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Research Article

The Mitochondrial Genome and Phylogenetic Relationships of Muong Lay Black Pig (*Sus scrofa*) in Vietnam

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Abstract

Muong Lay black pig is an indigenous breed of livestock and has recently been listed for conservation in Vietnam. The aim of this study was to investigate the phylogenetic relationship of the Muong Lay black pig with other pig breeds by sequencing its mitochondrial genome (mtDNA). The PCR products were sequenced by Applied Biosystems 3500 Genetic Analyzer. The complete mtDNA sequence was 16,742bp in length and includes two rRNA (12S and 16S), 22 tRNA, 13 mRNA genes, and one control region (D-loop). The phylogenetic analysis was performed by comparing both complete mtDNA and D-loop sequences of Asian and European pig breeds. The results of phylogeny reconstruction revealed that Muong Lay black pig belonged to an Asian clade and clustered with Bihu and Banmei pig from Yangtze River Region and Yellow River Region, while reporting distinctly different phylogenetic relationships with the European breeds.

Abbreviations: Mitochondrial DNA; Muong lay black pig; Pig breeds; Phylogenetic relationships; Sus scrofa

Introduction

Vietnam has numerous varieties of pig breeds distinct to each ecological region of the country. Muong Lay black pig is one of the local pig breeds with unique morphologic characteristics (e.g., dark back skin; straight back; 4-6 white spots on forehead, head, legs, and tail; and 14 teats) originally bred by the Muong community- a minority ethnic group residing in the mountainous region in Northern Vietnam. This is a voracious pig, well adapted to the harsh climate in term of high mountain zone, inadequate nutrition, and high disease resistance [1]. In addition, this pig breed provides excellent quality tenderloin and fragrant meat [2]. In recent years, the population size of Muong Lay black pig has been constantly decreasing [3] associated with high risks of losing rare allelic variations because of replacement by imported pig breeds. Consequently, in 2008, the Muong Lay black pig was included in the list of genetic resources that should be preserved in Vietnam [4]. The mitochondrial DNA (mtDNA) of vertebrates has become a common tool used in resolving phylogenetic relationships at different evolutionary depths levels related to particular properties, such as the presence of strictly orthologous genes, the lack of recombination, and an appropriate substitution rate [5]. For instance, mtDNA is deemed to strictly follow maternal inheritance and highly variable at the species level; the rate of mtDNA evolution is about 5 to 10 times higher than nuclear DNA, and hardly goes through gene recombination [6]. The fast rate of mutation produces more variance between sequences which gives an advantage when studying closely related species. Therefore, mtDNA sequences are useful markers for phylogenetic inference and also in studying genetic diversity [7]. The mitochondrial control region (Displacement or D-loop) is one of the fastest mutating sequence regions in animal DNA, and is also known to be more variable in sequence than other regions in either the nuclear or mitochondrial genomes [8]. There are several studies that have reported mutations in the D-loop sequence region for phylogenetic analyses [9-11]. For example, mtDNA from other Vietnamese pigs have been reported from I pig [12], Huong pig [13], Muong Khuong pig [14], and Mong Cai pig [15]. All of the previous studies reported the shortest genetic distance between the indigenous pigs of Vietnam and Asian pig breeds. The aim of this study was to derive the complete mtDNA sequence of the Muong Lay black pig breed to accurately assess the valuable genetic resources in Vietnam, and report on key implications for the maintenance and utilization of genetic diversity in Vietnamese livestock species. We constructed a phylogenetic tree of mtDNA data to investigate the evolutionary relationships between indigenous Muong Lay black pig and other wild boar and domestic pig breeds found in Asia and Europe.

Materials and Methods

Sample collection from all the Muong Lay black pigs used in this study was done in line with the guidelines of National Institute of Animal Sciences (Hanoi, Vietnam). Thirty blood samples (5ml per sample) were collected with anticoagulant from the jugular vein of Muong Lay black pigs reared in Muong Lay town, Dien Bien province (Vietnam). The experiments on animals were approved by the Ethics Committee at the Institute of Genome Research (Vietnam). Genomic DNA was extracted by standard phenol-chloroform method described by [16].

A total of 30 primer pairs (Table 1) was used to amplify the overlapping fragments of D-loop region and coding region sequences of pig mtDNA. The PCR reaction system was carried out in a 50µl volume, including 5µl of 10 x buffer (with $Mg2^+$), 1µl of dNTP Mix, 1µl of 10mmol/L each primer, 0.5µl of 5U/µl Taq DNA polymerase and 2µl of genomic DNA. PCR-amplifications included an initial denaturation at 94 °C for 5 min, followed by 25 cycles, each one consisting of 30sec denaturation at 94 °C, 30sec primer annealing in range 53-55 °C (depending on the composition of primers), 30-45sec extension at 72 °C, and then a final 8 min extension at 72 °C. Post amplified DNA was purified by GeneJET™ PCR Purification Kit (Thermo Fisher Scientific, Singapore, Singapore) and sequenced using ABI3500 Genetic Analyzer system (Applied Biosystems®, CA, USA).

Table 1: Primer pairs for Muong Lay black pig complete mitochondrial DNA.

Primer No.	Primers Sequences		Annealing Temperatute	Size of PCR Product (bp)
	Forward (Nucleotide Sequence 5'-3')	Reverse (Nucleotide Sequence 5'-3')		
1	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

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The sequences were edited and assembled by DNADragon v1.6.0 software (SequentiX, Germany). The mitogenome sequence was annotated using BLAST, MITOS, and DOGMA web servers [17]. Base-composition statistics of the mitogenome were calculated using DAMBE v6.3.17 software [18].

To analyse evolution of Muong Lay black pig breeds, 16 sequences were used, which were downloaded from the GenBank of the other domestic and wild board (WB) pigs in Vietnam and the rest of the world (Table 2). The mitochondrial sequence of African Warthog (Phacochoerus africanus)- a wild member of the pig family (Suidae) (accession number: DQ409327.1) was used as an outgroup. Multiple alignments of mtDNA sequences were performed using MUSCLE algorithm [19]. The best-fit models of nucleotide substitution was identified as TIM1+G for whole mtDNA sequence using jModelTest with NNI topology search in Cipres Gateway [20]. The alignment sequences were analysed in RAxML version 8.2.10 [21] to estimate the best maximum likelihood phylogenetic tree, with 1,000 bootstrap replicates, estimated GTRGAMMA model and the rapid bootstrapping algorithm. Finally, Fig. Tree v1.4.2 software was used to read exported format file for the phylogenetic tree construction.

Table 2: Pig breeds, their geographic definitions and sequence components.

Geographic Definitions	SeqName	Genbank ID	Full Sequence		
			%AT	GC Skew	AT Skew
Vietnam	Muong Lay black pig	KX147101	60.89	-0.332	0.148
	I	KX094894	60.65	-.0332	0.149
	Mong Cai	KU556691	60.77	-0.331	0.146
	Muong Khuong	KY432578	60.76	-0.332	0.148
	Huong	KY964306	60.77	-0.333	0.148
North East Asia	WB-Korea	AY574047.1	60.81	-0.33	0.15
Yellow River Valley	Bamei	EF545583.1	60.79	-0.33	0.15
Mekong Region	Banna mini	GQ220328.1	60.72	-0.33	0.15
	WB-Vietnam	EF545584.1	60.74	-0.33	0.15
South China	Lantang	KC250274	60.78	-0.33	0.15
	Lanyu	DQ518915.2	60.75	-0.30	0.12
Yangtze River Region	Bihu	EF545591.1	60.19	-0.31	0.14
	WB-Jiangxi	EF545579.1	60.78	-0.33	0.15
European Country	Duroc	AY337045.1	60.68	-0.33	0.15
	Hampshire	AY574046.1	60.67	-0.33	0.15
	Landrace	AF034253.1	60.72	-0.33	0.15
	Large White	KC250275	60.73	-0.33	0.15

Results and Discussion

The complete mtDNA genome of Muong Lay black pig sequence from this study was deposited to the NCBI GenBank with accession number KX147101. The genome of Muong Lay black pig breed was 16,742bp in total length, containing 13 protein-coding genes, 22 transfer RNA (tRNA) genes, 2 ribosomal RNA (rRNA) genes and non-coding regions. The overall base composition was Adenosine (A) (34.90%), Cytidine (C) (26.09%), Guanosine (G) (13.02%), and Thymidine (T) (25.99%), and the percentage of A and T (60.89%) was higher than G and C percentage. The D loop region was 1,314 bp in length and located between tRNA-Phe and tRNA-Pro. The lengths of twenty two tRNA genes ranged from 59 to 75bp. The locations, size and anti codon of genes in the mtDNA genome are given in Table 1. Like other vertebrate animals, the order and composition of full mtDNA sequence of this indigenous Vietnamese pig were similar to most mitochondrial genomes [22-25]. Genes were encoded on the heavy strand except for ND6 and eight tRNA genes, which were on the light strand.

The nucleotide content of the entire mitogenome sequence of Muong Lay black pig was biased toward A + T rich that could

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be explained by the initiation of replication and transcription in accordance with mitogenomes of other Asia pigs [26]. The AT content was used to study the nucleotide compositional behaviour of mitochondrial genomes, and related to phylogenetic analysis [27], in order to show the difference of Muong Lay black pig component indexes, which may be linked to the little genetic variation of Muong Lay black pig as compared to the taxa of other pig breeds. In this study, the GC skew and AT skew were also calculated and used as indexes to estimate the genetic variant in finding the relatedness of phylogeny [28,29]. But the little difference of the GC skew and AT skew in Muong Lay black pig as compared to other pig breeds was not sufficient in drawing significant conclusion. There were seventeen repeat sequences (5'-tacacgtgcg) in the non-coding region that were different from other pig breeds such as Japanese Wild boar (1 repeat), Large White (6 repeats), Landrace (13 repeats) and Duroc (10 repeats). The larger the repeat motif number, the more chances for hairpin structures, leading to errors in the copying process [30].

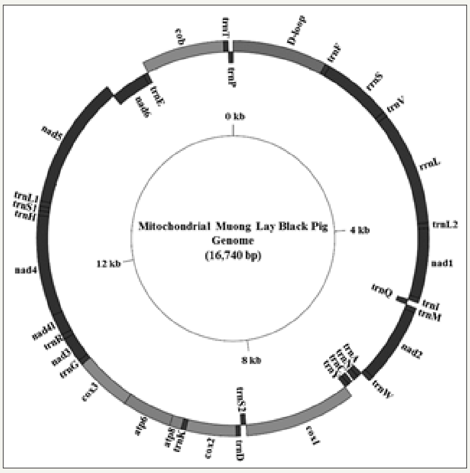

Figure 1: The circular map of the mt genome of Muong Lay Black pig used GenomeVx.

As illustrated in phylogenetic trees of the complete coding sequence (Figure 1), the overview of the maximum likelihood topology showed that the bootstrap of nodes was higher than 70 bootstrap percentage (BP) except for the node of Lantang and Huong pigs (but it was still more than 50BP). Therefore the branches of the_phylogenetic tree were well supported and the genetic relationships were certain [31]. The root of the phylogenetic tree was determined by using Warthog as an outgroup, which showed the independent development of two big groups of Sus scrofa depending on the geography, either in Asia or Europe [32,33]. In the Asian clade, Muong Lay and four other domestic pigs were joined to three subclades. In the first group, I pig was close to Banna mini pig and WB-Vietnam, which the bootstrap support of 100 and 91BP respectively. The second group contained Lantang pig and two Vietnamese pig breeds as Huong (bootstrap -54BP) and Mong Cai pig (bootstrap -97BP). The last group included Muong Lay, Muong Khuong pig (bootstrap -100BP) and two Chinese pig breeds are Bihu (bootstrap -83BP) and Bamei (bootstrap - 89BP). In addition, WB Jiangxi branch was between the first and second subclade (bootstrap - 97BP), while WB Korea and Lanyu pig fell out of three sub clades with bootstrap support of 98 and 95BP, respectively. These results showed the possibility that there may be other subclades.

Using the published sequence of pig breeds from different areas as reference points makes it easy to validate the results. Vietnam is an Asian country, so there is a high probability that Vietnamese domestic pigs were joined in the Asian clade. For instance, the I pig ("Ỉ" is the name of Vietnamese indigenous pig breed) had close genetic relationship with Banna mini - a pig breed from Mekong region in Vietnam. The Muong Lay black pigs and three other Vietnamese pig breeds were close to Chinese domestic pigs. While Huong and Mong Cai pig breeds were close to Lantang - a South China Region pig breed, other Muong Khuong and Muong Lay black pig breeds were close to Bihu and Bamei -breeds from two regions in Central China.

Based on the results shown in Figure 1, Muong Lay black pig breeds have close relationships with other pig breeds such as Muong Khuong (in Vietnam), and Bihu of Yangtze River Region (in China), with the bootstrap range of 83 to 100BP amongst these breeds. This study also found some interesting similarities in the morphological characteristics between the Muong Lay black pig and the pig breeds in the Yangtze River Region (e.g., small body weight around 50 to 60kg/individual mature; thin skin around 0.45cm; early maturing from 3 to 4 months old for female pigs or from 40 to 50 days old for male pigs; an average offspring size of 13 and seven pairs of teats) [34,35]. On the other hand, the European pig breeds (e.g., Landrace, Large-White, Hampshire, and Duroc) are usually over the 200kg body weight when mature, an offspring size of 10 to 12 individuals, and a maximum of six pairs of teats [36]. A hypothesis for these traits might be from thousands of years ago when pigs were traded by the nearby Vietnam-China border regions, where the Yangtze River Region pig breeds entered Vietnam and gradually developed into a Vietnamese domestic pig breed. However, it can be difficult to determine fully the original relationship between Muong Lay black pig, Bihu and pig breeds as genetic markers were not included in this study Figure 2 & Table 2,3.

Muong Lay black pig was also sister taxon with Bamei pig- a specific Yellow River region pig breed in East Asia region. These results may contribute additional evidence to the hypothesis about original East Asia of Vietnamese pig breeds [37]. In a previous study Hongo et al. [38] reported that Vietnamese pigs were genetically diverse and may be descendent of wild and domestic pigs from other regions of Asia. However, several wild boar subspecies inhabiting East Asia have been domesticated from WB, with such domestication occurring repeatedly from 6000 to 9000 years ago. Thus, our study suggest that Vietnamese pigs may share a common ancestor with other East Asian domestic pigs. However, further studies need to be carried out into genetic indicators such as synthetic biology, metabolic engineering, genome engineering, and genome editing [39] to affirm the origin of indigenous pigs in Vietnam.

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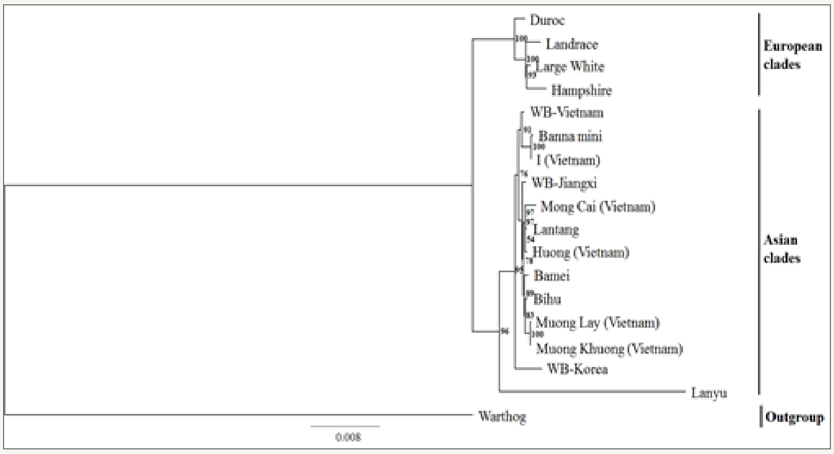

Figure 2: The phylogenetic relationship was analysed Maximum likelihood inference in RAXML software version 8.2.10 and Fig. Tree version 1.4.2 by comparison of the mitogenome of Muong Lay black breed with other pig breeds. Abbreviations of pig breeds are the same in the Table 2.

Table 3: Genes and their location in the mitochondrial genome of the Muong Lay black pig.

Name of Genes	Location		Length (bp)	Start Codon	Stop Codon	Anticodon	AT-Skew/ GT-Skew	Strand
	Start	Stop						
Dloop	1	1304	872					H
tRNA Phe	1305	1374	70			GAA		H
12s rRNA	1375	2336	962					H
tRNA Val	2336	2403	68			TAC		H
16S rRNA	2402	3972	1571					H
tRNA Leu2	3973	4047	75			TAA		H
ND1	4056	5000	945	ATG	TAG		0.14/-0.41	H
tRNA Ile	5005	5073	69			GAT		H
tRNA Gln	5071	5143	73			TTG		L
tRNA Met	5145	5214	70			CAT		H
ND2	5215	6253	1044	ATT	TAG		0.17/-0.43	H
tRNA Trp	6258	6325	68			TCA		H
tRNA Ala	6332	6399	68			TGC		L
tRNA Asn	6401	6475	75			GTT		L
tRNA Cys	6508	6573	66			GCA		L
tRNA Tyr	6574	6638	65			GTA		L
COX1	6640	8178	1539	ATG	TAA		0.08/-0.27	H
tRNA Ser2	8188	8256	69			TGA		L
tRNA Asp	8264	8331	68			GTC		H
COX2	8332	9012	681	ATG	T- -		0.15/-0.34	H
tRNA Lys	9020	9086	67			TTT		H
ATPase8	9088	9282	195	ATG	TAA		0.23/-0.57	H
ATPase6	9249	9923	675	ATG	TAA		0.08/-0.43	H
COX3	9929	10711	783	ATG	TA-		0.008/-0.28	H
tRNA Gly	10713	10782	70			TCC		H
ND3	10783	11127	345	ATA	TA		0.09/-0.41	H
tRNA Arg	11130	11198	69			TCG		H
ND4l	11214	11492	279	GTG	TAA		0.11/-0.42	H

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ND4	11489	12856	1368	ATG	T--		0.07/-0.45	H
tRNA His	12867	12935	69			GTG		H
tRNA Ser1	12936	12994	59			GCT		H
tRNA Leu1	12995	13064	70			TAG		H
ND5	13065	14880	1800	ATA	TAA		0.10/-0.42	H
ND6	14876	15391	516	ATG	TAA		0.48/-0.25	L
tRNA Glu	15398	15466	69			TTC		L
Cytb	15471	16604	1134	ATG	AGA		0.04/-0.34	H
tRNA Thr	16611	16678	68			TGT		H
tRNA Pro	16678	16742	65			TGG		L

bp: Base Pairs; Heavy strand: H and Light strand: L; rRNA: Ribosomal RNA; rrnL: Large rRNA Subunit (16S); rrnS: Small rRNA Subunit (12S); tRNA: Transfer RNA and italic Words are Replaced by One 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

TA- and T-- mean/ indicates the incomplete termination codon (the incomplete stop codon, that is, amino acid translation is terminated when the gene forms a stop codon by post-transcriptional polyadenylation).

In the phylogenetic tree, Muong Lay black pig and other Vietnamese pig breeds also fell in the same sub-group as Chinese pigs, whereas it showed a significant difference to European pig groups. The Chinese domestic pig populations were derived from multiple Asian ancestral origins whereas the European domestic pig populations represent a single ancestral European lineage [40]. Therefore, Muong lay black pig probably belongs to the Asian type which differs from the European breeds.

Conclusion

By studying the mitogenome sequences and phylogenetic analysis of Muong Lay black pig in comparison to other Asian and European pig breeds, this study revealed a close relationship between the Muong Lay black pig breed and the pigs from Yellow River Valley region and Yangtze River Region in East Asia. A hypothesis was posed that the Muong Lay black pig's origin belongs to East Asia region, but further research is needed for more clarity.

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