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Ancient DNA of pigs in Thailand: Evidence of multiple origins of Thai pigs in the late Neolithic period

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ABSTRACT: Pigs, the principal sources of meat for humans, have been crucial to cultures throughout Asia, especially in China and SE Asia, since prehistoric times. Several archaeological studies have used pig remains to elucidate the origin, culture, social evolution, and migration patterns of Asiatic people. However, ancient DNA of these remains in central SE Asia, and in Thailand in particular, has not been investigated to test the historical theories resulting from these archaeological studies. Here, we investigate ancient DNA of pig remains excavated from Pong Takhop archaeological site, central Thailand aged at least 3000 BP. The phylogenetic tree we obtained suggests that ancient Thai pigs were descended from ancient Chinese pigs. The tree topology further suggests that these ancient pigs had multiple origins, which were probably generated by multiple waves of migration of ancient Chinese pigs from 4000–3000 BP. Most of these ancient Thai pigs left their lineages as modern Thai pigs observed in northern Thailand. The contrasting cluster of pure modern Thai pigs suggested that these pigs might be descended from non-Chinese ancestors, possibly the native SE Asian ancestors.

KEYWORDS: *Sus scrofa*, hypervariable region, phylogenetic relationship

INTRODUCTION

Pig is one of the principal sources of protein for human beings. Native European and Asian wild boars have been brought in the process of domestication since 6000 BP[1] and 9000–5000 BP[2], respectively. In other regions including China, indigenous wild boars were brought in as early as several thousand years ago and their lineages still exist as modern domesticated pigs in the area. Domesticated pigs in one region would not always descend from local wild boars because domesticated pigs of a region could be relocated to another region via human migration. Previous studies reported that domesticated pigs from Near East were introduced into Europe during the Neolithic period[1] and 8000 BP[3]. In addition, there was indication that Chinese domestic pigs were distributed southward to South and Southeast Asian countries[4]. Hence historical studies regarding pig dispersal and pig domestication could imply human demographic history and socio-cultural evolution, especially the shift from the hunter-gatherer culture to the agricultural culture.

In China, the origin of pig husbandry happened around 8000 BP in various regions, ranging from north-east to south-east China[5–8]. Chinese domesticated pigs have been distributed southward during 5000–4000 BP. In Thailand, the discovery of several pig remains within human graves in many archaeological sites of Thailand suggests that pig had been involved in Thai people culture as ceremonies and rituals since prehistoric times[9]. Archaeological studies reported that the beginning of pig husbandry might have occurred as early as 4000 BP. These findings were not based on the evidence indicating the transition from the hunter-gatherer culture to the agriculture culture. No evidence of introducing native wild boars to initiate the process of pig domestication in Thailand has been observed. On the contrary, a number of archaeological evidence suggested the migration of domesticated pigs from other countries,

especially China, to Thailand.

In the context of molecular genetic analysis, mitochondrial control region (mtCR) sequences, mitochondrial cytochrome b sequences and microsatellite markers were used to study the relationship between Thai native pigs and other pigs. These studies independently indicate that Thai native and Chinese domesticated pigs are closely related. The findings correspond to the similarity of the phenotypic characteristics between these modern pigs. The results of phylogenetic tree analyses also further suggest that these pigs have common ancestors. However, the results of these molecular genetic analysis is only restricted to the relationships between modern Thai and Chinese pigs but not the prehistoric time.

The study of ancient DNA from human and animal remains has always been challenged by the extensive fragmentation of ancient DNA and the contamination of soil. Especially in the tropical area, this kind of study has been even more challenged by severely degraded ancient DNA because a high temperature and humidity in that accelerate the process of DNA degradation[10]. Only short DNA fragments with the size of 100–500 bp could survive[11] and the quantity of DNA fragments with the size larger than 141 bp has been reported to exponentially decrease[12]. Because human and animal remains were usually found underground, the contamination of these archaeological samples by soil is always observed. This contamination could cause complication in molecular study on ancient DNA by inhibiting PCR amplification[13]. Despite the impediment to study ancient DNA, the successful amplification of partial mitochondrial DNA (mtDNA) extracted from human and animal remains excavated from archaeological sites in tropical regions (for example, Latin America, Africa, and Asia) has been reported. In Thailand, a study reported successful amplified partial mtCR sequences from human remains dated 3200–1800 BP[14]. Until recently, no genetic study on animal remains excavated from archaeological sites in Thailand has been reported.

Even though the relationship between Thai and Chinese pigs was revealed in previous studies[15,16], no such relationship on the ancient Thai and Chinese pigs nor the ancient and modern Thai pigs have been reported. These findings are necessary to complete the picture of how Thai pigs are related to Chinese pigs. This study aims to provide the first step to understand these relationships by analysing DNA sequences of Thai pig remains excavated from Pong Takhop archaeological site located in central Thailand. The analysis consisted of two main parts: exploring the principal features of the ancient DNA sequences and analysing the association of these ancient Thai pigs to the ancient Chinese pigs previously reported by Larson et al[17] as well as to the modern Thai pigs reported by Charoensook et al[15] and Larson et al[1,18].

MATERIALS AND METHODS

Sample collection

Molar and canine of 13 pig remains were collected from the Pong Takhop archaeological site. The independence of each pig remains was verified by anatomical analysis. The approximated age of these samples was based on the age of the Pong Takhop archaeological site.

The Pong Takhop archaeological site is situated at 14° 50′ 08″ N, 101° 11′ 04″ E in Wang Muang district, Saraburi province, Thailand. This site was first excavated in May 2009 by the Department of Archaeology, Faculty of Archaeology, Silpakorn University, Thailand. Mammal remains discovered in this site included bones of pigs, deer, cattle, monkeys and dogs. Many prehistoric artefacts including pottery decorated with curvilinear incised and rocker-stamped designs, beads made of marine shell, ceramic assemblage, and polished stone adzes were observed at this site. These relics were considered as typical artefacts of the late 4th to the early 3rd millennium before Common era (BC) cultures in central Thailand[19]. In addition, the absence of bronze artefact and semi-precious stone beads indicated that this archaeological site was occupied by a large community aged at least 3000 BP (uncalibrated) or in the late Neolithic period (Natapintu, unpublished data).

Prevention of DNA contamination

During the step of sample preparation, the ancient pig remains were sterilized by soaking in 10% sodium hypochlorite solution for 15 min, washing twice with sterile distilled water, then exposing to UV light for 20 min on each surface side. The surface of these samples was further cleaned by sterilized mini drill before grinding by a sterilized mortar and pestle. This process was done to reduce soil contamination.

The steps of DNA extraction and PCR reaction preparation were performed in a laminar flow that was cleaned by 70% alcohol and DNA AWAY Surface Decontaminants (MBP-QSP, USA). The laminar flow was previously cleaned by exposing to UV light for two hours. All micropipettes and other instruments used in these processes were also cleaned by 70% alcohol and DNA AWAY Surface. The cleansed micropipettes, disposable gloves and boxes of filtered tips were exposed to UV light for 60 min. All PCR

reagents were aliquoted and used only once.

Disposable groves were used in every step of sample preparation, DNA extraction and PCR preparation. Filtered tips and all common reagents were separated for using in pre-PCR and post-PCR procedure.

DNA extraction

Approximately 1 g of tooth powder per sample was used for DNA extraction. Total DNA of each sample was extracted by ZymoBead Genomic DNA Kit (Zymo Research Corporation, USA) according to the manufacturer's instructions and directly used as PCR template. To ensure the devoid of external DNA contamination during DNA extraction, blank controls were used to compare with all samples.

PCR amplification and DNA sequencing

The hypervariable region of mitochondrial DNA, the partial mtCR, was amplified by PCR using a primer pair; L180 (5′-TGCTAGTCCCCATGCATA-TAA-3′) and R358 (5′-CCTGCCAAGCGGGTTGC-TGG-3′)[17]. PCR amplification was carried out in a reaction mixture of 20 µl containing 2 µl of ancient DNA, 10 µl of 2× QIAGEN Multiplex PCR Master Mix (QIAGEN, Germany), 20 µg of bovine serum albumin and 25 pmol of each primer. The PCR reaction mixture was incubated at 95 °C for 15 min; 40 cycles of 95 °C for 30 s, 51.3 or 54.5 °C for 90 s, 72 °C for 90 s; and final extension at 72 °C for 10 min. To authenticate the PCR products, blank extract and sterile distilled water were used as negative controls. The reproducibility of each PCR product was verified by independently repeating the PCR process. PCR products were examined on 2% agarose gel (Bio-Rad, USA), purified by HiYield Gel/PCR DNA Fragments Extraction Kit (Real Biotech Corporation, Taiwan), and sequenced by Macrogen (Korea) (www.macrogen.com).

Data analysis

The length of the PCR product is 179 bp including 41 bp of primer sequence, thus this primer sequence was removed prior to sequence analysis. The 138-bp sequence of each sample was blasted against GenBank data sequences to verify whether it was a sequence of partial mtCR. Then, the 138-bp sequences of these pig remains excavated from the Pong Takhop archaeological site were aligned with the partial mtCR sequences of 18 ancient Chinese pigs reported by Larson et al[17] and of 92 modern Thai pigs reported by Charoensook et al[15] and Larson et al[1,18] using CLUSTALW version 1.83[20]. The profiles of 18 ancient Chinese pigs and 92 modern Thai pigs are presented in Table 1. The phylogenetic tree was constructed using neighbour-joining method[21]. The substitution model used in this study is the Kimura 2-parameter model[22]. The phylogenetic tree was constructed using MEGA version 5.0[23] and bootstrap values were derived from 1000 replications[24].

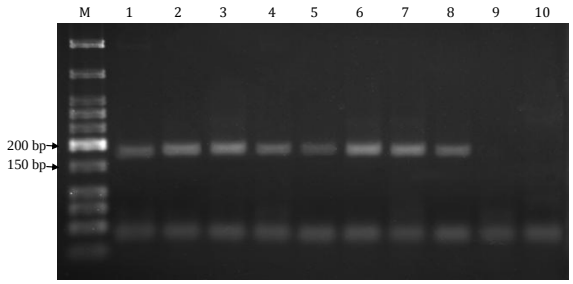

Fig. 1 The PCR products of ancient Thai pigs excavated from Pong Takhop archaeological site (PTKs). Lane M presented a low molecular weight DNA ladder. Lanes 1–8 presented PCR products (179 bp) of PTK01, 03, 04, 05, 07, 08, 09, and 10. Lanes 9 and 10 presented PCR product of blank extraction and blank control, respectively.

RESULTS

The hypervariable region of mtCR (179 bp) of 13 pig remains excavated from the Pong Takhop archaeological site was amplified and the results showed that only 10 out of 13 samples could be successfully amplified (Table 2). However, only 8 out of 10 samples could be well amplified (Fig. 1). These 8 samples were PTK01, 03, 04, 05, 07, 08, 09, and 10. No PCR product could be amplified from the negative control, while all 8 successful PCR products were reproducible. These partial mtCR sequences were blasted against GenBank database sequences and the results confirmed that they were partial mtCR of *S. scrofa* with the E-value of 2×10^{-85} to 7×10^{-84} (Table 2). Since the 179 bp sequences included 41 bp of primers, the 41 bp primer sequences were excluded prior to being analysed and submitted to Genbank database. The accession numbers of these partial mtCR sequences (138 bp) were presented in Table 2.

Besides these 8 partial mtCR sequences of PTKs, additional 18 sequences of ancient Chinese pigs[17] and 92 sequences of modern Thai pigs[1,15,18] were included in the phylogenetic analysis (Fig. 2). MH, JT, OK, Fa, CD, LP, CS, VC, NP, FT are name codes of Thai domesticated pigs from Amphur Muang in Mae Hongson province (MH), Amphur Jhom Thong (JT), Om Koi (OK), Fang (Fa), and Chiang Dao (CD) in Chiang Mai province, Amphur Tung Huang in

Table 1 Profiles of 110 mtDNA CR sequences of 18 ancient Chinese pigs, 78 modern Thai domesticated pigs, and 14 Thai wild boars from GenBank included in this study.

Code name	Accession no.	Country	Location	Status	Breeds/Age (BP)	Reference
GC05	FJ601530	China	Xinmi city/Henan	Ancient	4150–3950	17
GC06	FJ601531	China	Xinmi city/Henan	Ancient	4150–3950	17
GH01	FJ601532	China	Liulin county/Shanxi	Ancient	3500–3200	17
GH02	FJ601533	China	Liulin county/Shanxi	Ancient	3500–3200	17
GH04	FJ601534	China	Liulin county/Shanxi	Ancient	3500–3200	17
GH05	FJ601535	China	Liulin county/Shanxi	Ancient	3500–3200	17
GH06	FJ601536	China	Liulin county/Shanxi	Ancient	3500–3200	17
GH07	FJ601537	China	Liulin county/Shanxi	Ancient	3500–3200	17
JH01	FJ601538	China	Wuyang county/Henan	Ancient	9000–8600	17
JH02	FJ601539	China	Wuyang county/Henan	Ancient	8600–8200	17
TS01	FJ601540	China	Xiangfen county/Shanxi	Ancient	4350–3850	17
TS02	FJ601541	China	Xiangfen county/Shanxi	Ancient	4350–3850	17
TS06	FJ601542	China	Xiangfen county/Shanxi	Ancient	4350–3850	17
TS07	FJ601543	China	Xiangfen county/Shanxi	Ancient	4350–3850	17
WC05	FJ601544	China	Dengfeng/Henan	Ancient	3550–3100	17
WD01	FJ601545	China	Yuzhou city/Henan	Ancient	4150–3950	17
WD04	FJ601546	China	Yuzhou city/Henan	Ancient	4150–3950	17
WD05	FJ601547	China	Yuzhou city/Henan	Ancient	4150–3950	17
TWB01	AM779933	Thailand	Ban Luang/Nan	Wild		15
TWB02	AM779934	Thailand	Ban Luang/Nan	Wild		15
TWB03	AM779935	Thailand	Ban Luang/Nan	Wild		15
TWB04	AM779936	Thailand	Ban Luang/Nan	Wild		15
TWB05	AM779937	Thailand	Ban Luang/Nan	Wild		15
TWB06	FM244683	Thailand	Mae Sariang/Mae Hongson	Wild		15
TWB07	FM244684	Thailand	Mae Sariang/Mae Hongson	Wild		15
TWB08	FM244685	Thailand	Mae Sariang/Mae Hongson	Wild		15
TWB09	FM244686	Thailand	Mae Sariang/Mae Hongson	Wild		15
TWB10	FM244687	Thailand	Mae Sariang/Mae Hongson	Wild		15
TWB11	FM244688	Thailand	Mae Sariang/Mae Hongson	Wild		15
TWBG6	AY884630	Thailand	Trang Trong	Wild		18
TWBG7	DQ779403	Thailand	Klong Klung Camp	Wild		1
TWBG9	DQ779410	Thailand		Wild		1
CS01	AM774640	Thailand	Chiang San/Chiang Rai	Domestic	Thai native	15
CS02	AM774641	Thailand	Chiang San/Chiang Rai	Domestic	Thai native	15
CS03	AM774642	Thailand	Chiang San/Chiang Rai	Domestic	Thai native	15
CS04	AM774643	Thailand	Chiang San/Chiang Rai	Domestic	Thai native	15
CS05	AM774644	Thailand	Chiang San/Chiang Rai	Domestic	Thai native	15
CS06	AM777917	Thailand	Chiang San/Chiang Rai	Domestic	Thai native	15
CS07	AM777918	Thailand	Chiang San/Chiang Rai	Domestic	Thai native	15
CS08	AM777919	Thailand	Chiang San/Chiang Rai	Domestic	Thai native	15
VC01	AM777920	Thailand	Viang Chai/Uttaradit	Domestic	Thai native	15
VC02	AM777921	Thailand	Viang Chai/Uttaradit	Domestic	Thai native	15
VC03	AM777922	Thailand	Viang Chai/Uttaradit	Domestic	Thai native	15
Fa01	AM777923	Thailand	Fang/Chiang Mai	Domestic	Thai native	15
Fa02	AM777924	Thailand	Fang/Chiang Mai	Domestic	Thai native	15
Fa03	AM777925	Thailand	Fang/Chiang Mai	Domestic	Thai native	15
Fa04	AM777926	Thailand	Fang/Chiang Mai	Domestic	Thai native	15
MH01	AM778824	Thailand	Muang/Mae Hongson	Domestic	Thai native	15
MH02	AM778825	Thailand	Muang/Mae Hongson	Domestic	Thai native	15
MH03	AM778826	Thailand	Muang/Mae Hongson	Domestic	Thai native	15
MH04	AM778827	Thailand	Muang/Mae Hongson	Domestic	Thai native	15
MH05	AM778828	Thailand	Muang/Mae Hongson	Domestic	Thai native	15
MH06	AM778829	Thailand	Muang/Mae Hongson	Domestic	Thai native	15
JT01	AM779904	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
JT02	AM779905	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
JT03	AM779906	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
JT04	AM779907	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
JT05	AM779908	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
JT06	AM779909	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
JT07	AM779910	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
JT08	AM779911	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
JT09	AM779912	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
JT10	AM779913	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
JT11	AM779914	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
JT12	AM779915	Thailand	Jhom Thong/Chiang Mai	Domestic	Thai native	15
LP01	AM779916	Thailand	Tung Huachang/lamphun	Domestic	Thai native	15
CD01	AM779917	Thailand	Tung Huachang/lamphun	Domestic	Thai native	15
CD02	AM779918	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD03	AM779919	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD04	AM779920	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD05	AM779921	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD06	AM779922	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD07	AM779923	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15

Table 1 (Cont.)

Code name	Accession no.	Country	Location	Status	Breeds/Age (BP)	Reference
CD08	AM779924	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD09	AM779925	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD10	AM779926	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD11	AM779927	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD12	AM779928	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD13	AM779929	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD14	AM779930	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD15	AM779931	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
CD16	AM779932	Thailand	Chiang Dao/Chiang Rai	Domestic	Thai native	15
FT01	FM244466	Thailand	Fak Tha/Uttaradit	Domestic	Thai native	15
FT02	FM244467	Thailand	Fak Tha/Uttaradit	Domestic	Thai native	15
FT03	FM244468	Thailand	Fak Tha/Uttaradit	Domestic	Thai native	15
FT04	FM244469	Thailand	Fak Tha/Uttaradit	Domestic	Thai native	15
FT05	FM244470	Thailand	Fak Tha/Uttaradit	Domestic	Thai native	15
FT06	FM244471	Thailand	Fak Tha/Uttaradit	Domestic	Thai native	15
FT07	FM244472	Thailand	Fak Tha/Uttaradit	Domestic	Thai native	15
FT08	FM244473	Thailand	Fak Tha/Uttaradit	Domestic	Thai native	15
FT09	FM244474	Thailand	Fak Tha/Uttaradit	Domestic	Thai native	15
FT10	FM244475	Thailand	Fak Tha/Uttaradit	Domestic	Thai native	15
OK01	FM244491	Thailand	Om Koi/Chiang Mai	Domestic	Thai native	15
OK02	FM244492	Thailand	Om Koi/Chiang Mai	Domestic	Thai native	15
OK03	FM244493	Thailand	Om Koi/Chiang Mai	Domestic	Thai native	15
OK04	FM244494	Thailand	Om Koi/Chiang Mai	Domestic	Thai native	15
OK05	FM244495	Thailand	Om Koi/Chiang Mai	Domestic	Thai native	15
OK06	FM244496	Thailand	Om Koi/Chiang Mai	Domestic	Thai native	15
OK07	FM244497	Thailand	Om Koi/Chiang Mai	Domestic	Thai native	15
NP01	FM244672	Thailand	Nam Pat/Uttaradit	Domestic	Thai native	15
NP02	FM244673	Thailand	Nam Pat/Uttaradit	Domestic	Thai native	15
NP03	FM244674	Thailand	Nam Pat/Uttaradit	Domestic	Thai native	15
NP04	FM244675	Thailand	Nam Pat/Uttaradit	Domestic	Thai native	15
NP05	FM244676	Thailand	Nam Pat/Uttaradit	Domestic	Thai native	15
NP06	FM244677	Thailand	Nam Pat/Uttaradit	Domestic	Thai native	15
NP07	FM244678	Thailand	Nam Pat/Uttaradit	Domestic	Thai native	15
NP08	FM244679	Thailand	Nam Pat/Uttaradit	Domestic	Thai native	15
NP09	FM244680	Thailand	Nam Pat/Uttaradit	Domestic	Thai native	15
NP10	FM244681	Thailand	Nam Pat/Uttaradit	Domestic	Thai native	15
NP11	FM244682	Thailand	Nam Pat/Uttaradit	Domestic	Thai native	15

Table 2 Profiles of 13 ancient pig samples excavated from Pong Takhop archaeological site (PTK) in Wang Muang district, Saraburi province, Thailand.

Extraction	BLAST result		Element	PCR	Sequence	Accession
No.	Species	E-value			obtained (bp)	No.
PTK1	*Sus scrofa*	2×10^{-85}	Molar	Yes	179	JQ429497
PTK2	–	–	Molar	No	–	–
PTK3	*S. scrofa*	2×10^{-85}	Molar	Yes	179	JQ429498
PTK4	*S. scrofa*	2×10^{-85}	Molar	Yes	179	JQ429499
PTK5	*S. scrofa*	2×10^{-85}	Canine	Yes	179	JQ429500
PTK6	–	–	Molar	Yes	Poor quality	–
PTK7	*S. scrofa*	2×10^{-85}	Molar	Yes	179	JQ429501
PTK8	*S. scrofa*	2×10^{-85}	Molar	Yes	179	JQ429502
PTK9	*S. scrofa*	2×10^{-85}	Molar	Yes	179	JQ429503
PTK10	*S. scrofa*	7×10^{-84}	Molar	Yes	179	JQ429504
PTK11	–	–	Canine	Yes	Poor quality	–
PTK12	–	–	Canine	No	–	–
PTK13	–	–	Molar	No	–	–

Lamphun province (LP), Amphur Chiang San (CS) and Viang Chai (VC) in Chiang Rai province, Amphur Nam Pat (NP) and Fak Tha (FT) in Uttaradit province. GC, JH, WC, WD, GH, and TS are name codes for the ancient Chinese pigs from Guchengzhai (GC) archaeological sites in Xinmi city, Henan province, Jiahu (JH) archaeological sites in Wuyang county, Henan province, Wangchenggang (WC) archaeological sites in Dengfeng county, Henan province, Wadian (WD) archaeological sites in Yuzhou city, Henan province,

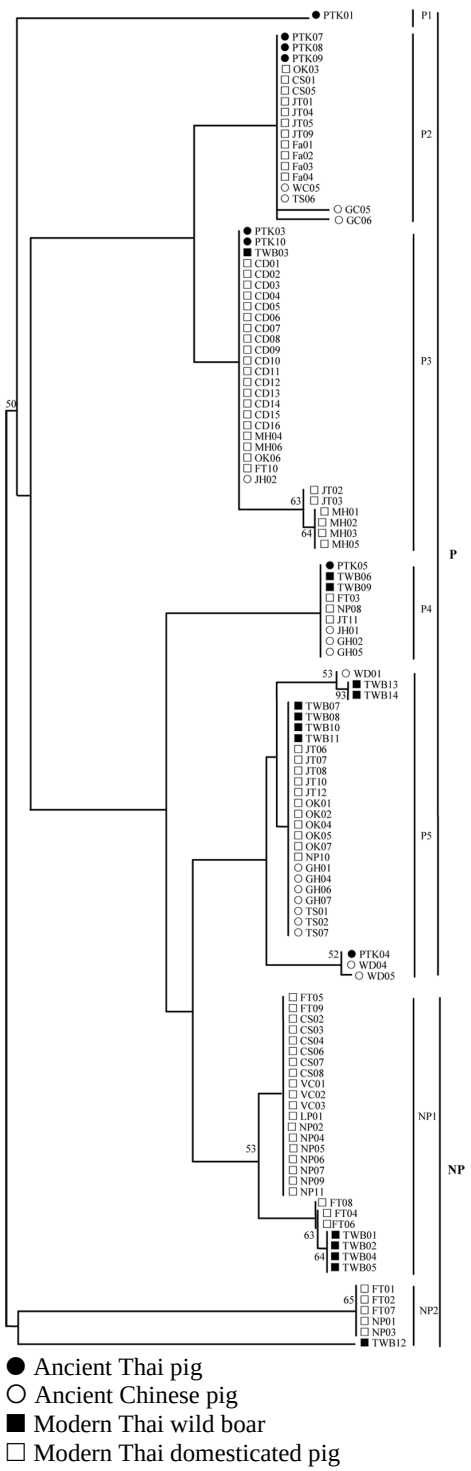

Fig. 2 A neighbour-joining phylogenetic relationship of the partial control region sequences amplified from 8 ancient Thai pigs (PTK), 18 ancient Chinese pigs, and 92 modern Thai pigs. Numbers presented at the nodes represented bootstrap values ($\geqslant$ 50%) derived from 1000 replications. See text for name codes.

Gaohong (GH) archaeological sites in Luilin county, Shanxi province and Taosi (TS) archaeological sites in Xiangfen county, Shanxi province. The tree topology suggested that these 118 partial mtCR sequences could be separated into two clusters. The separation of these clusters corresponded well to the association of modern Thai pigs to PTKs, so we named these clusters as PTK related (P) and non-PTK related (NP) clusters. The P cluster could be further divided into 5 subclusters: P1–P5 subclusters. Each P subcluster contained at least one PTK, ancient Chinese pigs, and modern Thai pigs, except for P1 subcluster that contained only PTK01. Interestingly, one branch of P5 subcluster contained only ancient pigs: PTK04, WD04, and WD05. The NP cluster was the pure modern Thai pigs cluster. This cluster could be further separated into two subclusters: NP1 and NP2. The haplotypes containing these 118 pigs were shown in Table 3.

DISCUSSION

The primary challenge of this study is the analysis of ancient DNA molecules extracted from animal remains excavated from the archaeological site located in the tropical area. These ancient DNA molecules tended to be severely fragmented due to the high temperature and humidity which accelerated the degradation process[10]. As shown in other studies on ancient DNA, the length of analysed sequences was limited to 100–500 bp[11]. In our study, the length of hypervariable region, a part of mtCR that has the highest sequence variation, was 179 bp, within the range of generally analysed ancient DNA sequences. The partial CR sequences of 8 out of 13 samples could be well amplified. This successful outcome would be the results of using mini-drill to remove surface soil particles from teeth samples during pretreatment steps. The mtDNA molecules of other 4 unsuccessfully amplified samples may be severely degraded. The purity and authenticity of 8 eight amplified sequences were confirmed by PCR and BLAST analysis.

Phylogenetic tree analysis was applied to disclose the relationship between these 8 ancient Thai pigs (PTKs) and 18 ancient Chinese pigs. Since 92 modern Thai pigs were also included in this analysis, the tree topology provided information regarding not only the relationship between ancient pigs but also the connection between these ancient pigs and modern Thai pigs. The tree topology contained two main clusters: PTKs-related (P) and non-PTKs-related (NP) clusters. All PTKs occurred in P subclusters, except PTK01 was found in subcluster P1, showing a tight

Table 3 Haplotype distributions of mtDNA control region of 8 ancient Thai pigs in this study and those of 18 ancient Chinese and 92 modern Thai pigs from GenBank database.

Group	Subgroup	Haplotype	Name code[†]
P	P1	H1	PTK01
	P2	H2	PTK07, PTK08, PTK09, OK03, CS01, CS05, JT01, JT04, JT05, JT09, Fa01, Fa02, Fa03, Fa04, WC05, TS06
		H3	GC05
		H4	GC06
	P3	H5	PTK03, PTK10, TWB03, CD01–CD16, MH04, MH06, OK06, FT10, JH02
		H6	JT02, JT03
		H7	MH01, MH02, MH03, MH05
	P4	H8	PTK05, TWB06, TWB09, FT03, NP08, JT11, JH01, GH02, GH05
	P5	H9	WD01
		H10	TWB13, TWB14
		H11	TWB07, TWB08, TWB10, TWB11, JT06, JT07, JT08, JT10, JT12, OK01, OK02, OK04, OK05, OK07, NP10, GH01, GH04, GH06, GH07, TS01, TS02, TS07
		H12	PTK04, WD04
		H13	WD05
NP	NP1	H14	FT05, FT09, CS02, CS03, CS04, CS06, CS07, CS08, VC01, VC02, VC03, LP01, NP02, NP04, NP05, NP06, NP07, NP09, NP11
		H15	FT08
		H16	FT04, FT06
		H17	TWB01, TWB02, TWB04, TWB05
	NP2	H18	FT01, FT02, FT07, NP01, NP03
		H19	TWB12

[†] Nomenclature of name codes of mtDNA control region of modern Thai pigs, both domesticated pigs (MH, JT, OK, Fa, CD, LP, CS, VC, NP, FT) and wild boars (TWB01–TWB11) followed by Charoensook et al [15].
Nomenclature of name codes of mtDNA control region of ancient Chinese pigs (GC, GH, JH, TS, WC, WD) followed by Larson et al [17].

relationship to at least one ancient Chinese pig. These findings support the previous analysis on modern Thai pigs that Thai and Chinese pigs are derived from a shared common ancestor [15]. A lack of correlation of PTK01 to any ancient Chinese pig indicated that this ancient Thai pig might have a different common ancestor from other ancient Thai and Chinese pigs included in this study.

Besides the tree topology, the tight relationship between PTKs and ancient Chinese pigs located in P2–P5 subclusters should be considered in the context of the relationship between the modern Thai pigs in these subclusters and the modern Chinese pigs in the D2 cluster of Larson et al [18]. The previous analysis by Charoensook et al [15] indicated that all modern Thai pigs belonged to these subclusters are members of D2 cluster [15]. The D2 cluster was originally reported as the group of Chinese pigs being widely distributed to East Asia and Southeast Asia [18]. Hence, the ancient Chinese pigs shown in this study would probably leave their lineages both in China and Thailand. Their lineages in China would be developed to modern Chinese pigs as analysed by Larson et al [18] and Tanaka et al [4], while at least parts of their lineages in Thailand would be developed to ancient Thai pigs (PTKs) that later left their lineages as modern Thai pigs.

This tight association between PTKs and ancient Chinese pigs located in P2–P5 subclusters is also supported by archaeological studies. Based on teeth morphology and archaeological evidence, the origin of pig husbandry had been proposed to happen in both northern and southern China around 8000 BP [5–8]. Once plant cultivation and animal domestication had been established, sedentary agriculture, including pig husbandry, had been distributed across regions in Northeast Asia and Central China around 6500–5000 BP and southward from the Yangtze region about 5000–4000 BP. The beginning of pig domestication in Thailand might have occurred as early as 4000 BP [25, 26], thus it would be possible that these domesticated pigs in Thailand were migrated from China around 4000 BP or earlier. This hypothesis is possible because the age of the Pong Takhop archaeological site dated by the typical artefacts observed in this area was at least 3000 BP [19].

According to the variation of ages and location

of excavated sites of the ancient Chinese pigs related to PTKs in P2–P5 subclusters, these PTKs would probably have multiple origins. PTKs in P2 and P5 subclusters were tightly associated with ancient Chinese pigs aged 4350–3100 BP from Wangchenggang archaeological site (dated 3550–3100 BP) in Dengfeng county, Guchengzhai archaeological sites (dated 4150–3950 BP) in Xinmi city, Henan province and Taosi archaeological sites (dated 4350–3850 BP) in Xiangfen county, Shanxi province, while PTKs in P3 and P4 subclusters were related to ancient Chinese pigs aged 9000–3200 BP from Jiahu archaeological sites (dated 9000–8200 BP) in Wuyang county, Henan province and Gaohong archaeological sites (dated 3500–3200 BP) in Luilin county, Shanxi province[17]. These variations of the ancient Chinese pigs related to PTKs in these subclusters suggested the possibility of either the migration of multiple populations or the multiple waves of migration of these ancient Chinese pigs to Thailand.

Several archaeological studies supported that the beginning of pig husbandry had happened independently in multiple regions of China, ranging from northern to southern China, around 8000 BP[5–8]. The analysis on mtDNA sequences of East Asian pigs also indicated that Chinese wild boars would have been independently domesticated mainly in the Mekhong and Yangtze regions[27]. These archaeological and molecular studies suggested that there might be multiple populations of ancient Chinese pigs. We might imply that these pigs migrated from China to Thailand as early as 4000 BP. Because we only knew that the time when the rice cultivation and pig domestication happened in Thailand around 4000 BP, we might further imply that these pigs migrated to Thailand only once.

It is also possible that these ancient Chinese pigs might be multiply migrated to Thailand. Archaeological studies that attempted to understand human migration from China to Thailand would support this alternative hypothesis. In north-east Thailand, pig husbandry might happen about 6000–3000 BP. The excavation studies in Non Nok Tha (Kon Kean province) and Ban Chiang (Udonthani province) archaeological sites in Thailand revealed that rice cultivation and pig domestication might happen in these areas approximately 6000 and 5600 BP, respectively. Some rice seeds and remains of domestic animal including cattle, dogs and pigs were discovered at Ban Na Di archaeological site in Udon Thani province, Thailand[28]. The age of this site was approximately 3400–3000 BP[28]. Archaeological evidence suggest that prehistoric agriculturalists, who had brought agricultural intellectuality from China, settled down in Ban Kao (Kanchanaburi province) and Non Pa Wai (Lopburi province) around 4500[29] and 4300[30] BP, respectively. Based on our results that all PTKs in P2–P5 subclusters were firmly associated with ancient Chinese pigs with various ages and the findings from these archaeological studies, we could imply multiple waves of migration of the ancient Chinese pigs along with immigrants to Thailand. Focusing only on the central Thailand, the multiple waves of migration would happen as early as 4000 BP.

Interestingly, the two main clusters, P and NP clusters, did not only separate by the relationship between PTKs and other pigs but also split by the association of modern Thai pigs with ancient pigs. The association between modern Thai pigs and ancient pigs, both Thai and Chinese pigs, was restricted to P cluster only; therefore, these 92 modern Thai pigs would possibly have multiple origins. Regarding the relationship among modern Thai pigs within each subcluster in both P and NP clusters, some subclusters that contain both domesticated pigs and wild boars could refer to the incorporation of wild boars into the process of pig husbandry. This incorporation would probably increase the fitness of the domesticated pig lineages by introducing new alleles from wild boars to domesticated pigs. Several research groups have shown that the incorporation of native wild boars into regional domesticated pigs had occurred in various regions including China and Southeast Asia.

The tightly relationship among ancient Chinese pigs, ancient Thai pigs and modern Thai pigs in P2–P5 subclusters suggested the continuation of maternal transmission of mtDNA molecules from the ancient Chinese pigs to ancient Thai pigs and down to modern Thai pigs. On the contrary, the NP cluster, the cluster of pure modern Thai pigs, suggests that these modern Thai pigs might descend from neither ancient Chinese pigs included in this study nor ancient Thai pigs (PTKs). This cluster could be further separated to NP1 and NP2 subclusters. All members of NP1 subcluster were previously reported as Thai haplogroup (THG)[15]. Charoensook et al[15] also claimed that this THG group could be formed by combining Southeast Asian pigs carrying Mountainous and Southeast Asian (MTSEA) haplotypes[15]. These MTSEA haplotypes have been considered as indigenous haplotype of Southeast Asia[4], thus this result indicated that the ancestor of modern Thai pigs in NP1 subcluster would be ancient native Southeast Asian pigs.

Within P1 and P5 subclusters, there were two PTKs: PTK01 and PTK04 that showed no connection to any modern Thai pigs. There are three possible

reasons for this lack of connection: (1) the genealogies of these ancient pigs were extinct, (2) the lineages of these ancient pigs did not live in the northern Thailand, and (3) the lineages of these ancient pigs did not live in Thailand. In the case of PTK01, every hypothesis is possible because there is no connection either to any ancient pigs or to modern Thai pigs. Hence the genealogy of this ancient Thai pig may have already been extinct or existed in other part of Thailand or other country. On the contrary, the first hypothesis is the least likely to explain the lack of connection of PTK04 to any modern Thai pigs because this ancient Thai pig carried haplotype H12 that was also detected in domesticated pigs in China, Laos, and Vietnam[17]. Even though this observation supported the third hypothesis, it could not completely exclude the second hypothesis because every modern Thai pig shown in this study was collected only from northern Thailand[15].

CONCLUSIONS

Here is the first analysis of the DNA of pig remains extracted from the late Neolithic pig remains excavated from the central part of Thailand. This study mainly focused on identifying the relationship between ancient Thai and ancient Chinese pigs. Our results suggested that all of the ancient Thai pigs, except PTK01, might descend from ancient Chinese pigs. The tree topology suggested that these ancient Thai pigs had multiple origins that generated by multiple waves of different ancient pig populations migrated from China during 4000–3000 BP. Most ancient Thai pigs left their lineages as modern Thai pigs; however, certain lineages may disappear from Thailand. The distinct cluster of pure modern Thai pigs suggested that the ancestors of these pigs live elsewhere. Some of these pigs, the members of NP1 subcluster, were possibly descended from the common ancestors of the pigs carrying MTSEA haplotypes. It is interesting to learn that genealogy of Thai pigs is more complicated than anticipated.

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