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Genetic Diversity between Bali Cattle (*Bos javanicus*) and It's Hybrids Using Microsatellite Markers

Research Article

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ABSTRACT

The aim of this study was to evaluate the Bali cattle genetic diversity and hybrid cattle using microsatellite markers. Blood samples (n=192) were collected from various cattle breed, i.e. Bali (n=96), Madura (n=48), and Peranakan Ongole (PO) Kebumen (n=48). The microsatellite locus used for assessing the genetic variation was INRA035, ILSTS006, ETH225, and HEL9, while the genetic profile was described using GenAlEX, Cervus, MEGA6, structure and R programs. As a result, 46 alleles were found at four microsatellite loci studied. The genetic diversity of Bali cattle from Bali island (H_o=0.337) and Nusa Penida island (H_o=0.375) were recorded lower than that of the hybrids, i.e. Madura (H_o=0.747) and PO Kebumen (H_o=0.567) cattle breeds. Specifically, we found 283 bp (locus ILSTS006), 194 bp (locus ETH225), 147 bp and 151 bp (locus HEL9) demonstrating the distinctive alleles for Bali cattle. Also, our experimental data showed that microsatellite markers used allowed us to produce an obvious differentiation of the cattle cluster between Bali cattle and the hybrids, which was meaningful for future cattle breeding program.

KEY WORDS Bali cattle, genetic diversity, microsatellite marker.

INTRODUCTION

Bali cattle, an Indonesian native cattle, is a domesticated descendant of banteng (*Bos javanicus*) (Martojo, 2012) and constitutes a beef cattle breed. The population is estimated to reach 27% (5 millionheads) of total cattle in Indonesia (Purwantara *et al.* 2012). The adaptive features of Bali cattle in marginal land are favorable for small holder farmers, while the reproductivity of the cattle is also high (Mohamad *et al.* 2009). Besides, Madura and PO Kebumen cattle breeds are also popular in small holder farmers; some studies reported that both cattle breeds were the hybrids of Bali cattle (Nijman *et al.* 2003; Hartati *et al.* 2015; Sutarno and Setyawan, 2016).

The existence of Bali, Madura, and PO Kebumen cattle as Indonesian native cattle breed has been legalized through the Indonesian Ministry of Agriculture. The genetic variation of Bali cattle and it's hybrid seemed to be partially documented. For further studies, microsatellite markers could be a noticeable approach in conducting the exploration of genetic diversity (Viryanski, 2019). Close breeding policy in Indonesia for Bali cattle in Bali and Nusa Penida island aims for maintaining the purity of Bali cattle in Indonesia. Madura cattle are the result of a crossbreeding between Zebu and Bali. Madura cattle have a native geographical distribution in Madura island and surroundings. Sapudi island is an isolated island that is concentrated as a close breeding area for Madura cattle.

PO Kebumen cattle are the result of selection in Kebumen Regency, Central Java. In 1806, Ongole cattle (*Bos indicus*) were brought by traders to East Java and mated with Javanese cattle (*Bos javanicus*) (Sutarno and Setyawan, 2016).

Many areas have extensively used microsatellite markers for evolution studies and genetic relationships in of cattle, including *Bos taurus* and *Bos indicus* (MacHugh *et al.* 1997), Niger cattle (*Bos indicus*) (Grema *et al.* 2017), Brazilian cattle (De Oliveira *et al.* 2012), Latin-American Creole (*Bos indicus*) (Delgado *et al.* 2012), Sanga cattle (Gororo *et al.* 2018), Blanco Orejinegro cattle (Martínez *et al.* 2013), and Vietnamese indigenous cattle (Pham *et al.* 2013).

The approach is also used for conducting pedigree verification (Jevrosima *et al.* 2009) and investigating the association with carcass traits in Hanwoo cattle (Choi *et al.* 2006). The method has favorable features such as locus-specific, co-dominant, highly polymorphic, rapid, reproducible, while it is also possible to conduct at various scales from individual to population (Viryanski, 2019).

Although the use of microsatellite markers on Bali cattle has been previously reported by researchers (Handiwirawan *et al.* 2003; Nijman *et al.* 2003; Sutarno *et al.* 2015), the number of samples and the methodology used seems to be limited.

In terms of the method, there is a shifting from polyacrylamide gel electrophoresis (PAGE) to fragment analysis (Viryanski, 2019). Currently, the microsatellite markers have been applied for molecular investigation of Indonesian beef cattle, including Bali, Madura, and PO cattle breeds (Agung *et al.* 2019). However, the study needs improvement, particularly focusing on the use of locus recommended by Measurement of Domestic Animal Diversity (MoDAD), International Society of Animal Genetics (ISAG) FAO with totally reaching 30 loci especially INRA035 and HEL9 due to their high diversity. Therefore, genetic diversity of Bali cattle (domesticated at Bali and Nusa Penida islands) and their hybrids (Madura and PO Kebumen) were investigated in this study using microsatellite markers.

MATERIALS AND METHODS

Sample collection and total DNA extraction

A blood sample (3-5 mL) of 192 individuals was collected through the jugular vein by a veterinarian (Table 1), using vacuum venojectcontaining anti-coagulant EDTA K3 (BD, United State). The DNA extraction was performed through standard procedures of DNA Mini Kit (GenAid protocol Cat. No. GB100).

Primer, amplification, fragment analysis

The four loci of microsatellite used in this study are presented in Table 2. Polymerase chain reaction (PCR) amplification of microsatellite primer was made in a final volume of 28 µL containing 2 × GoTaq® Green Master Mix (Promega, United States), primer (forward and reverse,25 ng/µL primer), 7.7 µL nuclease-free water, and DNA sample (25 to 50 ng/µL).

PCR program (Eppendorf, Germany) was operated as the following procedure. The initial denaturation was at 95 ˚C for 5 min (1 cycle). The amplification was conducted by 35 cycles: (1) denaturation 95 ˚C for 10 s, (2) annealing at 55-60 ˚C for 20 s (depending on microsatellite locus), and (3) extension at 72 ˚C for 30 s, and (4) finally elongation at 72 ˚C for 5 min. The PCR products were then observed by electrophoresis using 1.5% agarose gel. Multiplex fragment analysis was performed on fragment analysis services on 1st base (http://www.base-asia.com/fragment_analysis/).

Data analysis

Each microsatellite locus was determined for calculating the allele number (na) and effective allele number (ne), allele frequency, observed heterozygosity (H_o) and expected heterozygosity (H_e), F statistic (F_{IS}, F_{IT}, F_{ST}). Hardy-Weinberg (HW) equilibrium test and molecular variance analysis (AMOVA) were analyzed using GenAlEx 6.5 (Peakall and Smouse, 2012), while the polymorphic informative content (PIC) was analyzed using CERVUS version 3.0.7 (Kalinowski *et al.* 2007).

Cluster differentiation among populations, genetic distance as well as the genetic tree ware analyzed using MEGA version 6 (Tamura *et al.* 2013). Genetic structure and genetic admixture in cattle species were analyzed by using the procedure of Bayesian clustering in STRUCTURE version 2.2 (Pritchard *et al.* 2000). This study employed a total of 10 independent runs for each K between 2 and 10. A burn-in period used was 1000000 iterations, then followed by 1000000 iterations of the Markov chain Monte Carlo algorithm. The optimum K value was determined using Structure Harvester (Earl and vonHoldt, 2012), adopted from the Evanno method (Evanno *et al.* 2005). Finally, this study used the principal component analysis (PCA) using R version 3.2.0. (Jombart, 2008) for data analysis.

RESULTS AND DISCUSSION

Microsatellite diversity

The study successfully identified 46 alleles of the four loci, i.e. 8 alleles (INRA035), 12 alleles (ILSTS006), 12 alleles (ETH225), and 14 alleles (HEL9) (Table 1).

Table 1 Location of cattle breeds studied

No.	Breed	N (sex)	Location
1	Bali-1	48 (male and female)	Breeding center, Bali island, Bali province
2	Bali-2	48 (female)	VBC, Nusa Penida island, Bali province
3	Madura	48 (female)	VBC, Sapudi island, Madura island, West Java
4	Peranakan Ongole (PO) Kebumen	48 (female)	VBC, Kebumen district, Centre Java
Total sample		192	-

N: number of individual and VBC: village breeding centre.

Table 2 The description of microsatellite markers used

Locus*	Chr.	Motif	Primer sequence (Forward 5'-3'and Reverse 5'-3')	Size (bp)	Label	Ta (˚C)
INRA035	16	[1](TG)$_{16}$	ATCCTTTGCAGCCTCCACATTG TTGTGCTTTATGACACTATCCG	98-124	FAM	55
ILSTS006	7	[2](GT)$_{23}$	TGTCTGTATTTCTGCTGTGG ACACGGAAGCGATCTAAACG	277-309	FAM	60
ETH225	9	[3](CA)$_{11}$	GATCACCTTGCCACTATTTCCT ACATGACAGCCAGCTGCTACT	137-159	HEX	58
HEL9	8	[4](GT)$_{25}$	CCCATTCAGTCTTCAGAGGT CACATCCATCCATGTTCTCACCAC	141-173	NED	55

* Microsatellite marker recommended by MoDAD, ISAG FAO; Chr.: chromosome; Access number GenBank [1]X68049, [2]L23482, [3]Z14043 and [4]X65214; bp: base pair and Ta: temperature annealing.

The Bali cattle (from Bali and Nusa Penida islands) possessed a lower number of alleles compared to the Madura and PO Kebumen cattle, especially in INRA035 and HEL9 loci (Table 3). Clearly, the HEL9 served as the private allele in Bali cattle, i.e. 147 bp and 151 bp, specifically 147 bp with the highest allele frequency compared to151 bp. This private allele was not found in both Madura and PO Kebumen cattle breeds. Furthermore, Bali cattle from Nusa Penida island tend to have a higher uniformity compared to that from Bali island. Based on the observation on INRA035, ILSTS006 and ETH225 loci, the number of alleles in Bali cattle from Nusa Penida island was lower than that of Bali island (Table 4). In addition, the H_o and H_e values of Bali cattle from Nusa Penida island were the lowest, i.e. 0.375 ± 0.181 and 0.398 ± 0.170, respectively. On the contrary, the highest score of the H_o and $H_e$was attributed to the Madura cattle, i.e. 0.747 ± 0.111 and 0.763 ± 0.044. Regarding the total population, there was no difference between H_o (0.516±0.075) and H_e (0.579±0.073), with the exception of the scores of PO Kebumen, i.e. 0.567 ± 0.109 and 0.739 ± 0.070 (Table 4).

The population differentiation due to genetic structure (F_{ST}), inbreeding coefficient of individual relative to the total population (F_{IT}) and inbreeding coefficient of individual within-population (F_{IS}) and polymorphism information content (PIC) values were 0.246 ± 0.067, 0.316 ± 0.081, 0.070 ± 0.132 and 0.738 ± 0.102, respectively (Table 5).

This indicates that all microsatellite loci contribute to the genetic differentiation (F_{ST}=24.6%), primarily on the INRA035 and HEL9 loci.

Based on F_{IT}= 31.6%, the ILST006 and HEL9 loci greatly account for the deficiency of heterozygote (inbreeding coefficient) among populations reaching up to 49.1% and 39.1%, while, within-population (F_{IS}=7.0%), the ILSTS006 and HEL9 loci also contributed to deficiency of heterozygote, except for the INRA035 and ETH225 contributing to excess of heterozygote.

Based on the PIC analyses, all microsatellite markers were informative and in this study, the ETH225 was the most informative locus with the highest score of PIC (0.804).

Meanwhile, the HW equilibrium analysis showed that all microsatellite markers studied were in disequilibrium status (P<0.001).

Population structure and phylogenetic tree

Based on genetic distance analysis, the four cattle populations showed a different genetic distance (Table 6). The genetic tree shows that Bali cattle from Bali island and Nusa Penida islands differed, although they have high similarity.

In contrast, the Bali cattle had different cluster groups with Madura cattle and PO Kebumen populations. The results show two main clusters: *Bos javanicus* and *Bos indicus* (Figure 1). In addition, Structure Harvester's analysis exhibited the optimum value at K= 3, demonstrating that the Bali cattle from Bali and Nusa Penida islands possessed genetic similarities (red). On the other hand, Madura cattle (blue) and PO Kebumen cattle (green) located in different clusters (Figure 2).

Table 3 Frequency of allele and allele size (bp) for the four microsatellite markers*

Locus	Allele (bp) / N	Population			
		Bali-1	Bali-2	Madura	Peranakan Ongole (PO) Kebumen
INRA035	N	46	47	48	47
	98	-	-	0.521	0.340
	100	-	-	0.104	0.096
	102	0.967	0.989	0.229	0.074
	104	0.022	-	-	0.160
	106	-	-	-	0.181
	110	-	-	-	0.011
	116	0.011	0.011	0.146	0.128
	120	-	-	-	0.011
ILSTS006	N	41	43	45	32
	275	-	-	0.022	0.031
	277	-	-	0.022	
	279	0.110	0.035	0.022	0.016
	283	0.073	0.140	-	-
	285	0.341	0.570	0.033	0.016
	289	0.134	0.081	0.222	0.016
	291	0.317	0.174	0.267	0.391
	293	0.024	-	0.344	0.188
	295	-	-	0.022	0.063
	297	-	-	0.022	0.203
	299	-	-	0.011	0.078
	301	-	-	0.011	-
ETH225	N	46	47	48	47
	137	-	-	0.094	0.106
	139	-	-	0.104	0.011
	145	-	-	0.083	0.085
	147	-	-	0.073	0.011
	149	0.011	0.011	-	-
	151	-	-	0.042	0.053
	153	0.011	-	0.010	-
	155	0.011	0.117	0.229	0.660
	157	0.304	0.202	0.052	0.053
	159	0.022	0.043	0.042	-
	163	0.239	0.330	0.042	-
	165	0.402	0.298	0.229	0.021
HEL9	N	48	34	48	47
	143	-	-	0.063	-
	145	-	-	-	0.011
	147	0.906	0.882	-	-
	149	-	-	0.229	0.043
	151	0.094	0.118	-	-
	153	-	-	0.292	0.211
	155	-	-	0.021	0.064
	159	-	-	-	0.202
	161	-	-	0.125	0.138
	165	-	-	0.208	0.138
	167	-	-	0.010	0.032
	169	-	-	0.052	0.117
	171	-	-	-	0.032
	173	-	-	-	0.011

* Underline is a specific allele.
N: number of individuals.

Similarly, PCA analysis also demonstrated two main clusters, i.e. *Bos javanicus* (Bali cattle of Bali and Nusa Penida islands) and *Bos indicus* (Madura and PO Kebumen cattle), shown in Figure 3.Additionally, the AMOVA analysis results show that the between-population variation was 29.4% (P<0.000), shown in Table 7.

Table 4 Alleles number (Na), effective alleles number (Ne), heterozygosity (H_o) and expected heterozygosity (H_e)

Population	Locus	n	Na	Ne	H_o	H_e
Bali-1	INRA035	46	3	1.1	0.065	0.064
	ILSTS006	41	6	4.0	0.537	0.747
	ETH225	46	7	3.2	0.717	0.688
	HEL9	48	2	1.2	0.188	0.170
	Mean		4.5	2.4	0.377	0.417
	SE		1.2	0.7	0.151	0.175
Bali-2	INRA035	47	2	1.0	0.021	0.021
	ILSTS006	43	5	2.6	0.372	0.618
	ETH225	47	6	3.9	0.872	0.746
	HEL9	34	2	1.3	0.235	0.208
	Mean		3.8	2.2	0.375	0.398
	SE		1.0	0.7	0.181	0.170
Madura	INRA035	48	4	2.8	0.958	0.644
	ILSTS006	45	11	4.1	0.444	0.757
	ETH225	48	11	6.9	0.854	0.855
	HEL9	48	8	4.9	0.729	0.796
	Mean		8.5	4.7	0.747	0.763
	SE		1.7	0.9	0.111	0.044
Peranakan Ongole (PO) Kebumen	INRA035	47	8	4.9	0.787	0.795
	ILSTS006	32	9	4.2	0.313	0.759
	ETH225	47	8	2.2	0.468	0.54
	HEL9	47	11	7.2	0.702	0.862
	Mean		9.3	4.6	0.567	0.739
	SE		0.9	1.0	0.109	0.070
All population	Mean		6.5	3.5	0.516	0.579
	SE		0.8	0.5	0.075	0.073

SE: standard error.

Table 5 F-statistic (F_{ST}, F_{IS}, F_{IT}), PIC and HW test in Bali-1, Bali-2, Madura and Peranakan Ongole (PO) Kebumen cattle populations

Locus	Allele sizes (bp)	Na	F_{IS}	F_{IT}	F_{ST}	PIC	HW *test*
INRA035	98-120	8	-0.203	0.264	0.388	0.586	**
ILSTS006	275-301	12	0.422	0.491	0.120	0.793	**
ETH225	137-165	12	-0.029	0.118	0.143	0.804	**
HEL9	143-173	14	0.089	0.391	0.331	0.767	**
Mean		11.5	0.070	0.316	0.246	0.738	
SE		2.5	0.132	0.081	0.067	0.102	

Na: number of allele; PIC: polymorphic informative content and HW: Hardy-Weinberg equilibrium.
** ($P<0.001$).
SE: standard error.

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

Population	Bali-1	Bali-2	Madura	Peranakan Ongole (PO) Kebumen
Bali-1	-	0.9767	0.3273	0.1596
Bali-2	0.0236	-	0.2902	0.1582
Madura	1.1169	1.2371	-	0.7190
Peranakan Ongole (PO) Kebumen	1.8351	1.8441	0.3299	-

Variation in microsatellite markers

Based on the listed of microsatellite markers in MoDAD ISAG FAO, the four studies out of the 30 recommended loci exhibited high polymorphisms in the Bali cattle and it's hybrids (Madura and PO Kebumen cattle). Meanwhile, the high value of PIC was found in all loci, i.e INRA035 (0.586), ILSTS006 (0.793), ETH225 (0.804) and HEL9 (0.767) (Table 5). Agung *et al.* (2019) reported that the PIC of ILSTS006 and ETH225 was 0.869 and 0.935, respectively, which was applied to 10 Indonesian cattle, including Bali, Madura, and PO cattle. Gororo *et al.* (2018) also asserted the PIC of ILSTS006 and ETH225 reached 0.748 and 0.661, investigated in three Zimbabwean Sanga cattle breeds. Kale *et al.* (2010) also found the PIC of INRA035, ILSTS006, and ETH225, with a value of 0.684, 0.749 and 0.602 in three Indian cattle breeds (Gir, Deoni and Kankrej). PIC from various loci was also reported by Sodhi *et al.* (2007), with scores of INRA035 (0.815), ILSTS006 (0.555), ETH225 (0.554), and HEL9 (0.817) studied in Indian Kankrej cattle breeds.

Table 7 Analysis of molecular variance (AMOVA) among cattle breed populations

Source of variation	Df	SS	MS	Var. comp.	Variation (%)	P-value
Among populations	3	66.0	22.0	0.226 Va	29.4	0.000
Within populations	376	203.8	0.54	0.542 Vb	70.6	
Total	379	269.9	-	0.768	100.0	

Df: degree of freedom; SS: sum of squares; MS: mean of square and Var. comp.: variance component.

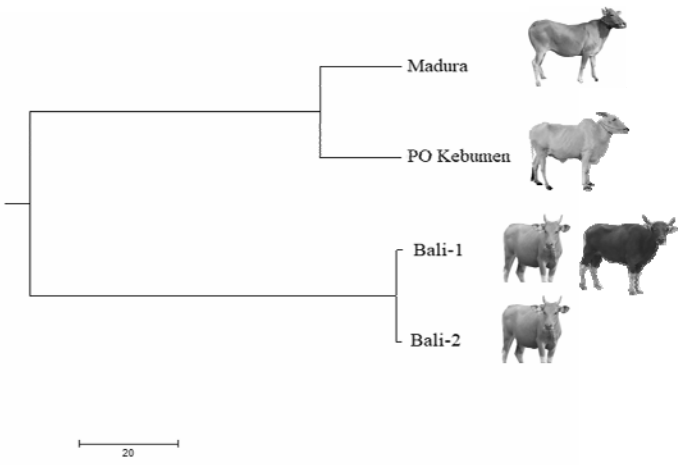

Figure 1 Dendogram of Bali cattle domesticated at Bali island (Bali-1), Bali cattle domesticated at Nusa Penida island (Bali-2), Madura cattle and PO Kebumen cattle using UPGMA method

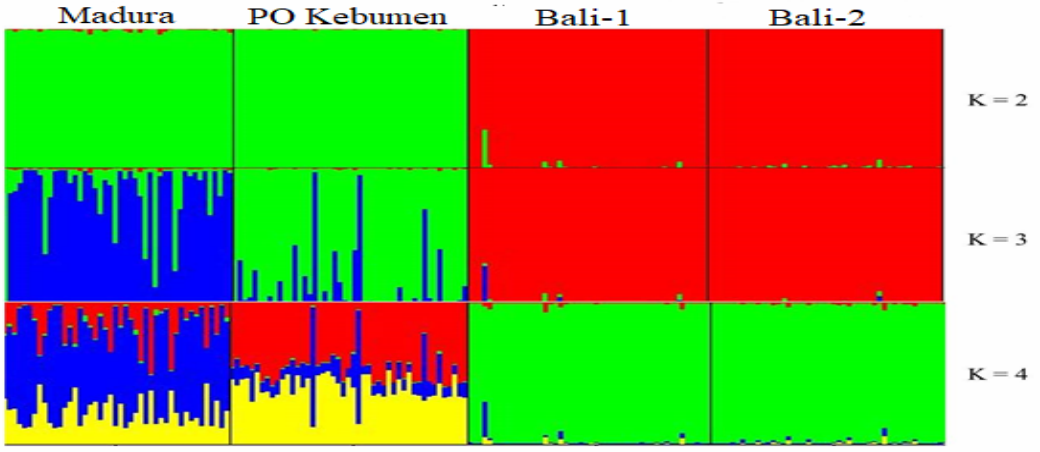

Figure 2 Genetic structures of the four population cattle breeds
K= 3 represents optimal genetic structure for Bali cattle (red), Madura cattle (blue), and PO Kebumen cattle (green)

Based on these findings, PIC obtained at loci could differ due to some factors, including the number of samples and cattle breeds. However, in this study, four microsatellite markers are considered as an ideal marker to investigate genetic diversity since they are informative and have high diversity, as represented by their high value of PIC, which is more than 0.5 (Viryanski, 2019).

Genetic diversity of Bali cattle and the hybrids

Microsatellite markers applied in Bali cattle (*Bos javanaicus*) and the hybrids demonstrated noticeable differences in genetic diversity, observed in both total populations and within the population for each microsatellite marker locus. The INRA035, ETH225, and HEL9 loci are a specific locus for Bali cattle which is not present in Madura and PO Ke-

bumen cattle, especially HEL9 allele 147 bp possessing the highest frequency. Previously, locus INRA035 and HEL9 were found as the specific allele for Bali cattle, primarily in HEL9 allele A (Handiwirawan *et al.* 2003), but the determination of allele for each locus was carried out by PAGE analysis, without using comparative cattle breeds in Indonesia.

The low rate of genetic diversity of Bali cattle (domesticated at Bali and Nusa Penida islands) compared to the hybrids may result from an artificial insemination (AI) program. This is evidenced by the results of the HW equilibrium test, showing the imbalance for each locus ($P<0.001$), high inbreeding depression (in F_{IS} and F_{IT}), as described in Table 5. Interestingly, such HW non-equilibrium and inbreeding depression were always found in commercial livestock, especially beef cattle (Grema *et al.* 2017; Gororo *et al*. 2018; Agung *et al*. 2019).

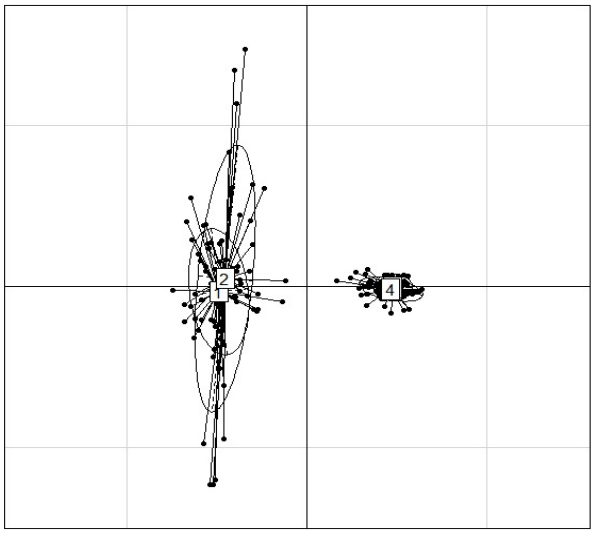

Figure 3 Principle component analysis (PCA) plot of four populations. (1) Madura cattle; (2) PO Kebumen cattle; (3) Bali cattle domesticated at Bali island and (4) Bali cattle domesticated at Nusa Penida island

Studies on genetic diversity of cattle are recommended to employ a larger quantity of microsatellite loci, as previously reported by Grema *et al*. (2017) using 27 loci, Gororo *et al.* (2018) using 16 loci, Agung *et al.* (2019) using 12 loci, Ilie *et al*. (2015) using 11 loci, and Özşensoy *et al.* (2014) using 7 loci. In this work, although the number of the locus in four microsatellite markers is relatively small the microsatellite enables to differentiation of the cluster clearly between the *Bos javanicus* cluster and the hybrids, using the genetic tree of UPGMA, genetic structure, and PCA analysis. Moreover, the result from D-loop analysis showed the same cluster for PO and Madura cattle (Abdullah *et al*. 2012). This study could produce satisfying information that can be meaningful for cattle breeding strategies in the future, mainly related to the conservation, utilization, and breeding of Bali cattle and it's hybrids.

CONCLUSION

Four microsatellite markers (INRA035, ILSTS006, ETH225, and HEL9) used in this research found 46 alleles. Furthermore, 283 bp of ILSTS006 locus, 194 bp of ETH225 locus and 147 and 151 bp of HEL9 locus were private alleles in Bali cattle that were not found in Madura and PO Kebumen cattle breeds. Microsatellite markers of INRA035, ILSTS006, ETH225, and HEL9 loci were informative and use full loci in genetic diversity study especially for Bali cattle and their hybrid.

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