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GENETIC DIVERSITY OF COMMERCIAL CHICKENS CONSUMED IN SELANGOR, MALAYSIA

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ABSTRACT

Chickens represent the most widely consumed meat in the world. Modern breeds are generally from a narrow genetic base. The genetic diversity of chickens consumed in urban areas of Malaysia has not been previously investigated. The aim of this study was to investigate the genetic diversity of chickens available for purchase in urban areas of Selangor adjacent to Kuala Lumpur. DNA of chickens were isolated from meats and livers. Seven microsatellite markers were selected and fluorescently labeled to allow the identification of each individual chicken from the seventeen populations based on the amplification of target DNA. A total of 52 different alleles was observed for the seven markers, giving a mean of 7.1 alleles per marker. The cumulative power of discrimination (CPd) of the seven microsatellites used was 0.999 based upon our population study. The data showed that most of the chickens consumed in the urban areas came from a very narrow genetic base. The supply is thus vulnerable to disruption caused by outbreaks of disease. Furthermore the data obtained illustrates the potential of this system to be used in chicken lineage identification. This would help to resolve uncertainties over the origin of the chickens. This system could be used for product assurance as well as safety.

Key words: Simple Tandem Repeats (STR), Halal assurance, Genotyping, Microsatellite, Individual identification

INTRODUCTION

Chicken (*Gallus gallus*) is a major source of protein in the world and the chicken population was more than 58 billion worldwide in 2011 (FAO, 2013). Chicken plays a very significant economic role in most countries as a source of food, as both their meat and eggs are consumed. Broilers (*Gallus gallus domesticus*) are the chickens that are bred and reared specifically to produce meat for human consumption.

The chicken population in Malaysia has undergone a dramatic shift in the last forty years. It is estimated by the FAO that the number has gone up from about 40,000 heads of chicken in 1973 to 270,000 in 2013 (http://faostat3.fao.org/browse/Q/QA/E). This is due to intensive farming systems for chickens, where outbreaks of disease could have a rapid and serious impact (IB Times, 2016; Chia, 2016) on chicken numbers. In addition, due to high demand, Malaysia imports chickens from Holland, Thailand and China with an average of 4806 tonnes of broilers from China alone annually (Mcintyre, 2008; Bernama, 2013). However, the smuggling of chickens from neighboring countries such as Thailand, Vietnam and Myanmar is a major problem (Regenstein & Chaudhry, 1994; Malaysia Competition Commission, 2014; The Star, 2015). This smuggling can also lead to the spread of highly

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pathogenic diseases to Malaysia (Mcintyre, 2008; Lim, 2013).

Molecular markers can also be used to distinguish chicken breeds. Genetic variation within a population can also be assessed. The simplest method of assessing genetic variability in a population is through the use of short tandem repeat (STR) typing and it is used for human as well as animal and plant populations (Butler, 2005). In STR typing, individual lineages can differ in the number of tandem repeats of a particular DNA sequence. This repeat unit is usually 2-10 bases (Goodwin, 2007). The number of repeats of a particular sequence can be determined by the polymerase chain reaction (PCR). By examining several such different repeats, lineages and individuals can be differentiated. The PCR for several markers can be conducted simultaneously and the process is known as multiplex PCR. Depending on the number of STR markers used, lineages or individual chickens can be identified and relatedness of populations can be determined. Several studies have been conducted to identify lineage using STR markers (Lee *et al.*, 2007; Eurlings *et al.*, 2010; White *et al.*, 2012; Lin *et al.,* 2014) in pigeons, big cats and cockatoos.

The genetic diversity of chickens available for consumption in Malaysia does not appear to have been previously reported. This is important because if there is close genetic identity then one would expect greater vulnerability to epidemics (Muir & Aggrey, 2003). In this paper we have developed a multiplex PCR assay based on seven markers to assay the diversity of chickens in retail outlets in Selangor. The markers were tested on DNA extracted from 114 chickens (from livers and meat) to provide an insight into the relatedness of chickens consumed in an urban area of Malaysia. Although the sample size is small, it is adequate to draw broad conclusions about the supply in Selangor (see Materials and Methods). The results have implications for vulnerability to microbial outbreaks and trace-ability of individual chickens and chicken meat samples.

MATERIALS AND METHODS

2.1 Sample Collections and STR Multiplex-PCR Genotyping

2.1.1 Sample collections and DNA extractions

A total of 114 samples from 95 individual livers and meats(breast) from 19 individual chickens (*G. gallus*) were collected from the following distributors: Aeon Co. Sdn Bhd, Bdr Sunway *(n=6)*, Aeon Big, Puchong Utama *(n=7)*, Cold Storage, Bandar Utama *(n=6),* Dindings *(n=7),* Econsave, Jalan Klang Lama *(n=7)*, Giant Superstore, Bandar Puteri Puchong *(n=7)*, Giant Brand Whole chicken, Bdr Puteri Puchong *(n=7)*, Hero Hypermarket Sdn Bhd, Batu 14 Puchong *(n=7),* Lay Hong, Subang *(n=7),* Mydin Mohamed Holdings Berhad, Subang *(n=7)*, NSK, Kuchai Lama *(n=7)*, PTS Poultry, Puchong Utama *(n=7)*, Tesco Puchong Jaya *(n=7)* and The Store (Malaysia) Sdn Bhd, Shah Alam. (*n=6)*. Samples of meat consists of Ayam Madu, Subang *(n=7),* Kopeda, Bdr Sunway *(n=7)* and NutriPlus, Bandar Puteri Puchong *(n=5)* as shown in Table 1. All of the livers or meat selected were certified Halal based on the presence of the Halal logo from JAKIM.

DNA from all the samples were extracted from the tissue using the DNeasy® Blood and Tissue Kit (Qiagen), following the manufacturer's protocol for purification of total DNA from animal tissues (Spin-Column Protocol).

2.1.2 Multiplex PCR Amplification and Genotyping

Seven polymorphic loci selected from previous chickens studies (McConnell *et al.*, 1999; Parmar *et al.*, 2007) are shown in Table 2. STR loci were amplified in a single heptaplex PCR reaction in a total volume of 25uL which contained approximately 10-40 ng of extracted DNA, the optimum concentration of each fluorescent primer (0.2 uM), 3 mM $MgCl_2$, 0.5 mM of each dATP, dTTP, dCTP, dGTP, 1 unit of MyTaq DNA (Bioline) and 5 uL of 10x reaction buffer (Bioline). This followed the

Table 1. Genetic variation for each chicken population included in the hepta-plex PCR system

Population	Ab	N	Na	Ho	He
Aeon	A	6	3.14	0.62	0.65
Aeon Big	AB	7	3.80	0.66	0.64
Ayam Madu	AM	7	3.71	0.54	0.67
Cold Storage	C	6	3.29	0.28	0.61
Dindings	D	7	3.33	0.73	0.82
Econsave	E	7	3.71	0.52	0.70
Giant	G	7	4.00	0.58	0.64
Giant Brand	GB	7	3.71	0.54	0.67
Hero	H	7	4.14	0.60	0.72
Kopeda	K	7	3.71	0.66	0.68
Lay Hong	LH	7	3.29	0.64	0.67
Mydin	M	7	3.25	0.48	0.61
NSK	N	7	3.29	0.52	0.75
NutriPlus	NP	5	3.71	0.71	0.65
PTS Poultry	P	7	4.29	0.64	0.73
Tesco	T	7	4.43	0.65	0.76
The Store	TS	6	3.83	0.51	0.73
Total		**114**			

[1]Abbreviations: Ab, abbreviation of breed; N, sample numbers; Na, mean number of alleles; Ho, mean observed heterozygosity; He, mean expected heterozygosity. *All of the numbers in this table are the average across all loci for each population.

Table 2. The size range, primer sequence, repeat motif and allele range of PCR products in each STR marker

Marker	Allele size range (bp)	Ch	Dye label	Primer Sequence (5'–3')	Repeat motif	Study
MCW-01	161	3	FAM	F: ACTGTCACAGTGGGGTCATGGACA R: ACACGTCCTGTGTCACATGCCTGT	$(TG)_9$	Parmar *et al* (2010)
MCW-07	313–349	1	FAM	F: AGCAAAGAAGTGTTCTCTGTCTCA R: TCACCCACGGGGACGAATAC	$(TG)_5$	Parmar *et al* (2010)
ADL-44	168	–	VIC	F: AAGTGGTTTATTGAAGTAGA R: CTGTGGTGTTGCGTTAGTTG	$(AC)_9$	Parmar *et al* (2010)
ADL-158	216	–	VIC	F: TGGCATGGTTGAGGAATACA R: TAGGTGCTGCACTGGAAATC	$(CA)_{12}$	Parmar *et al* (2010)
ADL-267	117	–	VIC	F: AAACCTCGATCAGGAAGCAT R: GTTATTCAAAGCCCCACCAC	$(CA)_{12}$	Parmar *et al* (2010)
LEI-246	250	1	FAM	F: TTGCACTGAGACCAAATGTC R: CATAGATTTTCCTTAGTAGGTAACTTG	$(CTTT)_{28}$	McConnell *et al* (1999)
LEI-248	248	2	VIC	F: TTTGAAAGTGACCATGATTCT G R: AAGCAGTTTCCAAGCTAAGAAC	$(TTTC)_{25}$	McConnell *et al* (1999)

Table 3. Allele frequency, Pd of each STR locus and Molecular variance of population

Allele	Locus							
	MCW01	LEI246	MCW07	ADL267	ADL44	ADL158	LEI248	Mean
Na	6	10	6	7	7	6	10	7.143
Pd	0.715	0.861	0.581	0.426	0.5681	0.7637	0.851	0.681
Ho	0.5	0.459	0.452	0.744	0.667	0.605	0.848	0.611
He	0.669	0.821	0.624	0.672	0.727	0.553	0.804	0.696
PIC	0.605	0.784	0.623	0.623	0.666	0.481	0.769	0.650
Pm	0.285	0.139	0.419	0.574	0.4319	0.2363	0.149	0.319

[2]Abbreviations: Na, number of allele; Pd, power of discrimination; Ho, observed heterozygosity; He, expected heterozygosity; PIC, polymorphic information content; Pm, match probability.

protocol recommended by the manufacturer. PCR amplification was conducted in a MyCycler thermal cycler (Biorad) at 95°C for 5 min; then 30 cycles – at 95°C for 30 s, 57°C for 90 s, and 72°C for 30 sec. with a final extension at 72°C for 30 min.

The seven fluorescently labeled PCR products were separated on an AB 3130XL Genetic Analyzer (Applied Biosystems, USA). Two different fluorescent dyes, FAM and VIC (Table 2) were used to tag one of the primers from each pair. The PCR products of loci MCW01, LEI246 and MCW07 were labeled with FAM; the loci ADL267, ADL44, ADL158 and LEI248 were labeled with VIC. Gene Scan LIZ 500 (Applied Biosystems) was used as the internal standard to score the alleles. Alleles were scored (Table 3) and individuals were genotyped using Gene Marker (Soft Genetics, LLC, State College, PA). The genotype of each sample was confirmed by comparison to the in-house prepared allelic ladder. The allelic ladder was generated by collecting alleles generated as a result of individuals from a wide range of samples and finally combining the different alleles.

2.1.3 Statistical Analysis

Linkage disequilibrium (LD) between the STR markers adopted in the multiplex amplification was analyzed with Arlequin v.3.5.1.3 software (Excoffier *et al.*, 2005). The allele frequency, observed heterozygosity (Ho), expected heterozygosity (He) and F-Statistics (F_{ST} and F_{IS}) were determined using Arlequin software Version 3.5.1.3. Polymorphic information content (PIC) was determined using Cervus software Version 3.0.3 (Kalinowski *et al.*, 2007) and deviation from Hardy-Weinberg equilibrium (HWE) was assessed using P value (P)

and this was determined using the GENEPOP software Version 4.0.10 (Raymond & Rousset, 1995).

RESULTS AND DISCUSSION

One hundred and fourteen samples were obtained from seventeen retail outlets in Selangor (Table 1). The retail outlets are from the urban area around the territory of Kuala Lumpur and were chosen as they represent the source of approximately 70% of the chickens bought in Malaysia for individual consumption (Malaysia Competition Commission, 2014).

Initially thirteen STR markers were tested on the meat from fourteen chicken's. Based on the data obtained from fragment analysis of these 13 STR markers, only seven STR markers were finally selected based on the number of potential alleles, and their chromosomal location. The PCR amplification efficiency and repeatability was also taken into consideration; only those primers that repeatedly produced clear and distinct products were used. The repeat motif and allelic range for each of the seven loci amplified by these primer pairs are shown in Table 2. All of the loci selected contain simple repeat motifs. Five of the seven loci are di-nucleotide repeats and two of them are tetra-nucleotide repeats. The primers sets used in this multiplex ensure that the size of the PCR products for the seven loci range from 100bp to 350bp making fragments more suitable for simultaneous amplification without bias (Sint *et al.*, 2012).

The results with the seven STR markers were first analyzed for linkage disequilibrium. None of the seven loci tested showed significant linkage disequilibrium after applying the Bonferroni correction for multiple testing ($p \leq 0.00238$). Based on the samples in this study, the combined power of discrimination (CPd) of the seven STR loci was 0.999 (Table 3). Complete STR profiles were generated from all the 114 samples. Each of the chickens could be distinguished from the other.

None of the seven loci investigated showed significant deviation from Hardy-Weinberg equilibrium (HWE) ($p<0.05$) (Table 1). There was low genetic differentiation between the different sub-populations (AMOVA test $F_{ST} = 0.00095$) but a moderate degree of fixation (Weir & Cockerham, 1984) (F_{IS} of 0.241) within each sub-population. A high degree of inbreeding is thus represented by the relatively high F_{IS} value, also known as inbreeding coefficient.

The parameters of average genetic variation and power of exclusion based on the seven loci for each population are shown in Table 1. All the populations had similar levels of observed heterozygosity except apparently those from the source 'Cold Storage' (H_o=0.28). However, the numbers from each source clearly needs to be increased to clarify the situation.

Interesting insights into the chicken supply market in Malaysia can be obtained from this study. The chickens used in this study come from sources that provide 70% of the chickens for consumption in Kuala Lumpur and Selangor (Malaysia Competition Commission, 2014). In Malaysia, the upstream segment of the broiler supply chain involves breeder farming of grandparent and parent stocks, and the farming of broilers for human consumption. Parent stock day-old chickens (DOC) are either sourced locally or imported and are placed in breeder farms according to a planned schedule. The parent stocks will lay eggs when they are about 25 weeks old, the hatching eggs are then sent to the hatcheries and will hatch into DOC in 21 days. DOCs are then immediately delivered to contract farmers or sold directly to customers and will be marketed as grown broilers when they are about 45 days old.

The very low F_{ST} value found in this study (0.0136) indicates that the sub-populations used by different suppliers are almost identical ($p < 0.1$). This is to be expected if the grandparent stock used by the various suppliers is closely related and probably originate from the same genetic material. Secondly the F_{IS} value 0.241 is quite high, showing a reduction in the number of observed heterozygotes compared to that expected at the loci tested in each sub-population. This confirms that the paternal and maternal stock are closely related. The genetic markers used are not related to broiler performance and therefore would not be selected for directly. Thus, the relative absence of heterozygotes indicated by F_{IS} suggests a long history of inbreeding. The F_{IT} value which measures the reduction in the average number of heterozygote loci observed for an individual in compared to that expected given the genetic make up for the entire population is very similar to the F_{IS} value, confirming that the sub-populations are very similar to one another and to the whole population. These are all worrying indicators for the Malaysian poultry industry as the genetic uniformity across the sub-populations makes the entire industry very vulnerable to epidemics. It is suggested that the industry urgently look into divergent genetic stocks that can produce broilers in order to be better prepared against epidemics.

We used seven microsatellite markers to conduct this study. In addition to providing insights into the genetic basis of the chicken consumed in suburban areas, the markers can also be used to establish a database of chickens for product quality assurance. For example, with the seven markers and the narrow genetic base surveyed, the data provides

a matching probability of 0.000118. The probability of two chickens having the same pattern is one in ten thousand in this very closely related population. With a more diverse population, the discrimination power would increase further. In addition, a larger number of microsatellites could be used. This would add more discriminatory power and reduced the matching probability but at increased cost. Moreover, a more comprehensive survey of chickens consumed in Malaysia could alter the genotype frequencies somewhat and also almost certainly further reduce the matching probability. Such a database could then be used to identify chickens of unknown provenance and hence unknown quality.

The nature of the chicken market in Malaysia can also be utilized to further improve the discriminatory power of the chicken markers. As described above the chicken market in Malaysia has transitioned from one of small suppliers providing individually bred local chickens to one where there are several large suppliers providing most of the chickens that are bought for consumption. These suppliers in turn buy parent day-old chickens. The genotyping of the grandparent stock – a relatively small number of chickens – would therefore provide a blueprint for all possible patterns for their progeny at the loci analyzed. A chicken that had a pattern that could not be produced from the grandparent stock at two loci – would therefore be from outside the market surveyed and may raise questions about its provenance and safety. This is analogous to paternity cases in humans where three exclusions (from using 12–14 markers) rule out the suspect from paternity.

In summary then, in this study, seven markers were multiplexed to provide a DNA profile. Use of the markers demonstrated that the chickens being sold at retail outlets have a very narrow genetic base with all its attendant risks. The markers also provide the power to discriminate among all of the chickens surveyed. A powerful approach, given the fast-evolving nature of the Malaysian chicken market, would be to determine the profile of the grandparent stocks and use them to exclude the possibility of a suspect chicken being descended from the grandparent genetic material. Thus more extensive testing and sampling may allow the establishment of a database that would allow one to check the genetic provenance and hence provide quality assurance of chickens being sold at retail outlets and this could also have utility in satisfying religious requirements such as Halal. For example, if the chickens are clearly not of Malaysian origin and yet have the Malaysian Halal logo, then a suitable explanation needs to be found.

It is recommended that the Malaysian poultry industry investigate routes to increasing the genetic diversity of the chickens used.

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