Poultry Science®

Identification and validation of novel breed-specific biomarker for the purpose of village chicken authentication using genomics approaches

Sara Nematbakhsh [*,§], Chong Pei Pei [†], Noordiana Nordin [*], Jinap Selamat, [*] Lokman Hakim Idris [‡], and Ahmad Faizal Abdull Razis [*,§,1]

[*]*Laboratory of Food Safety and Food Integrity, Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia (UPM), Serdang 43400, Selangor, Malaysia;* [†]*Faculty of Health and Medical Sciences, School of Biosciences, Taylor's University, Subang Jaya 47500, Selangor, Malaysia;* [‡]*Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM), Serdang 43400, Selangor, Malaysia; and* [§]*Natural Medicines and Products Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia (UPM), Serdang 43400, Selangor, Malaysia*

ABSTRACT Local village chicken, or "Ayam kampung" as it's known in Malaysia, is considered a premium chicken breed with a higher price than other chicken breeds. As a result of their comparable appearances and sizes, colored broiler chickens are often sold as village chickens, which is a form of food fraud that can result in a 3- to 4-fold rise in profit. Therefore, developing a breed-specific authentication method is crucial for preventing food fraud in the poultry industry. This study aims to investigate the genetic diversity of village chickens from other commercial chicken breed populations available in the market (broiler [Cobb], colored broiler [Hubbard], and layer [DeKalb]) to identify breed-specific DNA fragments as biomarkers for village chicken authentication. The Whole-genome sequencing and mutation calling of 12 chickens (3 chickens/breed) led to the identification of a total of 73,454,654 single nucleotide polymorphisms (**SNP**) and 8,762,338 insertion and deletions (**InDel**) variants, with more variants detected in the village chicken population (6,346,704 SNPs; 752,408 InDels) compared to commercial breeds. Therefore, this study revealed that village chickens were more genetically variable compared to other breeds in Malaysia. Furthermore, the breed-specific genomic region located on chromosome 1 (1:84,405,652) harboring SNP (C−T) with high discrimination power was discovered and validated which can be considered as a novel breed-specific biomarker to develop a method for accurate authentication of village chickens in Malaysia. This authentication method offers potentialw applications in the chicken industry and food safety.

Key words: village chicken, food fraud, chicken authentication, genetic variant, breed-specific biomarker

2024 Poultry Science 103:104128
https://doi.org/10.1016/j.psj.2024.104128

INTRODUCTION

Globally, the most widely consumed poultry meat is deemed to be chicken. The past several decades have seen a rise in the demand for chicken because of its health benefits, superior choice, and affordability compared to red meat, ease of processing, and lack of religious restrictions. In Asian nations, there has been a noticeable popularity for village chickens, where their cost are 2 to 4 times greater than commercial chickens (Feng et al.,2018). The demand for village chicken is particularly high because of its special flavor and superior flesh quality. The average live weight of a village chicken is between 1 and 1.5 kg, and its market age is approximately 4 to 5 mo (Tan et al., 2021; Azhan, 1994).

Underage-colored broilers are frequently offered as village chickens at a higher price to defraud people for financial benefit. Because of the size similarity of the entire carcass between the chicken breeds, manipulating the market age of chickens sold on the market could elevate the potential for food fraud and counterfeiting (Tan et al., 2021).

Moreover, consumers find it difficult to identify the breed of chicken according to retail cuts of breast meat and other chicken parts. While this kind of food fraud does not pose a health risk, finding a solution to protect consumer rights and promote fair trade is crucial (Fontanesi, 2017).

There are noticeable number of analytical methods applied in food authentication for species/breed detection

© 2024 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Received April 18, 2024.
Accepted July 24, 2024.
The nucleotide sequence data reported in this paper have been submitted to the National Center for Biotechnology Information (NCBI) and have been assigned the accession number PRJNA769554.

[1]Corresponding author: madfaizal@upm.edu.my

and identification; In which omics-based studies including genomics can compensate for the limitations of previous authentication methodologies (Böhme et al., 2019). The approach of whole genome re-sequencing has proven to be a powerful tool for genetic evaluation and genetic relationship exploration among different chicken breeds. For instance, Sun et al. (2022a) investigated the genetic diversity and selection of Guangxi indigenous chickens using whole genome resequencing data. Moreover, Boschiero et al. (2018) assessed the genome-wide genetic variants and putative regions under selection in meat and egg-type chicken lines through whole-genome resequencing. Zang et al. (2020) performed a genome-wide population genetic analysis of commercial, indigenous, game, and wild chickens.

On the other hand, in some prior studies, microsatellite markers and mitochondrial DNA sequence variants have been widely used in the genetic diversity, genetic structure, and origin analysis of local chicken breeds (Soglia et al., 2017). However, the coverage of these DNA markers in the whole chicken genome is extremely small. For example, there are only about 30 chicken microsatellite markers, and the D-loop region of chicken mitochondrial DNA is only about 1,200 bp in length. Furthermore, the available commercial chicken SNP arrays in the market are mostly driven from abroad commercial layers and broilers, lacking the specific genome variation information of Malaysian village chickens (Liu et al., 2019). Moreover, the universal primer sets which are commonly used for chicken identification in food products have a great limitation in the application for Malaysian village chicken authentication.

Therefore, in this study, in order to gain a greater knowledge of the genetic differences between village chickens and commercial chickens (broiler, colored broiler, and layer), a deep catalog of genetic variants (SNP and InDel) was produced by whole genome sequencing **(WGS)** of 12 chickens (3 chickens per breed). As far as we know, there are currently no recognized biomarkers or analytical methods that can differentiate village chickens from other commercial breeds in Malaysia. Therefore, the main aim of this study is to provide breed-specific genetic sits based on the whole-genome SNPs and InDels molecular marker identification which can be used as a breed-specific biomarker for village chicken differentiation and authentication for the first time in Malaysia. Furthermore, this discovered breed-specific biomarker can be applied to develop an authentication method for village chicken authenticity by the local authorities and industries. Therefore, this study aimed to develop the PCR assay kit based on the specificity of the discovered breed-specific biomarker and novel primer sets to differentiate village chicken from other commercial breeds available in the local market.

MATERIALS AND METHODS

Ethics Statement

The Institutional Animal Care and Use Committee (**IACUC**) at Universiti Putra Malaysia (**UPM**) gave their stamp of approval to all the animal experiments performed in this study (approval number: UPM/IACUC/AUP-R022/2019), and all the experiments were conducted following the guidelines set by this committee.

Experimental Population and Sample Preparation

In this study, 4 female chicken breeds were investigated, which varied in growth performance and other commercially essential features. Local farms which are located in Ladang Bukit Mertajam, Permatang Tinggi, and Butterworth, provided female broilers (Cobb, n = 15), colored broilers (Hubbard JA57, n = 15), and layers (DeKalb, n = 15) at their market age of 6, 10, and 48 wk (not functional for laying eggs), respectively. The Ladang Pahang Tua farm supplied the village chicken (Ayam Kampung, n = 15) population at the age of 4.5 mo. Chickens were selected on their market ages, and each line was raised under their specific environment and feeding regimes.

Genomic DNA was extracted from breast muscle (pectoralis major) using a Qiagen Tissue and Blood extraction kit according to the kit instruction, the gel electrophoresis analysis, and Qubit were used for checking degradation and concentration of extracted DNA, respectively. The extracted DNA was stored at −80 °C for further analysis.

It should be noted that a total number of 12 samples (3 female chickens/breed) were used for library preparation, NGS, and genetic differentiation. Subsequently, after high putative region ($0 > 0.99$) identification, a total number of 15 female chickens/breed were used for breed-specific biomarker validation.

Library Preparation and Sequencing

The high-quality genomic DNA was fragmented to an average size of ∼350 bp and subjected to DNA library creation using NEBNext Ultra II DNA library prep kit for Illumina according to the manufacturer's protocol, followed by a quality control test that included size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies, CA), and molarity measurement using RT-PCR (Kanzi et al., 2020). In the current study the sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA) to generate 2×150-bp paired-end reads with a 30x sequencing depth per sample.

Raw Data and Sequencing Quality Control

The original image data (raw binary file) from the sequencer in the form of .bcl file were converted into sequenced reads (raw reads) via base calling using Consensus Assessment of Sequence and Variation (**CASAVA**) v1.8.2. Then, raw data were stored in FASTQ (. fq) format files, which contain both raw

sequencing reads and corresponding base quality scores (Cock et al., 2009).

Sequencing Data Filtration

The sequencing raw reads were subjected to quality control (**QC**) to filter low-quality and unstable sequencing reads using fastp v0.20.0 with the parameters as " -g -q 5 -u 50 -n 15 -l 150 −min_trim_length 10 −overlap_-diff_limit 1−overlap_diff_percent_limit 10". In this study, the quality data filtration criteria include: (1) remove the paired reads when either read contains adapter contamination; (2) discard the paired reads when uncertain nucleotides (**N**) constitute more than 10 percent of either read; (3) discard the paired reads when low-quality nucleotides (base quality less than 5, $Q \leq 5$) constitute more than 50 percent of either read. The output from this step is a "cleaned" FASTQ file (Cock et al., 2009; Chen et al., 2018).

Sequencing Alignment and Mapping Statistics

Subsequently, filtered, and effective sequencing reads were aligned to the reference genome (Gallus gallus-GRCg6a) using Burrows-Wheeler Aligner (**BWA**) software v.0.7.8-r455 (parameters: mem -t 4 -k 32 -M). The alignment results were used to calculate mapping rates, which reflect the degree to which each individual's genome is similar to the reference genome, as well as depth and coverage, which reflect the degree to which each individual's genome is evenly distributed and similar to the reference genome (Li and Durbin, 2009).

Genetic Variants Detection and Annotation

Once alignment was complete, the original BAM files from BWA were sorted and indexed using SAMtools v.1.3.1 and then Picard v.1.111 was used to merge the BAM files from the same samples. In this step, mutations that do not match the reference genome are called from the BAM file; these calls are presented in the form of a text-based file in Variant Call Format (**VCF**) file.

SNPs and InDeLs identification were performed using SAMtools v.1.3.1, with the following parameter " mpileup -m 2 -F 0.002 -d 1000" (Li et al., 2009). In order to reduce the variant detection error rate, the filtered criteria were applied as follows: (1) the number of support reads for each SNP should be more than 4; (2) the Mapping Quality (**MQ**) of each SNP should be higher than 20. Subsequently, the annotation of detected SNPs was done through ANNOVAR (Dec 14 2015) (Wang et al., 2010).

Principal Component Analysis

The principal component analysis (**PCA**) analysis was performed based on the exonic genetic variants (VCF file) in all chicken populations using a vcf2PCA. Two principal components and their linear discriminants were extracted as horizontal and vertical coordinates using Plotly.

Population Differentiation

According to the produced quality-controlled exonic SNPs and InDels datasets, the fixation index (**Fst**) (Weir and Cockerham pairwise) (Weir and Cockerham, 1984) was calculated among village chicken and commercial chicken populations using VCFtools. Fst calculates the proportion of genetic variation that can be explained by population structure using Wright's F-statistics (Luo et al., 2019). The Fst value has a range from 0 to 1, where 0 reveals that the chicken breed population are not differentiated, whereas 1 indicates the highly and completely differentiated populations (Luo et al., 2019; Zhang et al., 2020). Accordingly, in order to achieve a greater understanding of the genetic differences between village chicken and commercial chicken groups using the deep catalog of genetic variants (SNPs, InDels), firstly there was a need to merge all separate BAM files belonging to the commercial chicken group (Broiler [Cobb], Colored-broiler [Hubbard], and Layer [Dekalb]) to re-arrange and create the 1 merged VCF file named commercial chicken group and another file named village chicken using Strelka software. Subsequently, Fst value calculation was performed using VCFtools v.0.1.15 software with SNPs (n = 1,103,949) and InDels (n =29,791) datasets.

It should be noted that in the current study, the Fst values were only calculated between village chicken (**VC**) and commercial chicken groups to evaluate the level of genetic differentiation between these 2 chicken groups.

Breed-Specific Biomarker Identification

The population differentiation followed by finding genetic variants with high Fst value (>0.99) to empower the significant differentiation of village chickens, provided a comprehensive array of genetic variants with high Fst value on autosomal, sex, and mitochondrial chromosomes that can be effectively used for the authentication of village chickens. Subsequently, further validation of the differentiation power of these genetic variants was performed using PCR, Sanger sequencing, HRM, and allele-specific PCR by randomly selecting variants (Fst > 0.99) on various autosomal chromosomes to identify and validate a significant breed-specific genomic region as a biomarker for village chicken authentication. The steps of breed-specific biomarker identification and validation were as follows:

Primer design for sanger sequencing based on the genomic region of identified specific genetic variants, the breed-specific primer sets were designed using primer 3.0 software. All the primers used in this study were provided by Apical Scientific Sdn Bhd and the sequences were presented in Table 1.

PCR amplification and detection PCR amplification of the target regions was performed at the total volume of 25 μl: 1 μl DNA template, 12.5 of μl 2 × PCR Taq master mix (ABM, Canada), 1 μl forward primer, 1 μl reverse primer, and 9 μl of Ranse/DNase free water. The PCR amplification procedure was performed at thermocycler as follows: predenaturation at 94°C for 3 min, denaturation at 94°C for 30 s, suitable annealing temperature and time was applied according to the primer sets, extension at 72°C for 1 min/1 kb template, 35 cycles, and final extension at 72°C for 5 min. The PCR products were first run through gel electrophoresis to check the specificity and sensitivity of the designed primer set to target the region of interest. Subsequently, the PCR products revealed the single and strong band on the gel for both chicken populations (VC and commercial chicken) were sent for sanger sequencing.

Sanger sequencing The purified and validated PCR products were sent for Sanger sequencing using 1st BASE Sequencing service. Subsequently, the produced electrogram or sequencing trace file can be viewed and checked for quality value by Biosystem Sequence Scanner 2.0 software. The samples with clear peaks and without background noise at the region of interest were considered for further validation.

High-resolution melting temperature (HRM) The highly trusted genomic region approved by PCR and Sanger sequencing was gone through the next validation method using High-resolution melting temperature (**HRM**) which is a highly sensitive method allowing discrimination of DNA sequencing differing of only 1 nucleotide substitution or 1 base pair InDels. The HRM assay was done in a total volume of 20 μl, containing 10 μl of HRM master mix (WisPure), 2 μl of each reverse and forward breed-specific primer (Table 1), 2 μl DNA template, and 4 μl of Ranse/DNase free water. The HRM analysis was carried out using LightCycler 480 (Roche, Switzerland) and the LightCycler version 1.5.1.62SP3 software was used to analyze the HRM data. In this software, normalized melting curves and temperature-shifted differential plots were acquired from the program's gene scanning module. The Conditions for the thermal cycling were as follows: predenaturation at 95°C for 5min followed by 45 cycles of amplification at 95°C for 10s and annealing at 60°C 30s and a final extension at 72°C for 10s. Subsequently, amplicons were subjected to HRM analysis followed by melting analysis in the same instrument using the temperature range of 75°C to 95°C with 15 acquisitions per every 1°C increment.

Allele-specific primer design to target novel biomarker Ultimately, the validated genomic region employing 3 validation methods can be considered as the novel breed-specific biomarker for village chicken authentication. As the last validation method, the allele-specific primer set was designed, with the help of the primer 5.0 software, to specifically target the discovered breed-specific biomarker which enable us to specifically differentiate village chickens from other breeds (Table 1). Subsequently, the allele-specific PCR was carried out at a total volume of 25 μl: 1 μl DNA template, 12.5 of μl 2 × PCR Taq master mix (ABM, Canada), 1 μl forward primer, 1 μl reverse primer, and 9 μl of Ranse/DNase free water. The PCR amplification procedure was performed at thermocycler as follows: predenaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 60°C for 20 s, extension at 72°C for 20 s, 35 cycles, and final extension at 72°C for 5 mins.

RESULTS AND DISCUSSION

Genetic Variants Identification and Annotation

A genome-wide SNP and InDels identification in the village chicken, broiler-Cobb, colored broiler-Hubbard, and layer-DeKalb was conducted to gain a detailed map of genetic variation in these lines as well as find genomic regions with high discrimination power to differentiate among VC and commercial chicken breed populations (Surya et al., 2019).

Through WGS and after quality control, a total of 1,828,405,668 clean raw reads were obtained from 4 breeds of chicken (3 female chickens/breed), corresponding to an average depth between 15.24x to 19.84x and coverage of more than 98%. Besides, the overall mapping rate was greater than 98%.

A genome-wide SNP and InDels identification in the VC and commercial chickens led to the identification of a total of 73,454,654 SNPs and 8,762,338 InDels from all 12 samples (3 female chickens/breed), in which the village chicken harbored a higher number of genetic variants as compared to commercial chicken groups, while layer revealed the lowest number of genetic variants. Moreover, village chicken showed a higher heterozygosity rate compared to other breeds, in which the higher heterozygous genetic variants in VC revealed its broad genetic backgrounds. Globally, the genetic characterization of different indigenous chicken breeds to investigate

Table 1. Sequence of primer sets used for biomarker identification and validation.

Primer sequence	Type	Product size	Experiment
5'-GTCATGTAGCAGTTATGGGAGG-3'	F	393	Sanger sequencing
5'-AGGCTCATGCATGCTTTTCT-3'	R		
5'-CCTTACCCTCAAGCAGGTATGA-3'	F	108	HRM
5'-TCGCAGTTAAAATGACTTAGGC-3'	R		
5'- AGGTATGAGATAGCATCCTACT-3'	F	318	Allele-specific PCR
5'-AGGCTCATGCATGCTTTTCT-3'	R		

Abbreviations: F, forward; R, reverse.

their population structure and diversity revealed the higher genetic diversity of indigenous chickens compared to commercial broilers (Buttie Machete et al., 2021; Ren et al., 2022; Yacouba et al., 2022), as well as high genetic diversity among local chicken populations come from separated geographic regions (Habimana et al., 2020). For instance, a study in Malaysia revealed that the genetic diversity of Kampung chickens was higher in Pahang and Terengganu compared to Kelantan (Ha et al., 2017). Furthermore, annotation of the variants exhibited that most of them belonged to noncoding regions of the genome and only 1.5% of the SNPs and 0.3% of the InDels were located in exonic regions, including 1,103,949 SNPs and 29,791 InDels that may alter protein function and have the highest phenotype impact. The transition and transversion (ts/tv) ratio for SNPs is 2.43 which is almost the same in all chicken breeds ($P < 0.05$). Similar TS/VS ratios in chicken were reported by different studies (Boschiero et al., 2018; Moreira et al., 2018; Qiu et al., 2020). The statistics of SNP and Indels detection and annotation can be seen in Tables 2 and 3, respectively.

Overall, indigenous chickens seem to be more genetically variable compared to commercial breeds (Boschiero et al., 2018; Li et al., 2020; Zhang et al., 2020; Wang et al., 2021; Sun et al., 2022b) since they have long breeding history compared to commercial breeds. Therefore, the preservation of local chicken, as a genetic resource, is essential to meet unexpected future breeding demand (Zhang et al., 2020). In the same way, a study by Wang et al. (2021) revealed that red jungle fowls and native chickens possessed more genetic diversity compared to commercial chickens. This result is consistent with known reductions in the genetic diversity of modern livestock compared to their wild ancestors. Poultry genetics resources, which include different types of chicken varieties, are disappearing quickly due to various reasons. Therefore, creating collections of genetic material such as germplasm collection can help prevent a total loss of genetic diversity. These collections ensure that a range of genetic options is available to revive and maintain the different chicken populations (Sun et al., 2022b).

Due to the poor annotation of functional noncoding regions of the chicken genome, it is challenging to determine the impact of variations found in these noncoding regions (Boschiero et al., 2018). Therefore, in this study, only genetic variants (SNP = 1,103,904 and InDel = 29,791) located in the coding region of the genome have been used for further finding the highly putative regions to use as biomarker for Malaysian chicken differentiation and authentication.

Principal Component Analysis

The PCA was performed using the total exonic SNPs and InDels datasets to characterize the pattern of individual samples as well as the genetic distance among 4 chicken breed populations. The population structure of the 4 chicken breed populations, comprising village

Table 2. Statistics of SNP detection and annotation.

Chicken ID	Upstream	Exonic				Intronic	Splicing	Downstream	Upstream/ Downstream	Intergenic	ts/tv	Het rate (%)	Total
		Stop gain	stop loss	Synonymous	Nonsynonymous								
VC	83,813	270.67	66.33	66,102	29,373	2,949,387	185	79,127	6,131.3	3,069,366	2.4407	3.778	6,346,704
CB	79,595	273.33	56.33	63,120	28,138	2,812,429	182.33	75,209	5,733.7	2,920,311	2.4413	3.4537	6,045,362
COB	79,818	272.67	57	62,535	27,888	2,835,430	174.67	75,623	5,735.7	2,958,034	2.4367	3.402	6,105,894
LA	78,301	258	57.33	62,060	27,453	2,776,421	171.33	74,288	5,629	2,902,876	2.4387	3.284	5,986,925

Abbreviations: COB, broiler (cobb); CB, colored broiler; LA, layer; VC, village chicken.

Table 3. Statistics of InDels detection and annotation.

Chicken ID	Exonic						Intronic	Intergenic	Insertion	Deletion	Het rate (%)	Total
	Stop gain	Stop loss	Frameshift deletion	Frameshift insertion	Nonframeshift deletion	Nonframeshift insertion						
VC	18.33	4.33	614	1042.3	496.67	434.7	357,219	381,608	404,735	346,876	0.33733	752,408
CB	19	3.33	564	969.3	472	442.67	341,953	363,949	388,941	330,654	0.3107	720,255
COB	16	4	589.7	970.67	457.33	423.33	350,121	375,180	398,377	338,998	0.308	738,123
LA	14	3.67	552.33	942.3	440	436.33	338,905	363,517	387,271	328,316	0.28967	716,228

Abbreviations: COB, broiler (cobb); CB, colored broiler; LA, layer; VC, village chicken.

chickens, broilers-Cobb, colored broilers-Hubbard, and Layers-Dekalb were analyzed using PCA. As can be seen, the PCA demonstrated (Figure 1) genetic differences between the village chicken and commercial chicken populations and revealed that all individuals are well clustered by breed, showing a consistent genetic relationship within the population. However, the village chickens were clustered more loosely together, indicating greater genetic variation within the population.

The result showed the top 2 principal components accounted for 1.58 % (PC1) and 1.37% (PC2) of the total variability (Figure 1). Accordingly, the differentiation between village chickens and commercial chicken breeds was clearly displayed on the x-axis, which showed 1.58 % of the total genetic variance. The village chicken population was clearly separated from other chicken breeds, confirming the results of Fst values measurement.

At the same time, the layer chicken population can also be differentiated via the second eigenvector (1.37% of total variance), revealing the genetic differentiation between the layers and the broiler breed populations. As previously mentioned, the layer population had the lowest genetic variation compared to other breeds, in which the PCA result was consistent with the result of genetic characterization. Furthermore, the broiler and colored broiler breeds were closely clustered together, exhibiting similarity in their genetic structure and lower genetic differentiation between these 2 breeds.

Overall, the PCA results presented the genetic differences among 4 breeds of chicken and disclosed that village chickens can be significantly differentiated from other breeds. Based on the PCA, the presence of 3 distinct clusters among the 4 chicken populations was observed: the village chicken population clearly differentiated from other populations (cluster 1) which approved the results of Fst value with a range of 0.99-1; revealing the high genetic differentiation between village chicken and commercial chicken. The layer population (cluster 2) with lower genetic variation, separating from the broiler population and cluster 3 in which broiler and color broilers were grouped together, showing a consistent genetic relationship (Figure 1).

The PCA in other studies also revealed the clear separation of indigenous chicken breeds. For instance, in the study of Zhang et al. (2020) the PCA revealed that indigenous Chinese breeds were clearly separated from commercial breeds, which may be related to their geographic location, selection targets, and production performance. Moreover, the Tibetan Silkies one of the indigenous Chinese breeds showed scattered distribution, indicating greater genetic variation within the breed (Zhang et al., 2020). Furthermore, identifying genetic differences between red jungle fowl and indigenous village chickens including Ethiopian, Arabian, and Seri Lankan domestic chickens presented a significant separation of domestic populations from red jungle fowl based on the PCA results (Lawal et al., 2018). The study of Sun et al. (2022a) also presented the genetic differentiation of Guangxi indigenous chickens from commercial chickens according to their clustering

Figure 1. PCA revealed the clear differentiation of village chicken from other breeds based on the SNPs and InDels datasets. Cluster 1: village chicken (VC); Cluster 2: layer (LA); Cluster 3: colored broiler (CB) and broiler-Cobb (COB).

trend in PCA analysis. The result of these studies was consistent with our study, revealing that village chickens were more genetically variable within a population and can be significantly differentiated from other commercial breeds.

Genetic Differentiation

The Fst statistic, which quantifies genetic differentiation, can be used to quantify the degree of genetic similarity between animal breeds (Hall, 2022). In fact, different genetic backgrounds and intensive artificial selection can cause a high degree of genetic differentiation between indigenous and commercial chickens in specific genomic regions (Zhang et al., 2020).

In this study, Fst values were obtained from quality-controlled SNPs and InDels datasets separately using an overlapping sliding window of 20 kb with 10 kb step sizes. A majority of the windows had low Fst value (<0.2), while a few windows showed significantly high Fst values (Fst>0.99). The genomic region revealed Fst > 0.99 considered as the significant putative regions which can be considered as a biomarker for village chicken authentication.

The results of our study support findings from other studies that applied Fst measurement for chicken breed differentiation. For instance, the genetic differentiation estimation among commercial, indigenous, and wild chicken lines, revealed the average Fst value ranged from 0.03 to 0.27; the higher chicken Fst values, underlined the larger genetic variability of the chicken population, in which the Fst of commercial breeds and Chinese indigenous breeds had the highest differentiation (Zhang et al., 2020). In another study, 28 microsatellite markers were used to differentiate between Italian indigenous chicken breeds, broilers, and layer lines. The data analysis of genetic differentiation using Fst value revealed moderate to great differentiation between Italian indigenous breeds and commercial lines (Soglia et al., 2017). Furthermore, Li et al. (2020) characterized the genetic variations and genetic relationships among Chinese indigenous chicken breeds and commercial lines (broilers and layers) using 28 SNP markers loci exhibiting that the 3 native chicken populations, shared close genetic relationship based on these 28 SNP markers, which is distinctively different from both commercial broiler and layer. In another investigation, the genome-wide weighted Fst distribution was calculated based on the 91,649 and 89,847 SNPs and InDels database between broiler and layer chicken populations, which revealed that the Fst value range between 0.3-0.9, in which the highest Fst value (0.976) was observed on several regions such as chromosome 8 (GGA8:28,220,001-28,240,000). Subsequently, these regions were considered as the selection signature regions to find genes potentially related to fat deposition and muscle development (Boschiero et al., 2018).

Overall, various marker loci have been applied for molecular traceability (Soglia et al., 2017), in which SNP is an applicable genetic marker based on the variability at the nucleotide level, to characterize the genetic variations and genetic relationship among the different populations (Li et al., 2020).

In this study, according to the SNP and InDels datasets and further genetic differentiation analysis, 9 genomic regions harboring genetic variants (SNP and InDel) with Fst value >0.99 and high differentiation power were discovered (Table 4) based on their visualization on the integrative genomic viewer (IGV). To further consider these genetic regions as a novel and strong breed-specific biomarker for village chicken authentication, we need to validate these bioinformatically discovered genomic regions in the laboratory through various methods of genetic variant detection including PCR, sanger sequencing, HRM, and allele-specific PCR.

Table 4. Selected genetic variants with Fst > 0.99 on different chromosomes.

Genetic variant	Chromosome	Position	Fst Value
SNP	GGA1	14,597,276	0.99
SNP	GGA1	84,405,652	0.99
SNP	GGA1	38,115,570	0.99
SNP	GGA2	4,850,494	0.99
SNP	GGA6	28,561,043	0.99
SNP	GGA8	20,027,787	0.99
SNP	GGA15	5,487,758	0.99
SNP	GGA18	9,780,975	0.99
InDels	GGA1	67,019,463	0.99

Abbreviations: InDels, insertion and deletion; GGA, chromosome; SNP, single nucleotide polymorphism.

Breed-Specific Biomarker for Village Chicken Authentication

Sanger Sequencing In this study, 4 breeds of chickens were divided into 2 separate groups, including the VC population and the commercial chicken population (broiler, colored broiler, and layer). Subsequently, the individual DNA samples of the village chicken group (15 chickens) and the commercial chicken group (15 from each breed) were separately pooled together, providing 2 sample representatives, VC, and commercial chicken groups. Furthermore, among all discovered genomic regions with high Fst values (Fst > 0.99) on different chromosomes, the total amount of 9 genomic regions, which significantly showed different genotypes between VC and commercial chicken populations were considered randomly (Table 4) from both SNP and InDel. Then the specific primer sets were designed based on the genomic sequence of these regions (Table 4). Subsequently, after PCR running, the amplicons that represented the strong bands on the gel electrophoresis for both chicken groups (VC, commercial chicken) were sent for Sanger sequencing.

Sanger Sequence Analysis The genomic region in which its chromatogram exhibited the minimum baseline noise, evenly-spaced peaks, and clearly showed the presence of targeted genetic variants in both VC and commercial chicken groups have been used for further validation analysis. Among these 9 genomic regions, the SNP located on chromosome 1 (1:84,405,652) (Figure 2) exhibited a strong band on the gel (Figure 3) and sharp peak with clear distinguishable genotypes in VC and commercial chicken groups on the chromatogram (Figure 4). Based on this genomic region, the VC population had an SNP with a CT genotype, while the commercial chicken population had the same SNP but with a CC genotype. The PCR results of the amplicon targeted with designed breed-specific primer (Table 1) according to breed-specific biomarker sequence (1:84,405,652) for all 4 breeds as well as the Sanger results of the same region were presented in Figures 3 and 4, respectively. As can be seen in the gel image, all samples positively produced the expected 393 bp amplicons, which confirmed the existence of genetic marker in both chicken groups (VC, commercial chicken). Subsequently, the chromatogram demonstrated the clear separation of these 2 chicken groups based on the different genotypes of targeted SNP. Therefore, this genomic region was chosen as a putative breed-specific biomarker among all 9 genomic regions to be further validated with HRM and allele-specific PCR.

Sanger sequencing of the PCR-generated amplicon using species-specific primers can be considered one of the DNA-based approaches for meat species identification by comparing the obtained sequence to other sequences of the same targeted DNA region (Fontanesi, 2017). For instance, the study of Xing et al. (2019) has used a short segment of 16S ribosomal RNA (16S-rRNA) mitochondrial gene as a good DNA metabarcoding for the authentication of animal species in complex meat and poultry using Sanger and next-generation sequencing. Accordingly, the amplification fragments of 16S rRNA from pig and chicken were verified by Sanger sequencing and then were used as a reference sequence for NGS analysis.

As mentioned earlier, in the current study, Sanger sequencing of genomic loci containing genetic variants

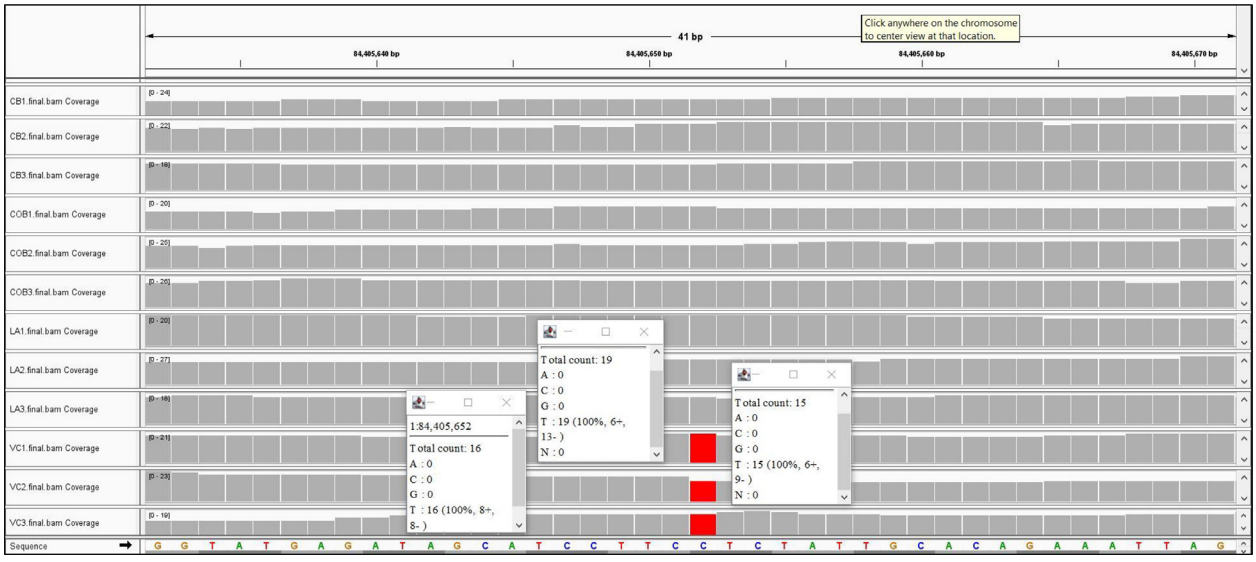

Figure 2. The representation of genomic region containing breed-specific biomarker on Integrative Genomics Viewer (IGV).

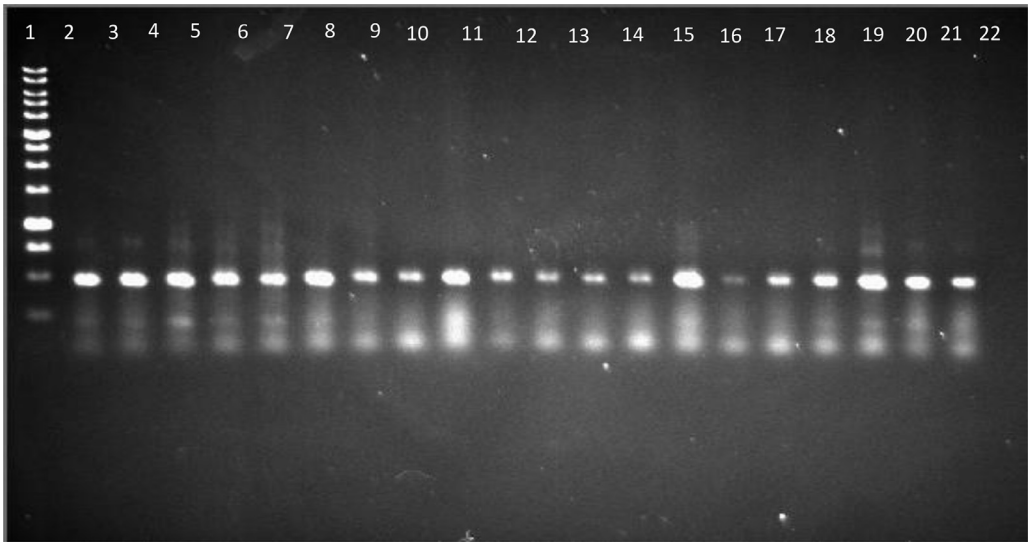

Figure 3. Representative image of PCR products on agarose gel. Lines 1: DNA ladder (1 kb), Lines 2 to 6: village chicken, Lines 7 to 11: Broiler-Cobb, Lines 12 to 16: Colored broiler-Hubbard, Line 17 to 21: Layer-Dekalb, 22: No template control (dH2O) Product size: 393 bp.

with high discriminating power, led to the discovery of a novel SNP located on chromosome 1 (1:84,405,652) with different genotype (CT in village chickens, CC in commercial breeds), which can be considered as a breed-specific biomarker for village chicken authentication in Malaysia. Accordingly, different studies tried to find the breed-specific genetic variant to use as the genotyping panel for specific chicken breeds. For instance, a novel chicken 55k SNP genotyping array was developed for the genetic diversity analysis based on whole genome sequencing of Chinese indigenous breeds, in which this discovered SNP dataset was specific to Chinese breeds (Liu et al., 2019). In other study, the high density 600k SNP was developed for chicken genotyping based on the whole genome sequencing of different lines of layer and broiler chickens which were commercially available to the public (Kranis et al., 2013). In the current research, a high-density SNP array for genotyping of local

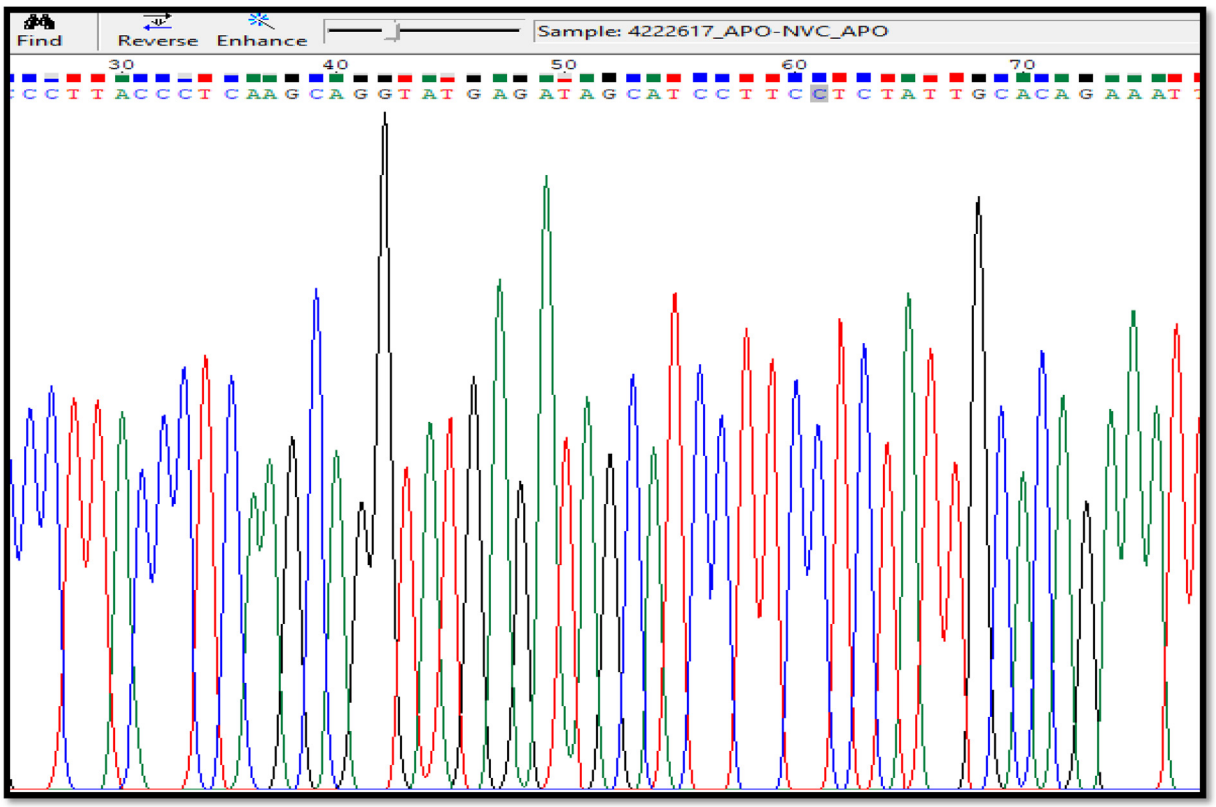

Figure 4. Sanger sequencing results for biomarker validation. the commercial chicken population showed CC genotype (Up); Village chicken showed CT genotype (Down) for the biomarker discovered on chromosome 1 (1:84,405,652).

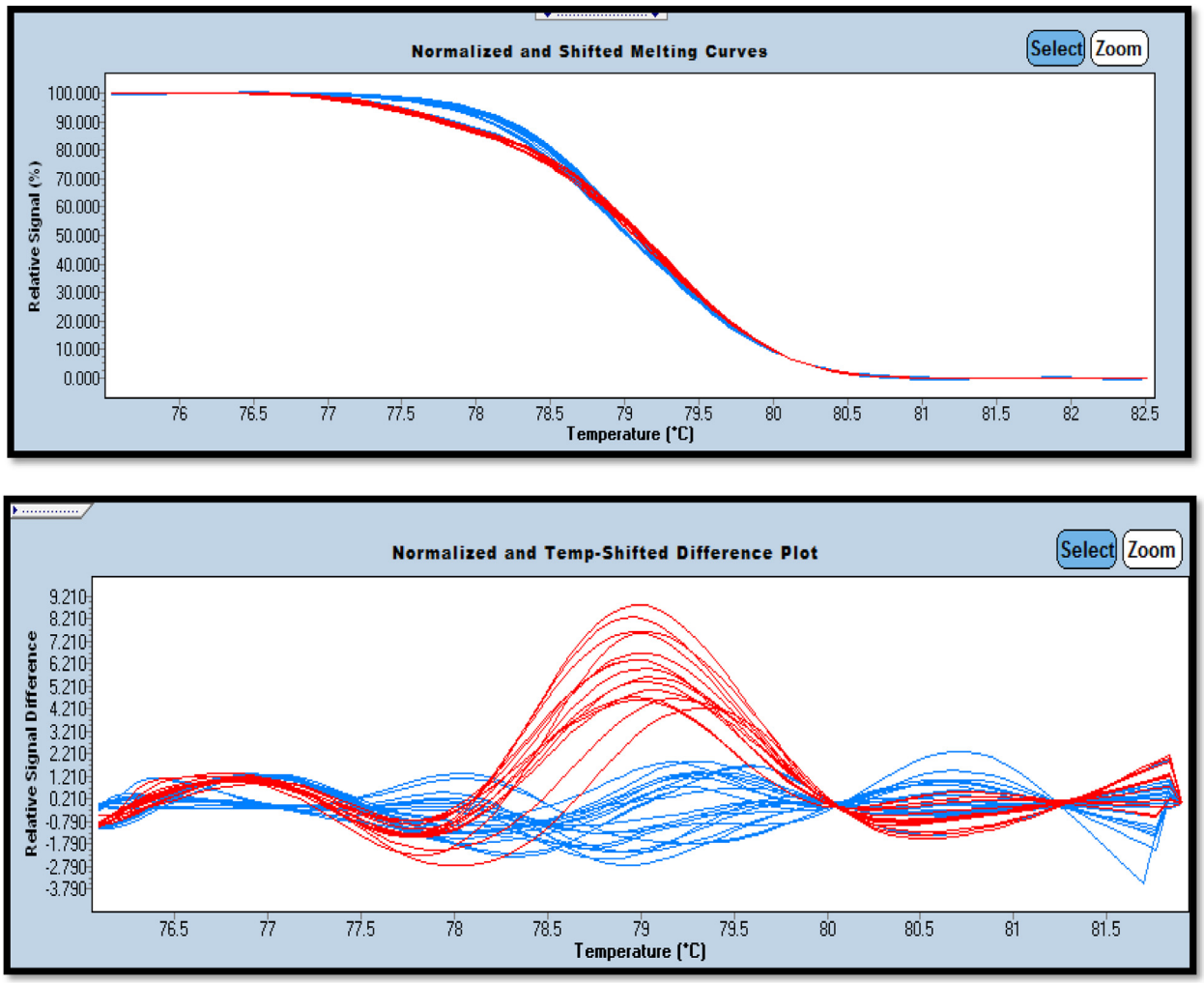

Figure 5. High-resolution melting temperature (HRM) validates the differentiation power of biomarker for village chicken authentication. red line = village chicken group, blue line = commercial chicken group.

Malaysian chickens can be developed, which requires validation of the discovered breed-specific biomarker in a larger sample of village and commercial chickens, as well as investigation of additional breed-specific markers to create a more reliable authentication panel.

Other investigations used SNPs or InDels datasets produced through WGS to find biomarkers associated with specific phenotypic traits or diseases (Shi et al., 2012; Wei et al., 2013; Boschiero et al., 2018; Zhao et al., 2018). On the other hand, some studies used the genetic variant datasets to study the population structure of different chicken breeds to conserve their genetic variability and the phenotypic features of these chicken breeds (Strillacci et al., 2017; Lawal et al., 2018; Zhao et al., 2018; Zhang et al., 2020).

High-Resolution Melting Temperature Assay After the identification of the genomic region (GGA1:84,405,652) harboring highly putative genetic variants with significant genotype variation among VC (CT) and commercial chicken group (CC), the HRM assay combined with designing the target-specific primers for HRM analysis was applied for the further validation of the genomic region for village chicken authentication. Since the sensitivity and specificity of HRM analysis will be affected by the length of the resultant DNA amplicon and shorter amplicons provide melt profiles that are typically less complex than longer amplicons (Biosystem, 2010); therefore, in this study a new primer set targeting the region of interest (GGA1:84,405,652) was designed (Table 1) with amplicon length of 108 pb to guarantee a sensitive detection of sequence variants between VC (15 female chickens) and commercial chicken groups (15 female chickens/breeds).

Subsequently, a qPCR assay using EvaGreen dye coupled with HRM analysis was developed targeting the region of interest (1:84,405,652) as a potential breed-specific biomarker to differentiate VC from the commercial chicken population. The result of HRM demonstrated the presence of distinct genotypes of region of interest among VC (CT) and commercial chicken (CC) populations. According to the normalized melting curves (Figure 5), 2 distinct melting curves were obtained for the targeted genomic region containing a breed-specific molecular marker which is based on differences in the melting temperature due to nucleotide substitution (CT genotype in

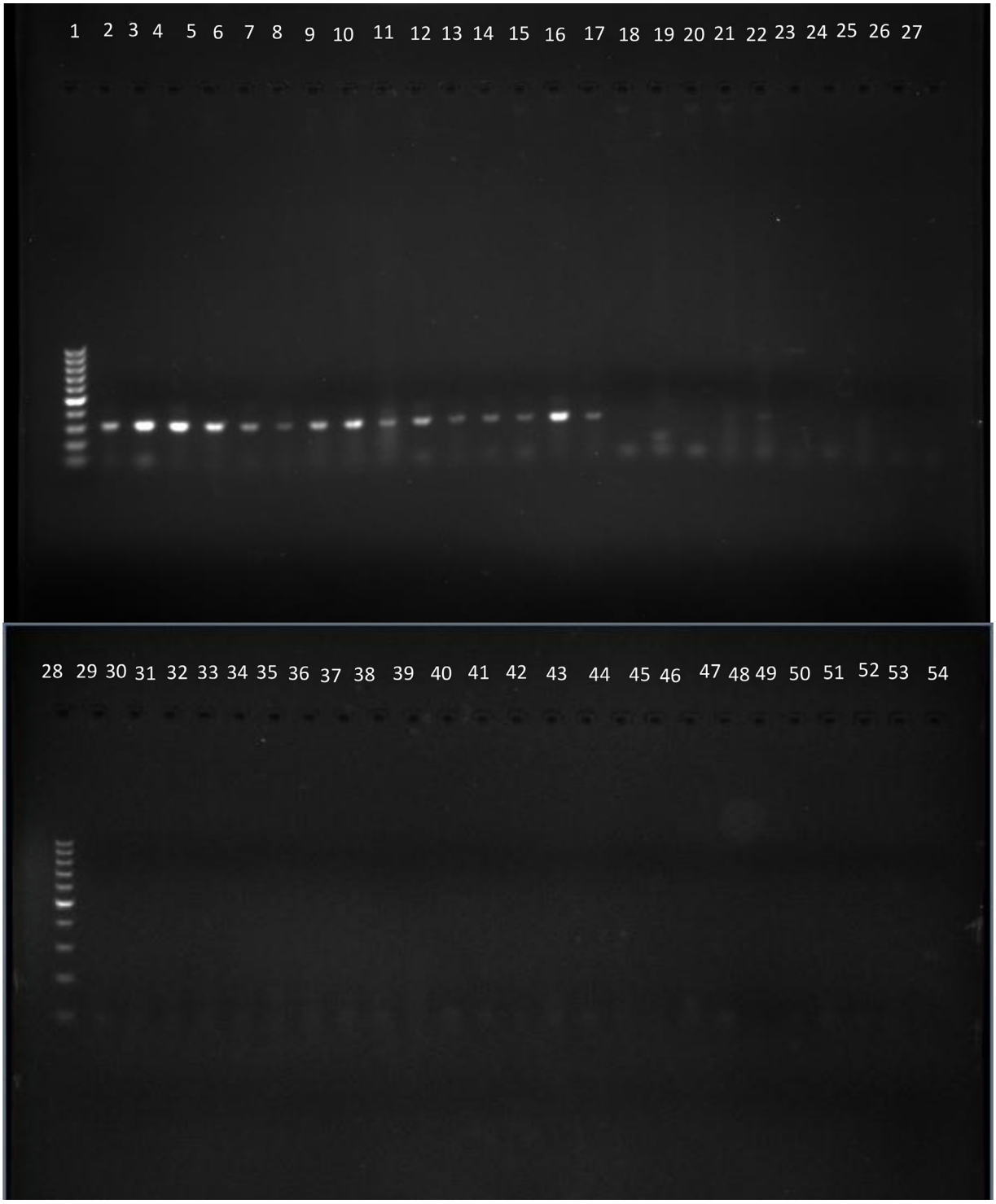

Figure 6. The result of allele-specific PCR. Lines 1: ladder; 2 to 16: village chicken; 17 to 27: broiler-Cobb; 28: Ladder; 29 to 38:Colored broiler-Hubbard, 39 to 48: layer-DeKalb; 49 to 50: Duck; 51 to 52: quail; 53 to 54: Nontemplate Control (NTC). Product size: 318 bp.

village chickens and CC genotype in commercial breeds). In fact, a clear segregation of the melt peaks at 79°C enabled the differentiation of village chickens from a commercial chicken population. According to the normalized melting curves and the temperature-shifted differential plots (Figure 5), 2 distinct melting curves were obtained for the targeted genomic region containing a breed-specific molecular marker which is based on differences in the melting temperature with a level of confidence greater than 99.2%. Therefore, this genomic region which is located on chromosome 1 (1:84,405,652) contains the SNP with mutant genotype (T/T) in village chicken breeds and wild genotype (C/C) in commercial chicken breeds were discriminated by changes in the melting temperature, in which this result was consistent with Sanger sequencing outcome. Therefore, this discovered and validated genomic region was further used as the novel breed-specific biomarker to differentiate village chickens from commercial chicken breeds using the specifically designed breed-specific primer.

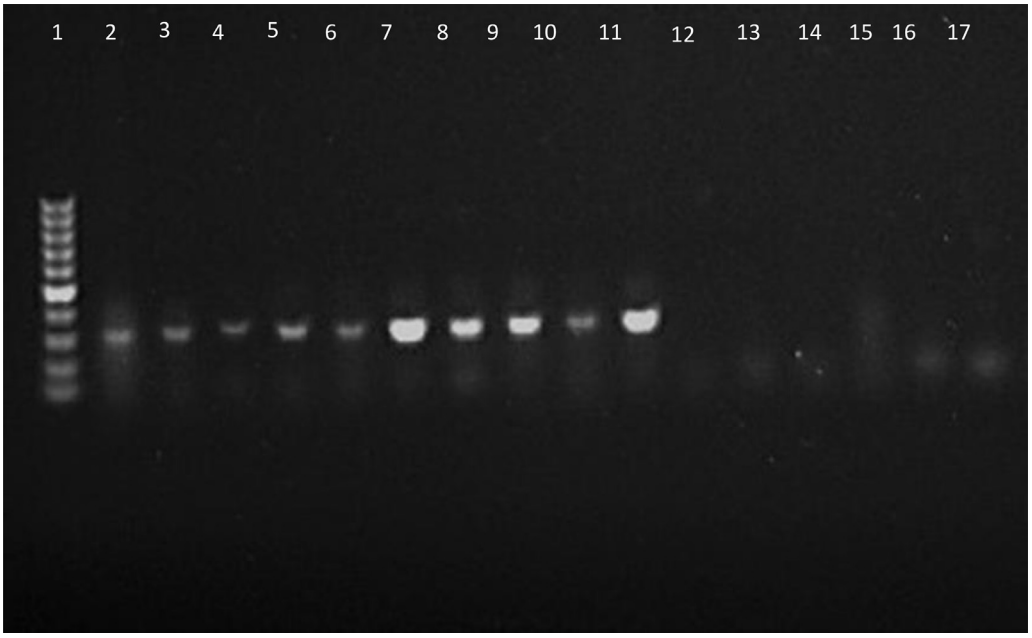

Figure 7. The specificity of allele-specific primer. Lines 1: ladder, 2 to 11: village chicken, 12 to 14: duck, and 15 to 17: quail. Product size: 318 bp.

Overall, our HRM result further verified the discovered breed-specific biomarker, in which HRM analysis has been considered a superb tool for the identification and differentiation of closely related species. Several studies have applied HRM for animal species/ breed authentication. For instance, in the study of Fernandes et al. 2017, the HRM using *cytochrome b* mini-barcode with high variability among species was applied for the Gadidae fish species authentication. The HRM results revealed the clear discrimination of 4 fish species in different clusters, which can be used as an authentication method to successfully identify gadoid species in commercial fish-containing foods (Fernandes et al., 2017). Another study performed HRM analysis for the species identification of minced meat samples as a method for meat fraud detection (Gholamnezhad et al., 2021). Moreover, HRM was considered as the reliable, fast, and affordable detection method that can be used for discrimination of Common types of meat utilized in the meat industry by using universal primer pair targeting mitochondrial 16S rRNA. In this method, separate melting patterns were produced for each species which enabled species discrimination (Jafar et al.,2023).

Allele-Specific Polymerase Chain Reaction Allele-specific polymerase chain reaction (**AS-PCR**), also known as PCR amplification of specific alleles, is a PCR-based approach that can be used to detect recognized SNPs (Darawi et al., 2013; Liu et al., 2012).

An allele-specific PCR method was developed for further validation of discovered breed-specific biomarker which was approved as a biomarker for differentiation of VC (15 female chickens) and commercial chicken populations (15 female chickens/breed). Allele-specific primers were designed (Table 1) based on different criteria 1) Permit the PCR amplification only if the nucleotide at the 3' end of the primer complemented the base at the homozygous wild-type variant that exists in VC samples 2) A single artificial mismatch nucleotide was placed within the 3 bases nearest to the 3′end (SNP location), to overcome low SNP detection efficiency (Liu et al., 2012). Accordingly, primer design was applied by introducing 2 types of mismatches in the forward primer sequence, 1 at the 3' end and the second site near the 3' end of the forward primer sequence. Based on the study of Cha et al. (1992) mismatch sites and mismatch bases were normally chosen closest to the SNP site. Therefore, in this study, the mismatch sites are close to the SNP site and the designed forward primer only can amplify the DNA samples harboring the mutant-type genetic variant in the VC population. Furthermore, in the present study, the DNA samples from ducks (3 females) and quails (3 females) as other poultry species have been used to guarantee the specificity of the discovered breed-specific biomarker and the designed allele-specific primer. The allele-specific PCR was carried out on the conventional thermocycler using the designed allele-specific primers and DNA templates and detected by agarose gel electrophoresis (Figure 6). As can be seen in Figures 6 and 7, the allele-specific primer could generate 318 bp amplicon by successfully targeting the SNP (C to T) at position 84,405,652 in village chicken groups which led to clear differentiation of village chickens from other chicken breeds and poultry species (duck and quail). Therefore, the discovered and validated genomic region can be considered as the breed-specific biomarker for village chicken authentication. In various food authentication methods, there is a need for designing species/ breed-specific primers to target the species/breed of interest in food and food products. For instance, 1 study by designing a specific primer based on the Cytochrome b (***CYTB***) gene aimed to authenticate pork in commercial meatballs, in which this primer was validated by

RT-PCR that has the specificity and sensitivity for pork identification in the commercial samples (Orbayinah et al., 2019). Besides, Allele-specific primers have variety of applications in the chicken industry, for example, an RT-PCR using an allele-specific primer was done to genotype the Myxovirus resistance protein (***Mx***) gene G2032A SNP (nonsynonymous polymorphism [G to A]), which was demonstrated to be related to antiviral activity resistance; in this method, the resistant A allele was the preferred allele which needs to be detected (Ye et al., 2010). In our study, the detection of the mutant-type T allele (village chicken) is the preferred allele of breed-specific biomarker to be targeted and detected by allele-specific primer which enables us to authenticate village chicken.

Even though HRM and allele-specific PCR have some limitations in meat authentication, such as needing technical expertise and specialized equipment, these assays are among the most effective molecular tests to detect fraudulent meat at a percentage of very small-sized DNA sequences in the mix and highly processed meat samples. These molecular test can offer us with precise and reliable results that could improve food safety and protect consumer rights (Adenuga et al., 2023; Azad et al., 2023).

However, as technology continues to advance, this discovered breed-specific genomic region with an improved primer set is going to be considered as the novel breed-specific biomarker for developing and fabricating lateral flow nucleic acid-based assay (**LFNAA**) strips as an on-site method for Malaysian village chicken authentication. The unique aspect of this work will be its novelty regarding the genomic region that can be considered as a biomarker to differentiate village chicken from commercial breeds. Moreover, this developed portable lateral flow assay will be the first assay for the purpose of chicken breed authentication which can be beneficial for the industry for on-site detection of chicken breed fraud in Malaysia.

CONCLUSION

The present study demonstrated the differentiation of the village chicken population from the commercial chicken population by genomic approaches using NGS and further bioinformatics analysis and validation methods. The WGS and mutation calling revealed that the village chicken population has more genetic variants (6,346,704 SNPs; 752,408 InDels) compared to commercial breeds.

Moreover, the NGS and further validation methods contributed to the discovery of a putative genomic region located on chromosome 1 (1:84,405,652) harboring SNP (C to T) that can be considered a novel breed-specific biomarker for village chicken authentication.

In conclusion, the discovered and validated breed-specific biomarker and novel primer set with high discriminating power have successfully differentiated village chickens from commercial breeds. Therefore, this novel breed-specific biomarker coupled with PCR assay can serve as an authentication method, which can be applied as a baseline method for fraud identification in the Malaysian chicken industry. In addition, our team is in the process of applying this novel breed-specific biomarker to develop LFNAA strips to enable rapid and on-site detection of village chickens in Malaysia.

ACKNOWLEDGMENTS

The authors would like to thank the Ministry of Higher Education for the HICoE rendered to the Institute of Tropical Agriculture and Food Security (ITAFoS), Universiti Putra Malaysia (UPM); Institute of Bioscience, UPM, and the Faculty of Food Science and Technology, UPM, for the facilities rendered. This project also extends its sincere appreciation to Mr. Eddy Shahriza Jamein for his invaluable support and guidance in conducting the animal study and sampling. This research was supported by the Ministry of Higher Education of Malaysia (MOHE) under (UPM/800-4/11/MRUN/2019/5539130); Putra Grant-IPS of Universiti Putra Malaysia (GPIPS/2020/9683500) and Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA) (Ref. No. GBG19-3260).

DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

Adenuga, B. M., and M. Montowska. 2023. A systematic review of DNA-based methods in authentication of game and less common meat species. Comprehen. Rev. Food Sci. Food Safety 22:2112–2160.

Applied Biosystem. (2010). A guide to high resolution melting (HRM) analysis.

Azad, M. A. K., M. Dey, F. Khanam, B. Biswas, and S. Akhter. 2023. Authentication of meat and meat products using molecular assays: A review. J. Agric. Food Res. 12:100586.

Azahan, E. E. 1994. The red and black-red native chickens of Malaysia. Malaysian Agric. Res. Dev. Inst. Res. J 22:73–78.

Böhme, K., P. Calo-Mata, J. Barros-Velázquez, and I. Ortea. 2019. Recent applications of omics-based technologies to main topics in food authentication. TrAC Trends Anal. Chem 110:221–232.

Boschiero, C., G. C. M. Moreira, A. A. Gheyas, T. F. Godoy, G. Gasparin, P. D. S. C. Mariani, …, L. L. Coutinho. 2018. Genome-wide characterization of genetic variants and putative regions under selection in meat and egg-type chicken lines. BMC Genom. 19:1–18.

Buttie Machete, J., P. M. Kgwatalala, S. J. Nsoso, N. L. Hlongwane, J. Cassius Moreki, and J. B. Machete. 2021. Genetic diversity and population structure of three strains of indigenous tswana chickens and commercial broiler using single nucleotide polymormophic (SNP) markers. Open J. Anim. Sci. 11:515–531.

Cha, R. S., H. Zarbl, P. Keohavong, and W. G. Thilly. 1992. Mismatch amplification mutation assay (MAMA): application to the cH-ras gene. Genom. Res. 2:14–20.

Chen, S., Y. Zhou, Y. Chen, and J. Gu. 2018. fastp: An ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34:i884–i890.

Cock, P. J. A., C. J. Fields, N. Goto, M. L. Heuer, and P. M. Rice. 2009. The Sanger FASTQ file format for sequences

with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Res. 38:1767–1771.
Darawi, M. N., C. Ai-Vyrn, K. Ramasamy, P. P. J. Hua, T. M. Pin, S. B. Kamaruzzaman, and A. B. A. Majeed. 2013. Allele-specific polymerase chain reaction for the detection of Alzheimer's disease-related single nucleotide polymorphisms. BMC Med. Genet. 14:1–8.
Fernandes, T. J. R., J. Costa, M. B. P. P. Oliveira, and I. Mafra. 2017. DNA barcoding coupled to HRM analysis as a new and simple tool for the authentication of Gadidae fish species. Food Chem. 230:49–57.
Feng, Y., Y. Cai, X. Fu, L. Zheng, Z. Xiao, and M. Zhao. 2018. Comparison of aroma-active compounds in broiler broth and native chicken broth by aroma extract dilution analysis (AEDA), odor activity value (OAV) and omission experiment. Food Chem. 265:274–280.
Fontanesi, L. 2017. Meat authenticity and traceability. Lawrieś Meat ScienceWoodhead Publishing, 585–633.
Gholamnezhad, P., H. Ahari, G. Nikbakht Brujeni, S. A. A. Anvar, and A. Motallebi. 2021. Evaluation of high resolution melting (HRM) analysis for meat species identification of raw and cooked meat. Separations 8:116.
Ha, H. C., S. I. Nguang, S. Zarizal, C. F. Komilus, I. Norshida, and K. Ahmad-Syazni. 2017. Genetic diversity of kampung chicken (Gallus gallus domesticus) from selected areas in East Coast Peninsular Malaysia inferred from partial control region of mitochondrial DNA. Malays. Appl. Biol. 46:63–70.
Habimana, R., T. O. Okeno, K. Ngeno, S. Mboumba, P. Assami, A. A. Gbotto, C. T. Keambou, K. Nishimwe, J. Mahoro, and N. Yao. 2020. Genetic diversity and population structure of indigenous chicken in Rwanda using microsatellite markers. PLoS One 15:e0225084.
Hall, S. J. G. 2022. Genetic differentiation among livestock breeds—values for Fst. Animals 12:1115.
Kanzi, A. M., J. E. San, B. Chimukangara, E. Wilkinson, M. Fish, V. Ramsuran, and T. de Oliveira. 2020. Next generation sequencing and bioinformatics analysis of family genetic inheritance. Front. Genet. 11:544162.
Kranis, A., A. A. Gheyas, C. Boschiero, F. Turner, L. Yu, S. Smith, R. Talbot, A. Pirani, F. Brew, P. Kaiser, P. M. Hocking, M. Fife, N. Salmon, J. Fulton, T. M. Strom, G. Haberer, S. Weigend, R. Preisinger, M. Gholami, and D. W. Burt. 2013. Development of a high density 600K SNP genotyping array for chicken. *BMC Genom.* 14:1–13.
Lawal, R. A., R. M. Al-Atiyat, R. S. Aljumaah, P. Silva, J. M. Mwacharo, and O. Hanotte. 2018. Whole-genome resequencing of red junglefowl and indigenous village chicken reveal new insights on the genome dynamics of the species. Front. Genet. 9:264.
Li, H., and R. Durbin. 2009. Fast and accurate short read alignment with Burrows−Wheeler transform. Bioinformatics 25:1754–1760.
Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, and R. Durbin. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079.
Li, J. J., L. Zhang, P. Ren, Y. Wang, L. Q. Yin, J. S. Ran, ..., Y. P. Liu. 2020. Genotype frequency distributions of 28 SNP markers in two commercial lines and five Chinese native chicken populations. BMC Genet. 21:1–10.
Liu, J., S. Huang, M. Sun, S. Liu, Y. Liu, W. Wang, X. Zhang, H. Wang, and W. Hua. 2012. An improved allele-specific PCR primer design method for SNP marker analysis and its application. Plant Methods 8:1–9.
Liu, R., S. Xing, J. Wang, M. Zheng, H. Cui, R. P. Crooijmans, and J. Wen. 2019. A new chicken 55K SNP genotyping array. BMC Genomics 20:1–12.
Luo, Z., J. Brock, J. M. Dyer, T. Kutchan, D. Schachtman, M. Augustin, Y. Ge, N. Fahlgren, and H. Abdel-Haleem. 2019. Genetic diversity and population structure of a Camelina sativa spring panel. Front. Plant Sci. 10:184.
Moreira, G. C. M., C. Boschiero, A. S. M. Cesar, J. M. Reecy, T. F. Godoy, F. Pértille, ..., L. L. Coutinho. 2018. Integration of genome wide association studies and whole genome sequencing provides novel insights into fat deposition in chicken. Scientific Rep. 8:16222.
Orbayinah, S., H. Widada, A. Hermawan, S. Sudjadi, and A. Rohman. 2019. Application of real-time polymerase chain reaction using species-specific primer targeting on mitochondrial cytochrome-b gene for analysis of pork in meatball products. *J.* Adv. Vet. Anim. Res. 6:260–265.
Qiu, M., C. Yang, H. Du, Q. Li, Z. Zhang, X. Xiong, C. Yu, X. Song, C. Hu, B. Xia, L. Yang, H. Peng, L. Liu, and X. Jiang. 2020. Whole-genome resequencing reveals aberrant autosomal SNPs affect chicken feathering rate. Anim. Biotechnol. 33:884–896.
Ren, T., M. Nunome, T. Suzuki, and Y. Matsuda. 2022. Genetic diversity and population genetic structure of Cambodian indigenous chickens. Anim. Biosci. 35:826–837.
Shi, D., P. Li, L. Ma, D. Zhong, H. Chu, F. Yan, Q. Lv, C. Qin, W. Wang, M. Wang, N. Tong, Z. Zhang, and C. Yin. 2012. A genetic variant in pre-miR-27a is associated with a reduced renal cell cancer risk in a chinese population. PLoS One 7:e46566.
Soglia, D., P. Sacchi, S. Sartore, S. Maione, A. Schiavone, M. De Marco, M. T. Bottero, A. Dalmasso, D. Pattono, and R. Rasero. 2017. Distinguishing industrial meat from that of indigenous chickens with molecular markers. Poult. Sci. 96:2552–2561.
Strillacci, M. G., M. C. Cozzi, E. Gorla, F. Mosca, F. Schiavini, S. I. Román-Ponce, F. J. Ruiz López, A. Schiavone, M. Marzoni, S. Cerolini, and A. Bagnato. 2017. Genomic and genetic variability of six chicken populations using single nucleotide polymorphism and copy number variants as markers. Animal 11:737–745.
Sun, J., T. Chen, M. Zhu, R. Wang, Y. Huang, Q. Wei, M. Yang, and Y. Liao. 2022a. Whole-genome sequencing revealed genetic diversity and selection of Guangxi indigenous chickens. PLoS One 17: e0250392.
Sun, Y., Y. Li, Y. Zong, G. M. K. Mehaisen, and J. Chen. 2022b. Poultry genetic heritage cryopreservation and reconstruction: advancement and future challenges. J. Anim. Sci. Biotechnol. 13:115.
Surya, T., M. R. Vineeth, J. Sivalingam, M. S. Tantia, S. P. Dixit, S. K. Niranjan, and I. D. Gupta. 2019. Genomewide identification and annotation of SNPs in Bubalus bubalis. Genomics 111:1695–1698.
Tan, C., J. Selamat, N. N. Jambari, R. Sukor, S. Murugesu, and A. Khatib. 2021. Muscle and serum metabolomics for different chicken breeds under commercial conditions by GC−MS. Foods 10:2174.
Wang, K., H. Hu, Y. Tian, J. Li, A. Scheben, C. Zhang, Y. Li, J. Wu, L. Yang, X. Fan, G. Sun, D. Li, Y. Zhang, R. Han, R. Jiang, H. Huang, F. Yan, Y. Wang, Z. Li, and X. Kang. 2021. The chicken pan-genome reveals gene content variation and a promoter region deletion in IGF2BP1 affecting body size. *Mol. Biol.* Evol. 38:5066–5081.
Wang, K., M. Li, and H. Hakonarson. 2010. ANNOVAR: Functional annotation of genetic variants from high-throughput sequencing data. Nucl. Acids Res. 38.
Wei, Y., H. Huang, Z. Meng, Y. Zhang, J. Luo, G. Chen, and H. Lin. 2013. Single nucleotide polymorphisms in the leptin-a gene and associations with growth traits in the orange-spotted grouper (Epinephelus coioides). Int. J. Mol. Sc. 14:8625–8637.
Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370.
Xing, R. R., N. Wang, R. R. Hu, J. K. Zhang, J. X. Han, and Y. Chen. 2019. Application of next generation sequencing for species identification in meat and poultry products: A DNA metabarcoding approach. Food Contr. 101:173–179.
Yacouba, Z., H. Isidore, K. Michel, G. B. Isidore, T. Boureima, M. Vinsoun, K. Maurice, B. Ousseni, Z. Moussa, B. Y. M. C. Valerie, R. Romdhane, and N. A. Joseph. 2022. Genetic diversity and population structure of local chicken ecotypes in burkina faso using microsatellite markers. Genes 13:1523.
Ye, X., Y. Zhang, Z. Tan, and K. Li. 2010. Single-tube real-time multiple allele-specific pcr for genotyping chicken mx gene g2032a snp. Br. Poult. Sc. 51:361–367.
Zhang, J., C. Nie, X. Li, Z. Ning, Y. Chen, Y. Jia, J. Han, L. Wang, X. Lv, W. Yang, and L. Qu. 2020. Genome-wide population genetic analysis of commercial, indigenous, game, and wild chickens using 600K SNP microarray data. Front. Genet. 11.
Zhao, Q. B., R. R. Liao, H. Sun, Z. Zhang, Q. S. Wang, C. S. Yang, X. Z. Zhang, and Y. C. Pan. 2018. Identifying genetic differences between dongxiang blue-shelled and white leghorn chickens using sequencing data. *G3:* Gen. Genom. Genet. 8:469–476.