ORIGINAL ARTICLE

GENETIC DIVERSITY OF INDIGENOUS CHICKEN FROM THE EAST COAST OF PENINSULAR MALAYSIA INFERRED FROM CONTROL REGION OF MITOCHONDRIAL DNA

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ABSTRACT

Background: Genetic variation within and among populations is important for conservation, genetic improvement and species adaptability. The effectiveness of selection and crossbreeding depend on the amount of genetic variation. In the current study, genetic diversity of indigenous chicken (*Gallus gallus gallus*) from East Coast Peninsular Malaysia (ECPM) was estimated using mitochondrial DNA (mtDNA) analysis. **Method**: First 546 bp of the control region of mtDNA were analyzed in 59 chickens collected from three populations of Kelantan, Pahang and Terengganu. **Result**: Eleven haplotypes were detected from all samples. Hap-1 is the most common among the three populations and comprised 45.8% of the total samples used. The average haplotype and nucleotide diversity were 0.7645 and 0.00836 respectively. Non-significant Tajima's *D* and Fu's F_S values were observed in Pahang and Terengganu populations. The significant Tajima's *D* value ($p<0.05$) observed in Kelantan indicated significant departure from neutrality model. The haplotype neighbour joining (NJ) tree revealed shallow topology, and no significant genealogical clusters of samples corresponding to sampling locations was observed. **Conclusion**: Higher genetic diversity was observed in this study. Low gene diversity observed in Kelantan might indicate the evidence of population bottleneck due to flood disaster in the area. Additionally, based on the NJ tree, indigenous chicken from ECPM are closer to the *Gallus gallus gallus* and relatively far away from *Gallus gallus bankiva.*

***Key words**: indigenous chicken, mitochondrial DNA, control region, genetic diversity.*

1. INTRODUCTION

Chickens are the most common and widespread domestic animals. In Malaysia, indigenous chicken production has been a business among the rural families centuries ago. It serves as sources of immediate income and food security to the low income farmers. In Peninsular Malaysia, more than three-quarter out of million rural communities still keep these chickens at free range operation [22]. Until recently, there was no any study that quantified the genetic diversity of indigenous chicken in Malaysia. Genetic variation within and among population is important for conservation, genetic improvement and species adaptability. The effectiveness of selection and crossbreeding depend on the amount of genetic variation. Molecular characterization provides reliable Information for assessing the level of genetic diversity among the populations of the animals [19]. It reveals the rates of distribution of diversity between the populations [17], and distribution of diversity in populations found in various locations [7]. Additionally, it allows the comparison of genetic variation within and between populations of individuals of the same species and reconstruction of the phylogenetic tree to trace the history and ancestral populations [10]. mtDNA is commonly used as molecular marker in population genetics because of its high copy number in the cell, maternal inheritance, haploidy and its rapid rate of evolutionary changes [34]. The rate of evolutionary changes in mtDNA is 5 to 10 times compared with nuclear DNA [3]. The fast evolutionary rate of mtDNA control region together with its maternal inheritance has made it an extremely suitable marker for the study of the population level phenomena and most utilized segment of mtDNA genome for population genetic study [11]. In the study of genetic diversity, relatively high genetic diversity was reported among Bangladesh indigenous chickens [2], Sri Lankan indigenous chickens [25], and Vietnamese indigenous chickens [4]. Therefore, the aim of the current study was to evaluate the genetic diversity and phylogenetic relationship among the indigenous chickens in East Coast Peninsular Malaysia (ECPM).

2. Materials and Methods

A total 59 samples of indigenous chicken were randomly collected from 14 villages in three states ofEast Coast Peninsular Malaysia (Figure 1). Among the 59 samples, 20 were from Kelantan, 17 from Pahang and 22 from Terengganu (Table 1).

2.1 DNA extraction and PCR amplification

Genomic DNA was extracted from wing tissue using Geneaid DNA extraction kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) following the manufacturer's protocols. PCR amplification of the partial sequence of mtDNA control region of 59 sample was carried out with pure DNA extract, using L16759 5'-AGGACTACGGCTTGAAAAGC-3' [26] as forward primer and H547 5'-ATGTGCCTGACCGAGGAACCAG-3' [31] as reverse primer. PCR was conducted using thermal cycler GENEAMP SYSTEM9700 (Applied Biosystems, California, USA), in 20 µl reaction volume containing 0.2 mM of each dNTPs, 0.25 µM of each primer, 1.5 mM $MgCl_2$, 1X PCR buffer and 1.25 U *Go*Taq® DNA polymerase (*GoTaq* Promega, Madison, USA). Genomic DNA was 20 ng/µl. PCR thermal condition were; initial denaturation 95^0C for 2 min, followed by 35 cycles at 95^0C for 20 sec, 61.5^0C for 20 sec and 72^0C for 30 sec. Final extension step at 72^0C for 3 min. The PCR products were electrophosed on a 1% agarose gel in 1X TBE to check the yield. The detection of the amplified fragments was done under UV light using a transilluminator.

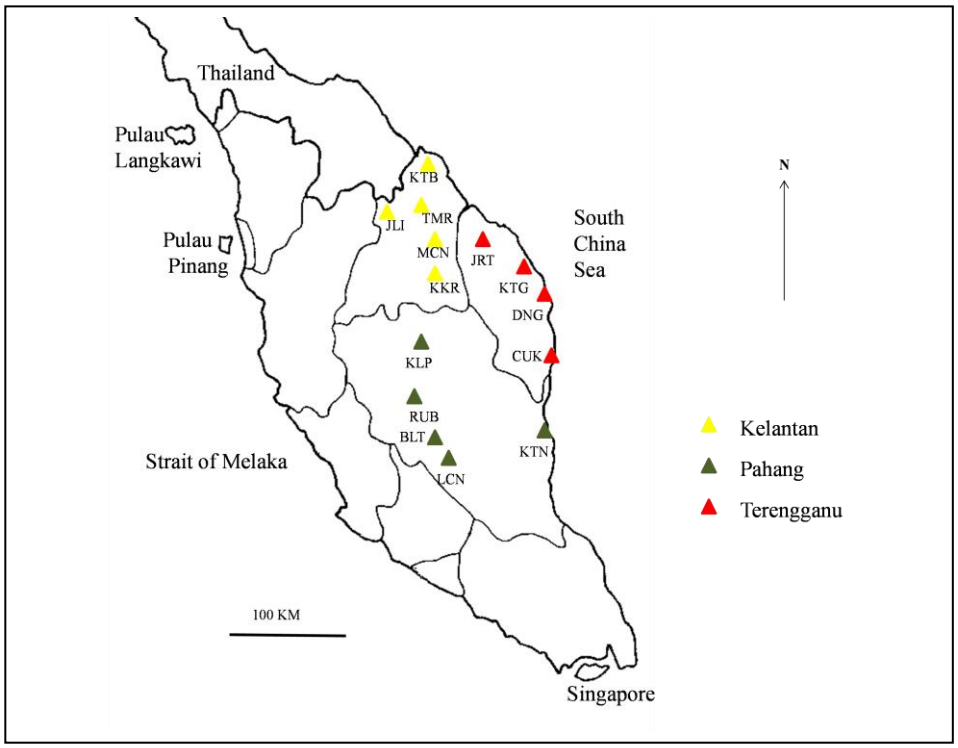

Figure 1: Map of Peninsular Malaysia indicating the fourteen sampling locations of the indigenous chickens from three states in East Coast Peninsular Malaysia.

Table 1: List of Populations, Sampling Locations, Abbreviations and Sample Size.

Populations	Sampling Locations	Abbreviations	Sample Size
Kelantan	Kuala Krai	KKR	2
	Kota Bharu	KTB	7
	Manchang	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
3	14		59

2.2DNA sequencing

Purified PCR products were used for sequencing using the same pair of primers. The sequence analysis was performed using the BigDye® Terminator version 3.1 cycle sequencing kit (Applied Biosystem, California, USA), with a total volume of 20 µl comprising 20 ng of the DNA template. And the sequence data analyzed using ABI Prism 3730xl Genetic Analizer (Applied Biosystem, California, USA).

2.3 Data analysis

The successful sequence data for the first 546 nucleotides of the control region were used for the analyses. The sequence data was analyzed using computer software programs. The sequence chromatograms were successfully viewed with Chromas version 2.4 (Technelysiumpty, ltd., Queensland, Australia). Multiple alignments and editing of the sequences were performed using program Genetyx version 9 (Genetyx Co., Tokyo, Japan).

2.4 Genetic diversity indices

Nucleotide composition and number of variable sites were assessed using MEGA version 6 [29]. The control region sequences diversity indices were determined to elucidate the sequence polymorphism and the content of genetic variability in the populations. Diversity indices as explained by Nei (1987), include number of segregation sites (S), number of haplotypes (H), haplotype diversity (Hd), and nucleotide diversity (Pi) [18]. DnaSP software version 4.0 [24] was used to evaluate the sequence polymorphism and genetic diversity in the populations. Both Tajima's *D* [28] and Fu's *FS* [8] tests of neutrality were carried out using ARLEQUIN version 3.5 [6].

2.5 Molecular evolution genetic analyses (MEGA)

Phylogenetic analyses were conducted using the program Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 [29]. Genetic distances of the haplotypes were calculated using Kimura's 2-parameters model with 1,000 bootstrap replicates to construct a neighbor joining phylogenetic tree. Two control region sequences of jungle fowls; *Gallus gallus gallus* GenBank accession number AB007720, and *Gallus gallus bankiva* GenBank accession number AB007718, were included in the tree as out-group.

3. RESULTS

3.1 Sequence variation

A total of 546 bp of mtDNA control region fragments were successfully sequenced from 59 chickens in East Coast Peninsular Malaysia (ECPM). The variability in the sequences was concentrated between nucleotide 167 to 446 (Figure 2). The composition of the examined control region sequences of the indigenous chicken in the current study shows that the A/T base contents were higher than the C/G base content (A=26.54%, T=29.76%, C=30.31%, G=13.39%). Among the 546 nucleotides analyzed, 23 are polymorphic (variable) sites, and they are equivalent to 4.21% of total nucleotides used, while the 523 nucleotides are monomorphic sites. The sequences from Pahang and Terengganu have 21 and 16 polymorphic sites respectively, while Kelantan contained 14 polymorphic sites.

3.2 Genetic diversity

The genetic diversity as expressed by haplotype and nucleotide diversities were estimated using 59 partial control region sequences. The genetic diversities of all populations were shown in Table 2. The highest number of haplotypes (7) was found in each Pahang and Terengganu populations, while the lowest number of haplotypes (5) was observed in Kelantan. The average haplotype diversity (Hd) and nucleotide diversity (Pi) among the entire populations were 0.7633 and 0.00818, respectively. And they are higher in Pahang (Hd: 0.8676, Pi: 0.01037) and Terengganu (Hd: 0.7316, Pi: 0.00938), and lower in Kelantan (Hd: 0.5579, Pi: 0.00363).

Tajima's *D* and Fu's *Fs* were used in the neutrality test. Tajima' *D* can be used to study demography in such a way that, negative value indicate departure neutrality while the positive value is expected in the population under equilibrium. But Fu's *Fs* statistic was proposed to detect the effect of recent population growth. Hence the combined usage of the two analyses may explain the likely mechanism responsible for the deviation from model [34]. Non-significant departure from neutrality was observed in Pahang and Terengganu using both of neutrality tests. Significant negative Tajima's *D* ($P<0.05$) was observed in Kelantan (Table 2).

	1 6 7	1 7 1	1 9 9	2 1 2	2 1 7	2 2 5	2 4 3	2 4 6	2 5 6	2 6 1	2 8 1	2 9 6	3 0 6	3 0 9	3 1 0	3 1 5	3 2 2	3 4 2	3 5 4	3 6 3	3 9 1	4 1 7	4 4 6	5 1 7	N
Hap1	T	T	T	G	T	C	C	C	C	T	G	C	C	T	T	C	T	G	T	C	C	C	C	C	27
Hap2	.	.	C	.	C	.	.	.	.	.	A	.	T	.	.	.	.	A	.	.	.	.	T	.	5
Hap3	.	.	.	.	C	.	.	.	.	.	A	.	T	.	.	.	.	A	.	.	.	.	T	.	2
Hap4	.	.	.	A	.	.	T	T	T	C	A	T	T	.	C	T	.	A	.	.	.	.	.	.	5
Hap5	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.		.	.	.	.	.	.	4
Hap6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	7
Hap7	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
Hap10	.	C	.	A	.	.	.	.	.	.	A	T	T	.	.	.	A	A	C	T	T	T	T	.	1
Hap11	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	1

Figure 2: Nucleotide polymorphism observed in partial control region of 59 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. The abbreviations indicate the haplotype names.

Table 2: Nucleotide sequence data of three populations based on the partial sequence of mtDNA control region, haplotype and nucleotide diversity, and neutrality tests.

Populations	Sample size	Number of polumorphic site	Number of haplotype	Haplotype diversity	Nucleotide Diversity	Tajjiam's *D*	Tajjim's *D* *P*-value	Fu's *Fs*	Fu's *Fs* *P*-value
Kelentan	20	14	5	0.55790	0.00363	-1.82002	0.0340*	0.66200	0.23300
Pahang	17	21	7	0.86760	0.01037	-0.26488	0.42500	1.54700	0.17400
Terengganu	22	16	7	0.73160	0.00938	0.33338	0.67000	2.04200	0.12900

3.3 Phylogenetic relationships

The haplotype neighbour joining (NJ) tree was constructed from the 11 haplotypes of mtDNA control region using the Kimura 2-parameter model (Figure 3). Two genus of *Gallus gallus* retrieved from National Centre of Biotechnology Information (NCBI) were also included in the tree as out-groups. Reliability of the tree topology was accessed by 1000 bootstrap. The numbers at the node indicate the percentage of bootstrap values for the interior branches (Figure 3). The tree showed no obvious genealogy among the 11 haplotypes. The topology of the tree was shallow, and there were no significant genealogical clusters of samples corresponding to sampling locations.

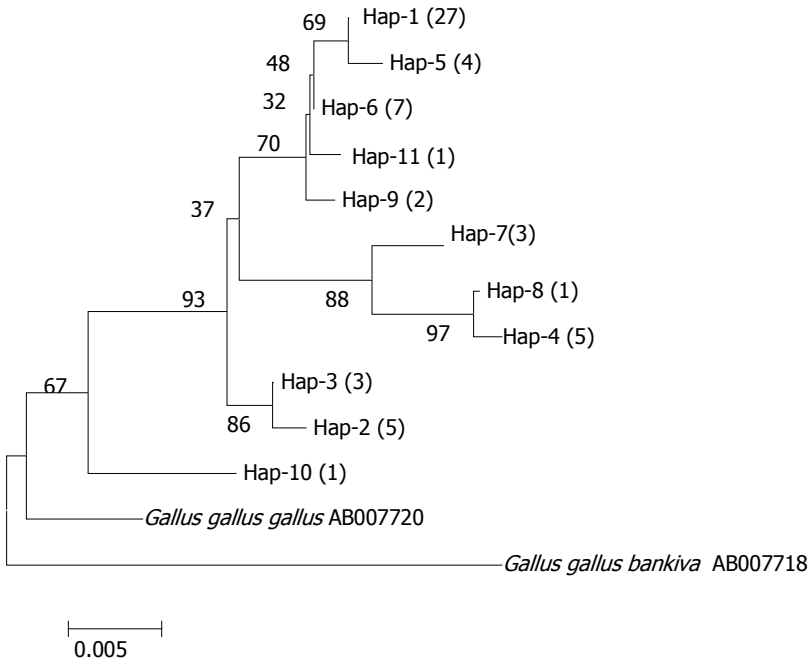

Figure 3: Neighbour joining tree constructed using MEGA package version 6 from 11 haplotypes identified in the indigenous chickens in East Coast Peninsular Malaysia. Two haplotypes of the genus *gallus gallus* were retrieved from NCBI (*Gallus gallus gallus*; Genbank accession number AB007720 and *Gallus gallus bankiva*; Genbank accession number AB007718) and used as out-groups.

4. DISCUSSION

Genetic variation within and among populations is important for domestic animal improvement and species adaptability. The effectiveness of selection and crossbreeding depend on the amount of genetic variation. The genetic diversity as expressed by haplotype and nucleotide diversities was estimated using 59 control region sequences. Both haplotype and nucleotide diversity can be use to estimate the genetic diversity in the population. But the nucleotide diversity parameter is more effective because it addresses both frequencies of haplotypes and nucleotides differences among the haplotypes [14]. Higher level of genetic diversity was observed in this study Table 2. And it higher in Pahang population followed by Terengganu. According to Savolainen (2002), genetic diversity may be higher in ancient population compared to derived population [27]. This suggests that, Pahang population might probably be the ancient or ancestral population of indigenous chickens in ECPM. Higher value of nucleotide and haplotype diversity observed in the current study could be as a result of natural selection [33]. Other possibilities of high nucleotide diversity as reported by Komiyama (2003) are higher mutation rates or multiple maternal ancestral populations have contributed to the genetic background of the population [25].

Low nucleotide diversity observed in Kelantan population may indicate the loss of gene diversity, which might occur from relatively population bottleneck [28]. Based on the available evidence in the area, low nucleotide diversity in Kelantan might be attributed to the loss of chickens due the environmental disasters such as flooding which occurred annually.

The genetic diversity observed in the current study was higher than that of 232 Nigerian indigenous chickens (Hd: 0.4217, Pi: 0.00157) [16], 81 Sudanese indigenous chickens (Hd: 0.5405, Pi: 0.00282) [30]. High genetic diversity observed in this study was quite expected, and it might be due to the proximity of the study region to the ancestral area (Thailand) which was proposed to be one of the places where chickens were first domesticated [2]. Additionally the study of Granevitze (2007) reported that, higher genetic diversity among the indigenous chickens in Asian countries might be due to the history of the region as a center of chicken domestication especially South and Southeast Asia [12].

Non-significant departure from neutrality were observed in Pahang and Terengganu using both of neutrality tests (P<0.05), which signified that the two populations are in genetic equilibrium [13]. This implied that the allele or genotype frequency in these populations were stable and do not change from one generation to another. This condition normally occurred when the evolutionary forces acting upon the allele are equal, thus, resulting in non-evolving population even after several generations [26]. Significant negative Tajima's D (P<0.05) observed in Kelantan population indicated departure from neutrality which illustrates population expansion (Table 2). The population expansion is a clear indication that the Kelantan population starts recovering after previous bottleneck. This might probably occur due to the gene flow from neighbouring populations as a result of trade activities and free movement of the farming community [16-25]. Therefore, low gene diversity observed in Kelantan (Table 2) might be due the increase in haplotype diversity and segregating sites without corresponding increase in the nucleotide diversity. This is because; nucleotide diversity in an expanding population is more heavily affected by the previous bottleneck than the haplotype diversity and number of segregating sites. Therefore, in a population that is increasing from a genetic bottleneck, the number of variable sites will increase rapidly more than that of nucleotide diversity, hence resulting in the negative Tajima's D. This is in consistent with the conclusion of Tajima (1987) that said; in an expanding population, the number of segregating sites is influenced by the size of the current population while the average numbers of nucleotide differences are more strongly affected by the size of the original population [28]. Contrarily, the Fu's Fs signified non-significant value. Although the Fu's Fs was proposed to be more sensitive to population growth, Rozas (2003) reported that the behaviour of Fu's Fs test is better when the sample sized is bigger [24]. Hence, non-significant value revealed Fu's Fs test might be attributed to the small sample size used in this study. Low nucleotide diversity observed in Kelantan might be due to the small sample size used. The use of large sample from more sampling location may help to understand the actual population divergence.

Phylogenetic analysis provided an important guideline for making conservation initiatives among the indigenous chickens [1]. Phylogenetic pattern among the haplotypes gives summary about the general pattern of variation at gene level. The phylogenic tree constructed using 11 haplotype found in this study illustrated shallow topology Figure 3.2. There were no significant genealogical clusters of samples corresponding to sampling locations. In fact, all the haplotypes in the populations were randomly scattered throughout the tree. This might probably suggested a common maternal lineage among the indigenous chickens in the ECPM [32]. In addition, The Figure 3.2 showed that the indigenous chickens in ECPM are genetically closer to *Gallus gallus gallus* and relatively far away from *Gallus gallus bankiva*. This finding is compatible with studies of Fumihito et al, (1994) and Wani et al. (2014) which suggested that based on the information obtained from mtDNA, the maternal origin of all indigenous chickens is from red jungle fowl (*Gallus gallus gallus*) [9-29].

5. CONCLUSIONS

The results of the current study provided basic genetic information which will help for conservation, selection and breeding plan of the indigenous chicken in ECPM. High level of genetic diversity was observed in the current study. The phylogenetic tree using 11 haplotypes identified in this study revealed single maternal ancestry among the indigenous chicken in ECPM. Low nucleotide diversity was observed in Kelantan population. And it is an evidence of population bottleneck. Hence, conservation initiatives on Kelantan population should be carried out to prevent the loss of these natural gene resources.

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