Malays. Appl. Biol. (2017) 46(1): 63–70

GENETIC DIVERSITY OF KAMPUNG CHICKEN (*Gallus gallus domesticus*) FROM SELECTED AREAS IN EAST COAST PENINSULAR MALAYSIA INFERRED FROM PARTIAL CONTROL REGION OF MITOCHONDRIAL DNA

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Accepted 26 January 2017, Published online 31 March 2017

ABSTRACT

After the 2014 Malaysia massive flood, genetic variation of the *kampung* chicken *Gallus gallus domesticus* in East Coast Peninsular Malaysia (ECPM) was investigated for a better understanding of their genetic diversity for its conservation. A total of fifty-nine samples were collected from three states; Kelantan, Terengganu and Pahang for mitochondrial DNA analysis. Partial mtDNA control regions were amplified, sequenced, and analyzed to determine the genetic variation among the states. Eleven haplotypes were detected among all the samples. Hap-1 was the most widespread haplotype among the three states and comprised of 45.8% of all samples. Genetic variation was the highest in Pahang (Pi = 0.01037, Hd = 0.8676), followed by Terengganu (Pi = 0.00938, Hd = 0.7316) and Kelantan (Pi = 0.00363, Hd = 0.5579). Low nucleotide diversity in Kelantan indicated the loss of genetic resources, which might be due to the population bottleneck phenomenon. Both the non-significant values of Tajima's D and Fu's F_S in Pahang and Terengganu, suggested that they were at genetic equilibrium. Significant deviation from Tajima's D neutrality test ($P<0.05$) in Kelantan indicated the possibility of population expansion, which might be a result of population recovery from the population bottleneck due to the massive flood in December 2014.

Key words: *Gallus gallus domesticus*, *kampung* chicken, mtDNA, control region

INTRODUCTION

Kampung chicken *Gallus gallus domesticus* is reared at home backyard by most of the villagers in East Coast Peninsular Malaysia (ECPM) for a small business and own consumption. It becomes an important poultry species among the villagers, because of its delicious taste, hardiness against the harsh environment and disease. However, the *kampung* chicken production is given less consideration and undermanaged breeding program compared to the commercial broiler and layer chickens, mainly due to their slow growth and low feed conversion efficiency (Ramlah, 1996; Henning *et al*., 2007; Daghir, 2008). Although the *kampung* chicken production is not intensively practiced at the commercial level, they contribute significantly to the economy and food security of the rural families that cannot be overlooked at ECPM. Moreover, the sudden 2014 Malaysia massive flood at ECPM most probably could have caused the reduction of the *kampung* chicken diversity (Agamuthu *et al*., 2015; Ibrahim & Muhidin, 2015).

Very rare of biodiversity studies were carried out on the *kampung* chicken in Peninsular Malaysia. Yap *et al* (2011) only able to investigate genetic variations using RAPD markers among the 20 *kampung* chickens, and 7 commercial chickens which were collected from Peninsular Malaysia but without ECPM region.

Therefore, this study was carried out at ECPM to analyse the genetic diversity in order to conserve these *kampung* chicken from extinction. The goal of this study was to determine the genetic diversity of the *kampung* chicken in ECPM using partial sequence analysis of the control region of the mtDNA. As mtDNA is maternally inherited, the distinct haplotypes can be used as indicators or

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DNA markers to identify the *kampung* chicken. According to Silva *et al* (2009), the distinct haplotype is very important to minimize the risk of animal inbreeding and increase the biological diversity in a population.

MATERIALS AND METHODS

Wing Samples

Fifty-nine samples were collected from fourteen sampling sites from January 2015 until July 2015, as shown in Table 1. A small portion of the chicken wing was cut from each individual and preserved in 90% ethanol for DNA extraction.

DNA Extraction, PCR and mtDNA Sequencing

Flesh samples were digested using proteinase K, followed by the DNA extraction using the Genomic DNA Mini Kit (GENEAID, New Taipei City, Taiwan). The DNA was precipitated with 70% ethanol, and then resuspended in 50 µl of TE buffer before stored it in a freezer at -20°C.

A pair of primers, forward primer L16750 5'-AGGACTACGGCTTGAAAAGC-3' (Desjardins & Morais, 1990), and reverse primer H547 5'-ATGTGCCTGACCGAGGAACCAG-3' (Komiyama *et al.*, 2003) were used to amplify the partial mtDNA control region using polymerase chain reaction (PCR). PCR was performed using a thermal cycler, GENEAMP SYSTEM 9700 (Applied Biosystems, California, USA) in 20 µl reaction volumes containing approximately 20 ng of DNA, with 1.25U GoTaq DNA polymerase (Promega, Madison, USA), 1X PCR buffer, 200 µM each dNTP and 0.25 µM of each primer. The PCR cycling conditions were 95°C for 2 min; 35 cycles at 95°C for 20 s, 61.5°C for 20 s and 72°C for 30 s; followed by a final extension for 3 min at 72°C.

Table 1. List of sample sizes and sample abbreviations for mtDNA analysis according to the sampling sites

States	Sampling Sites	Abbreviations	Sample Sizes
Kelantan	Kuala Krai	KKR	2
	Kota Bharu	KTB	7
	Machang	MCN	1
	Tanah Merah	TMR	5
	Jeli	JLI	5
Pahang	Bilut	BLT	2
	Lanchang	LCN	2
	Kuala Lipis	KLP	6
	Kuantan	KTN	5
	Raub	RUB	2
Terengganu	Cukai	CUK	5
	Dungun	DNG	5
	Jerteh	JRT	10
	Kuala Terengganu	KTG	2
Total	14		59

The PCR products were analysed by electrophoresis (70V for 90 min) on a 1% agarose gel containing Gel-Red dye (Biothium, California, USA) and visualised by UV fluorescence. Briefly, the same amplified products were purified by using QIAQuick Gel Extraction Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The same PCR primers were also used for sequencing reactions using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) according to the manufacturer's instructions. The nucleotide sequences were determined for both ends of the PCR products using ABI 3730xl DNA Analyser (Applied Biosystems, California, USA) automated sequencer.

Data Analysis

The sequence chromatograms from the automated sequencer were checked with the Chromas version 2.4 (Technelysium Pty. Ltd., Queensland, Australia). Nucleotide composition and number of variable sites were assessed using MEGA version 6 (Tamura *et al.*, 2013). All the aligned sequences were used to analyze the genetic variation using ARLEQUIN version 3.5 (CMPG, University of Berne; Excoffier *et al.*, 2005). Genetic diversity in each state was measured as haplotypic diversity (Nei, 1987) and nucleotide diversity (Tajima, 1983). Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) tests, as implemented in ARLEQUIN version 3.5, were used to evaluate the neutrality of the investigated sequences. Fu's F_S was used to detect an excess of mutation in case of any population expansion occurred. A neighbour-joining tree of the haplotypes was constructed under the model of the Kimura 2-parameter using MEGA version 6, and evaluated with 1,000 bootstrap replicates. The level of genetic population differentiation was tested using analysis of molecular variance (AMOVA) as implemented in ARLEQUIN version 3.5 (Excoffier *et al.*, 2005), using the genetic distance matrix to estimate the components of variance that are attributable to differences among populations and among individuals within populations. Due to the difficulties of collecting samples, the small sample numbers from sampling sites that were geographically close were pooled and treated as a single "population" for the comparison between populations. Significance of variance components was tested by a nonparametric permutation procedure with 1,000 permutations (Excoffier *et al.*, 1992). The pairwise fixation index (F_{ST}) was employed to check the genetic differentiation between populations. The correlation of genes of

Table 2. Nucleotide polymorphism observed in partial control region of 59 *kampung* chicken sequences and their frequencies (N). Vertically oriented numbers indicate the site position and the sequences shown are only the variable sites. Dots (.) indicate identity with reference sequence and different base letters denote substitution

Haplotype	Nucleotide substitutions																							N
	167	171	199	212	217	225	243	246	256	261	281	296	306	309	310	315	322	342	354	363	391	417	446	
Hap-1	T	T	T	G	T	C	C	C	C	T	G	C	C	T	T	C	T	G	T	C	C	C	C	27
Hap-2	.	.	C	.	C	.	.	.	.	.	A	.	T	.	.	.	.	A	.	.	.	.	T	5
Hap-3	.	.	.	.	C	.	.	.	.	.	A	.	T	.	.	.	.	A	.	.	.	.	T	3
Hap-4	.	.	.	A	.	.	T	T	T	C	A	T	T	.	C	T	.	A	.	.	.	.	.	5
Hap-5	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	4
Hap-6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	7
Hap-7	C	.	.	.	.	T	T	.	T	C	A	.	T	.	C	.	.	A	.	.	.	.	.	3
Hap-8	.	.	.	A	.	.	T	T	T	C	A	.	T	.	C	T	.	A	.	.	.	.	.	1
Hap-9	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	2
Hap-10	.	C	.	A	.	.	.	.	.	.	A	T	T	.	.	.	A	A	C	T	T	T	T	1
Hap-11	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	1

different individuals in the same population and the genetic differences among populations were also tested by the F_{ST}.

RESULTS AND DISCUSSION

Sequence Variation

A total of fifty-nine 546 bp fragment were sequenced and aligned successfully from the samples. The variable sites of each haplotype sequences were shown in Table 2. Among the examined sequences, the A/T base contents were higher than the C/G base content (mean: A=26.5%, T=29.8%, C=30.3%, G=13.4%), which is consistent with the results of Brown *et al* (1986), who found that the control region is an A/T rich region of the mtDNA. Among all the 546 nucleotides sequences, 23 or 4.21% of them were variable sites. The sequences from Kelantan, Terengganu and Pahang contained 14, 16, and 21 variable sites, respectively (Table 3). Niu *et al* (2002) reported the similar percentage of variable sites (4.27%) in 539 nucleotides of the mtDNA control region from 25 China indigenous chickens.

Genetic Diversity

Among all the samples, eleven haplotypes were detected (Table 3); five haplotypes were found in Kelantan, seven haplotypes in each Terengganu and Pahang. Five haplotypes were shared between more than one states. Among the shared haplotypes, Hap-1, Hap-4 and Hap-6 were shared in all the three states. Hap-1 comprised 45.8% of all samples. It was clear that Hap-1 was the most widespread haplotype in the ECPM region and the most common haplotype among the control region sequences. A total of six distinct haplotypes were found in one single state only. Kelantan contained a distinct haplotype, while Terengganu and Pahang had two and three distinct haplotypes respectively. These distinct haplotypes have no introgression stemming from other populations, which usually due to the natural geographic boundaries and different maternal ancestors (Weiss *et al*., 2001).

Table 3. Relative haplotype frequencies of *kampung* chicken by states

Haplotypes	Kelantan	Pahang	Terengganu
Hap-1	13	3	11
Hap-2		2	3
Hap-3	1		2
Hap-4	1	3	1
Hap-5*	4		
Hap-6	1	5	1
Hap-7*			3
Hap-8*			1
Hap-9*		2	
Hap-10*		1	
Hap-11*		1	
Total	20	17	22

* Distinct haplotype

Both nucleotide diversity (Pi) and haplotype diversity (Hd) were used to estimate the genetic diversity of all sequences is shown in Table 4. Genetic diversity was higher in Pahang (Pi: 0.01037, Hd: 0.8676) and Terengganu (Pi: 0.00938, Hd: 0.7316) compared to Kelantan (Pi: 0.00363, Hd:

Table 4. Nucleotide sequence data of three states based on the partial fragments of mtDNA control region, haplotype and nucleotide diversity, and neutrality tests

State	Sample size	Number of polymorphic site	Number of haplotype	Haplotype diversity (Hd)	Nucleotide diversity (Pi)	Tajima's *D*	Tajima's *D* *P*-value	Fu's *Fs*	Fu's *Fs* *P*-value
Kelantan	20	14	5	0.5579	0.00363	-1.820	0.034*	0.667	0.233
Terengganu	22	16	7	0.7316	0.00938	0.333	0.670	2.042	0.129
Pahang	17	21	7	0.8676	0.01037	-0.265	0.425	1.547	0.174

*Significant level (P < 0.05) of Tajima's D value. No significant values of Tajima's D and Fu's Fs were observed in the neutrality tests, except in Kelantan (P<0.05), signifying that all the populations were in genetic equilibrium with exception of Kelantan population.

0.5579). Genetic diversity might be higher in ancient population compared to the derived population, therefore, Pahang might probably be the ancestral of *kampung* chickens in ECPM (Savolainen *et al*., 2002).

Low nucleotide diversity in Kelantan indicated the loss of genetic diversity, which might be due to the population bottleneck phenomenon (Wani *et al*., 2014). Factors that might lead to population bottleneck include reduction of a population size due to natural disasters, disease outbreak, and human activities; consequently cause a great loss of genetic diversity in a population. Excessive rainfalls annually during the monsoon raining season that occurred from November until February of the following year have caused the floods at low-lying areas in ECPM (Muqtada *et al*., 2014; Baharuddin *et al*., 2015). Current low nucleotide diversity in Kelantan might be mainly attributed to the reduction of *kampung* chickens caused by the torrential rain fall and the massive flood in December 2014 (Shakirah *et al*., 2016). Similar incident of flood was also reported in Sudan and Malolwane, which caused the low level of nucleotide diversity of their indigenous chickens (Wani *et al*., 2014).

Tajima's D and Fu's F_S neutrality tests were performed to determine departures from neutrality in the sequence data. No significant deviations from neutrality were detected in Pahang and Terengganu using both of neutrality tests (P>0.1). This condition normally occurred when the evolutionary forces acting upon the allele are equal, thus, resulting in non-evolving population even after several generations (Shriner, 2011). The significant negative Tajima's D (P<0.05) in Kelantan indicated a possibility of population expansion, even though Fu's F_S in Kelantan showed no significant deviation from neutrality (Table 4). Fu's Fs is more sensitive than Tajima's D in detection of population growth, but it required larger sample size (Ramos-Onsins & Rozas, 2002). Hence, no significant values of Fu's Fs test in this study might be due to the small sample size.

The population expansion in Kelantan might be as a result of slowly recovery from a population bottleneck. This result further explains the low nucleotide diversity (0.00363) in Kelantan (Table 4) and support the loss of genetic diversity caused by the natural disaster of the massive flood. Furthermore, samples from Kelantan were collected from month of March to July 2015 after the massive flood. This might probably occur due to the gene flow from neighbouring regions as a result of trade activities and free movement of the farming community into the population (Makanjuola, 2010; Silva *et al*., 2009). Therefore, population expansion observed in Kelantan might be due to the geographical location of the population in the Northern part of ECPM which probably facilitate the gene flow from the neighbouring region such as Northern Peninsular Malaysia, and Thailand. To further study the relatedness of *kampung* chicken among these regions, a more rigorous study using larger samples sizes is recommended to help elucidate the population genetic of the *kampung* chicken.

Phylogenetic Relationships

The neighbour-joining (NJ) tree was constructed from the 11 haplotypes of mtDNA control region using the Kimura 2-parameter model (Figure 1). Two genus of *Gallus gallus*; *Gallus gallus gallus* GenBank accession number AB007720 and *Gallus gallus bankiva* GenBank accession number AB007718, that were retrieved from the National Centre of Biotechnology Information (NCBI) were included in the tree as out-groups. The tree showed no obvious genealogy among the eleven haplotypes. The topology of the tree was shallow and there were no significant genealogical clusters of samples corresponding to sampling sites. All the haplotypes were randomly scattered throughout the tree.

Figure 1 showed that the *kampung* chickens in ECPM are genetically closer to *Gallus gallus gallus* and relatively far away from *Gallus gallus bankiva*. Similar result was reported by Niu *et al* (2002) that

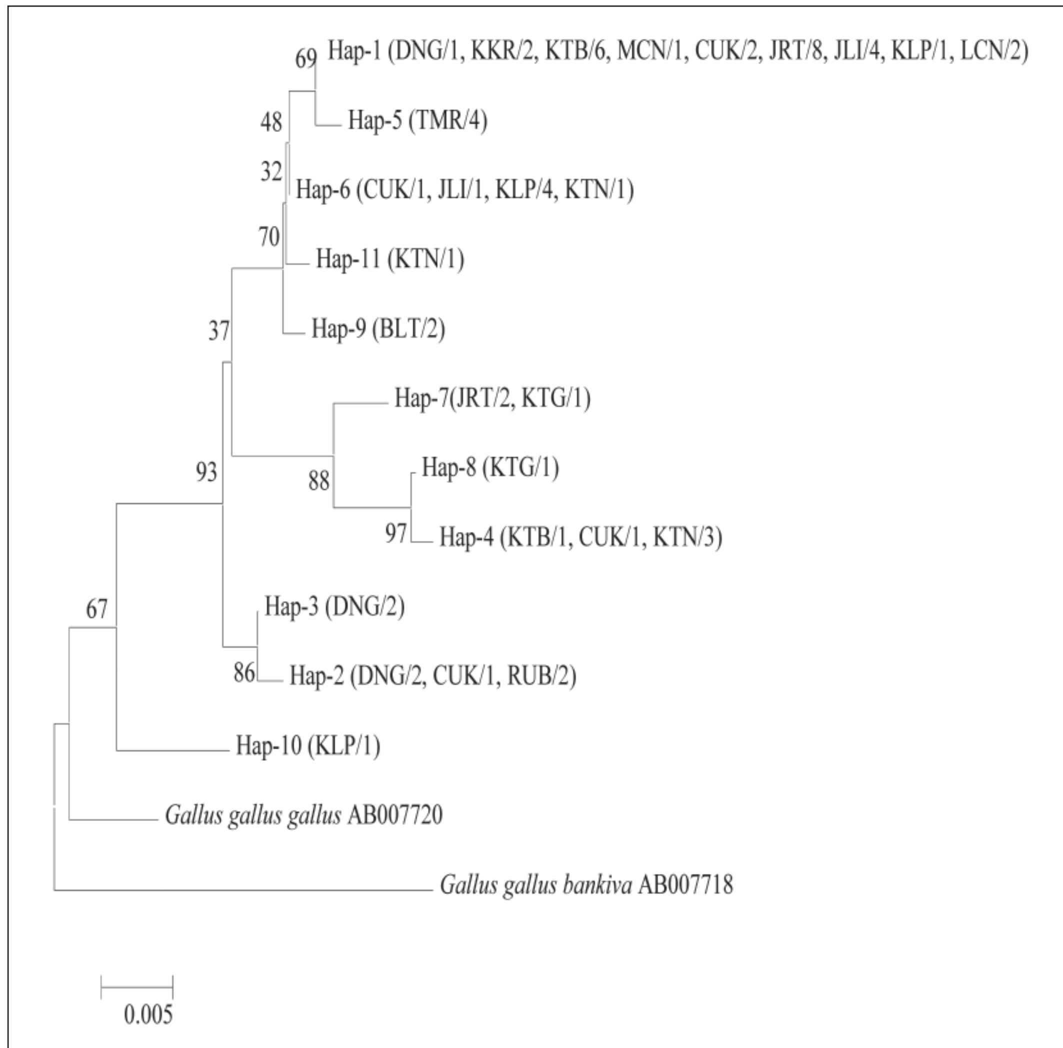

Fig. 1. Neighbour-joining tree constructed using MEGA version 6 from 11 haplotypes identified in the *kampung* chicken in East Coast Peninsular Malaysia. *Gallus gallus gallus*; Genbank accession number AB007720 and *Gallus gallus bankiva*; Genbank accession number AB007718 were retrieved from NCBI as out-groups. The abbreviations and numbers in the brackets show the sampling sites and the number of samples.

the China indigenous chickens were most probably originated from the Thailand red jungle fowl *Gallus gallus gallus*. Hap-10 is unique to Pahang population and has a close relationship to *Gallus gallus gallus*. This Hap-10 might represent the indigenous of Pahang population, which is worth to be conserved as a local breeder.

Population Structure

Maternal genetic differentiation within and among population of *kampung* chicken in ECPM was estimated using hierarchal analysis of molecular variance (AMOVA). The analysis generated an estimate of variance components showing the correlation of haplotype diversity at different levels of hierarchal division. *F*-statistics estimated from AMOVA revealed significant differences among populations (F_{ST} = 0.08175, P<0.05) (Table 5). In other words, 8.17% of the significant variance was attributable to among the populations. The remaining 91.83% of the variance was attributed to the among individuals within populations. Lower percentage of genetic variance among the populations (8.17%) might be due to the absent geographical barriers among the states.

Low F_{ST} value in the current study might be due to the small sampling coverage which constituted only ECPM with total land of 64,000 km^2. Sampling from many sites is more likely to provide the evidence of multiple gene pools (Appleyard *et al*., 2001).

In pairwise comparison, non-significant negative pairwise F_{ST} was observed between Pahang and Terengganu (Table 6). This indicated a remarkable gene flow that could prevent the genetic differentiation; hence, resulted in the lower genetic

Table 5. Analysis of molecular variance (AMOVA) base on the partial control region sequences of the *kampung* chicken in East Coast Peninsular Malaysia

Source of variation	df	Sum of squares	Variance components	Percentage of variation	*F*-statistics
Among populations	2	12.357	0.20066	8.17	0.08175*
Within populations	56	126.219	2.25391	91.83	
Total	58	138.576	2.53958		

* The significant level (P< 0.05) of the F-statistics.

Table 6. Pairwise comparison F_{ST} values of populations differentiation base on the partial control region of mtDNA sequences

	Terengganu	Pahang	Kelantan
Terengganu	–		
Pahang	-0.00211	–	
Kelantan	0.11580*	0.15615*	–

*Significant level (P< 0.05) of F_{ST} value, with sequential Bonferroni correction (Rice, 1989).

differentiation between the two populations (Slatkin, 1987; Cai *et al.*, 2008). Gene flow between the two populations might be due to intensive genetic intermixing among the populations following human migration and trade activities (Granevitze *et al*., 2007; Leroy *et al*., 2012).

The significant pairwise F_{ST} (P<0.05) observed in Kelantan illustrated the population differentiation. In other word, Kelantan could be population genetically differentiated from Terengganu and Pahang. This scenario normally occurs when there is populations' fragmentation due to geographical barriers. But there is no distinct geographical barrier between Kelantan and other two states. Therefore, genetic differentiation observed in Kelantan might be due to the geographical location of the population in the Northern part of ECPM which probably facilitate the gene flow from the neighbouring areas such as Thailand into the population and make it to become unique population from Pahang and Terengganu (Dancause *et al*., 2011; Leroy *et al*., 2012). Further study using more samples from Southern Thailand and Northern Peninsular Malaysia is recommended to elucidate the population structure and the relatedness of *kampung* chickens in ECPM and Thailand.

The results of the present study have important implications for the conservation of *kampung* chicken in ECPM. This study revealed significant differences in the partial mtDNA control region of Kelantan compared to other populations (by F_{ST} values and genetic distances). Low nucleotide diversity in Kelantan population is an evidence of population bottleneck, which is probably due to the massive flood disaster in 2014. But the neutrality test revealed population expansion, which means the population is recovering from the previous bottleneck. Hence, conservation initiatives on Kelantan population should be carried out to prevent the loss of these natural gene resources, such as the cross-breeding program using different populations outside of ECPM. Even though our data showed Pahang and Terengganu were closely related, Pahang with the most number of distinct haplotype could be the ancestral population in ECPM. The distinct haplotypes, especially Hap-10 could be used as DNA markers to identify local breeders in ECPM.

ACKNOWLEDGEMENTS

Our highest gratitude to the Universiti Sultan Zainal Abidin for funding this study under the Research Fund of the University (UniSZA/2015/DPU/1). We thank the staff of Molecular Biology Laboratory in Faculty of Bioresources and Food Industry, UniSZA Besut Campus, especially Syaidatul Najiah binti Zakaria, Azifah binti Yahya and Fariha binti Yahaya for their cooperation. Special thanks to Mustapha Jamilu Adamu for his assistance on the DNA extraction and PCR works in the laboratory.

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