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Genetic Diversity of Bali Cattle Base on Two Microsatellite Loci - INRA032 and BM2113

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Abstract: Microsatellites are short tandem repeat (STR) sequences that consist of simple repeats and exhibit a high number of alleles at each genomic locus. The aim of this study was to examine genetic variation in Bali cattle and the population dynamics using microsatellite markers. Two microsatellite loci, INRA032 and BM2113 were amplified using PCR Total DNA samples from the genome of 60 Bali cattle, then The PCR products were analyzed through agarose gel electrophoresis, followed by staining with Ethidium Bromide (EtBr). The number and size of alleles that appeared on the gel, while the diversity and population dynamics were analyzed using Popgene version 1.31 and Gene Calc. In this study, The effective number of alleles for the two microsatellite loci in the Bali cattle population analyzed was 6.53, with an average of 3.27. The average PIC of the two microsatellite loci was 0.70, while the observed heterozygosity (Ho) exceeded the expected heterozygosity (He), with values of 0.975 and 0.695, respectively. This suggests an excess of heterozygosity in the population and indicates that the populations are not in Hardy-Weinberg equilibrium. The inbreeding coefficient was high, with negative Fis values of -0.38 for INRA032 and -0.43 for BM2113.

Keywords: Bali cattle, diversity, inbreeding, microsatellite, PCR.

Introduction

In recent years, notable advancements have been achieved in individual identification and breed certification for local livestock species in several countries. This has primarily been accomplished through the use of microsatellites, which are internationally recognized as the preferred molecular markers for tracking information at the breed registration level, from species to individual. Microsatellites are spread less densely but uniformly across the eukaryotic genome and are highly variable due to differences in the number of repeat units (Vargas et al., 2016). Microsatellite markers, due to their high polymorphism and relatively consistent coverage across the genome, have proven to be essential molecular tools for a wide range of genetic studies in humans, model organisms, wild vertebrate populations, and livestock (Nishimaki et al., 2013), including cattle (Vargas et al., 2016), horses (Cortés, et al., 2017), goats (Bulut et al., 2016), and pigs (Montenegro, et al., 2014).

The International Society for Animal Genetics (ISAG) and the Food and Agriculture Organization (FAO) of the United Nations have recommended the use of microsatellites in the genetic studies of livestock breeds population, especially for genetic diversity and distance, structure of population, purity and origin of breed, effective population size, and other related characteristics analyzing (Barker et al., 1993). The different degrees of multilocus heterozygosity and allelic diversity identified through microsatellite polymorphism analysis in cattle have sparked a range of additional research in this field. One such study led to the development of a meat traceability system for indigenous Chinese cattle, aimed at ensuring meat safety and

tackling issues of adulteration (Zhao et al., 2017). The various ecotypes of Nguni cattle formed due to adaptation to the extremely different environments in South Africa have been found to have close genetic relationships (Sanarana et al., 2015). The Macabea cattle were identified as part of the American Creole group and are believed to have originated from southern Spain (Vargas et al., 2016). Finally, genetic distinctiveness and allelic variability have been observed in Korean native cattle breeds, setting them apart from both international and regional cross-border cattle breeds (Suh et al., 2015).

Indonesia has many types of native cattle, one of which is Bali cattle. Because of its very important role for Indonesia's socio-economy, the breeding program of Bali cattle and conservation efforts should be carefully planned, taking into account their potential and genetic data. Genetic diversity information is essential for implementing conservation programs, particularly regarding the genetic resources of local Indonesian cattle and their contribution to the development of community-based livestock businesses. Molecular genetic analysis has enabled the examination of the potential of specific cattle breeds at the deoxyribonucleic acid (DNA) level. Microsatellites are considered nearly perfect genetic markers due to their abundance, high diversity, codominance, and widespread distribution throughout the euchromatic regions of the genome (Hillel et al., 2003). Microsatellite markers can serve as a tool to assess genetic distance (Rehman et al., 2009), relationships between cattle breeds (Maretto et al., 2012), paternity testing (Stevanovic et al., 2010); Nishimaki et al., 2013). and genetic diversity (Seo et al., 2017; Olschewsky et al., 2021).

This study seeks to evaluate the genetic variability, genetic changes at the individual level and population dynamics of Bali cattle using microsatellite markers, which can inform the development of breeding and conservation strategies for Indonesian cattle breeds in the future.

Material and Method

Blood sample and DNA collection

All animal-related procedures were approved by the Ethics Committee of the Faculty of Medicine at Mataram University, Indonesia. Blood samples for cattle genotyping were collected as much as 3 to 5 mL through the coccygeal vein using Venoject and stored in a Vaccutainer tube with EDTA as anticoagulant. DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's method.

Microsatellite Amplification

Microsatellite amplification was performed using the Nexus Mastercycler PCR machine, with a reaction composition of 1 µL DNA, 0.5 µL primer, 5.25 µL MyTaq HS Red Mix (2x), and 6.25 µL dH2O. The amplification protocol included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation (95°C; 10 sec), annealing (60°C; 20 sec), and elongation (72°C; 30 sec) with one cycle of final elongation was carried out at 72°C for 5 minutes. The visualization of microsatellite alleles from PCR products was achieved through electrophoresis on a 2% agarose gel, followed by SyBr staining. The results were then captured using the Gel Documentation System (Syngene, Cambridge, UK).

Statistic Analysis

The position, size, and number of alleles on the agarose gel were manually identified. All DNA alleles with identical migration rates on the gel are considered homologous, with the fastest migrating allele designated as allele A, followed by allele B, and so on (Leung et al., 1993). Genetic parameters, including the effective number of alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), Shannon Information Index (I), fixation index (Fis), and differentiation index (Fst), were calculated using PopGene software (Version 32).

Results and Discussion

Genetic parameters of microsatellite loci

In this research, two microsatellite loci (BM2113 and INRA032) were selected for a more in-depth analysis of the genetic variability within the Bali cattle population as a native breed of Indonesian cattle.The genetic diversity of the 2 microsatellite loci was simulated. Some of the

amplification results are shown in Figures 1-2. The distribution of alleles and genotypes of the two microsatellite loci BM2113 and INRA032 in each individual was determined by amplifying genomic DNA using the PCR technique. The results of microsatellite amplification of the BM2113 and INRA032 loci in each individual obtained fingerprints of both loci each having 4 alleles (A, B, C and D) in Bali cattle. The polymorphism pattern of both microsatellite loci is shown in the following figure 1 and 2.

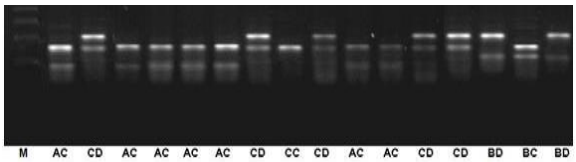

Figure 1. Fingerprint of INRA 032 Loci. M is 100 bp Marker

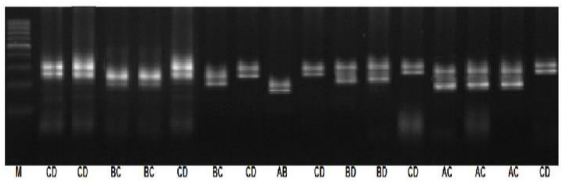

Figure 2. Fingerprint of BM2113 Loci. M is 100 bp Marker

The balance of allele distribution/allele segregation pattern at both microsatellite loci (INRA 023 and ETH 225) in Bali cattle population was analyzed using chi-square test (X2) with degree of freedom (db) = k - 1 at 95% confidence interval (α = 0.05) (Clamp et al., 1992). The results of the chi square significance test (X2) can be seen in tables 2 and 3.

Table 1. The frequencies of allele and genotype of two microsatellite loci in the population of Bali cattle.

LOCUS	Number of Sample	**Allele**		**Genotype**	
		Type	frequencies **(%)**	**Type**	frequencies **(%)**
INRA032	60	A	23,33	AC	46,7
		B	11,67	BC	8,33
		C	45,00	BD	15,0
		D	20,00	CC	5,0
				CD	25,0
BM2113	60	A	A = 29	AB	1,67
		B	B = 15	AC	46,67
		C	C = 52	BC	11,67
		D	D = 24	BD	11,67
				CD	28,33

The analysis of the two microsatellite loci in the Bali cattle population revealed a total of 8 alleles, with an average of 4 alleles per locus (Table 1). Barker (1994) states that microsatellite markers utilized for genetic distance estimation should contain more than four alleles to minimize the standard error in the estimation process. In accordance with FAO standards, at least four different alleles per locus are necessary for evaluating genetic diversity and differences within and across populations. Therefore, both INRA 032 and BM 2113 loci can be utilized to assess genetic variation within and between Bali cattle populations in Indonesia.

The results of PCR analysis of INRA 032 and BM 2113 loci in 60 Bali cattle obtained 5 genotype variations. The results of measuring the balance of genotype distribution in the population using the chi square (X^2) test showed that the genotype distribution of both loci in the population of Bali cattle was in equilibrium (X^2 count > X^2 table) meaning that the alleles of the locus did not segregate with the pattern in the "Mandel" hypothesis.

Genetic diversity within bali cattle population

Genetic diversity in a population is represented by the effective number of alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), and the values of polymorphic information content (PIC) which are fundamental measures that provide essential information for distinguishing individuals and populations (Seo et al., 2016). The outcomes of assessing genetic diversity values in the Bali cattle population are listed in Table 4. The INRA032 and BM2113 loci

showed high variation in the population. The results of the calculation of genetic parameters (Table 4) were carried out using the Microsatellite-Toolkit PopGene32 software.

Table 2. The equilibrium of Genotype Distribution of INRA 032 Locus in Bali cattle population

Genotype	Genotype Frequencies (%)	Observed Value (O)	Expected Value (E)	$O-E)^2/E$	χ^2 table
AC	46,7	28	7.5	56.03	12.59
BC	8,3	5	7.5	0.83	
BD	15	9	7.5	0.30	
CC	5,0	3	3.75	0.15	
CD	25	15	7.5	7.50	
AD/AA/BB/DD/AB	0	0	26.25	26.25	
	100	60	60	91.07	12.59

Table 3. The equilibrium of Genotype Distribution of BM2113 Locus in Bali cattle population

Genotype	Genotype Frequencies (%)	Observed Value (O)	Expected Value (E)	$O-E)^2/E$	χ^2 table
AB	1.67	1	7.5	5.63	12.59
AC	46.67	28	7.5	56.03	
BC	11,67	7	7.5	0.03	
BD	11.67	7	7.5	0.03	
CD	28.33	17	7.5	12.03	
AD/AA/BB/DD/CC	0	0	22.5	22.50	
	100	60	60	96.27	12.59

The effective number of alleles denotes the total number of alleles with frequencies that fulfill the conditions for expected heterozygosity in a population. It is frequently utilized to assess allele effects in populations and to capture genetic variation as shown by inverse homozygosity. Estimating heterozygosity values is crucial for understanding genetic variability (Marson et al., 2005) and determining the allele polymorphism level and the population's future prospects (Falconer and Macay, 1996). Genetic diversity can be assessed by calculating the average heterozygosity level in a population, the number of alleles per locus, and the percentage of polymorphic loci (Soysal, 2004).

In the Bali cattle population analyzed, the effective number of alleles for the two microsatellites was 6.53, with a mean value of 3.27 (Table4). The result of this study are consistent with those reported by Septian *et al.*, (2019) thet the average number of effective alleles of bali cattle in BPTU Sapi Bali, BPT-HMT Sumbawa and VBC Barru Distric (Ne = 3,58). Alawiyah et al. (2021) also reported the effective number of alleles observed in the Bali cattle population was 3.92 (SPS115); 3.20 (ETH225) and 6.16 (INRA037). Dalam penelitian ini, 2 penanda mikrosatelit yang dipilih sangat informatif untuk ras sapi Bali dan juga sesuai untuk diskriminasi individu.

The fixation index (Fis) of both microsatellite INRA032 and BM2113 in this study were negative (-0.38 and -0.43). This indicates excess heterozygosity, which can occur due to several factors such as outbreeding and the absence of selection pressure or the both locus are under pressure of heterozygosity selection. There is a possibility of outbreeding occurring in the population which allows for the introduction of genetics from outside the population. This is very likely to occur in smallholder farms, where farmers increase their livestock productivity through crossbreeding with exotic breeds. The findings of this study contrast with those reported by Septian et al. (2019) that the average Fis or inbreeding coefficient in Bali cattle at BPTU Sapi Bali, BPT-HMT Sumbawa and VBC Barru Distric was positive (0.296) which indicates inbreeding and selection pressure in the population. Selection and inbreeding programs are indeed carried out to increase productivity and maintain breed purity. Fis

is a metric in population genetics to assess the degree of inbreeding in a subpopulation. It quantifies the reduction in heterozygosity (genetic variation) in individuals compared to the expected levels under random mating. A positive fixation index (FIS) value (> 0) indicates a heterozygote deficit, often caused by inbreeding in the population (Eusebi et al., 2020).

The most accurate parameter for measuring genetic variation is the heterozygosity value. By definition, Observed heterozygosity refers to the proportion of heterozygous individuals at each locus, while expected heterozygosity indicates the chance that a randomly selected individual from a population in Hardy-Weinberg equilibrium will be heterozygous. (Rutledge et al., 2010). The findings of this study suggest that the INRA032 and BM2113 loci show higher observed heterozygosity (Ho) than expected heterozygosity (He) which indicates an excess of heterozygosity and confirms that the population is not in equilibrium of Hardy-Weinberg. The HWE test revealed a significant divergence of the two loci in the population.

Table 4. Genetic diversity of Bali cattle population base on INRA032 and BM2113 loci

LOCUS	N	Na	Ne	I	Ho	He	HWE	PIC	Fis
INRA032	60	4.00	3.22	1.27	0.95	0.69	55.73	0.69	-0.38
BM2113	60	4.00	3.31	1.29	1.00	0.70	53.37	0.71	-0.43
Avarege		4.00	3.265	1.28	0.975	0.695	54.55	0.70	-0.405

N: Sample number; Na: number of alleles; Ne: Effective alleles number; I: Shannon's index; Ho: Observe heterozygosity; He: Expected heterozygosity; PIC: polymorphic information content; HWE: Hardy-Weinberg equilibrium; and Fis: Wright's fixation index

PIC quantifies the information content in microsatellites and is influenced by the number of identified alleles and their frequencies (Oscar et al., 2022). This refers to the capacity to detect polymorphisms among individuals within a population and is frequently used in linkage studies to evaluate the informativeness of genetic markers. We found that the average PIC value of INRA032 and BM2113 loci in the population was higher than 0.70, meaning that these two loci are highly informative. meaning that these two loci are highly informative (Bolstein et al., 1980). The results of this study are consistent with those observed in Pesisir, Madura, and Kuantan cattle for the three microsatellite loci, namely INRA35, HEL9, and ETH225 (Misrianti et al.), and were higher than those observed in Egyptian Cattle (El-Sayed et al., 2016) and Punganur Cattle (Devi et al., 2017), but lower than those found in Turkey Cattle (Demir et al., 2019).

Conclusions

Identification of genetic diversity in the Bali cattle population, using a panel of two microsatellite loci, demonstrated an adequate level of genetic diversity for the purposes of genetic conservation. The microsatellites examined exhibited moderate to high PIC values, and the number of alleles aligned with the minimum standard of FAO. The observed heterozygosity is higher than expected, indicates excess heterozygosity, which can occur due to several factors such as outbreeding and the absence of selection pressure or the loci is under pressure of heterozygosity selection. Outbreeding can happen within a population, leading to the introduction of genetic material from external sources. In this research, we found that the Wright inbreeding coefficient (Fis) was negative, which indicated an excess of heterozygosity resulting from outbreeding or heterozygosity selection pressure. The population genetic index based on microsatellite data can provide valuable insights into the genetic structure and dynamics of a population as basic information in formulating breeding strategies, namely crossbreeding and conservation. However, in the utilization of genetic resources, especially in increasing livestock productivity through crossbreeding, it is necessary to consider sustainable conservation to prevent extinction.

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