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Determining the hybrid diversity between Bali cattle (*Bos javanicus*) and Wagyu cattle (*Bos taurus*) breeds using specific microsatellite markers

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Abstract. *Jakaria, Dairoh, Setyani NMP, Sutikno, Ulum MF, Priyanto R. 2024. Determining the hybrid diversity between Bali cattle (*Bos javanicus*) and Wagyu cattle (*Bos taurus*) breeds using specific microsatellite markers. Biodiversitas 25: 3048-3055.* This study aimed to determine hybrid diversity between Bali and Wagyu cattle using microsatellite markers. Total blood samples were collected from Bali purebreds (n=32 heifers), Wagyu×Bali crossbreeds, which consist of 2 bulls and 9 heifers (n=11), and Wagyu purebreds (n=6 dams). The four microsatellite markers (INRA035, ILST006, ETH225, and HEL9) were used to determine genetic diversity among cattle populations, which is part of what is recommended by FAO (Food and Agriculture Organization). GenAlex, CERVUS, MEGA10, UPGMA, and RStudio software were used for data analysis. Thirty-one alleles were identified at the four microsatellite loci used in this study. All identified alleles were polymorphic and informative. This study found 283 bp and 300 bp (ILSTS006) and 139 bp (ETH225), demonstrating the private alleles for Wagyu×Bali crossbreeds; 104 bp (INRA035), 284 bp (ILST006), 155 bp, 159 bp, and 167 bp (ETH225) were private alleles for Bali purebred, while 288 bp (ILST006), 145 bp (ETH225), and 162 bp (HEL9) were private alleles for Wagyu purebred. Based on Neighbor Joining and PCA analysis, the Wagyu purebred exhibited at the different clusters in both Wagyu×Bali crossbred and Bali purebred. These findings highlight that microsatellite markers effectively distinguished between Bali purebred, Wagyu x Bali crossbred, and Wagyu purebred cattle, providing valuable insights for breeding programs tailored to each population.

Keywords: Bali cattle, crossbreeding, genetic hybrid, microsatellite marker, Wagyu cattle

INTRODUCTION

Animal breeding in the economy of a country is considered one of the most important economic branches and is of special importance (Norouzy et al. 2005; Ahsani et al. 2022). Animal breeding is a very profitable job and it is considered as a means of raising the economy of countries (Rohallah et al. 2007; Ahmadabadi et al. 2023). Most of the people of the world are engaged in cattle breeding and use its products. In addition, cattle breeding has an important role (Askari et al. 2011).

Crossbreeding plays a crucial role in enhancing the genetic quality of local livestock, particularly Bali cattle that are indigenous to Indonesia. Crossbreeding has improved the genetic quality of Bali cattle, known for their adaptation to tropical climates, high fertility, and disease resistance, making them ideal for Artificial Insemination (AI) (Widyas et al. 2022). Since its introduction in the 1970s (Muslimiah et al. 2023), AI has involved mating Bali cows with superior breeds like Limousin, Simmental, and Brangus (Kocu et al. 2019; Nubatonis and Dethan 2021). However, despite decades of AI implementation, no crossbreeds have been recognized as new breeds in Indonesia, highlighting the need for further research.

Genetic variety is crucial for promoting the development of more sophisticated genes, safeguarding existing populations, advancing evolutionary processes, and enabling adaptation to changing conditions in the natural environment (Javanmard et al. 2008; Mohammadabadi et al. 2021). Conversely, the identification of gene polymorphisms is crucial in the process of breeding farm animals (Mohammadabadi and Tohidinejad 2017; Saadatabadi et al. 2023). Moreover, the study of breeds, using molecular techniques is very important and useful for their characterizing (Mohammadifar and Mohammadabadi 2017; Noori et al. 2017; Ahmadabadi et al. 2023). Microsatellites are genetic marker tools extensively applied in livestock genetics studies to determine genetic diversity, divergence within and among populations, relationship studies, and genetic mapping (Abdul-Muneer 2014). Microsatellite markers have high polymorphism, co-

dominant inheritance, abundance, high reproducibility, high variability, and cost-effectiveness, all of which make them suitable for studying conservation genetics, paternity testing, and breeding programs (Veira et al. 2016).

Genetic diversity is based on microsatellites using 30 microsatellite markers, as recommended by the United Nations' Food and Agriculture Organization (FAO) (FAO 2011). These 30 markers have a high diversity of repeat motifs, making them highly effective for genetic analysis. Various studies based on microsatellite markers in crossbred cattle have been reported for the Simmental-Brahman crossbreed (Van Der Nest et al. 2021), Mongolian (Tseveen et al. 2019), Senegal (Ndiaye et al. 2015), and Indonesian cattle breeds (Septian et al. 2015; Sutarno and Setyawan 2015; Agung et al. 2019). Crossbreeding in cattle has been utilized to enhance desirable traits by integrating the beneficial characteristics or genes from their purebred parent breeds (Paim et al. 2020).

Crossbreeding Wagyu and Bali cattle represents a preliminary step towards achieving the optimal combination for producing a new breed that offers high-quality meat and heat tolerance. Wagyu cattle are renowned for their superior meat quality, intense marbling, and tenderness (Gotoh et al. 2014; Gotoh et al. 2016; Motoyama et al. 2016). In contrast, Bali cattle are well-suited to tropical climates and exhibit high disease resistance and superior reproductive capabilities (Pribadi et al. 2015; Sudrajad et al. 2020; Freitas et al. 2021). The Wagyu-Bali crossbreeding program aims to capitalize on the strengths of both breeds to produce robust, productive, and well-suited cattle to Indonesia's environmental and economic conditions. However, further research is required to fully understand the genetic potential of these crossbreeds. This includes detailed genetic analysis using specific microsatellite markers to assess the genetic diversity and stability of crossbreed populations. Wagyu×Bali crossbreeds with a 50%:50% blood ratio have been produced, but no study has been conducted on the microsatellite DNA diversity of these first-generation hybrids. This research gap presents an opportunity to explore the genetic diversity and potential advantages of these crossbreeds, providing valuable data to enhance future breeding programs and genetic improvement strategies. This study is the first to utilize microsatellite markers to investigate the genetic diversity between Bali cattle, Wagyu cattle, and their crossbreeds.

MATERIALS AND METHODS

Animals and DNA extraction

Blood samples were collected from 49 cattle aged 1-2.5 years from various beef cattle breeds. Blood samples were collected from the jugular vein of each animal using a venoject and stored in a 10 ml vacuum tube containing EDTA as an anticoagulant. These included animals from female Bali cattle purebred (n=32 heifers) obtained from BPTP East Nusa Tenggara and UPT Livestock Breeding and Animal Feed Production East Nusa Tenggara; Wagyu×Bali crossbred (n=11, consisting of 2 bull and 9 heifers) obtained from UPT Livestock Breeding and Animal Feed Production East Nusa Tenggara, Indonesia; and Wagyu purebred (n=6 dams) populations obtained from BET Cipelang West Java. Blood samples from these animals were collected using a venoject of 3-5 mL and stored in EDTA tubes containing an anticoagulant. The DNA of the animals in this study was obtained from blood using the Geneaid^{SM} protocol (Geneaid Biotech Ltd. New Taipei City, Taiwan).

Microsatellite primers amplification and genotyping

Four microsatellite primers were amplified using a PCR System (Applied Biosystem). These microsatellite primers included INRA035, ILSTS006, and ETH225, and as specific markers, they are among the 30 primers recommended by the FAO. The four microsatellite primers used in the present study are listed in Table 1. The PCR reaction for the four microsatellite labels was conducted in a 25 µL reaction mixture, which included 2 µL of DNA and 23 µL of PCR mix. This PCR mix contained 0.6 µL of each forward and reverse primer, 12.5 µL of MyTaq HS RedMix, and 9.9 µL of nuclease-free water (NFW). The amplification process was performed under these conditions: starting with predenaturation at 95°C for 1 minute, then 35 cycles with 10 seconds at 95°C, 15 seconds at 72°C, and 15 seconds at 55°C. The process concluded with a final extension of 5 minutes at 72°C. The amplified products were then analyzed by running them on a 1.5% agarose gel and staining with FluoroSafe dye. Documentation was performed using a UV Transilluminator 100 Voltage (AlphaImager). All amplified products were analyzed by fragment analysis conducted by the 1st BASE Laboratory, Selangor Malaysia. Genotype identification was performed using the GeneMapper 5.0 version (Applied Biosystems, Carlsbad, CA, USA).

Table 1. Description of the primary sequences of the 4 microsatellite loci used based on FAO

Locus	Chromosome	Motif	Primer sequence	Accession no./reference	Label	Size of allele (bp)
INRA035	16	$(TG)_{16}$	ATCCTTTGCAGCCTCCACATTG TTGTGCTTTATGACACTATCCG	X68049 (Vaiman et al. 1994)	FAM	98-124
ILST006	7	$(GT)_{23}$	TGTCTGTATTTCTGCTGTGG ACACGGAAGCGATCTAAACG	L23482 (Brezinsky et al. 1993)	FAM	277-309
ETH225	9	$(CA)_{11}$	GATCACCTTGCCACTATTTCCT ACATGACAGCCAGCTGCTACT	Z14043 (Steffen et al. 1993)	HEX	131-185
HEL9	8	$(GT)_{25}$	CCCATTCAGTCTTCAGAGGT CACATCCATCCATGTTCTCACCAC	X65214 (Kaukinen et al. 1993)	NED	141-173

Data analysis

Various analytical tools have been used to estimate the genetic variability of microsatellite loci among the Bali purebred, Wagyu×Bali crossbred, and Wagyu purebred populations. Allele sizes in all populations were determined using multiplex DNA fragment analysis. GenAlEx 6.51 was used to estimate allele frequencies, the observed number of alleles (Na), effective number of alleles (Ne), observed and expected heterozygosity (Ho and He), inbreeding rate between population (F_{IS}), total inbreeding rate (F_{IT}), genetic differentiation (F_{ST}), gene flow (Nm), and genetic distance (Smouse et al. 2017). Hardy-Weinberg equilibrium (HWE) was analyzed and converted using CERVUS version 3.0.7. The dendrogram result from the genetic distance value was used to illustrate the relationship among Bali purebred, Wagyu×Bali crossbred, and Wagyu purebred populations using MEGA version 11 with Neighbor-Joining Methods. Principal component analysis (PCA) was performed using RStudio version 4.4.1.

RESULTS AND DISCUSSION

Microsatellite variability

Four microsatellite markers were used to amplify DNA from 49 samples from three cattle populations: Bali purebred, Wagyu×Bali crossbred, and Wagyu purebred. The result of PCR product and fragmentation analysis of cattle blood samples in each population for four microsatellite markers is clearly shown in Figure 1. The amplified fragments of the INRA035, ILST006, ETH225, and HEL9 markers ranged in length from 98-124bp, 277-309bp, 131-185bp, and 141-173bp, respectively. The amplification results indicated the presence of single bands (homozygous genotypes) and double bands (heterozygous genotypes).

Four markers of this study produced a total of 49 alleles, which were distributed as follows (i) 4 alleles at the INRA035 locus; (ii) 10 alleles at the ILSTS006 locus; (iii) 12 alleles at the ETH225 locus; and (iv) 5 alleles at the HEL9 locus. Allele frequencies for each locus in each population are presented in Table 2. Based on analysis using GenAlEx 6.5, it was found that all microsatellite loci in the three populations produced more than one allele, indicating significant genetic variation. The INRA035 locus had the highest allele frequency among all populations, whereas the ETH225 locus had the lowest allele frequency among the populations tested. The distribution of the resulting alleles demonstrated that each population had a unique allele pattern, with some alleles appearing only in a certain population. This finding supports the conclusion that significant genetic diversity exists among the Bali, Wagyu×Bali, and Wagyu populations, indicating that they are polymorphic.

The presence of specific alleles at certain loci in particular populations indicates significant and distinct genetic differences between populations (Table 3). The Wagyu population had private alleles 288 at locus ILST006, 145 at locus ETH225, and 162 at locus HEL9, which were not found in the Bali and Wagyu×Bali populations. These private alleles support the notion that the three populations exhibit high genetic diversity, which suggests that each population possesses distinct genetic characteristics.

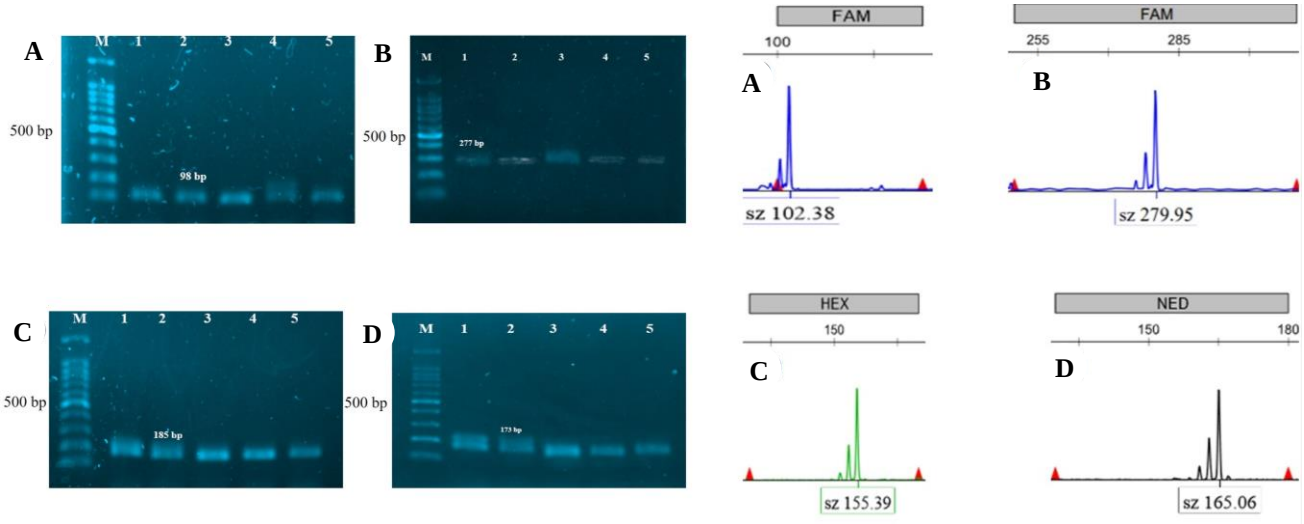

Figure 1. Results of PCR product and fragmentation using: A. INRA035, B. ILST006, C. ETH226, D. HEL9 markers

Table 2. Allele frequencies and sample size by populations

Locus	Allele (bp)/N	Wagyu-Bali	Bali	Wagyu
INRA035	N	11	32	6
	98	0.000	0.016	0.833
	100	0.409	0.016	0.167
	102	0.591	0.891	0.000
	104	0.000	0.078	0.000
ILST006	N	11	32	6
	280	0.045	0.125	0.000
	284	0.000	0.109	0.000
	286	0.273	0.438	0.000
	288	0.000	0.000	0.250
	290	0.000	0.031	0.083
	292	0.364	0.266	0.500
	296	0.045	0.000	0.000
	298	0.045	0.031	0.000
	300	0.136	0.000	0.000
	301	0.091	0.000	0.167
ETH225	N	11	32	6
	139	0.136	0.000	0.000
	141	0.136	0.000	0.167
	143	0.182	0.000	0.167
	145	0.000	0.000	0.583
	147	0.000	0.016	0.083
	155	0.000	0.047	0.000
	157	0.091	0.188	0.000
	159	0.000	0.063	0.000
	161	0.136	0.109	0.000
	163	0.182	0.453	0.000
	165	0.136	0.109	0.000
	167	0.000	0.016	0.000
HEL9	N	11	32	6
	148	0.500	0.859	0.000
	152	0.045	0.125	0.000
	156	0.000	0.016	0.667
	162	0.000	0.000	0.250
	164	0.455	0.000	0.083

Note: N: Number of samples

Heterozygosity and inbreeding analysis across three cattle populations

Heterozygosity and fixation indices are presented in Table 4. The mean value of observed heterozygosity in each population ranged from 0.398 (Bali cattle) to 0.841 (Wagyu×Bali cattle). Wagyu×Bali and Wagyu populations had higher observed heterozygosity (0.841 and 0.625, respectively) than expected heterozygosity (0.659 and 0.549, respectively), indicating high genetic diversity with heterozygosity values greater than 0.5 (Table 4). Conversely, the Bali population had lower observed heterozygosity (0.398) than expected heterozygosity (0.471). Analysis of the four microsatellite loci indicated a significant rate of inbreeding in all three populations. As shown in Table 5, the mean values were -0.203 for within-line inbreeding F_{IS} and 0.173 for the total inbreeding rate (F_{IT}).

Table 3. Summary private alleles by population

Populations	Locus	Allele	Frequency
Wagyu-Bali	ILST006	296	0.045
Wagyu-Bali	ILST006	300	0.136
Wagyu-Bali	ETH225	139	0.136
Bali	INRA035	104	0.078
Bali	ILST006	284	0.109
Bali	ETH225	155	0.047
Bali	ETH225	159	0.063
Bali	ETH225	167	0.016
Wagyu	ILST006	288	0.250
Wagyu	ETH225	145	0.583
Wagyu	HEL9	162	0.250

Table 4. Number of alleles, number of effective alleles, information index, observed and expected heterozygosity

Populations	Locus	N	Na	Ne	I	Ho	He
Wagyu-bali	INRA035	11	2	1.936	0.677	0.818	0.483
	ILST006	11	7	4.172	1.633	0.636	0.760
	ETH225	11	7	6.722	1.925	1.000	0.851
	HEL9	11	3	2.180	0.845	0.909	0.541
	Mean		4.750	3.753	1.270	0.841	0.659
	SE		1.315	1.109	0.302	0.078	0.088
Bali	INRA035	32	4	1.250	0.432	0.188	0.200
	ILST006	32	6	3.430	1.432	0.656	0.708
	ETH225	32	8	3.690	1.603	0.531	0.729
	HEL9	32	3	1.326	0.455	0.219	0.246
	Mean		5.250	2.424	0.981	0.398	0.471
	SE		1.109	0.658	0.312	0.116	0.144
Wagyu	INRA035	6	2	1.385	0.451	0.333	0.278
	ILST006	6	4	2.880	1.199	0.833	0.653
	ETH225	6	4	2.483	1.119	0.667	0.597
	HEL9	6	3	1.946	0.824	0.667	0.486
	Mean		3.250	2.173	0.898	0.625	0.503
	SE		0.479	0.325	0.170	0.105	0.083

Note: N: number of samples, Na: number of allele, Ne: number of effective allele, I: information index, Ho: observed heterozygosity, He: expected heterozygozity

Table 5. F-statistics (F_{IS}, F_{IT}, and F_{ST}) and estimates of Nm overall populations for each locus

Locus	F_{IS}	F_{IT}	F_{ST}	Nm
INRA035	-0.393	0.299	0.496	0.254
ILST006	-0.002	0.090	0.092	2.463
ETH225	-0.009	0.156	0.164	1.278
HEL9	-0.410	0.146	0.394	0.384
Mean	-0.203	0.173	0.287	1.095
SE	0.114	0.044	0.095	0.510

Note: Nm: gene flow

Table 6. Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Populations	Wagyu-Bali	Bali	Wagyu
Wagyu-Bali		0.252	1.531
Bali	0.777		2.533
Wagyu	0.216	0.079	

Genetic distance and relationships among three cattle populations

The genetic distance and relationship analyses of the three populations are presented in Table 6 and Figure 2. Based on the results of this study, the four microsatellite loci successfully separated Bali, Wagyu×Bali, and Wagyu populations. Table 5 shows that the three populations had genetic distances ranging from 0.252 to 2.533 across the four microsatellite loci used. The Wagyu×Bali population had the closest genetic distance to Bali, whereas Bali had the furthest genetic distance from Wagyu. The relationships among the three populations are explained by the phylogenetic tree shown in Figure 2. The phylogenetic tree based on the four microsatellite loci showed that Wagyu cattle were in a different cluster from the Wagyu×Bali crossbreed and Bali cattle. Additionally, a close relationship was found between Wagyu×Bali and Bali, whereas a more distant relationship was found between Wagyu×Bali and Wagyu.

Distinct clustering of cattle populations by PCA

PCA analysis revealed three distinct clusters among the populations (Figure 3). The first principal component (PC) distinguished the Wagyu population from the remaining populations, highlighting its unique genetic characteristics. The second PC separated the Bali and Wagyu×Bali populations, indicating a significant genetic differentiation between these groups. These findings provide valuable insights into the genetic structuring and relationships among the three cattle populations.

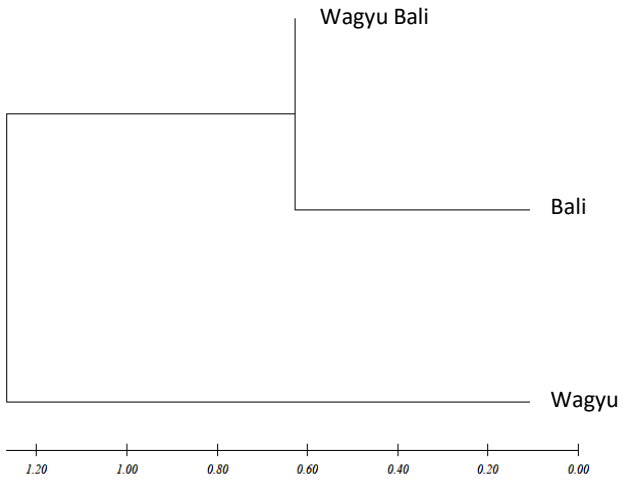

Figure 2. Dendogram of Wagyu-Bali crossbred, Bali purebred, and Wagyu purebred using Neighbour Joining method

Figure 3. Principal component analysis among three populations

Discussion

Artificial Insemination (AI) and changes in production systems have become integral to the cattle-farming industry. This technology enhances the genetic quality of livestock by controlled and efficient crossbreeding. Crossbreeding between Bali and Wagyu cattle, which are known for their high-quality meat, is an effort to improve the productivity and meat quality of local cattle. Since the introduction of AI biotechnology, there has been an urgent need to monitor and understand the genetic diversity of livestock populations. Currently, there are no comprehensive reports detailing the genetic diversity of Bali cattle and their crossbreeds, namely Wagyu×Bali, which should be addressed in this study.

This study utilized four microsatellite loci to determine hybrid diversity between Bali cattle (*Bos javanicus* d'Alton 1823) and Wagyu cattle (*Bos taurus* Linnaeus 1758). These loci, including INRA035, ILST006, ETH225, and HEL9, have been previously reported in Indonesian cattle breeds such as Kuantan, Pesisir, Madura, and Bali (Jakaria et al. 2020; Misrianti et al. 2022). This study is the first to employ microsatellite markers to ascertain the polymorphism of genetic diversity and population structure in Indonesian cattle breeds, specifically examining Bali cattle, Wagyu cattle, and their cross breeds. The microsatellite markers used showed polymorphism when applied to the Bali, Wagyu Bali, and Wagyu cattle populations. This polymorphism indicates a significant genetic diversity between and within these populations.

The analysis of microsatellite polymorphisms showed that the average number of alleles per locus (Na=4.417) was lower than the reported average for Bali cattle (Septian et al. 2015) in BPT-HMT Sumbawa and for Ongole grade cattle at RC Biotech Farm, West Java (Agung et al. 2016). This discrepancy in the number of alleles per locus suggests potential differences in the genetic diversity among these cattle populations. Various factors can contribute to this variation, including differences in breeding practices, population sizes, and environmental conditions (Pérez-Pereira et al. 2022). In this study, the locus ILST006 allele 284 bp was a private allele for the Bali purebred cattle and was not found in either Wagyu purebred or Wagyu×Bali crossbred cattle.

This private allele was not detected in a previous study at the same locus, that is, Simmental (Agung et al. 2019), and Indian indigenous cattle (Strucken et al. 2021), East Eurasian cattle (Svishcheva et al. 2020), Bali, PO and Kebumen (Jakaria et al. 2020). ILST006 (allele 288 bp), ETH225 (allele 145 bp), and HEL9 (allele 162 bp) were the candidates for a private allele in Wagyu purebred, with allele 145 having the highest allele frequency (0.583). For the Wagyu×Bali crossbreed in this study, ILST006 (allele 296 bp and 300 bp) and ETH225 (allele 139 bp) were observed.

These differences are a combination of breeding history, population size, population structure, environmental adaptation, and gene flow, all of which contribute to the variation in private alleles observed in each cattle population at microsatellite loci (Mwai et al. 2015; Edea et al. 2017; Bertolini et al. 2018; Decker et al. 2018). Private alleles (potentially breed-specific) define the genetic uniqueness of a breed. The presence of private microsatellite alleles with frequencies exceeding 0.01 in indigenous cattle breeds indicates that each breed probably possesses a distinct gene pool (Al-Jub et al. 2023). These private alleles could be valuable for accurately identifying different breeds (Svishcheva et al. 2020). This result showed that the Wagyu and Bali cattle hybridization conferred higher genetic diversity (Ho=0.841) than that of the purebred cattle. This result was in accordance with Agung et al. (2019), who found that simmental crossbreeds had higher genetic diversity than simmental purebreds.

This is evident from the negative inbreeding coefficient estimates, which indicate that the population has higher heterozygosity than expected under the Hardy-Weinberg equilibrium (Maiorano et al. 2018). Thus, hybridization between subspecies remains a vital method for introducing new genetic variations and enhancing genetic diversity (Cavani et al. 2018; Utsunomiya et al. 2019). Researchers can identify the genetic distances and similarities between cattle populations by constructing phylogenetic trees based on genetic data (Limpiti et al. 2014; Telles et al. 2018). This approach helps understand the historical lineage and genetic diversity of breeds, which is crucial for effective breeding programs and conservation strategies (Liu et al. 2022). Regarding Nei genetic distance, which reconstructed a rooted phylogenetic tree, the Wagyu×Bali crossbred had the greatest dissimilarities with the Wagyu purebred. The phylogenetic results also showed the greatest phylogenetic distance between the Wagyu×Bali crossbred and Wagyu purebred breeds.

The Wagyu×Bali crossbreed demonstrated the greatest similarity with the Bali purebred. High genetic similarities between Wagyu×Bali crossbred and Bali purebred breeds in this study. These results indicate that although Wagyu×Bali is a crossbreed, its genetics are more similar to those of Bali than Wagyu. This may be due to the dominant genetic influence of Bali cattle in the crossbred population. The significant genetic distance between Bali and Wagyu indicates substantial genetic differences between these two populations, which can be used in breeding programs to enhance genetic variation. Bali cattle are distinguished by their unique morphometric characteristics, which contribute to their adaptability and productivity. Bali cattle are relatively small and compact, making them well suited for adaptation to various environmental conditions, particularly in the tropics (Sutarno and Setyawan 2015). According to a previous study, Bali cattle have an average wither height of approximately 115 cm in males and 105 cm in females. Their average body length is approximately 120 cm for males and 110 cm for females (Hafid et al. 2020).

Principal Component Analysis (PCA) confirmed the genetic relationship between the Wagyu×Bali crossbred and Bali purebred cattle. The analysis, based on four microsatellite loci, revealed that the Wagyu×Bali crossbreed was genetically closer to the Bali purebred, but distinct from the Wagyu purebred. This suggests that the genetic contribution of Bali cattle is dominant in crossbred populations. Bigirwa et al. (2019) reported that Hanwoo

purebreds were genetically separated from Holstein hybrid (Korean×Holstein and Ugandan×Holstein). Based on using thirty microsatellite loci, Korean native cattle, also known as *Hanwoo*, were genetically differentiated from the two exotic breeds, that is, *Heugu* and *Jeju* black cattle (Suh et al. 2014).

A prior study, along with principal component analysis and a neighbor-joining tree, demonstrated a clear distinction between *Jeju* Black cattle and other local Korean and Japanese cattle as well as taurine breeds in their evolutionary development. Additionally, *Jeju* Black cattle exhibited a unique admixture pattern (Alam et al. 2021). The crossbreeding program between local Indonesian cattle and *B. taurus*, as reported by Sutarno and Setyawan (2015), resulted in crossbreeds such as Limpo (Limousin crossed with PO), Madrasin or Limad (Limousin crossed with Madura) Simpo (Simmental crossed with PO), Limbal (Limousin crossed with Bali), and Simbal (Simmental crossed with Bali). These hybrids faced challenges, such as male sterility and reduced reproductive capacity in females, suggesting potential genetic incompatibilities or hybrid vigor issues. Therefore, further research is needed to assess the reproductive performance and overall viability of the Wagyu×Bali crossbreeds.

In conclusion, the use of INRA035, ILSTS006, ETH225, and 197 HEL9 loci was highly informative and polymorphic in detecting the genetic diversity study, especially for Bali purebred, Wagyu purebred, and their hybrid. The genetic diversity within each population could serve as evidence for identifying certain specific alleles not found in other beef cattle breeds. Based on the genetic distance value, the Wagyu×Bali crossbred population was exceptionally close to the Bali purebred. This study's genetic diversity evaluation can be considered the initial (base) population for the Bali cross-cattle breeding programs.

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