



Microsatellite Markers Reveal Genetic Diversity and Population Structure of Village Chicken in Peninsular Malaysia

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HIGHLIGHTS

- Malaysian village chicken populations in Peninsular Malaysia exhibited a moderate level of genetic diversity (mean H_o of 0.58), potentially influenced by factors such as small sample size, non-random mating, and population sub-structuring.
- Population structure, phylogenetic tree and PCA analyses identified three genetic groups of Malaysian village chicken ecotypes corresponding to geographic regions: northern, central and southern.
- Village chickens retain a distinct genetic pool, with most genetic variation occurring among individuals, highlighting their potential importance for conservation and future breeding programs.

Microsatellite Markers Reveal Genetic Diversity and Population Structure of Village Chicken in Peninsular Malaysia

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Abstract: The emphasis on high-yield poultry breeds has contributed to the decline and underutilisation of indigenous chickens. In Malaysia, the “kampung chicken” or Malaysian Village Chicken (MVC) is often viewed as low in productivity. Yet, it remains culturally important and is well adapted to local environments, making it a valuable resource for sustainable poultry production. As selective breeding and improvement efforts, such as the development of the dual-purpose Saga chicken, expand, comprehensive genetic characterisation of MVC is essential to safeguard native diversity, guide future breeding strategies and prevent genetic erosion. This study aimed to ascertain the genetic diversity of MVC ecotypes across Peninsular Malaysia and examine their genetic relationships with commercial and jungle fowl breeds using simple sequence repeat (SSR) markers. 15 village chicken ecotypes, including Saga chicken, were sampled from multiple regions, together with four commercial breeds (Cobb, Ross, Sasso and Hy-Line) and jungle fowl chickens. 20 SSR markers were used, of which 17 polymorphic markers successfully amplified. Cluster analyses using Unweighted Pair Group Method With Arithmetic Mean (UPGMA) and Neighbour-Joining dendrograms revealed two major genetic groups. MVC formed a distinct lineage separate from commercial breeds and further segregated into three subclusters reflecting geographical origins. Although Sasso broilers resemble MVC phenotypically, SSR analysis confirmed clear genetic differentiation. These findings

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underscore the unique genetic structure of Malaysian Village Chickens, which likely supports their adaptability to tropical regions and efficient utilisation of local feed resources. The findings provide a strong foundation for conservation planning, genetic resource management and the strategic optimisation of future breeding programmes.

Keywords: Simple Sequence Repeats (SSRs), Indigenous Breed, Genetic Differentiation, Polymorphism

Abstrak: Penekanan terhadap baka ayam berproduktiviti tinggi telah menyumbang kepada kemerosotan serta kurangnya penggunaan ayam asli tempatan. Di Malaysia, “ayam kampung” atau Ayam Kampung Malaysia (MVC) sering dianggap rendah produktiviti, namun ia tetap penting dari segi budaya dan sangat sesuai dengan persekitaran tempatan, menjadikannya sumber berharga bagi pengeluaran ayam yang mampan. Dengan usaha pembiakbakaan terpilih dan penambahbaikan seperti pembangunan ayam Saga dwifungsi yang semakin berkembang, pencirian genetik menyeluruh terhadap MVC adalah penting untuk melindungi kepelbagaian asli, membimbing strategi pembiakbakaan masa hadapan dan mencegah hakisan genetik. Kajian ini bertujuan untuk menentukan kepelbagaian genetik ekotip MVC di seluruh Semenanjung Malaysia serta meneliti hubungan genetiknya dengan baka komersial dan ayam hutan menggunakan penanda ulangan jujukan ringkas (SSR). Sebanyak lima belas ekotip ayam kampung termasuk ayam Saga, telah disampel dari pelbagai kawasan bersama empat baka komersial (Cobb, Ross, Sasso dan Hy-Line) serta ayam hutan. Dua puluh penanda SSR digunakan, yang mana 17 berjaya diamplifikasi dan menunjukkan polimorfisme. Analisis kluster menggunakan dendrogram kelompok berpasangan tak berpemberat dengan min aritmetik (UPGMA) dan Neighbour-Joining menghasilkan dua kumpulan genetik utama. MVC membentuk keturunan tersendiri yang berbeza daripada baka komersial dan seterusnya terbahagi kepada tiga subkluster yang menunjukkan asal usul geografi. Walaupun ayam pedaging Sasso menyerupai MVC dari segi fenotip, analisis SSR mengesahkan perbezaan genetik yang jelas. Penemuan ini menekankan struktur genetik unik Ayam Kampung Malaysia yang berkemungkinan menyokong keupayaannya untuk menyesuaikan diri dengan kawasan tropika serta penggunaan sumber makanan tempatan secara cekap. Hasil kajian ini menyediakan asas kukuh bagi perancangan pemuliharaan, pengurusan sumber genetik dan pengoptimuman program pembiakbakaan strategik masa depan.

Kata kunci: Ulangan Jujukan Ringkas (SSR), Baka Tempatan, Perbezaan Genetik, Polimorfisme

INTRODUCTION

The Food and Agriculture Organization’s (FAO) Domestic Animal Diversity Information System (DAD-IS) identifies over 15,000 national breed populations and more than 8,800 breeds across 38 species worldwide (FAO 2024). This diversity is vital for food security and sustainable livelihoods. Native breeds, which are well adapted to their local environments, offer significant economic, scientific and cultural value due to their resilience and disease resistance. However, commercial selective breeding has reduced genetic variability, placing 29% of local breeds at risk of extinction (FAO 2021). This loss is particularly severe in developing countries, where high-yielding international breeds often replace native ones, threatening livestock diversity.

Village chicken refers to chicken reared and bred under small-scale backyard farming systems in rural villages. These chickens provide an alternative to broilers, reducing farmers' reliance on imported breeds and costly feed, while attracting consumers seeking better nutrition and taste. In 2017, the global chicken population exceeded 22 billion (FAO 2024), with approximately 1,600 recognised local chicken breeds (FAO 2020). In Malaysia, the chicken population reached 296 million in 2022, accounting for 96% of the country's total livestock (DOSM 2022). This statistic comprised of 63% broilers, 47% egg-layers, 4% village chickens, 1% quails and 0.01% other poultry. Despite their unique traits, village chickens remain underexplored, with limited information on their ecotypes. They are characterised by slender bodies, tall stature, strong chests and agility. They can jump or fly moderate distances, exhibit scavenging behaviour, show high immunity and are able to withstand tropical heat.

Malaysia's poultry industry exemplifies successful agricultural self-sufficiency, achieving a 99.9% rate by 2021 and exporting surplus products to neighbouring countries such as Singapore (DOSM 2022). Poultry is a staple in the national diet, with Malaysia having one of the highest per capita consumption rates globally. Nonetheless, the industry faces challenges, including dependence on imported chickens, rising feed costs, geopolitical tensions, increasing demand and fluctuating exchange rates. Today, Malaysia's poultry industry balances hardy local breeds like the Ayam Kampung (Village Chicken) with imported breeds such as Cobb, Ross and Sasso, ensuring a steady supply of fresh and nutritious poultry products for consumers across the country.

The Malaysian Agricultural Research and Development Institute (MARDI) has developed a new village chicken breed known as "Ayam Saga" or Saga chicken. This dual-purpose breed was created by crossing two pure village chicken lines (Farahiyah *et al.* 2022). Saga chicken retains the traditional village chicken's characteristics while exhibiting improved growth and laying performances. The breed is known for its uniformity in body size and feather colour. Saga chickens reach marketable weights within 10 to 12 weeks, with males reaching 1.8 kg and females 1.5 kg.

In recent years, the Sasso hybrid—a French coloured broiler adapted for free-range farming—has often been mistakenly marketed as authentic village chicken and sold at a premium price, raising concerns among traditional farmers who argue that it lacks the unique qualities of pure village chickens. Such misrepresentation threatens farmers' livelihoods and disrupts the market for genuine village chickens, posing a challenge to the sustainability of the industry.

As part of efforts to legally define the Malaysian Village Chicken breed, a genetic study using simple sequence repeat (SSR) markers is essential. This molecular approach helps clarify the genetic makeup and distinguish village chickens from commercial breeds. SSR markers are highly polymorphic and reproducible, providing crucial insights into genetic diversity, parentage, population genetics, evolutionary relationships and breed identification in livestock (El Nahas *et al.* 2008; Öner *et al.* 2014; Zhang *et al.* 2020). Selecting SSR markers recommended by organisations such as FAO and the International Society of Animal Genetics (ISAG) ensures standardised and reliable analyses (FAO 2004).

The objectives of this study were to evaluate the genetic diversity of village chicken ecotypes in Peninsular Malaysia and investigate their relationships with commercial chicken breeds using SSR markers. The aim was to elucidate the genetic relationships among local chicken populations in Malaysia, providing information that will support future breeding programmes.

MATERIALS AND METHODS

Chicken Blood Samples and DNA Isolation

A total of 15 village chicken ecotypes were collected from various regions in Peninsular Malaysia, including Kelantan (D, $n = 30$), Kedah (K, $n = 128$), Pahang (C, $n = 53$), Perak (A, $n = 30$), Selangor (Saga, $n = 29$), Negeri Sembilan (NS, $n = 48$), Malacca (M, $n = 57$) and Johore (J, $n = 29$) (Fig. 1, Table 1). Additionally, four commercial chicken breeds were obtained: broiler Cobb ($n = 30$) and Ross ($n = 30$), Sasso ($n = 22$) and laying hen Hy-Line ($n = 30$), along with 21 jungle fowls. The village chicken ecotypes, each displaying distinct characteristics, were identified and randomly selected based on their uniform appearance within a flock, predominantly from small-scale farmers who kept at least 250 chickens. Several ecotypes have been morphologically classified into groupings, namely *Arab* chicken (M1, M2), cockfighting chickens (D, K1, K2, K3), *Kurik* chickens (C1, C2), Naked Neck chickens (NS1, NS2), and multicolour chickens (A, SAGA, K4, K5, J) (Abu Bakar *et al.* 2025). Individual photographs and phenotypes of each chicken were taken and recorded.

Blood samples were collected from the brachial vein into EDTA tubes containing anticoagulant and stored at -4°C before being transported to the Animal Molecular Biology facility at MARDI Serdang. Genomic deoxyribonucleic acid (DNA) was isolated using the DNeasy Blood and Tissue kit (Qiagen, Germany). DNA integrity and concentration were evaluated using 0.7% agarose gel electrophoresis and ND-1000 NanoDrop

spectrophotometer (NanoDrop Technologies, USA). All animal sampling and experimental procedures were approved by the MARDI Animal Ethics Committee (Approval Number: 20210827/R/MAEC0097).

TABLE 1. Information on the chicken population studied.

No.	Chicken breed	Ecotype/ Breed code	Ecotype name	Location state/ District	Sample size
1	Village	D	AK_Kelantan	Kelantan/ Kota Bharu	30
2		C1	AK_Pahang1	Pahang/ Temerloh	25
3		C2	AK_Pahang2	Pahang/ Temerloh	28
4		A	AK_Perak	Perak/ Slim River	30
5		M1	AK_Malacca	Malacca/ Kg. Telok Mas	28
6		M2	AK_Malacca2	Malacca/ Kg. Telok Mas	29
7		Saga	AK_Saga	Selangor/ Serdang	29
8		K1	AK_Kedah1	Kedah/ Yan	26
9		K2	AK_Kedah2	Kedah/ Yan	19
10		K3	AK_Kedah3	Kedah/ Yan	30
11		K4	AK_Kedah4	Kedah/ Yan	25
12		K5	AK_Kedah5	Kedah/ Yan	28
13		J	AK_Johore	Johore/ Rengit	29
14	Commercial	NS1	AK_N.Sembilan1	N. Sembilan/ Tampin	23
15		NS2	AK_N.Sembilan2	N.Sembilan/ Tampin	25
16		Cobb	-	Selangor/ Serdang	30
17		Ross	-	N. Sembilan/ Mantin	30
18		Sasso	-	Selangor/ Shah Alam	22
19		Hy-Line	-	Selangor/ Kalumpang	30
20		Jungle Fowl	-	Selangor/Jenderam Hilir/Zoo Negara	21
Total					537

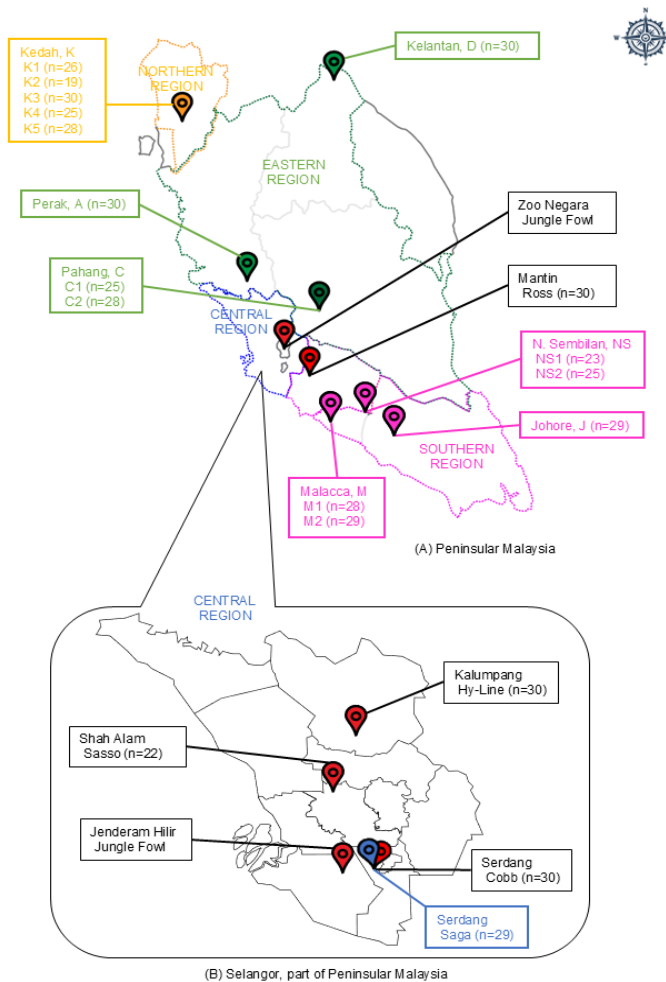


FIGURE 1: Geographical locations of chickens sampled across Peninsular Malaysia. (A) Map showing the regional divisions (Northern, Central, Eastern and Southern) and the distribution of Malaysian Village Chicken (MVC) ecotypes: Kelantan (D), Perak (A), Selangor (Saga), Johore (J), Pahang (C1, C2), Negeri Sembilan (NS1, NS2), Malacca (M1, M2) and Kedah (K1-K5); (B) Sampling locations of commercial chickens: Selangor (broiler Cobb, Sasso, and Hy-Line layers) and Negeri Sembilan (broiler Ross). Jungle fowls were collected from two locations within the Central Region: Zoo Negara and Jenderam Hilir, Selangor.

Primer Optimisation and Genotyping

A total of 50 SSR markers were sourced from the ISAG/FAO and previous studies demonstrated their utility in genetic diversity assessment of domestic animals (FAO 2011). Of these, 20 SSRs were selected based on their reported high polymorphism information content (PIC) and 17 markers were successfully optimised and amplified (Supplementary Table S1).

Polymerase chain reaction (PCR) was performed following Schuelke (2000), including the ligation of primers (forward or reverse) with fluorescent dyes (FAM, PET, NED or VIC) and the *M13* sequence (TGTAACGACGCGCCAGT). Approximately 30 ng–50 ng of genomic DNA was used for amplification. Each 10 µL PCR reaction contained 1 µL genomic DNA, 10 × buffer (Invitrogen, United States), 2.5 mM MgCl₂, 10 µM reverse and forward primer, 5 µM fluorescence-labelled M13 adaptor, 2 µM dNTPs (Invitrogen, United States), 0.1 µL bovine serum albumin (BSA) as a PCR enhancer and 1 U of *Taq* polymerase (Invitrogen, United States). PCR amplification was carried out using a Thermal Cycler GeneAmp® 9700 (Applied Biosystems, United States). The PCR profile began with initial denaturation at 94°C for five minutes, followed by 37–40 cycles of 94°C for 60 sec, 46°C–66°C for 60 sec and 72°C for 60 sec, with a final extension at 72°C for 7 min. Post amplification, PCR products were multiplexed by combining up to four different fluorescent primers, mixed with a size standard (GeneScan 500 LIZ) and Hi-Di formamide and resolved on an ABI 3130XL Genetic Analyser (Applied Biosystems, United States).

Allele Scoring and Statistical Analysis

Fragment analysis for allele sizing was performed using Gene Mapper version 5 (Thermo Fisher Scientific, United States). Allele peaks were scored and analysed following Arif *et al.* (2010), then exported to Microsoft Excel. Monomorphic loci were excluded from downstream SSR analyses because they lack genetic variation and thus provide no discriminatory power or informative value for assessing population structure or genetic diversity (Arca *et al.* 2021). Power Marker software was used to compute SSR marker metrics, including allele numbers, major allele frequency, gene diversity, heterozygosity and PIC and pairwise genetic distance based on shared alleles (Liu & Muse 2005). Validation and null allele detection were performed using Cervus to identify the presence and estimate the frequency of null alleles, large allele dropout and other potential genotyping errors. Null alleles that fail to amplify due to mutations in the primer binding regions can lead to underestimation of heterozygosity, as heterozygotes may be misclassified as homozygotes (Gill *et al.* 2005; Foley *et al.* 2024).

A heatmap of Hardy-Weinberg Equilibrium population-wise p-values per locus was illustrated using RStudio 4.4.1 (packages *pegas* and *lattice*) (Paradis 2010; Sarkar 2008). Analysis of Molecular Variance (AMOVA) was conducted using GenAlEx 6.5 software to partition total genetic variation within and among populations (Peakall & Smouse 2006; 2012). Both Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Neighbour-Joining (NJ) phylogenetic trees based on shared allele matrices of Nei's genetic distance were constructed in RStudio 4.4.1 (package *poppr*) (Kamvar *et al.* 2014; 2015) and visualised using FigTree (Rambaut 2018). Principal Component Analysis (PCA) was then carried out using RStudio 4.4.1 with *FactoMineR*, *factoextra* and *ggplot2* packages (Lê *et al.* 2008; Kassambara & Mundt 2020; Wickham 2009), grouping chicken ecotypes/populations into 4 ellipses (CI = 95%). A PERMANOVA test was then performed using the *vegan* package (Oksanen *et al.* 2024).

Population structure analysis was conducted using STRUCTURE version 2.3.4 based on a Bayesian clustering model (Pritchard *et al.* 2000). To ascertain the optimal K value, the range was set from 1 to 10, with a burn-in period of 5,000 generations followed by 50,000 Markov-Chain Monte Carlo (MCMC) replicates. The true number of populations was inferred using two methods:

1. A nonparametric test comparing $\text{LnP}(D)$ for each K value, as outlined by Pritchard *et al.* (2000).
2. Calculation of Delta K (ΔK) = $\text{mean}([L''(K)]/\text{sd}[L(K)])$ following Evanno *et al.* (2005).

Both methods were conducted using the STRUCTURE SELECTOR website (Li & Liu 2017) (<https://lmme.ac.cn/StructureSelector/>).

RESULTS

Assessment of SSR Markers

Among 20 SSR markers studied, 17 were found to be polymorphic, while two loci (*MCW0043* and *MCW0050*) were monomorphic, and one locus (*MCW0295*) proved difficult to optimise, resulting in a non-specific peak pattern. Analysis of these 17 polymorphic SSR markers across 20 chicken ecotypes identified a total of 289 alleles. The number of alleles per locus ranged from 8 (*ADL0112*) to 25 (*ADL0136*), with an average of 17 alleles per locus (Table 2). PIC values ranged from 0.46 to 0.89, with a mean of 0.74. Gene diversity also averaged 0.74. Observed heterozygosity (H_o) ranged from 0.25 to 0.87, indicating differences in the presence of different

alleles within individuals, while expected heterozygosity (H_e) ranged from 0.47 to 0.90, reflecting the variety of alleles within the population. All H_e values were higher than H_o , except for locus ADL0171.

TABLE 2. Genetic parameters of 17 polymorphic SSR markers obtained across 20 ecotypes of chicken.

SSR marker	N_o	H_o	H_e	F	PIC (%)	MAF
ADL0102	17	0.68	0.88	0.22	0.86	0.19
ADL0112	8	0.55	0.62	0.08	0.55	0.47
ADL0136	25	0.33	0.87	0.62	0.86	0.23
ADL0158	11	0.48	0.55	0.13	0.50	0.61
ADL0171	9	0.87	0.59	-0.47	0.51	0.47
ADL0172	15	0.63	0.81	0.22	0.79	0.31
LEI0094	23	0.77	0.84	0.08	0.82	0.32
LEI0192	24	0.62	0.81	0.24	0.80	0.36
LEI0234	19	0.44	0.90	0.51	0.89	0.18
MCW0005	18	0.60	0.88	0.32	0.87	0.20
MCW0016	21	0.44	0.84	0.47	0.82	0.30
MCW0034	17	0.77	0.85	0.09	0.83	0.30
MCW0049	18	0.25	0.47	0.47	0.46	0.72
MCW0069	18	0.69	0.82	0.16	0.80	0.31
MCW0078	14	0.52	0.71	0.27	0.67	0.45
MCW0183	16	0.47	0.80	0.41	0.77	0.31
MCW0295	16	0.68	0.81	0.17	0.79	0.28
Mean	17	0.58	0.77	0.25	0.74	0.35
Total	289					

Notes: N_o = observed allele numbers; H_o = observed heterozygosity; H_e = expected heterozygosity; F = inbreeding coefficient; PIC = polymorphic information content and MAF = major allele frequency.

Genetic Variability of Chicken Ecotypes

Genetic diversity parameters for each chicken ecotype are summarised in Table 3. Overall, the chicken populations included in this study exhibited a moderate level of genetic diversity, with an average observed

heterozygosity (H_o) of 0.58, which was lower than the average expected heterozygosity (H_e) of 0.77. Among the 15 MVC ecotypes, the AK_N. Sembilan2 (NS2) showed the highest observed heterozygosity ($H_o = 0.605 \pm 0.066$), while the AK_Kedah3 (K3) population had the lowest H_o (0.461 ± 0.077) (see Table 3). All MVCs and other chicken populations showed higher H_e than H_o , indicating heterozygous deficiency, except for Sasso and Hy-Line. These two populations also exhibited negative F_{is} values, while all other populations showed positive values. Overall, the mean inbreeding coefficient (F) across 20 chicken populations was 0.132, ranging from -0.092 in commercial Hy-Line to 0.266 in AK_Pahang1. The AK_Pahang1 had the highest F value (0.266 ± 0.074), while the AK_N. Sembilan1 (NS1) displayed one of the lowest F values among village chickens (0.06 ± 0.092).

TABLE 3. Genetic diversity among 20 chicken ecotypes based on 17 SSR markers.

Ekotype/Population	H_o	H_e	F
AK_Perak	0.509 ± 0.065	0.660 ± 0.036	0.254 ± 0.092
AK_Pahang1	0.568 ± 0.060	0.746 ± 0.035	0.266 ± 0.074
AK_Pahang2	0.596 ± 0.054	0.739 ± 0.030	0.186 ± 0.071
AK_SAGA	0.527 ± 0.070	0.617 ± 0.057	0.153 ± 0.098
AK_Kelantan	0.559 ± 0.055	0.671 ± 0.036	0.153 ± 0.084
AK_Johore	0.543 ± 0.052	0.628 ± 0.053	0.177 ± 0.062
AK_Kedah1	0.539 ± 0.063	0.657 ± 0.043	0.206 ± 0.103
AK_Kedah2	0.587 ± 0.065	0.638 ± 0.047	0.068 ± 0.080
AK_Kedah3	0.461 ± 0.077	0.582 ± 0.072	0.211 ± 0.088
AK_Kedah4	0.469 ± 0.070	0.557 ± 0.070	0.159 ± 0.076
AK_Kedah5	0.540 ± 0.069	0.578 ± 0.039	0.122 ± 0.097
AK_Malacca1	0.524 ± 0.056	0.651 ± 0.047	0.168 ± 0.067
AK_Malacca2	0.524 ± 0.054	0.648 ± 0.040	0.194 ± 0.071
AK_NS1	0.503 ± 0.072	0.557 ± 0.065	0.065 ± 0.092
AK_NS2	0.605 ± 0.066	0.667 ± 0.041	0.110 ± 0.089
Broiler_Cobb	0.536 ± 0.068	0.623 ± 0.047	0.115 ± 0.091
Broiler_Ross	0.580 ± 0.053	0.644 ± 0.046	0.075 ± 0.076
Sasso	0.688 ± 0.072	0.642 ± 0.052	-0.040 ± 0.088
Hy-Line	0.615 ± 0.080	0.547 ± 0.051	-0.092 ± 0.103
Jungle fowl	0.653 ± 0.055	0.740 ± 0.044	0.097 ± 0.064

Notes: H_o = observed heterozygosity; H_e = expected heterozygosity and F = inbreeding coefficient.

Hardy-Weinberg Equilibrium (HWE) analysis using a chi-square test identified loci that deviated from equilibrium across the populations. The HWE “heat map” (Fig. 2) shows population-wise *p*-values for each locus. Locus *ADL0736* locus deviated from HWE in 18 of the 20 chicken ecotypes, indicated by white and light blue squares (0.0 ~ 0.1, *p* < 0.05). Only AK_NS2 and Hy-Line, shown in dark blue, conformed to HWE. Conversely, the *ADL072* locus demonstrates greater alignment with HWE, indicating suitability for genetic studies. At the population level, the Hy-Line and Saga chicken populations deviated substantially from HWE, with 11 and 10 loci, respectively, showing significant deviations (0.0 ~ 0.2). In general, village chicken populations displayed mixed patterns, reflecting natural mating systems and higher genetic diversity.

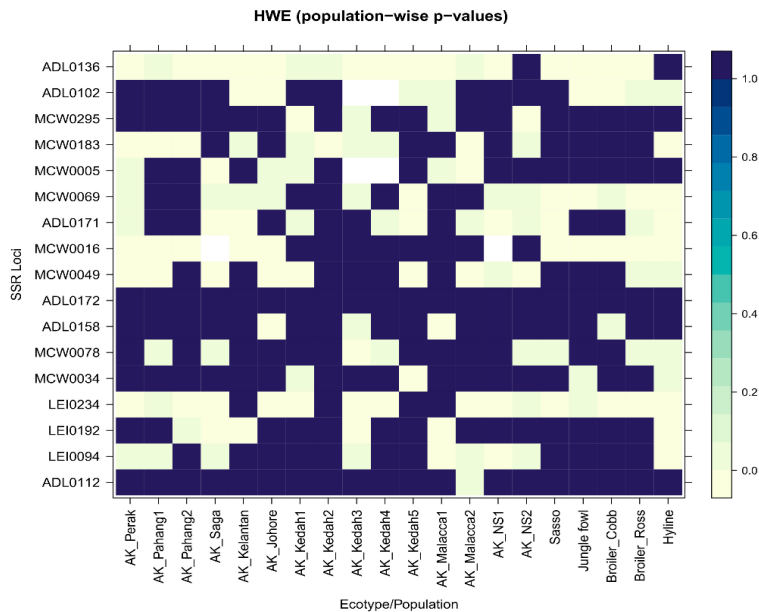


FIGURE 2: Heat map showing Hardy-Weinberg Equilibrium for all 20 chicken ecotypes across 17 SSR markers.

Degree of Genetic Differentiation Within and Among Village Chicken Ecotypes

A key objective of this study was to evaluate the genetic relatedness of selected ecotypes comprising 15 MVCs and several commercial breeds. AMOVA was performed to assess how genetic variation was distributed within and among populations (ecotypes). Results showed that 84% of the total genetic variation occurred within population (ecotype), including intra-individual variation (58%) and inter-individual chicken variation

(26%), while 16% of the variation occurred among populations (Table 4). Wright's F -statistics (F_{ST} , F_{IS} , F_{IT}) were also calculated. The fixation index for genetic differentiation between populations (F_{ST}) was 0.158. The global inbreeding coefficient (F_{IT}) and inbreeding within ecotypes or individuals (F_{IS}) were 0.305 and 0.415, respectively.

TABLE 4. Analysis of molecular variance (AMOVA) of 537 individual genotypes in 20 chicken ecotypes.

Source of variation	Df	SS	MS	Var. comp.	%T	F (p -value)
Among populations	19	1236.1	65.060	1.074	16	$F_{ST} = 0.158$ (0.001)
Among individuals	517	3851.0	7.449	1.739	26	$F_{IS} = 0.305$ (0.001)
Within individuals	537	2132.0	3.970	3.970	58	$F_{IT} = 0.415$ (0.001)
Total	1,073	7219.1		6.783	100	

Notes: Df = degree of freedom; SS = sum of squares; MS = mean squares; Var. comp. = variance component; %T = percentage of total variance; F = Fixation index; F_{ST} = fixation index between population; F_{IS} = fixation index between individuals and F_{IT} = fixation index within individuals.

Genetic Relationships Between Malaysian Village Chicken and Commercial Breeds

Dendrogram based on shared allele matrices using UPGMA and Neighbour-Joining method (Fig. 3) revealed that the 20 studied chicken populations formed two major clusters. All 15 village chicken accessions, including AK_Saga, were clustered together, while the remaining five accessions - commercial chickens and jungle fowl - formed a separate cluster (red). In the UPGMA clustering (Fig. 3A), several subclusters were observed within the MVC clade, corresponding broadly to province location in Peninsular Malaysia. These clades of MVC originate from North-Central (coloured in green) or Eastern-Southern regions (coloured in blue). The NJ tree (Fig. 3B) showed a similar pattern, although one population (AK_Johore) could not be confidently assigned to either clade. Furthermore, the longest branch length among native chickens was observed in AK_Kedah5, while within the commercial clade, Sasso and Broiler_Ross shared the most recent common ancestor, consistent with the UPGMA results. This suggests greater evolutionary change or genetic differentiation in these two populations, potentially through natural selection, artificial selection or mutations. The lowest pairwise genetic distance analyses occurred between AK_Malacca1 and AK_Malacca2, likely due to geographical

proximity (Figs. 3A and 3B). The phylogenetic tree further illustrated the relationships among the 20 chicken populations, including village chickens, commercial breeds and jungle fowl.

The principal component analysis (PCA) plot is shown in Fig. 4. Each point represents an individual chicken, colour-coded by MVC regions or breed type (for commercial, improved Saga and original jungle fowl). PCA indicated that the AK_Saga ecotype (black-coloured points) lies closely together on the right side of the X-axis, overlapping with the clustering of other native chickens. Saga chicken formed a compact ellipse nested within the larger MVC cluster along the X-axis, with slight overlap with the commercial breed. Commercial chickens clustered more centrally towards the right end of the X-axis, forming three distinct distributions along the Y-axis corresponding to Broiler_Ross, Hy-Line and Sasso populations. Some overlap between commercial chickens and jungle fowl was also observed.

Population structuring was examined using the Bayesian approach described by Evanno *et al.* (2005). The highest mean Delta K (ΔK) corresponded to $K = 3$ (Fig. 5A), indicating that 537 chicken individuals comprised of three genetically distinct groups (Fig. 5B). In this study, village chicken ecotypes were divided into two groups:

1. One group was composed of AK_Perak (A), AK_Pahang1 (C1), AK_Pahang2 (C2), AK_Kelantan (D), AK_Johore (J), AK_Malacca1 (M1), AK_Malacca2 (M2) and AK_N. Sembilan2 (NS2).
2. Another group consisted of village chickens from Kedah (K1–K5), N. Sembilan1 (NS1) and Saga chicken.

Meanwhile, the third group consisted of commercial breeds (broiler Cobb and Ross, Sasso and Hy-Line) together with jungle fowl. Results from STRUCTURE corroborate findings from the phylogenetic tree and PCA, showing that MVC ecotypes clustered according to three broad geographical regions: east, north and south.

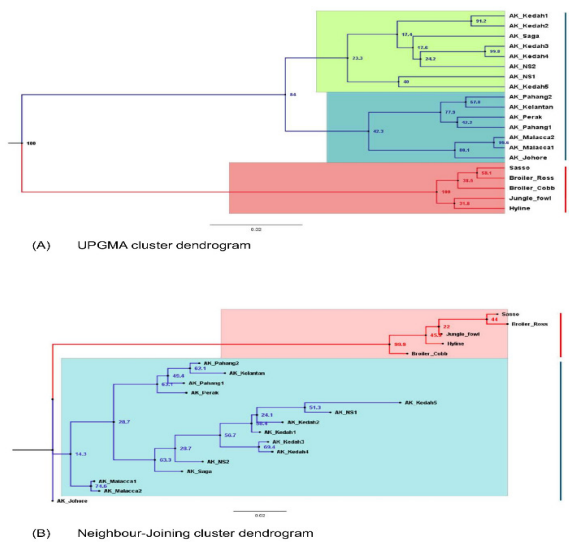


FIGURE 3: Dendrogram constructed using different clustering to depict genetic relationship among 20 chicken ecotypes based on 17 polymorphic SSR markers. (A) UPGMA cluster dendrogram; (B) Neighbour-Joining cluster dendrogram.

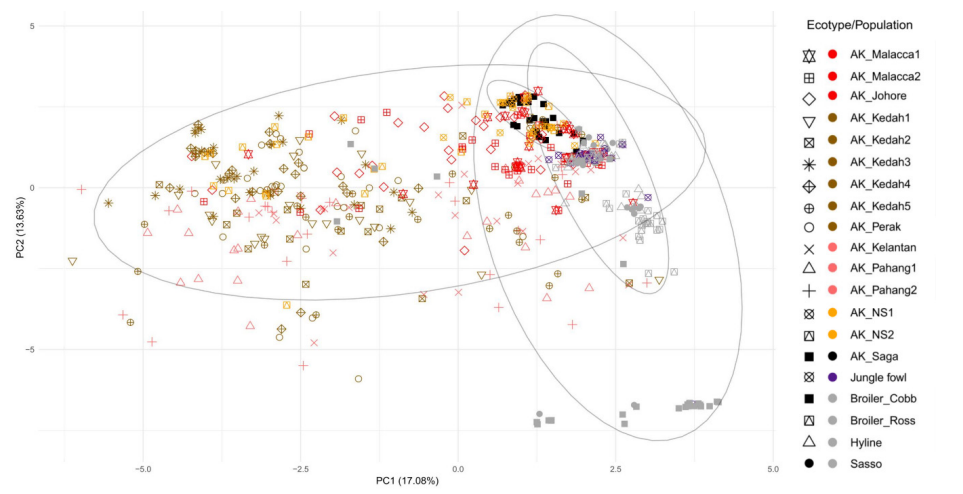


FIGURE 4: Principal Component Analysis (PCA) for all 20 chicken ecotypes to highlight the genetic similarities.

Notes: Populations are colour-coded according to the following classes: Grey = 4 commercial chickens, purple = Jungle Fowl (JF), black = AK_Saga chickens, and orange, pink, red and brown for the rest of 14 MVCs, representing the Central, Eastern, Southern and Northern regions, respectively, based on their geographic locations.

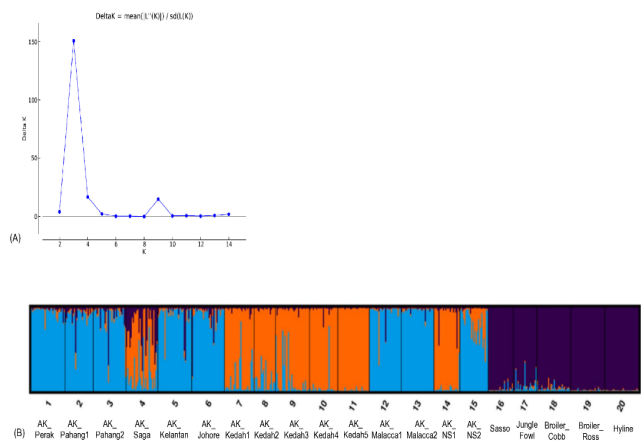


FIGURE 5: Analyses of population structure among chickens. (A) Estimation of the number of populations, K where $\Delta K (\Delta K) = 3, (\Delta K = \text{mean}([L''(K)]) / \text{SD}[L (K)])$ where K refers to the highest value of ΔK ;(B) Analysis of population structure using the STRUCTURE software with K = 3 as determined by ΔK based on 17 SSR markers representing the 20 groups of studied chicken: 15 village chicken ecotypes, four commercial breeds and a jungle fowl strain.

Notes: 1 = AK_Perak; 2 = AK_Pahang1; 3 = AK_Pahang2; 4 = AK_Saga; 5 = AK_Kelantan; 6 = AK_Johore; 7 = AK_Kedah1; 8 = AK_Kedah2; 9 = AK_Kedah3; 10 = AK_Kedah4; 11 = AK_Kedah5; 12 = AK_Malacca1; 13 = AK_Malacca2; 14 = AK_N.Sembilan1; 15 = AK_N.Sembilan2; 16 = Sasso; 17 = jungle fowl; 18 = broiler_Cobb; 19 = broiler_Ross; 20 = Hy-Line laying hen.

DISCUSSION

Domestic chickens can be categorised into three major groups: commercial strains, indigenous village chickens and fancy breeds. Commercial chickens, classified by productivity traits, include broilers, egg layers and dual-purpose breeds (Lawal & Hanotte 2021). Indigenous village chickens, often referred to as native or ecotypes, are adapted to specific agro-ecological systems (Desta et al. 2013). Fancy breeds, recognised for

cultural and aesthetic value, are traditional or standard breeds (Scrivener 2006; 2009). This framework highlights the diverse roles and importance of domestic chickens across contexts.

Our study focuses on assessing genetic diversity in MVC, conventionally bred by smallholders in Peninsular Malaysia. Recognising their potential, MARDI has implemented breeding programmes since 2008, culminating in the development of the Saga chicken breed in 2019. Saga chickens, with uniform traits and adaptability to local conditions, are suited for commercial production (Muhammad *et al.* 2019; Farahiyah *et al.* 2022). Previous MVC genetic studies, relying on Random Amplified Polymorphic DNA (RAPD) and partial mtDNA genes (Yap *et al.* 2010; Ha *et al.* 2017), provided limited insights due to marker constraints. In this study, we employ cost-effective and versatile SSR markers, known for their ability to enhance accuracy in distinguishing genotypes, assessing genetic diversity and supporting evolutionary research (Zhao *et al.* 2023).

Allelic Diversity and Microsatellite Marker Informativeness and Suitability

In this study, 17 polymorphic SSR markers were used to analyse 20 chicken ecotypes in Malaysia and yielded an average of 17 alleles per marker. The large allele number and size range indicate high allelic polymorphism, as evidenced by the high polymorphic information content (PIC) value of 0.74 averaged across all markers. Comparatively, the mean allelic diversity (N_e) of MVC exceeded those reported in other studies, such as Turkish Denizli chickens (5.53) (Özdemir & Cassandro 2018), Brazilian Northeast chickens (5.0) (Carvalho *et al.* 2020) and even lower allele numbers in Italian chicken breeds (Ceccobelli *et al.* 2013) and Hungarian indigenous chicken breed (2.9–4.2) (Bodzsár *et al.* 2009). The value obtained here, however, was lower than that of the local Vietnamese and southern Chinese breed, which averaged at 20.944 alleles per locus (Zhuang *et al.* 2023). Overall, this highlights the higher genetic diversity in Malaysian chickens, with commonly used markers like *ADL0112*, *LEI0094*, *MCW016*, *MCW034*, *MCW069*, *MCW078*, *MCW183* and *MCW295* in chicken diversity research. We speculate that this diversity may be attributed to the presence of private alleles maintained across a stable population (Peng *et al.* 2024). The population stability of MVC may be partly related to better adaptation to heat-stress conditions, especially in regions with tropical climates as opposed to temperate regions (Habimana *et al.* 2020; Rachman *et al.* 2024). Over a long period, such stability can help preserve higher genetic diversity. However, other ecological and demographic processes should also be considered.

The high PIC value observed in this study, with an average of 0.74 across 17 SSR markers, indicates their effectiveness for genetic analysis. These highly polymorphic markers are particularly useful for detecting genetic variation, distinguishing individuals or subpopulations and analysing genetic relationships (Azhar 2019). Their ability to efficiently differentiate between alleles makes them valuable tools for linkage mapping, identifying genes associated with important traits and conducting accurate parentage testing. Additionally, markers with high PIC values play a crucial role in assessing genetic diversity and inbreeding, making them essential for conservation efforts. Overall, these markers provide robust and reliable insights for genetic research and population studies.

Hardy-Weinberg Equilibrium (HWE) patterns provide valuable insights at both the marker and population levels. At the marker level, loci-specific deviations highlight potential genetic dynamics. For instance, the *ADL0736* locus consistently deviates from HWE in most populations, possibly indicating selection pressures or genotyping errors. In contrast, loci like *ADL072* align well with HWE, demonstrating their reliability for genetic analyses. At the population level, deviations from HWE often reflect broader evolutionary and management influences. For example, commercial populations such as Hy-Line frequently deviate from HWE due to the effects of artificial selection and inbreeding, which reduce genetic diversity and disrupt equilibrium. In contrast, MVC populations exhibit mixed HWE patterns, likely shaped by natural mating systems, genetic drift and local adaptation. Population-specific deviations, such as those observed in Saga village chickens, further underscore the impact of local adaptation and breeding practices. These findings emphasise the importance of analysing both marker-specific and population-specific patterns to support genetic studies and conservation strategies.

Diversity of MVC Ecotypes Across Peninsular Malaysia

In this study, all chicken ecotypes exhibit lower observed heterozygosity (H_o) than expected heterozygosity (H_e) and therefore showed positive F-values, except for the commercial Sasso and Hy-Line breeds, which had negative F values. Heterozygote deficiency is usually associated with a high inbreeding coefficient (F), which reduces genetic diversity. Nonetheless, the genetic diversity across the 20 chicken colonies is considered healthy, as all H_o values are above 0.5, except for AK_Kedah3 (0.461) and AK_Kedah4 (0.469). The lower H_o compared to H_e in the studied population suggests a potential deficit in genetic variation, possibly resulting from factors such as small sample size, non-random mating, population sub-structuring, or the Wahlund effect (El Nahas et al. 2008; Al-Barzinji et al. 2011). The Wahlund effect occurs when a population is divided into subgroups

that do not interbreed freely, often due to geographic, behavioural or ecological barriers. This phenomenon highlights the impact of population sub-structuring on genetic variation and the importance of considering subpopulations in genetic analyses.

In the case of MVC, all chicken ecotypes can be loosely classified under a large group of indigenous village chickens. We speculate that the observed sub-structuring might suggest that chickens from different ecotypes, although currently geographically separated, likely originated from a similar ancestral population but later dispersed throughout Peninsular Malaysia due to farming practices, exchange and transportation. Additionally, high F values indicate selective practices that have increased homozygosity within the population over the years.

Subsequently, AMOVA was performed to further partition genetic variation into different components attributable to among populations, within populations and within individuals. The AMOVA results indicate that most genetic variation exists among individuals within ecotypes, highlighting their potential as a valuable genetic resource for developing new commercial strains and improving breeding practices. The high genetic differentiation (58%) at the individual level suggests improved fitness and adaptability due to the availability of a broader range of genetic options to respond to environmental changes, disease threats, or other risks. This reflects high heterozygosity within individuals and the presence of multiple alleles at individual loci.

The F_{ST} value categorises genetic differentiation as low (0 to 0.05), moderate (between 0.05 and 0.15), high (from 0.15 to 0.25) and very high (above 0.25) (Hartl & Clark 2007). In this study, the F_{ST} value for chickens is considerably high (0.158), indicating substantial genetic differentiation among the studied populations. This is comparable to Italian local chicken breeds (0.225) but contrasts with the lower F_{ST} (0.029) of Brazilian Creole chicken breeds (Ceccobelli *et al.* 2013; Carvalho *et al.* 2020). The values also reflect shared relatedness due to their classification under the same subspecies, *Gallus gallus domesticus*.

Phenotypic Relationship, Distinctness and Genetic Structure of MVC Compared to Its Commercial Counterpart

Village chicken populations formed distinct genetic groups separate from commercial breeds, aligning with previous research using partial *cytochrome b* gene showing different origins for village and commercial chickens (Yap *et al.* 2010). Despite the commercial Sasso breed resembling village chickens phenotypically, MVC showed unique genetic clustering

when evaluated with SSR markers. MVC ecotypes in Peninsular Malaysia were clustered by region: east (Perak, Kelantan and Pahang), north (Kedah) and south (Johore and Malacca) (see Fig. 1), though ecotypes from N. Sembilan were unexpectedly in the northern group, likely due to transportation and crossbreeding. The highest H_o value observed for Sasso in this study further supports its distinction from village chickens, reflecting non-random mating and stable crossbreeding used in the development of Sasso globally. Commercial chickens like Ross, Cobb, Sasso and Hy-Line were distinctly separated based on SSR markers, unlike earlier studies using RAPD markers and mitochondrial DNA (Yap et al. 2010), which could not clearly distinguish the groups due to markers limitations.

The development of Saga chicken, derived from two pure lines of village chickens, shows the closest genetic similarity to four ecotypes from Kedah while maintaining traditional village chicken characteristics. Through artificial selection, the Saga lineage was developed from MVC populations to create a superior dual-purpose breed suitable for both egg production. The grouping of Saga chickens with other MVC ecotypes, as shown in the PCA analysis, highlights their preservation of MVC genetic resources. This contrasts sharply with the Sasso broiler, which, despite its resemblance to village chickens, forms a distinct outgroup cluster.

An unexpected finding showed that jungle fowls (*Ayam Hutan*) are closely related to commercial chickens, particularly Hy-Line layers. This contrasts with earlier studies that linked village chickens more closely with red jungle fowls (Ha et al. 2017). The purity of red jungle fowls remains uncertain due to habitat destruction, hunting and hybridisation with domestic chickens, though they are still occasionally observed in oil palm plantations and forest edges (Mazlan et al. 2023). Red jungle fowls contribute significantly to the genetic diversity of chicken breeds, and interbreeding with other jungle fowl species has complex effects on domestic chickens depending on geography and historical breeding (Lawal et al. 2020). The close relationship between jungle fowls and commercial chickens in this study suggests a more intricate evolutionary history and gene flow, possibly reflecting past breeding events and ongoing introgression. Recent genomic studies support a model of chicken domestication involving recurrent admixture between wild and domestic populations (Lawal et al. 2018; Buehler 2023). These dynamics can blur genetic boundaries between wild and commercial lines, thereby explaining the unexpected clustering observed in the present dataset. Further high-resolution genomic analyses will be necessary to disentangle the extent and direction of introgression and to resolve demographic histories. Such insights will be crucial for conservation strategies for wild jungle fowls and optimising the use of native genetic resources in future breeding programmes.

CONCLUSION

The phylogenetic tree and structure analysis collectively reveal that chicken ecotypes in Peninsular Malaysia, regardless of type, share a similar origin as indicated by their topology. The results show that ecotypes from each region have unique genetic structures, highlighting the genetic richness of the species *Gallus gallus* in this country. The findings suggest that village chickens likely retain a distinctive genetic pool that enables adaptation to their environment and locally sourced feed more effectively than commercial breeds. Expanding these studies offers downstream applications, including breed identification, proper management, conservation of genetic resources and exploitation for utilisation in the poultry industry.

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AUTHORS' CONTRIBUTIONS

Amie Marini Abu Bakar: Project leader, conception and planning of the study, experimental design, statistical analysis and writing the main manuscript.

Shahril Ab Razak and Lim Wen Hui: Data analysis and statistical analysis.

Siti Norhayati Ismail: Laboratory work and data analysis.

Azlina Azma Ismail Affendee, Roziatul Erin Abdul Razak, Nooraisyah Saharani, Mohd Hafizzudin Ayob, Mohd Firdaus Othman, Mohd Hishammfariz Mohd Amin: Execution of the study, data collection and field support.

Noraini Samat and Nadiatur Akmar Zulkifli: Research supervision, manuscript review and co-editing.

Shairah Abdul Razak: Research supervision, data interpretation, manuscript review, co-editing and approval of the final version of the manuscript.

All authors have read and approved the final manuscript.

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APPENDIX

TABLE S1. List of microsatellite loci recommended by FAO and other studies for analysing genetic diversity in chickens.

No.	Microsatellite loci	Primer sequences (5'-3')	Ta (°C)	Nucleotide repeats	Chromosome location	Allele size range (bp)
1	ADL0102	F: TTCCACCTTTCTTTTTATT R: GCTCCACTCCCTTCTAACCC	47	(GT) ₁₈	C30E29	91–107
2	ADL0112	F: GGCTTAAGCTGACCCATTAT R: ATCTCAAATGTAATGCGTGC	58	-	10	120–134
3	ADL0136	F: TGTC AAGCCCATCGTATCAC R: CCACCTCCTTCT CCTGTTCA	52	(TG)10TC(TG) ₁₀	E5C10	148–154
4	ADL0158	F: TGGCATGGTTGAGGAATACA R: TAGGTGCTGCACTGGAAATC	52	(CA) ₁₂	E29C09W09	189–217
5	ADL0171	F: ACAGGATTCTTGAGATTTTT R: GGTCTTAGCAGTGTGTTT	46	(TG) ₁₈	E43	92–107
6	ADL0172	F: CCCTACAACAAGAGCAGTG R: CTATGGAATAAAATGGAAAT	49	(AC) ₁₈	E42	134–160
7	LEI0094	F: GATCTCACCAGTATGAGCTGC R: TCTCACACTGTAACACAGTGC	60	-	4	247–287
8	LEI0192	F: TGCCAGAGCTTCAGTCTGT R: GTCATTACTGTTATGTTTATTGC	60	T8N6(CTTT) ₁₁	6	244–370
9	LEI0234	F: ATGCATCAGATTGGTATTCAA R: CGTGGCTGTGAACAAATATG	60	(TTTC) ₁₈	2	216–364
10	MCW0005	F: ACCTCTGCTGGCAAATAAATTGC F: TCACTTTAGCTCCATCAGGATTCA	55	(TG) ₁₄	C11E5	189–259
11	MCW0016	F: ATGGCGCAGAAGGCAAAGCGATAT R: TGGCTTCTGAAGCAGTTGCTATGG	50	(TG) ₁₅	3	162–206
12	MCW0034	F: TGCACGCACTTACATACTTAGAGA R: TGTCTTCCAATTACATTCATGGG	60	(TG) ₂₂	2	212–246

(continued on next page)

Table S1: (Continued)

No.	Microsatellite loci	Primer sequences (5'-3')	Ta (°C)	Nucleotide repeats	Chromosome location	Allele size range (bp)
13	MCW0049	F: AGCGGCGTTGAGTGAGAGGAGCGA R: TCCCAACCCGCGGAGAGCGCTAT	55	(GCA) ₁₁	C6E1	116–127
14	MCW0069	F: GCACTCGAGAAAACCTCCTGCG R: ATTGCTTCAGCAAGCATGGGAGGA	60	(CA) ₁₁	E60C04W23	158–176
15	MCW0078	F: CCACACGGAGAGGAGAAGGTCT R: TAGCATATGAGTGTACTGAGCTTC	60	(TG)	5	135–147
16	MCW0183	F: ATCCCAGTGTCTGAGTATCCGA R: TGAGATTACTGGAGCCTGCC	58	(CA)4T(AC) ₁₁	7	296–326
17	MCW0295	F: ATCACTACAGAACACCCTCTC R: TATGTATGCACGCAGATATCC	60	(CA) ₁₀	4	88–126