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The Use of HEL9 and INRA035 Microsatellites as Specific Markers for Bali Cattle

Dedicated to Prof. Dr. sc. Günter Herrendörfer on the occasion of his 65th birthday

Abstract

Bali cattle are one of Indonesian native cattle's that famous for their ability to adapt to tropical harsh conditions. For the last 25 years, indiscriminate crossbreeding using artificial insemination (AI) organizing mainly by government might be has contaminated the purity of Bali cattle. In order to utilize as well as to conserve Bali cattle it is necessary to develop an accurate and simples method to detect the purity of Bali cattle. This experiment is the continuation of a long term experiments in developing methods to detect the purity of Bali cattle i.e. phenotypic variations, blood protein polymorphisms, hair structure, chromosome and DNA microsatellite variation analyses. The specificity of HEL9 and INRA035 microsetellites in Bali cattle as well as in Banteng (*Bos sondaicus*) as their ancestor was tested.
The results show that A and B alleles at INRA035 microsatellite locus are monomorphic and can be used for a specific markers for Bali cattle. Allele A at locus HEL9 that has high frequency (92.90%) in Bali cattle and 100% in Banteng can also be used as a supporting marker.

Key Words: Bali cattle, DNA microsatellite

Zusammenfassung

Titel der Arbeit: **Die Anwendung von Mikrosatelliten HEL9 und INRA035 als spezifische Marker für das Balirind**
Das Balirind ist eine einheimische Rasse in Indonesien, die durch seine gute Angepasstheit an tropische Bedingungen gekennzeichnet ist. In den letzten 25 Jahren wurde unter Verwendung der künstlichen Besamung die Kreuzungszucht im Rahmen staatlicher Programme durchgeführt und so die Rasse verändert. Um das Balirind als reine Rasse zu nutzen und diese in Genreserven zu erhalten, sind einfache und genaue Methoden zu entwickeln, um die Reinheit der Balirinder zu überprüfen. Als geeignete Methoden zur Einschätzung der Reinheit der Rasse wurden die phänotypischen Varianzen von Merkmalen, die Blutgruppenpolymorphien, die Haar- und Chromosomenstruktur und die Anwendung von DNA Mikrosatelliten geprüft. Die Spezifität des HEL9 und INRA035 Mikrosatelliten im Balirind ebenso wie im Bantengrind (*Bos sondaicus*), der Stammform des Balirindes, wurden analysiert.
Die Ergebnisse der Untersuchungen zeigten, dass A und B Allele des INRA035 Mikrosatelliten monomorph sind und für das Balirind verwendet werden können. Das Allel A des HEL9 Mikrosatelliten tritt mit einer Frequenz von 92,40% im Balirind und mit 100% im Bantengrind auf und kann als zusätzlicher weiterer Marker genutzt werden.

Schlüsselwörter: Balirind, DNA-Mikrosatelliten

Introduction

Bali cattle are one of several Indonesian native cattle that plays major role for providing meat. Bali cattle can be found in most parts of Indonesia and they can easily

adapt new environmental conditions (MARTOJO, 1990). Comparing with other breeds, Bali cattle have better adaptation, especially in poor environment (MASUDANA, 1990), because they can utilize low quality of feedstuffs (SASTRADIPRAJA, 1990), have high reproduction rate (80% calving percentage), have high carcass percentage (52-57.7%) (PAYNE and ROLLINGSON, 1973), have high meat quality and low fat percentage (4%) and resistance to internal and external parasites (PAYNE and HODGES, 1997; NRC, 1983).

Pure breeding of Bali cattle can be found at Bali Island, Sumbawa Island, Flores Island and Bone district of South Sulawesi province (PANE, 1991). Indonesian Government has allocated those islands as the main sources of pure Bali cattle. Crossbreeding can only be conducted outside of areas. However, due to indiscriminate crossbreeding, the pure Bali cattle at those areas have been contaminated with other breeds, i.e. Simmental, Limousine, Brangus, Charolais, indicating by high frequency of abnormal appearances, including abnormal colour patterns and horn shapes. So it is necessary to develop accurate and simple methods that can detect the pure Bali cattle.

The methods for identifying the pure of Bali cattle has been developed and pioneered by NOOR et al. (2000). They used iso-electrical focussing blood protein polymorphism analyses, DNA microsatellite analyses, chromosome structure analyses and hair structure analyses using scanning electron microscope. They suggested that the Bali cattle in Artificial Insemination Centre at Singosari, Malang, West Java and in Bali island might had been contaminated by other species of cattle. However, due to the small sample size used the conclusion has not comprehensive yet.

NOOR et al. (2000) found that out of 15 microsatellite loci tested in Bali cattle, Simmental, Brangus and Limousine, only two loci, i.e. HEL9 and INRA035 were specific for Bali cattle. Based on the result, only these two loci were used in this experiment. WINAYA (2000) using limited number of animal found that HEL9 microsatellite locus is monomorphic in Bali cattle, but not for Madura, Ongole and Brangus breeds. NOOR et al. (2000) were also found the same result using Bali cattle, Simmental, Brangus and Limousine. In addition, INRA035 locus in Bali cattle consistently has two alleles, so it is possible to use both loci as specific markers for Bali cattle. Based on this finding, it is necessary to test the specific alleles using a larger sample at the major areas of Bali cattle distribution in Bali Island. It is expected that the results can be used to differentiate between the pure and un-pure Bali cattle.

Materials and methods

The whole blood of 300 Bali cattle was randomly collected from 8 major areas of Bali cattle distribution in Bali Island. Blood sample of two Banteng were collected from Ragunan Zoo Jakarta. The locations where the blood sample where collected are shown in Table 1 and Figure 1. HEL9 and INRA035 loci were used and the primer sequences and the size of locus are shown at Table 2.

For each individual, 10 ml of blood sample was taken from *vena jugularis* using venojeck contain heparin. The sample was stored at mobile cooler contain ice cube (2^oC) and then remains in a low temperature freezer until the DNA was extracted.

Before the blood sample collection was conducted, additional phenotypic information, i.e. colour patterns were also documented. The data then compared with normal colour patterns described by NAMIKAWA et al. (1982) and abnormal colour patterns described by HARDJOSUBROTO and ASTUTI (1993) and MASUDANA (1990).

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Table 1
Locations and number of individuals of whole blood sample collection (Herkunft und Anzahl Tiere mit Blutproben)

Locations	Samples	Name of Location	Individuals (heads)
1.	Bali cattle	Siyut village, Gianyar district	67
2.	Bali cattle	Tulikup village, Gianyar district	65
3.	Bali cattle	Kesiut village, Tabanan district	32
4.	Bali cattle	Mengesta village, Tabanan district	8
5.	Bali cattle	Batumadeg village, Klungkung district	31
6.	Bali cattle	Klumpu village, Klungkung district	25
7.	Bali cattle	Denpasar slaughter house	47
8.	Bali cattle	Ngurah Rai quarantine center, Denpasar	25
9.	Banteng	Ragunan zoo Jakarta	2
	Total		302

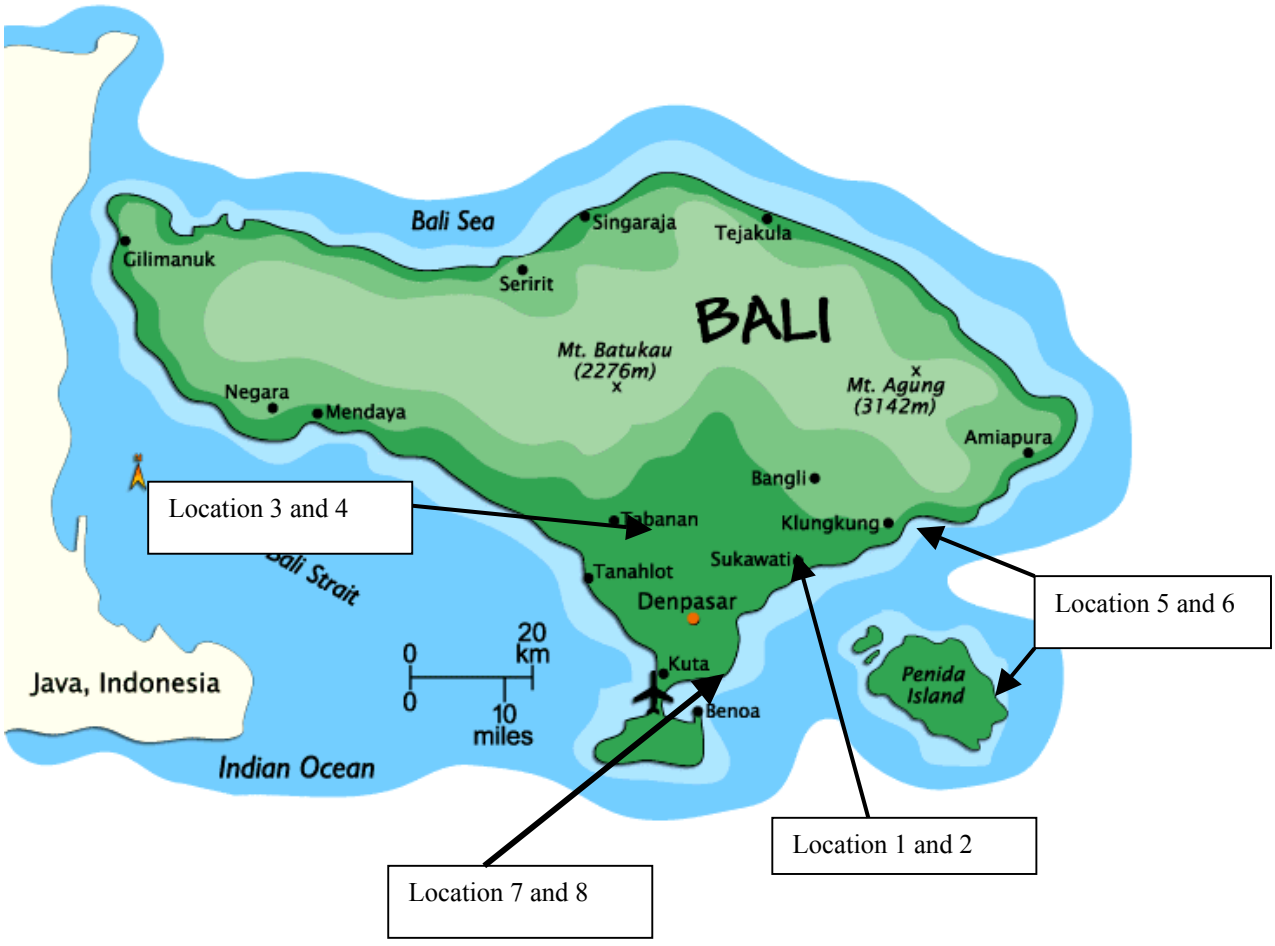

Fig. 1: Location of blood collection (Herkünfte der Blutproben auf Bali)

DNA isolation and extraction were conducted using *GenominPrep*^{TM} Blood DNA Isolation Kit procedures (product of Amersham Pharmacia Biotech). 50 ng of DNA sample was put into eppendorf PCR tube and mixed with 200 ng primer, 200 µM dNTP, 1 x buffer, 1.5 unit taq polymerase enzyme and deionised water until the volume reach 12.5 µl. One drop of mineral oil then added to the tube. The cycle, temperature and duration of thermal circler are shown in Table 3.

Table 2
Sequences and the size of HEL9 and INRA035 (Sequenzen und Größe der Mikrosatelliten HEL9 und INRA035)

Loci	Primer sequences (5' - 3')	Number of allele	T°C	Size (bp)	Sources
HEL9	F: CCCATTCAGTCTTCAGAGGT	12	55	143-	Bishop et
	R: CACATCCATGTTCTCACCAC			165	al. (1994)
INRA035	F: ATCCTTTGCAGCCTCCACATTG	7	55	120	Vaiman et
	R: TTGTGCTTTATGACACTATCCG				al. (1994)

F = *Forward*, R = *Reverse*

Table 3
Reaction conditions for amplification of microsatellite (Reaktionsbedingungen für die Amplifizierung der Mikrosatelliten)

Cycle	Steps	Temperature (°C)	Duration (Minutes)	No. of Cycle
1	1	94	05 : 00	1
2	1	94	00 : 30	35
	2	55	00 : 30	
	3	72	01 : 00	
3	1	72	05 : 00	1
4	1	4	–	–

PCR products and microsatellite allele detection were conducted by running the sample on 2% agarose gel with ethidium bromide colouration. The microsatellite polymorphism was detected by running the PCR product on 5% acrylamide gel and followed by silver staining procedure.

Allele frequency, the heterozygosity and the variance of heterozygosity for each locus were calculated by using the NEI (1987) and NEI and KUMAR (2000) formula.

Results

Only some of HEL9 and INRA035 allele were amplified. According to BISHOP et al. (1994), the sequence of HEL9 allele has 143 and 165 bp. On the other hand, INRA035 allele has 120 bp (VAIMAN et al., 1994). So based on this standard, HEL9 primer

Table 4
Number of amplified sample using HEL9 primer (Anzahl der amplifizierten Proben des HEL9 Primers)

Origin	Number of sample with positive band	Number of sample with negative band	Total
Tabanan district	19	21	40
Klungkung district	30	26	56
Gianyar district	88	36	124
Slaughter House	41	6	47
Quarantine Centre	16	9	25
Ragunan Zoo (Banteng)	2	0	2
Total	196 (66,7%)	98 (33,3%)	294

has only amplified 66.7% of the total sample, while INRA035 primer amplified 45.2% (Table 4 and 5).

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Table 5
Number of amplified sample using INRA035 primer (Anzahl der amplifizierten Proben des INRA035 Primers)

Origin	Number of sample with positive band	Number of sample with negative band	Total
Tabanan district	8	32	40
Klungkung district	26	30	56
Gianyar district	48	76	124
Slaughter House	31	16	47
Quarantine Centre	18	7	25
Ragunan Zoo (Banteng)	2	0	2
Total	133 (45,2%)	161 (54,8%)	294

At HEL9locus, there were four genotypes exist, i.e. AA, AB, AC and CC, where the AA genotype is the most common in Bali cattle. Banteng only has AA genotype in this locus (Table 6).

Table 6
Genotype frequencies at HEL9 locus (Genotypenfrequenz des HEL9 Locus)

No.	Locations	Genotypes	Number of Individual (heads)	Genotype frequencies	undetected
1.	Tabanan [1)]	AA	4	1	36
2.	Klungkung [1)]	AA	14	0,78	38
		AB	2	0,10	
		AC	1	0,06	
		CC	1	0,06	
3.	Gianyar [1)]	AA	53	0,91	66
		AB	2	0,04	
		AC	4	0,05	
4.	Slaughter house [1)]	AA	23	0,86	20
		AB	2	0,07	
		AC	2	0,07	
5.	Quarantine centre [1)]	AA	3	0,60	20
		AB	1	0,20	
		AC	1	0,20	
	Total	AA	97	0,87	180
		AB	7	0,06	
		AC	7	0,06	
		CC	1	0,01	
6.	Ragunan zoo [2)]	AA	2	1	0

[1)] Bali cattle; [2)] Banteng

There were five genotypes exist at INRA035 locus, i.e. AA, BB, AC, CC and CD. In general Bali cattle has AB genotypes (89%) (Table 7). All Banteng only has AB genotype.
There are some alleles that have high frequency at HEL9 and INRA035 loci. At HEL9 locus, the frequency of allele A was 92.9%. On the other hand, the frequency of A and B alleles were 96.8% and 52.6% respectively (Table 8). Those alleles are also exist in

Banteng. This result supports the hypothesis that Banteng is the ancestor of Bali cattle because they have common alleles.

Table 7
Genotype frequencies at INRA035 locus (Genotypenfrequenz des INRA035 Locus)

No.	Locations	Genotypes	Number of individuals (heads)	Genotype frequencies	undetected
1.	Tabanan [1)]	AA	1	0,33	37
		AB	1	0,33	
		CC	1	0,33	
2.	Klungkung [1)]	AA	4	0,17	32
		AB	20	0,83	
3.	Gianyar [1)]	AA	1	0,03	94
		AB	27	0,91	
		AC	1	0,03	
		CD	1	0,03	
4.	Slaughter house [1)]	AB	9	1	38
5.	Quarantine centre [1)]	AB	11	1	14
	Total	AA	6	0,08	215
		AB	68	0,89	
		AC	1	0,01	
		CC	1	0,01	
		CD	1	0,01	
6.	Ragunan zoo [2)]	AB	2	1	0

[1)] Bali cattle; [2)] Banteng

Table 8
The number of allele, frequency and heterozygosity of HEL9 and INRA035 loci (Anzahl der Allele, Frequenz und Heterozygotie der HEL9 und INRA035 Loci)

Parameters	HEL9 locus	INRA035 locus
Number of allele	3	4
Frequency of allele A (x_i)	0,929	0,526
Frequency of allele B (x_i)	0,031	0,442
Frequency of allele C (x_i)	0,040	0,026
Frequency of allele D (x_i)	-	0,006
Degree of monomorphic (%)	92,9	96,8
Heterozygosity ($\hat{h}$)	0,135	0,530
Heterozygosity variance ($V_{s1}(h)$)	0,00094	0,00024
Average Heterozygosity ($\hat{H}$)	0,3325	

Based on the colour pattern comparison with previous report, the percentage of abnormal colour pattern is 17% while the rest have normal colour pattern. The highest number of abnormal colour pattern is the red, brown or black colour in the lower parts of the legs. The colour of these parts for normal colour pattern should be white. The highest number of this abnormal pattern is in Klungkung district. The percentage of

spotted individuals is 0.67% and 0.33% for black coloured female. The normal and abnormal colour patterns are shown in Figure 2.

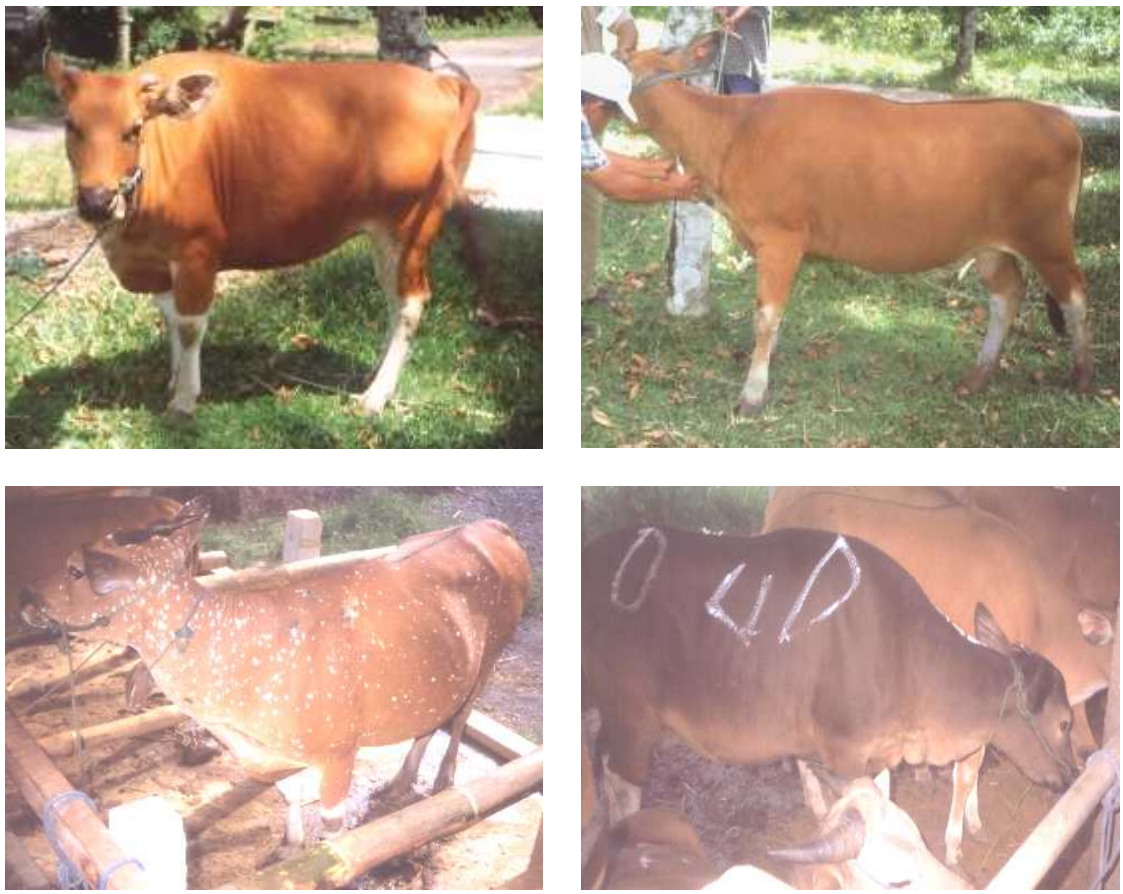

Fig 2: Female with normal colour pattern (top left); Abnormal female colour pattern with brown colour at the lower parts of the legs (top right); spotted female (left below); Black female (bellow right) (Kuh mit Standardfarbe (oben links); Abnormale Fellfarbe mit braunen Flecken an den unteren Beinen (oben rechts);Gefleckte Kuh (unten links); Schwarze Kuh (unten rechts))

Discussion

Some samples that cannot be amplified by using HEL9 and INRA035 primers could indicate that the individuals do not have HEL9 and INRA035 alleles. The other factors that could cause it, is the existence of null allele phenomena that had been reported by CIAMPOLINI et al. (1995) and LEHMANN et al. (1996). The null allele at microsatellite locus cannot be visualised on gel because of the mutation on the sequence that complement to the primer. As a result this locus cannot be amplified.

The high frequency of the null allele on microsatellite locus has been reported on cattle, human and mosquito. Previous study by using INRA25 microsatellite on four different breeds of cattle indicates the existence of point mutation in null allele with the frequency of 34% (CIAMPOLINI et al., 1995). In human the existence of null allele accident could reach 30% (7 out of 23 loci). The frequency is even higher in *A.*

gambiae (mosquito) at locus AG2H46 (LEHMAN et al., 1996). The null allele can cause false genotype because heterozygote individuals are categorized as homozygote because only one allele is amplified.

The result indicates that the alleles of Banteng and Bali cattle are not identical. In Bali cattle there are some alleles that have low frequency. At HEL9 locus, exist B allele that have the size of ± 161 bp and C allele with the size of ±169 bp. On the other hand, INRA035 has C and D alleles that have the size of ±121 bp and ±138 bp, respectively. It is suggested that these alleles could be the product of mutation due to replication slippage that result a longer sequence (LEVINTON and GUTMAN, 1987; LI and GRAUR, 1991). The other possibility is the existence of gene flow from outside of the population or from other breeds into the population in Bali Island. Other breeds of cattle that commonly present in Indonesia that also has B allele in HEL9 locus are Madura, Ongole, Brangus, Simmental and Limousine. On the other hand, C allele can also be found in Madura and Simmental (WINAYA, 2000 and NOOR et al., 2000). The breed of cattle that also has C allele at INRA035 locus is Madura and Ongole. However, in order to determine whether those alleles are identical with those in Bali cattle, the size of allele should be compared using the same electrophoresis medium i.e. polyacrylamide gel.

The previous experiment result indicated that the A allele at HEL9 locus is monomorphic (all Bali cattle has AA genotype) (WINAYA, 2000; NOOR et al., 2000). The same result is also found for INRA035 locus, because all Bali cattle have AB genotype (WINAYA, 2000). These two loci are excellence candidate to be re-tested in this experiment to determine whether they are specific allele in Bali cattle. According to HARTL and CLARK (1989), if the number of allele is ≥0.95, then this allele can be categorize as monomorphic and specific. In this experiment, the A and B alleles at INRA035 locus are monomorphic because they have high frequency (96.8%). On the other hand, the A allele at HEL9 locus cannot be categorized as monomorphic, because the frequency is only 92.9%.

The abnormal colour patterns were found in all locations, except for Tabanan district. The highest number of abnormal colour patterns was found at Klungkung district, especially at Nusa Penida Island. In Klungkung district, the percentage of abnormal colour patterns was 53.6%, while in Quarantine Centre the percentage was 56%. The most common abnormal colour pattern is the red or brown colour of the lower parts of the legs. It is speculated that these abnormal cattle was originated from Klungkung district, because it was quite often that Klungkung local government distributed the cattle to other districts in Bali island.

The abnormal colour patterns could be as a result of homozygous recessive genotype due to inbreeding. It is suggested that the inbreeding level at Nusa Penida Island (Klungkung district) is quite high because this island is closed for Bali cattle from outside of the island. PAYNE and ROLLINGSON (1973) mentioned that the number of black female cattle in 1939 was 8% of the total population. On the other hand, the percentage in the Nusa Penida Island was higher (24%). In general, the farmers do not like the cattle with abnormal colour patterns. As a result the frequency will decrease.

We found no relation between the abnormal colour patterns and the polymorphism of two loci studied. This result indicates that HEL9 and INRA035 loci do not close to the marker genes for colour patterns.

In order to obtain accurate identification, the use of allele A and B at INRA035 locus as specific marker for Bali cattle should be supported by HEL9 locus and combined by other methods that had been mentioned by NOOR et al. (2000).

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