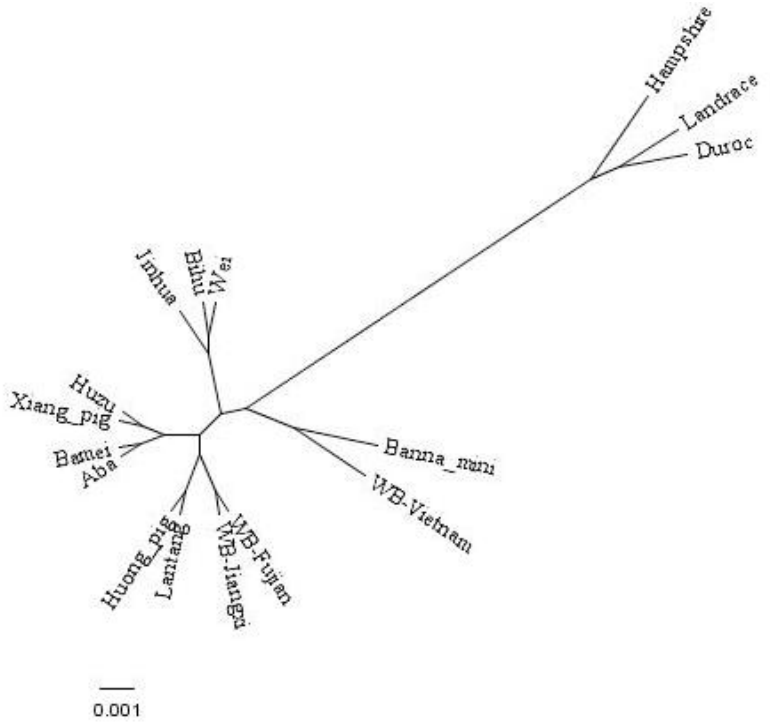

Figure 3 Phylogenetic relationships developed by performing the Bayesian analysis in BEAST version 1.8.3 and Figure Tree version 1.4.2 and obtained by comparing the mitochondrial D-loop region of different pig breeds. Abbreviations of pig breeds are the same in Table 3.

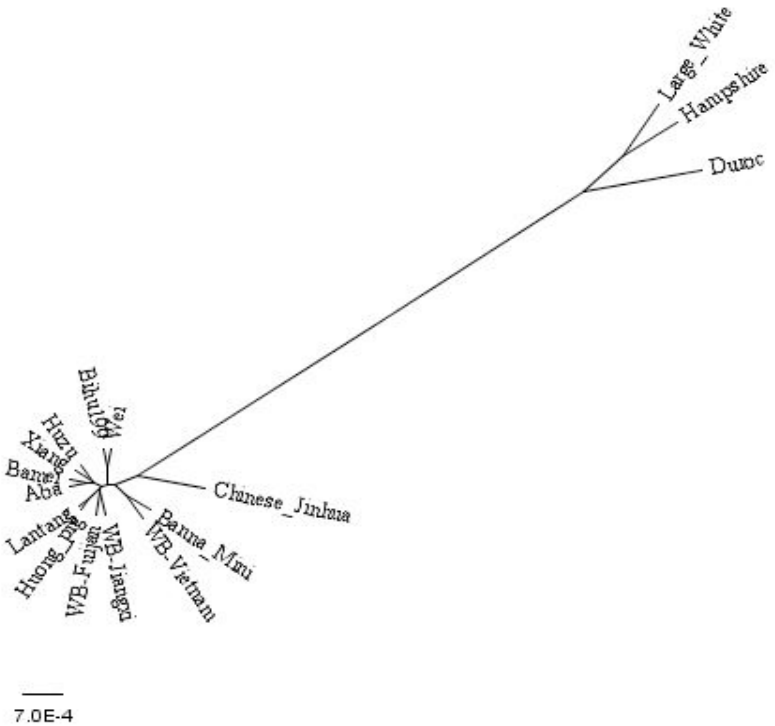

Figure 4 Phylogenetic relationships developed by performing the Bayesian analysis in BEAST version 1.8.3 and Figure Tree version 1.4.2 and obtained by comparing the mitochondrial genome sequences of the Huong pig and other breeds. Abbreviations of pig breeds are the same in Table 3.

Lantang pigs are also reported to be similar (Rothschild and Ruvinsky, 2011). It is hypothesized that perhaps thousands of years ago there were pigs traded or sold in the Vietnam-China border regions, thus the Lantang pig breed entered Vietnam and gradually developed into a Vietnamese domestic pig breed. However, it can be difficult to determine fully the original relationship between Huong and Lantang pigs as genetic markers were not included in this study.

The mtDNA phylogenetic trees also revealed results indicating a close relationship between the Huong and Lantang pigs. Those two breeds had the shortest genetic distance (0.0002) between them. There was also close clustering between the Huong pig and other Asian indigenous pig breeds (e.g., WB-Fujian, WB- Jiangxi, Aba, Xiang, Bamei, Huzu) with short genetic distances being observed among these pig breeds (from 0.0011 to 0.0025); such results may be explained by the nearby geographic regions in which those breeds occur. Several wild boar subspecies inhabiting East Asia have been domesticated from WB, with such domestication occurring repeatedly from 6000 to 9000 years ago (Hongo et al., 2002). Wu (2007) studied the mtDNA control region sequences of 567 domestic pigs and 155 WB across China, South East Asia, and India and reported monophyletic positioning of East Asian domestic pigs and WB (Wu et al., 2007). Kim (2002) had revealed that despite geographic separation, pig populations in China, Korea, and Japan were genetically similar to each other (Kim et al., 2002). Our results indicate that the Vietnamese domestic Huong pig is genetically closely related to the Lantang pig and other domestic pigs of the South China, Yangtze River Region, and Yellow River Valley regions. These results differ from those in other previous studies, which confirmed that the Asian domestic pig population derived from multiple sources in Asia (Luetkemeier et al., 2010; Megens et al., 2008); thus, this is a need for more research into genetic indicators in order to elucidate fully the origins of the indigenous pigs in Vietnam.

CONCLUSIONS

In conclusion, the results suggest that Huong and other Asian pigs have similar mtDNA sequences, but have different sequences from European type pigs. The close clustering of the Huong and Lantang pig breeds with other Asian pigs clearly demonstrate that Asian pigs were involved in the original development of these breeds. Finally, there is a need to apply mtDNA analyses in genetic studies of pig breeds in order to understand the selective mechanisms that can affect the characteristics and evolution of natural populations of Vietnamese indigenous pig breeds.

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