

Genetic Variation and Population Structure of Thai Indigenous Pig Populations Based on Mitochondrial and Microsatellite DNA Markers

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Abstract: To understand molecular genetic characteristics of Thai indigenous pig populations, researchers compared the genetic variation at 12 microsatellite loci and the mitochondrial DNA *Cyt b* gene in two populations of Thai indigenous pigs including Northeast Thai (NT) and South Thai (ST) pigs, a Wild Boar (WB) population and a Chinese Qianbei Black (CQB) pig population from Guizhou province of China. The higher effective number of alleles were detected in NT (7.09) and ST (6.3) pig compared to CQB (5.97) and WB (5.19) pigs. NT pigs had the highest PIC value (0.82) among all populations. The average expected Heterozygosity (HE) was 0.72 in NT and 0.71 in ST and 0.69 in WB pigs which was lower than that observed in CQB pigs (0.82). Hardy-Winberg (HWE) test showed of all 12 microsatellite loci, 7 loci were deviated from HWE ($p < 0.05$). Seventy four polymorphism sites were detected among 35 *Cyt b* gene sequences of 1046 bp in four populations. Eighteen *Cyt b* haplotypes were detected and haplotype H9 was found in CQB, NT and ST pigs. Both the F_{ST} and Nei's standard genetic Distances (Ds) from the microsatellite data indicated genetic differentiation between Thai pigs and Chinese Southwest pigs but UPGMA tree showed that Thai pigs had close relationship with Chinese pigs. Structure analysis indicated that NT populations were identified as the separate clusters while few ST individuals was assigned into the CQB population at $K = 2$. Median-joining network analysis showed that the NT population constituted predominated haplogroups (54.28%). These results suggest that Thai indigenous populations have high genetic diversity in both nuclear and mtDNA data. The close relationship between Chinese Southwest pigs and Thai pigs implies that Thai native pigs might have the same origin as pigs from South or Southwest China. These findings can be used as valuable genetic information for the preservation and further genetic improvement of the Thai indigenous pig population.

Key words: Microsatellite, mtDNA *Cyt b*, Thai indigenous pig, genetic diversity, improvement, China

INTRODUCTION

The Thai pig industry became increasingly commercialized in the 1980s. Since, that time, village production of pigs has declined and commercial units have become the main source of supply of pork in Thailand (Tisdell and Harrison, 1997). Accordingly, the number of Thai indigenous pigs has been rapidly decreasing since exotic pig breeds were introduced for breeding improvement. Thai indigenous pigs are classified into several different phenotypic groups distributing in the Northern and the Southern areas of Thailand (Kazue, 1974). Some of Thai wild boars (*Sus scrofa jubatus*) were crossed with Chinese Meishan pig for commercial requirements. These indigenous pig populations are

genetically adapted to endure food and water scarcity and resist extreme tropical disease and climate conditions. Although, some pigs have the same appellations as Chinese native pigs, there are no evidences that whether these Thai pigs have the common ancestors with Chinese pigs. The classification for Thai pigs is based on historic appellations and phenotypic characteristics. However, environmental factors have probably influenced phenotypic characteristics, so this classification is no longer inappropriate for the formulation of breed improvement and utilization strategies (Dadi *et al.*, 2008). The analysis of genomic DNA variation among and within populations allows the optimum utilization of animal genetic resources and permits efficient genetic improvement for production and conservation needs.

Effective species conservation programs require the evaluation of intra-species genetic diversity (Haig, 1998). Although, genetic relationship among several pig populations including Thai indigenous pigs in East Asia has been analysed by blood groups and serum protein polymorphism (Tanaka *et al.*, 1983), no studies have been reported on the genetic diversity and population structure of Thai Indigenous pigs using mitochondrial and microsatellites DNA markers. Information on Thai indigenous pigs is also very scarce and to the knowledge, limited to one report in which genetic variation of the Thailand indigenous pig populations were analysed using microsatellite markers (Chaiwatanasin and Chantsavang, 2002). In this study, researchers investigated genetic variation and structure of several Thai pig populations using sequence data from the mitochondrial *Cyt b* gene and 12 microsatellite loci.

MATERIALS AND METHODS

Samples and DNA extraction: The study samples comprised 112 individuals representing the four pig populations distributed in Thailand and China: Northeast Thai pig (NT, $n = 61$), Nouth Thai pig (ST, $n = 23$) wild boar (WB, $n = 7$), Chinese Qianbei black pig (CQB, $N = 21$) (Fig. 1). NT samples were collected from Northeastern Thailand including the following six provinces: Sakorn Nakhon, Nakorn Panom, Mokdahan, Si Saket, Loei and Surin. All hair samples were taken from farmer's backyards in these provinces. ST samples were collected from Nakorn Si Thammarat province, one of the representative provinces in Southern Thailand. WB samples were also gathered from Northeastern Thailand

and number of individuals sampled in this population was quite low due to sampling difficulties and inconveniences. In addition, CQB samples were collected from Chinese Qianbei Black Pig Conservation Farm in Zunyi city, Guizhou province of China.

Wizard Genomic DNA Purification kit (Promega, Madison, WI) was used to extract DNA from hair roots samples. For 100 hair root samples, 10 μ L of proteinase K was added for each tube after adding 200 μ L Nucleic lysis solution.

Microsatellite analysis: Twelve primers were selected from 27 pairs of microsatellite primers in swine recommended by FAO/ISIG in 2004 and PCR were performed in 10 μ L reaction mixture containing 2.5-5.0 ng template DNA, 1 μ L of 10 \times buffer (10 mM Tris, 50 mM KCl, 0.1% gelatin pH 8.4), 0.8 μ L of 2.5 mM dNTP mixture, 1.5-4.0 mM $MgCl_2$ (0.6-1.6 μ L), 1 μ L of 10 pmol primer and 0.25 U Taq DNA polymerase.

The amplification was performed in iCycler PCR system (BIO-RAD, USA) with an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at optimum temperature 55-62°C for 30 sec and extension at 72°C for 30 sec with a final extension at 72°C for 5 min. PCR reactions were performed according to recommended annealing temperatures and concentrates of $MgCl_2$ with suitable adjustments, so as to acquire the good quality of PCR products for running Polyacrylamide Gel Electrophoresis (PAGE). About 3 μ L denatured PCR products were loaded into 6% denaturing polyacrylamide sequencing gel, molecular marker Ph1 \times 174/HinfI and sequencing makers M13 ladder were loaded into middle of each panel gel.



Fig. 1: Sample representatives; a) South Thai pig; b) Northeast Thai pig; c) wild pig (male); d) wild pig (female) and e) Chinese Qianbei black pig

Preparations for M13 ladder solution, silver staining solution according to the methods described by Promega Corporation. Scoring of microsatellite genotypes is straight forward.

mtDNA sequencing: The 1046 bp fragment of *Cyt b* gene was amplified and sequenced in 35 individuals from three Thai pig population and one Chinese pig population (NT = 19, ST = 7, CQB, N = 7 and WB = 2). To amplify the *Cyt b* region, the primers mtL1 (5'-ATCGTTGTCATTC AACTACA-3' (Watanobe *et al.*, 1999) in the L strand of the tRNA for glutamic acid and mtH2 (5'-CTCCTTCTCGG TTTACAAG-3') in the H strand of the tRNA for threonine were used. PCR were performed in iCycler PCR system (BIO-RAD, USA) with an initial cycle at 95°C for 30 sec followed by 35 cycles at 95°C for 45 sec and 55°C for 30 sec and followed by 72°C for 1 min 72°C extension for 7 min. PCR products were purified and directly sequenced by MacroGen Co., in South Korea using Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

Statistical analysis: For microsatellite, the numbers of alleles (No), effective number of alleles (Ne), expected (HE) and observed heterozygosity (HO), allele frequencies were computed using GENEPOP 3.4 package (Raymond and Rousset, 1995) according to Kimura and Crow (1964), Polymorphism Information Content (PIC) values were calculated using little program PIC-CALC 0.6 (<http://www.bbiox.com>).

The classic Wright indices (FIT, FIS and F_{ST}) were calculated (Wright, 1965; Weir and Cockerham, 1984) using POPGENE Version 1.3 (Yeh *et al.*, 1997). Hardy-Weinberg Equilibrium (HWE) was also tested using a χ^2 -test for each pair of locus/population by the Fisher's Method implemented in the GENEPOP 3.4 package.

Expected homozygosity and heterozygosity were computed according to Levene (1949), Nei's expected heterozygosity was computed according to Nei (1972)'s. Several diversity parameters were computed from mtDNA *Cyt b* sequences using ARLEQUIN 3.0 software (Excoffier and Schneider, 2005) including nucleotide and mtDNA haplotype diversity, measures of polymorphism as number of haplotypes, exclusive haplotypes, number of polymorphic sites, nucleotide differences per sites, haplotype diversity and nucleotide diversity.

Genetic relationships among populations were established using three methods. First, the nuclear pairwise F_{ST} were compared among the populations under the hypothesis that genetic drift is the main factor causing genetic differentiation between populations. Nei's

standard distance (Nei, 1972) based on data of 12 microsatellite markers among the four populations were computed. An unrooted phylogenetic tree was also constructed using UPGMA Method (Sneath and Sokal, 1973) based on 12 microsatellite locus using PHYLIP Version 3.67 (Felsenstein, 1993). Bootstraps of 1000 replicates were performed in order to test the robustness of tree topology (Efron *et al.*, 1996). Second, a Bayesian maximum likelihood approach to infer the number of populations without prior information of the sampling location was used through the software Structure 2.2 (Pritchard *et al.*, 2000; Falush *et al.*, 2003). This method estimated the proportion of the individual genome derived from each inferred ancestral population and the number of ancestral populations represented in the samples. The genetic cluster analysis was performed assuming an Admixture Model (Pritchard *et al.*, 2000). Third, a network analysis was used to visualize the spatial distribution of sequence variation among the different mtDNA *Cyt b* haplotypes using the program Network 4.2 (Bandelt *et al.*, 1999). This procedure reveals possible evaluation pathways (Bandelt, 1995) and is based on a maximum parsimony procedure (Templeton *et al.*, 1992).

RESULTS

Genetic diversity: The average effective number (Ne) of alleles, the average expected Heterozygosity (HE) and PIC at 12 studied loci in four pig populations are shown in Table 1. The higher observed mean number of alleles and higher effective number of alleles were detected in NT (10.75; 7.09) and ST (9.92; 6.3) populations compared to CQB (9.17; 5.97) and WB (6.5; 5.19) populations. The PIC value for single population ranged from 0.77 (WB) to 0.82 (NT), a relatively low Ne value in WB population (5.19). The expected Heterozygosity (HE) was mainly considered because it is regarded as a better estimator of the genetic variability presented in a population. As shown in Table 1, the HE values ranged from 0.69 (WB) to 0.82 (CQB). Hardy-Weinberg test showed of all 12 microsatellite loci, 7 loci were deviated from HWE ($p < 0.05$). In general, observed HWE deviations were not consistent; they occurred with different microsatellites in different populations.

FIS is an estimate of variation within populations that measures the reduction in heterozygosity in an individual due to nonrandom mating within its subpopulation. In present study, FIS parameter ranged from 0.03 (CQB) to 0.22 (WB) (Table 1) indicating CQB had lower inbreeding degree compared to wild boar population from Thailand. The closed FIS values for ST and NT populations indicated similar heterozygous genotypes.

Table 1: Within-population diversity values and population structure derived from the microsatellite loci and mtDNA diversity estimates

Population	Locality	Sample size	Microsatellite data				mtDNA data		
			H _E	PIC	N _e	F _{IS}	N _h	h	π
ST	South, Thailand	23	0.71	0.81	6.30	0.16* (6)	4	0.714	0.0027
NT	Northeast, Thailand	61	0.72	0.82	7.09	0.16* (6)	9	0.819	0.0053
WB	North, Thailand	7	0.69	0.77	5.19	0.22 (1)	2	1.000	0.0009
CQB	Guizhou, China	21	0.82	0.79	5.97	0.03 (4)	6	0.952	0.0045

H_E = Expected heterozygosity, PIC = Mean Polymorphic Information Content, N_e = Effective number of alleles, F_{IS} = Inbreeding coefficient; N_h = Number of haplotype, h = Haplotype diversity, π = Nucleotide diversity; CQB = Chinese Qianbei Black pigs, ST = South Thai pigs, NT = Northeast Thai pigs; WB = Wild Boars; Number in parentheses indicates the number of loci showing a significant departure (p<0.05) from Hardy-Weinberg equilibrium after correction for multiple tests

Table 2: Characterization of the 12 microsatellites analyzed in four pig populations

Locus	F _{IS}	F _{IT}	F _{ST}
S0227	0.21	0.28	0.09
S0090	0.26	0.34	0.11
S0226	-0.05	0.01	0.05
S0005	0.25	0.29	0.06
S0068	0.04	0.13	0.09
S0225	0.12	0.20	0.09
S0155	0.05	0.13	0.09
SW122	0.02	0.09	0.08
S0355	-0.08	0.04	0.11
S0386	0.29	0.36	0.10
SW911	-0.11	-0.01	0.09
SW24	0.09	0.19	0.11
Mean	0.09	0.17	0.09

F_{ST} is measures of the genetic differentiation over subpopulations. Bonferroni correction (p<0.05/12 = 0.0041) *p<0.05

About 74 polymorphism sites were detected from 35 *Cyt b* gene sequences of 1046 bp in four populations, there were 62 transitions and 12 transversions, no insertions and deletions were observed. Estimates of mtDNA genetic diversity are shown in Table 1.

About 18 mtDNA haplotypes were detected and denoted by H1-H18 showing a high genetic maternal diversity within and among the populations. Among all the haplotypes, H1-H9 were produced from NT population, H9 was found not only in CQB pigs but in NT and ST populations, H8 was detected both in NT and WB populations, the remaining haplotypes such as H1-H7_CH10-H18 occurred only in single population.

Among the all populations analysed, NT had 9 haplotypes and higher diversity for all estimates (h = 0.819, π = 0.0053) while WB had only 2 haplotypes and the lowest diversity (h = 1 π = 0.0009) because only two *Cyt b* sequences of WB samples were analysed and two independent haplotypes were detected for this population.

Genetic differentiation and relationships among populations: The statistic F_{ST} an estimate of variation due to differences among populations which is the reduction in heterozygosity of a subpopulation due to genetic drift. Wright's F-statistic (F_{ST}, F and F_{IS}) values for each locus are shown in Table 2. The F_{ST} per locus varied from 0.05 (S0226)-0.11 (S0090, S0355, SW24) and the average F_{ST} of all loci was 0.09. Multilocus F_{ST} values indicated that around 9% of the total genetic variation was explained by

Table 3: Nuclear Pair-wise F_{ST} values (below the diagonal) and Nei's standard genetic distances (above the diagonal) among 4 pig populations

Pig population	ST	NT	WB	CQB
ST	-	0.71	0.76	0.95
NT	0.06**	-	0.51	0.98
WB	0.06**	-0.01**	-	0.99
CQB	0.12**	0.07**	0.04**	-

Nei's D_A (Nei, 1983) and pairwise F_{ST} (Weir and Cockerham, 1984) are measures of genetic distance and genetic differentiation between populations, respectively; **p<0.01 from a multi-locus test that there is no genetic differentiation in population pair after corrections for multiple comparisons

population differences with the remaining 91% corresponding differences among individuals within population.

Population genetic analysis showed no evidence for genetic differentiation between CQB and WB (F_{ST} = 0.04; p = 0.07) and also between NT and WB populations (F_{ST} = -0.01; p = 0.81) (Table 3). However, significant differentiation was found between the ST and CQB, NT and CQB, NT and ST, WB and ST populations (F_{ST} = 0.12; F_{ST} = 0.07, F_{ST} = 0.06, F_{ST} = 0.06, respectively p<0.001).

Nei's standard genetic distances among four populations are shown in Table 3. CQB and WB had the largest distance while NT and WB had the smallest. Nei's unbiased genetic distance was also measured considering that a comparatively smaller pig population. A similar result could be obtained although, the absolute values of genetic distances were different from Nei's standard genetic distances. The UPGMA tree (Fig. 2a-c) was constructed based on the standard genetic distance. The numbers at the nodes are values for 1,000 bootstrap resampling of the 12 genotyped loci.

Graphical displays of the results from the structure analysis are shown in Fig. 2a, the most likely K value was that where in Pr (G/K) is maximized. The maximum value of in Pr (G/K) was acquired at K = 2 suggesting that the most probable number of populations inferred was two. At K = 2, genetic population 1 (red) was mainly constituted from CQB individuals and a few of ST individuals while genetic population 2 (green) from NT and WB individuals. When K = 3, CQB and NT populations were identified as separate clusters while WB shared a small fraction with ST population. At K = 4, CQB and NT populations

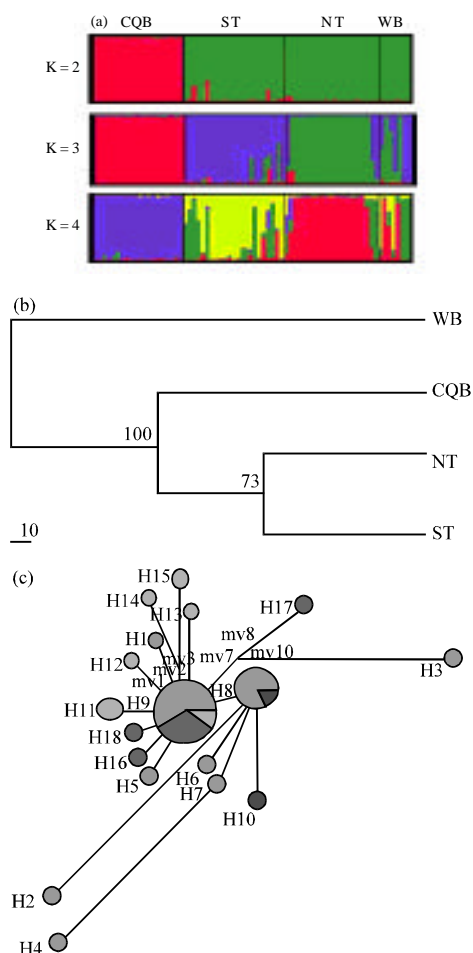


Fig. 2: a) Estimated membership fractions of pig populations for each of K inferred clusters ($K = 2-4$) using the Structure program (Pritchard *et al.*, 2000). Each individual was represented by a vertical line partitioned into K colors while K was the number of clusters assumed and the length of the colored segment represents the individual's estimated proportion of membership to a particular cluster; b) UPGMA tree showing the genetic relationships among four pig populations from Nei's standard distance (Nei, 1972) based on data of 12 microsatellite markers. The numbers at the nodes were percentage bootstrap values from 1,000 replications of re-sampled loci; c) Median-joining network analysis of eighteen mtDNA Cyt b haplotypes found in this study. Circles sizes were proportional to the observed frequency. Pig populations were defined based on geographical origins and were represented according to the following: south Thai pig (green), Northeast Thai pig (purple), Chinese Qianbei black pig (red), wild Boar (yellow)

were still identified as the separate two clusters. Median-joining network analysis showed the spatial distribution of eighteen haplotypes from 35 *Cyt b* gene sequences (Fig. 2-c). The detection of 18 haplotypes in 35 *Cyt b* sequence suggested that abundant genetic diversity existed in Thai indigenous pig populations also in Chinese pig population. Thai indigenous populations did not cluster together and were rather interspersed among the rest of the sequences. Overall, the network spatial distribution tended to form three clusters and showed a starlike phylogenetic pattern in which two higher frequencies of haplotypes, H9 and H8 were located in the center of three clusters. The haplotypes from the NT population constituted predominated ones (54.28%, 19/35). Haplotypes from the ST and CQB populations were in 20%, respectively while haplotype from WB was 5.7%.

DISCUSSION

In this study, the mean allele number and the genetic diversity of Thai indigenous pig populations sampled from the Northeast to the South across the country was assessed. A Chinese CQB pig population was studied to compare the level of genetic diversity. The observed heterozygosities and expected heterozygosities were detected among four pig populations. Thai indigenous pigs exhibited moderate levels of allelic diversity and heterozygosity whereas Chinese Qianbei black pigs had a relatively high degree of genetic diversity. Moderate genetic diversity of Thai indigenous pigs is consistent with the previous report (Chaiwatanasin and Chantsavang, 2002). The levels of allelic diversity and heterozygosity of Thai pigs are similar to those reported for Chinese Southwest pigs (Fan *et al.*, 2002; Fang *et al.*, 2005) and Vietnamese pigs (Thuy *et al.*, 2006). However, H_e and N_e of Thai pigs were higher than that of Korean and European pigs (Kim *et al.*, 2005; Laval *et al.*, 2000; Li *et al.*, 2004; Martinez *et al.*, 2000; Lemus-Flores *et al.*, 2001).

The Thai indigenous pig population showed relatively high levels of genetic diversity which is presumably to be a consequence of introducing exotic breeds to crossbreed with Thai pigs and lack of systematic artificial selection. In this study, all samples including NT, ST and WB populations were taken from peasants' backyards of villages and no intensive farms were found for these indigenous pigs. Randomly mating and non-artificial selection have been therefore, made for many years which may explain why Thai indigenous pig populations including NT and ST exhibited high H_e levels ($H_e = 0.72, 0.71$, respectively). This result could be

illustrated by high PIC values in NT and ST populations. The HE value (0.82) in the Chinese Qianbei Black pig population was slightly higher than that of Chinese Rongchang pig populations reported by Fang *et al.* (2005). This may be due to less controlled mating and artificial selection. The lowest HE observed in WB pigs may be due to a relatively small number of wild boars with inbreeding. Deviation from HWE in 7 microsatellite loci could be the result of the sampling of a single population with different allele frequencies in the subpopulations (Wahlund effect), nonrandom sampling, and/or inbreeding. In present study at least two populations, NT and ST, showed evidence of significant inbreeding (Table 1).

Among four pig populations, Chinese Qianbei Black pig population possessed the lowest FIS value ($FIS = 0.03$) which may be due to randomly mating without the intensively artificial selection within the population. In contrast, wild boar population with the highest FIS value ($FIS = 0.22$) could be on account of a relatively small population. WB individual was so scarce that it was quite difficult to acquire samples. Inbreeding was hard to avoid occurring within WB population. Hardy-Winberg test has shown no heterozygote excess in all loci in all populations ($p < 0.05$). Deficiency of heterozygotes was probably caused by the Wahlund effect which has been proposed in other domestic pigs such as Mexican hairless pig population (Lemus-Flores *et al.*, 2001) and Iberian cattle breeds (Martin-Burriel *et al.*, 1999). Throughout the 1960s and 1970s Thai pig production was dominated by the backyard raising of pig cross-breeds for consumption. Since, 1980s, Thai pig industry became increasingly commercialized, village production of pigs has declined and commercial units have become the main source of supply of pork in Thailand (Murphy and Tisdell, 1995). Researchers found some Thai pig populations had similar callings with Chinese pigs during sampling process, a survey for this was made and result indicated that some Chinese breeds were introduced to Thailand in the middle of 20th century, these similar calling had been remained. This is the reason why CQB population was selected to be a sample in this research. From geographically speaking, Thailand is closed to the South and the Southwest of China where are possible regions for gene exchange. In fact, structure analysis may confirm this deduction to some extent because CQB had a low percentage assigned to the ST and NT populations (Fig. 2a).

Eighteen haplotypes could be detected from 35 *Cyt b* gene sequences, indicating that a high mtDNA genetic diversity existed in four pig populations. Proportion of haplotypes from Thai indigenous pigs

including NT and ST populations reached to 74.28%, reflecting that mtDNA polymorphism within these two populations is relatively high. It is worth mentioning that haplotype H9 was shared among CQB, NT and ST populations, implying certain close correlation existed in these populations.

This inference also could be supported by a phylogenetic tree based on Nei's D_s standard genetic distances using UPGMA method (Fig. 2b). NT was grouped into the same branches with ST population with a 73% of bootstrap support value. CQB and two Thai indigenous populations (NT, ST) were clustered into the same branches with a 100% of bootstrap support value but WB was clustered into another branch. This result is consistent with that from the structure analysis.

When deciding conservation priorities, several factors should be taken into account such as adaptation to specific environments or diseases, possession of special traits of cultural and scientific or future economic value (Ruane, 1999). Conservation decisions will depend on future plans for the breeds in question. If the purpose is to use them in crossbreeding or introgression plans, the diversity between subpopulations should be prioritized. However, if the purpose is to preserve a closed population capable to coping with future challenging environments or with diversified production conditions, the within-population diversity should be prioritized (Thirstrup *et al.*, 2008). From the data, the F_{ST} statistic revealed that a high significant level of genetic differentiation among the study populations although, a lack population differentiation was found between the two populations from WB (Chinese Qianbei Black and Northeast Thai pigs) with nuclear markers ($F_{ST} = 0.04$ and $F_{ST} = -0.01$, respectively), estimation of the overall F_{ST} in present study was 0.09 which was between European pig populations $F_{ST} = 0.27$; Laval *et al.*, 2000) and Chinese populations ($F_{ST} = 0.077$; Yang *et al.*, 2003). Nevertheless, these differences are most likely to be explained by the different types of markers analysed as well as the source of population sampled.

CONCLUSION

In this study, Thai indigenous populations exhibited high genetic diversity in both nuclear and mtDNA information. However, Thai indigenous pig populations were distinct from Thai wild pig and Chinese Southwest pig populations. Haplotype network with mtDNA data showed that most of the Thai indigenous pig haplotypes were distinctly different from those of Chinese pig and Thai wild pig populations although, haplotype H9 was shared among Chinese Qianbei black pigs, Northeast Thai

pigs and South Thai pigs. These findings suggested that the introgression might have been occurred between Chinese pig and Thai pig populations. Today, Thai indigenous pig populations are at risk of gradually losing their genetic diversity unless effective and appropriate breeding management practices are implemented.

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