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Genetic Diversity of Eight Native Indonesian Chicken Breeds on Microsatellite Markers

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ABSTRACT

Indonesia has diversity in native chickens based on phenotypes. This diversity is utilized for economic purposes such as meat, eggs, fancy, crowing, and fighting. This study aimed to determine the genetic structure of eight native Indonesian chicken breeds with microsatellite markers, the genetic distance, and inbreeding coefficient of each breed of chicken used for crossbreeding programs to obtain a positive heterosis effect for selection programs. The samples used were Arab, Merawang, Pelung, Sentul, Cemani, KUB, Black Kedu and White Kedu. Broiler chickens (Cobb) were used as the outgroup in this study. A total of 192 DNA samples from eight breeds were used in this study. A total of 24 microsatellite markers were used in this study to observe the genetic diversity of 8 native breeds. The POPGENE, Cervus, and FSTAT were used to generate the observed number of alleles, the effective number of alleles, observed heterozygosity value, expected heterozygosity value, the heterozygote deficit within the breed (FIS), gene flow (Nm), Hardy-Weinberg equilibrium, Polymorphism Information Content (PIC), and UPGMA tree. The principal component analysis (PCA) was performed using adegenet package of R software. Bayesian clustering assignments were analyzed using the STRUCTURE program. This study revealed a very close genetic relationship between seven native chickens and broilers. We also found Arab chickens separated from other Indonesian native chickens and no inbreeding in eight native Indonesian chicken breeds. In conclusion, we found two clusters among eight native Indonesian chicken breeds. Twenty microsatellite markers have a high PIC value in this study.

1. Introduction

Indonesia has many genetic resources related to chickens. There are 31 breeds of Indonesian native chicken (Hidayat and Asmarasari 2015). Merawang is a native chicken originating from Merawang village, Bangka Belitung province, with the potential for meat and eggs, which are determined as one of Indonesian germplasm (Irmaya *et al.* 2021). Sentul is a native Indonesian chicken from Ciamis, with the potential for meat and eggs (Depison *et al.* 2020). Black Kedu is a native chicken that can be distinguished visually because it has a black plumage color and white skin color, while White Kedu has white plumage color and white skin color (Ismoyowati *et al.* 2012). Cemani has fibromelanosis, which causes black skin color (Dharmayanthi *et al.* 2017), whereas the plumage color is black (Ismoyowati *et al.* 2012).

Many studies on molecular genetics in Indonesian chickens have been carried out. Based on whole genome sequencing, Ulfah *et al.* (2016) found red junglefowl have a genetic contribution to Kedu, Sumatera, American Black Sumatera, American Black Java, Rhode Island Red, White Plymouth Rock, and White Leghorn. Indonesian crowing chickens have three maternal lineages based on D-loop. Indonesian crowing chickens infer from Bekisar chicken, a crossbred of the green junglefowl (Ulfah *et al.* 2017). Sulandari *et al.* (2008) suggest multiple maternal origins for Indonesian native chickens using D-loop. Furthermore, using nine microsatellite markers, Sartika *et al.* (2004) identified four native Indonesian chickens (KUB, Pelung, Sentul, and Black Kedu).

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Microsatellites can be used to reveal inbreeding, hybridization, and genetic structure (Honka *et al.* 2022). Furthermore, the microsatellite is often used in animal conservation to see the impact of conservation on genetic factors (Walter *et al.* 2021). Furthermore, microsatellites are extremely useful with low-quality samples, become inexpensive, and take time to develop (Castoe *et al.* 2012). Moreover, microsatellite markers are often used in population genetic studies because of their codominant transmission, highly polymorphic, and higher mutation rate. Therefore, this research aimed to observe the genetic structure of eight native Indonesian chicken breeds using microsatellite markers and evaluate the genetic distance and inbreeding coefficient of each breed of chicken used for crossbreeding or selection programs.

2. Materials and Methods

2.1. Sampling and DNA Extraction

This study was conducted following the guidelines of research implementation included in the Indonesian Agency of Agricultural Research and Development Regulation about the ethical clearance of research and scientific publication. Ethical Clearance Committee of the Indonesian Agency of Agricultural Research and Development, Jakarta, Indonesia, has approved all procedures related to the use of animals in this study (Registration No. Balitbangtan/Balitnak/A/03/2020). A total of 192 chickens including Arab (n = 24), Merawang (n = 24), Pelung (n = 24), Sentul (n = 24), Cemani (n = 9), KUB (n = 48), Black Kedu (n = 25), White Kedu (n = 10), Broiler (Cobb, Parent Stock) (n = 4) were used for blood sampling. Blood samples (1 ml) were taken from the ulnaris vein using 3 ml syringe and collected in 1.5 ml tubes containing an anticoagulant. DNA extraction was carried out using a SEPAGENE kit (Sancko-Junyaku Co., Ltd, Tokyo, Japan). Morphological characteristics of eight native Indonesian chickens were described in Table 1 and Figure 1.

2.2. Microsatellite Genotyping

Template DNA (10 ng/µl) was 3µl, and master mixture PCR solution was 3 µl, containing: 1.362 µl pure water, 0.6 µl 10 x buffer, 0.6 µl 2 mM dNTP, 0.288 µl 25 mM $MgSO_4$, each 0.0125 µl primer microsatellite (Forward and Reverse primer, 200 pmol/µl), and 0.125 µl KOD plus enzyme polymerase.

PCR conditions: Denaturizing at 94°C for 1 minute 15 seconds, three stages: (94°C for 15 seconds, 60°C for 30 seconds, 68°C for 1 minute), 10 cycles; (94°C for 15 seconds, 55°C for 30 seconds, 68°C for 1 minute), 10 cycles; and (94°C for 15 seconds, 50°C for 30 seconds, 68°C for 1 minute), 10 cycles; Elongation at 68°C for 9 minutes and hold temperatures at 4°C. D.N.A. fragments from PCR were used as DNA templates for genotyping, mixed with deionized formamide solution and GeneScan standard size. DNA samples were denatured at a temperature of 90°C for 2 minutes, then run using the ABIPRISM 3100 automatic sequencer (Applied Biosystems). Data analyzed using Gene Mapper 2.0 software (Applied Biosystems). The information of microsatellite markes that used are shown in Table 2.

2.3. Genetic Diversity

The data was processed using CONVERT version 1.3.1 (Glaubitz 2004) for converting to other data analysis. The converted data was processed using the POPGENE version 1.32 program (Yeh and Boyle 1997), Cervus version 3.0 (Kalinowski *et al.* 2007), and FSTAT version 2.9.4 (Goudet 1995) to generate the observed number of alleles (na), the effective number of alleles (ne), observed heterozygosity value (Ho), expected heterozygosity value (He), the heterozygote deficit within the breed (FIS), gene flow (Nm), Hardy-Weinberg equilibrium (HW), Polymorphism Information Content (PIC) and UPGMA tree.

2.4. Genetic Relationships

Bayesian clustering assignments were analyzed using STRUCTURE version 2.2 (Pritchard *et al.* 2000). Ten independent runs were performed for each K between 2 and 20, with a burn-in period of 100,000 iterations followed by 100,000 iterations of the Markov Chain Monte Carlo algorithm. Best optimal groups (K) were identified by STRUCTURE HARVESTER (Earl and vonHoldt 2012). The Principal Component Analysis (PCA) was performed using adegenet package (Jombart 2008) of R version 4.0.5 (R Core Team 2021) (2021.3.21).

Table 1. Morphological characteristics of eight native Indonesian chickens in the current study.

Breed	Morphometric features						
	Comb type	Plumage	Color pattern	Skin	Shank	Feather distribution	Utilization
Arab	Single and pea	Silver, golden, gold silver	Laced pattern	Black, white	Black, white	Normal	Egg
Merawang	Single	Brown, brown gold, red buff	Columbian pattern	White, yellow	Black, white, yellow	Normal	Dual propose
Pelung	Single	Black, yellow, brown	Orange/ yellow, red color in hackles	White	Black, white, yellow, grey	Normal	Fighting, meat and fancy
Sentul	Pea	White, grey	White and black spotted, Solid Gray cover with a golden red	White	Black, white, yellow, grey	Normal	Egg and meat
Cemani	Single	Black	Dark black	Black	Black	Normal	Culture, traditional medicine
KUB	Single, pea, rose walnut,	Black,brown, red buff, black and white	Black and laced with a golden color in hackles Orange/ yellow, red color in hackles	Yellow, white	Black, white, yellow, grey	Normal	Egg and meat
Black Kedu	Single	Black	Solid black	White, grey, white	Black	Normal	Egg and meat
White Kedu	Single	White	Solid white		Yellow, white	Normal	Egg and meat

A. Arab, courtesy by Trubus magazine 2006

B. Merawang, courtesy by Sartika 2006

C. Pelung, courtesy by Iskandar 2006

D. Sentul, courtesy by Iskandar 2006

E. Cemani, courtesy by Sartika 2006

F. KUB, courtesy by Sartika 2006

G. Black Kedu, courtesy by Trubus magazine 2006

H. White Kedu, Trubus magazine 2006

Figure 1. Eight native Indonesian chicken in this study

Table 2. Microsatellite marker that used in this experiment

Marker	Dye	Forward primer (5'→3') / Reverse primer (5'→3')	Size range	Chromosomal location	References
ABR0258	FAM	GCATGACAGAAATGCCAATA GATCAGAACTTAACCTCCCT	113-145	Chr. 1	(Takahashi *et al.* 2005)
ABR0645	HEX	TATTGTCCTTCCAATTACAT CACGCACTTACATACTTAGA	211-239	Chr. 2	(Takahashi *et al.* 2005)
ABR0297	NED	ATGTTCCTTCATTTCCAGAG GGTATCCATAGCAAGTTAGT	161-167	Chr. 3	(Takahashi *et al.* 2005)
ABR0075	FAM	CATGAAGACCACAGCAAAGGG CAGAACTGCAACAAATTCCAGAG	158-182	Chr. 4	(Takahashi *et al.* 2005)
ABR0046	FAM	GTGGTCCCGCCGTTTGCTCT GCCGTGGGGAAACCGAAAGCA	133-149	Chr. 5	(Takahashi *et al.* 2005)
ABR0209	FAM	CTGCCAAACATCAGGAACCG AGCGCATGACGTGTAGAAAA	176-212	Chr. 5	(Takahashi *et al.* 2005)
ABR0541	NED	GCCTCAGCTCAACTTAACCA TTTGCAGGGAATGTGAAACT	333-399	Chr. 5	(Takahashi *et al.* 2005)
ABR0028	NED	GTGCGAGGGCTTCGGATGTG TGTGCTTGGGCTGCCGTTGG	207-243	Chr. 6	(Takahashi *et al.* 2005)
ABR0419	NED	TTAAACTGGAGAATATTTAACAGC TGCTTATTTCCATTCACCAA	338-360	Chr. 7	(Takahashi *et al.* 2005)
ABR0228	HEX	TCTGACAATCGGAGAAAGAACTCG CCCTCCTTTGTTATCCCTCGT	81-105	Chr. 8	(Takahashi *et al.* 2005)
ABR0604	HEX	ATTAACAAATCTACACGTTTTCC CACTAACAACTCGTTTATGGG	213-233	Chr. 8	(Takahashi *et al.* 2005)
ABR0526	HEX	TCAATTCAGTACGTCCCACA GCAGGAGCTGCCTATTACAT	174-184	Chr. 9	(Takahashi *et al.* 2005)
ABR0343	NED	AGGACAATTTCTCAAAGGTT TTTCAAAGCAATATGAACAC	103-129	Chr. 11	(Takahashi *et al.* 2005)
ABR0506	FAM	ATCTTTATGGCTCCATCATA TAACCATCAGGGATTACTGT	135-151	Chr. 13	(Takahashi *et al.* 2005)
MCW0080	HEX	GAAATGGTACAGTGCAGTTGG CCGTGCATTCTTAATTGACAG	264-278	Chr. 15	(Groenen *et al.* 1998)
ABR0257	HEX	AGACAGCAGTAGCCACCCAT GCTCTGTTCTGAGGAGGAAG	323-331	Chr. 17	(Takahashi *et al.* 2005)
MCW0217	HEX	GATCTTTCTGGAACAGATTTC CTGCACTTGGTTCAGGTTCTG	141-177	Chr. 18	(Takahashi *et al.* 2005)
MCW0304	NED	TCAGTATGAGAGCTTCTCAAG TTGTTACAAGGTCTTCTGGAG	268-308	Chr. 19	(Groenen *et al.* 1998)
ABR0223	HEX	TTTCTCCCAGTCTTAGCAGT ATTTCACAGGCTTGACATCC	263-291	Chr. 20	(Takahashi *et al.* 2005)
ABR0624	HEX	GAGCCTGAGGACAGAGTTCCA CCATAGAGGTCGGCATTGTTT	67-87	Chr. 21	(Takahashi *et al.* 2005)
ADL0262	FAM	GTGCAGACACAGAGGGAAAG TCACATGCACACAGAGATGC	102-108	Chr. 23	(Takahashi *et al.* 2005)
ABR0617	NED	CCAAGAACTCACATCAACGAGCAA TGGAAGACTGGCAGGGAAGC	158-176	Chr. 26	(Takahashi *et al.* 2005)
ABR0015	NED	AGTGCTGGCTGCATGGGTTA CCGCCGCTTCCATTACAAAC	250-268	Chr. 27	(Takahashi *et al.* 2005)
ABR0366	FAM	GTTAGTTGGATTTGGGTTTT CTGGGTGACAGCAAGGATTA	134-172	Chr. 1	(Takahashi *et al.* 2005)

3. Results

Using 24 microsatellite loci, we found the number of alleles ranging from 4 to 26. ABR297 had the least number of observed alleles (4 alleles), while ABR343 had the highest number of observed alleles (26 alleles) in this study (Table 3). The observed heterozygosity ranged from 0.374 to 0.884, and the expected heterozygosity ranged from 0.433 to 0.901. The Hardy-Weinberg results showed all the loci were not in Hardy-Weinberg equilibrium. The Fis value at 24 loci ranges from -0.098 (ABR223) to

Table 3. Summary statistic of the number of observed allele, observed heterozygosities, expected heterozygosities, PIC, hardy-weinberg equilibrium, Fis, and Nm based on locus

Locus	N_a	N_e	N	H_o	H_e	PIC	HW	Fis	Nm
ABR0015	8	1.7599	190	0.374	0.433	0.412	NS	0.0156	2.2771
ABR0028	7	3.2693	190	0.689	0.696	0.646	NS	0.0045	3.4403
ABR0046	10	3.9744	184	0.739	0.750	0.707	NS	-0.0321	1.9938
ABR0075	9	5.4396	190	0.700	0.818	0.792	NS	0.1298	2.9995
ABR0209	15	4.0093	191	0.738	0.753	0.717	NS	0.0014	4.1266
ABR0223	13	2.7383	188	0.638	0.636	0.595	NS	-0.0980	6.5695
ABR0228	10	3.2039	190	0.647	0.690	0.648	NS	0.0286	2.0756
ABR0257	5	2.8016	173	0.561	0.645	0.575	NS	0.0443	1.7187
ABR0258	17	9.8365	190	0.884	0.901	0.890	NS	-0.0524	3.2434
ABR0297	4	2.1165	164	0.518	0.529	0.475	NS	0.0399	1.4676
ABR0343	26	10.0176	185	0.832	0.903	0.892	NS	-0.0055	1.7917
ABR0366	18	6.4254	183	0.732	0.847	0.830	NS	0.0899	2.5320
ABR0419	8	2.0382	190	0.416	0.511	0.454	NS	0.1077	2.8910
ABR0506	6	3.3179	183	0.645	0.701	0.660	NS	0.0228	2.6888
ABR0526	11	3.4489	191	0.602	0.712	0.693	NS	0.1125	2.6725
ABR0541	17	5.3003	187	0.754	0.814	0.793	NS	0.0378	2.4741
ABR0604	7	2.9968	190	0.626	0.668	0.622	NS	0.0251	5.7296
ABR0617	10	6.2354	190	0.753	0.842	0.821	NS	0.0711	4.1482
ABR0624	6	1.6565	192	0.396	0.397	0.362	NS	-0.0735	3.6294
ABR0645	12	5.2307	190	0.784	0.811	0.785	NS	-0.0393	3.3557
ADL0262	5	2.5386	189	0.624	0.608	0.532	NS	-0.0828	4.2875
MCW0080	9	4.3969	178	0.725	0.775	0.748	NS	0.0513	3.6322
MCW0217	18	4.5911	189	0.672	0.784	0.765	NS	0.1106	3.3333
MCW0304	17	7.3980	188	0.729	0.867	0.850	NS	0.0965	1.7961

Number of allele (N_a), number of effective allele (N_e), observed heterozygosities (H_o), expected heterozygosities (H_e), polymorphism information content (PIC), the heterozygote deficit within the breed (Fis), hardy-Weinberg equilibrium (HW), gene flow (Nm)

Table 4. Fis value of of eight native Indonesian chickens

	Arab	Merawanga	Pelung	Sentul	Cemani	KUB	Black Kedu	White Kedu	Broiler
All locus	0.032	0.008	0.025	0.033	0.086	0.025	0.049	0.064	0.319

0.1298 (ABR75). The Nm values ranged from 1.4676 (ABR297) to 6.5695 (ABR223). Nm>1.0 indicates a constant gene flow and little differentiation among populations. The Fis values of 9 breeds ranged from 0.008 (Merawang chicken) to 0.319 (Broiler) (Table 4). That means there is no inbreeding found in nine breeds. In this study, Polymorphism information content for 24 microsatellites markers is moderate to high (0.25 < PIC < 0.5 to PIC > 0.5). The polymorphism information content (PIC) value varied from 0.362 (ABR624) to 0.892 (ABR343). High PIC values (PIC>0.5) were found at 20 microsatellite loci. While four loci (ABR15, ABR297, ABR419, and ABR624) had a moderate PIC value. UPGMA showed two clades; clade 1 only consists of Arab chicken. Clade 2 consists of Merawang, Pelung, Sentul, Cemani, KUB, White Kedu, Black Kedu, and Broiler (Figure 2) and could be measured for the genetic distance between breeds for creating the breeding program crossbreeding or selection. Principle component analysis (PCA) described two clusters among 8 native Indonesian chicken breeds and Broiler (Figure 3). The first two

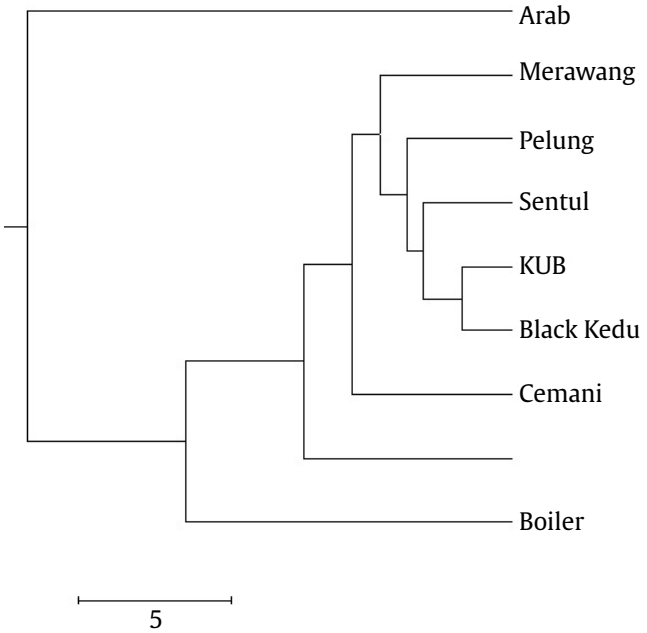

Figure 2. UPGMA of eight native Indonesian chicken breeds

components explained 3.6% and 2.3% of the total variance. The results of STRUCTURE clustering from K = 2 to 4 are displayed in Figure 4. The highest value for ΔK was obtained for K = 2. ΔK determines the correct number of clusters. So, the STRUCTURE result showed two clusters for Indonesian native chicken. At K = 3 to K = 4, the result showed 3 to 4 cluster components and also explained Arab chicken in the separated cluster.

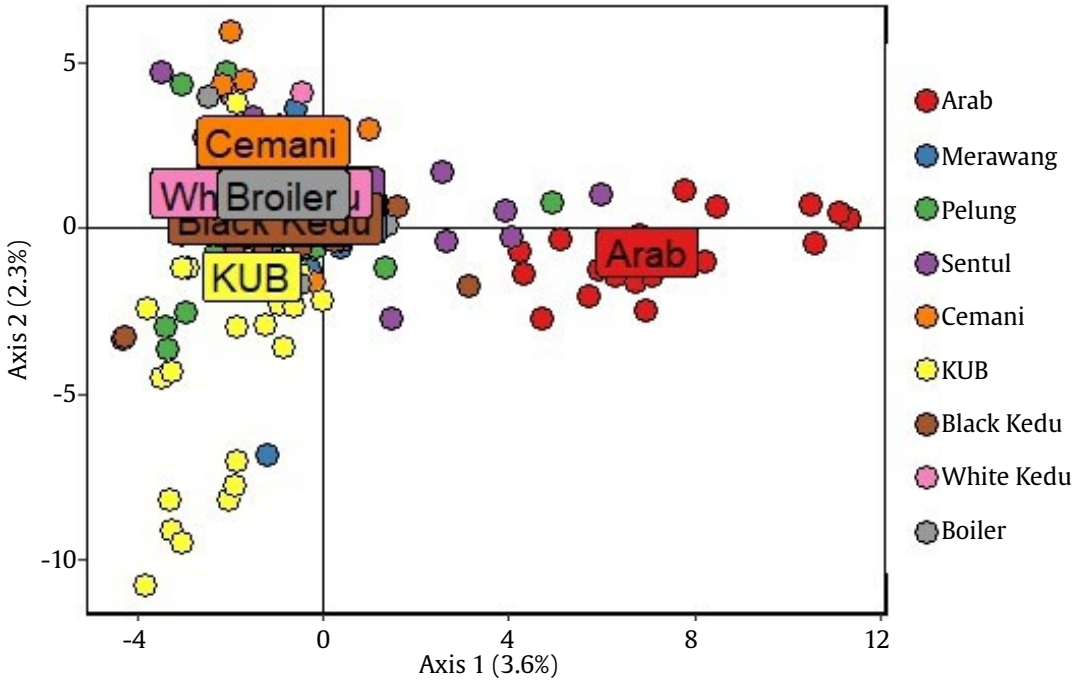

Figure 3. Principal component analysis of eight native Indonesian chicken breeds

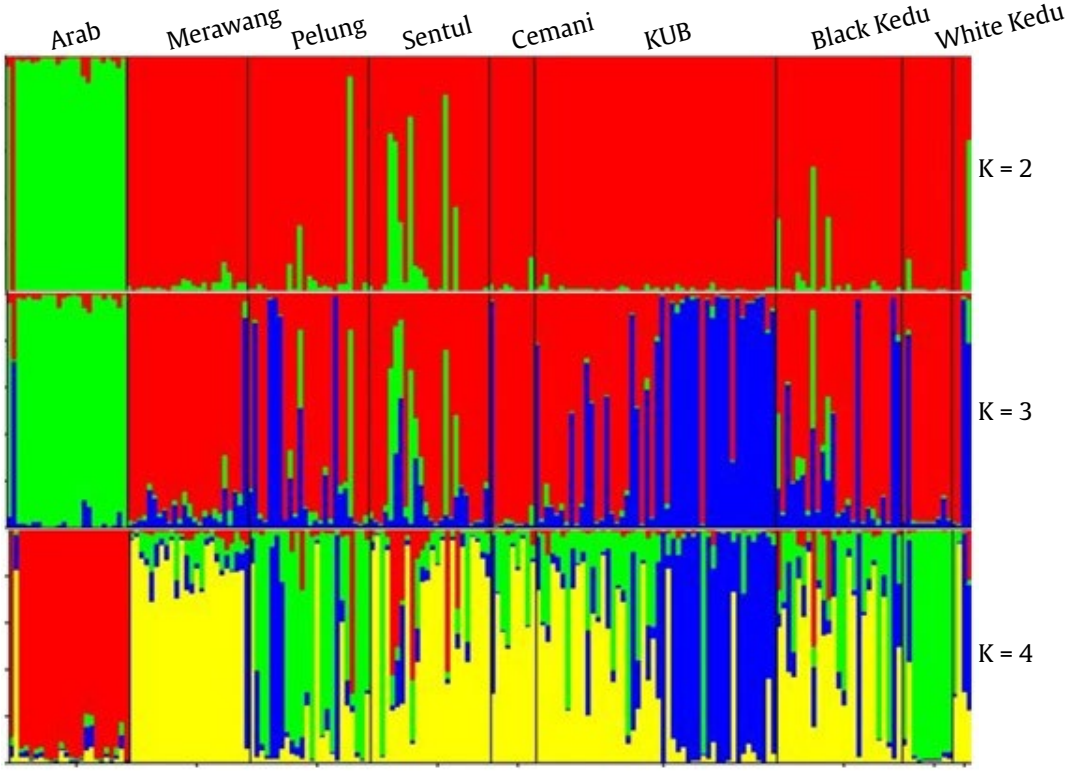

Figure 4. Genetic structures of eight native Indonesian chicken breeds. Black lines separate individual populations whose names were indicated

4. Discussion

Based on the UPGMA., it can be concluded that broilers have genetic closeness with native Indonesian chickens (Figure 2). Arab chickens are separated from the clade with Indonesian native chickens and broilers. Arab chicken historically from silver Brakel chicken originally from Belgium (Sulandari *et al.* 2007). Mention as an Arab chicken because of the neck feather-like using white Jilbab and the eyes have black circles like "Arabic women." It was about more than 20 years reared in Indonesia. Currently, the Arab chicken is thought to be crossbred from Silver Brakel and Indonesian native hen chicken (Sulandari *et al.* 2007). Indonesian native chickens, except Arab chickens, are in the same clade as Broiler Furthermore, Arab chickens have high egg production compared to other Indonesian native chickens, with egg production reaching 69.1%/hen/period (Syafwan and Noferdiman 2020). KUB chicken is a selected Kampung chicken for egg production (Superior KUB chicken of Balitbangtan), with egg production reaching 61.5%/hen/6 month period (Sartika and Iskandar 2019). The Indonesian Minister of Agriculture launched KUB with decree number 274/Kpts/SR.120/2/2014.

Using the principal component analysis (PCA) method, this study found two clusters, namely Arab chickens, which were in separate clusters. In contrast, the Merawang, Pelung, Sentul, Cemani, KUB, Black Kedu, White Kedu, and Broiler chickens were grouped into the second cluster (Figure 3). In Italian chickens, 6 breeds formed separate clusters using 580,961 SNP markers (Strillacci *et al.* 2017). Nxumalo *et al.* (2020) found 3 clusters in 7 South African native chickens using microsatellite markers. On the other hand, Abebe *et al.* (2015) found two clusters of 5 Swedish chicken breeds using 24 microsatellite markers. Zimmerman *et al.* (2020) found three main advantages of significant SNPs over microsatellites: more accurate estimates of diversity at the population level, a greater ability to identify groups in clustering methods, and the ability to consider local adaptation. Therefore, we suggested further research using large SNPs for clustering and identifying genetic structure in Indonesian native chickens.

Based on the analysis using Structure Harvester, it is known that the best K is 2 (Figure 4). This result means that there are two clusters of Indonesian native chickens that are similar to the PCA results, where the first cluster is Arab chicken. This cluster is thought to be the same European cluster found by Granevitze *et al.* (2009), where there are Brakel chickens in the European cluster. Brakel chicken is a main source of Arab chickens (Sulandari *et al.* 2007). The second cluster consists of Merawang, Pelung, Sentul, Cemani, KUB, Black Kedu, White Kedu and Broiler. Therefore, this cluster is most likely of Asian and other chickens found by Granevitze *et al.* (2009). White Plymouth Rock (WPR) is considered the main source of broilers in the industry (Gordy 1974). WPR is the result of crossing the paternal line from Dominique chickens with the maternal line from Black Java and Cochin chickens Guo *et al.* (2019). The crossing is probably why broilers are in a cluster with native Indonesian chickens.

The PCA analysis produced similar results as Structure (K = 2), finding two clusters. This result implied the existence of several independent domestication events in Asia (Miao *et al.* 2013) and Europe (Tixier-Boichard *et al.* 2011). To prove the domestication of chickens, the result should be supported by archeological and historical evidence. Meanwhile, red jungefowl is still considered the ancestor of domestic chicken, which is also suspected of having a mixture of grey junglefowl so that many domestic chickens have yellow skin color (Eriksson *et al.* 2008). We also suggest that 22 microsatellite markers from Takahashi *et al.* (2005) and two microsatellite markers from Groenen *et al.* (1998) can be additional markers from FAO recommendation due to the PIC value of microsatellite markers is moderate to high in this study.

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