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Genetic Profile of Local Buffalo (*Bubalus bubalis*) Populations in Pacitan and Tuban, East Java, Indonesia Measured by the Molecular Marker of INRA032 Locus

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ABSTRACT

The genetic profile of buffaloes is important information to support their breeding efforts, therefore the assessment of genetic profile needs to be carried out continuously. This study aimed to analyze the application of microsatellite marker at the INRA032 locus for genetic profile assessment in buffalo (*Bubalus bubalis*) populations in Pacitan and Tuban Regencies, East Java, Indonesia. The total number of samples used was 16, with each population represented by 8 samples. Genetic profile assessment parameters include allele frequency, the Polymorphism Information Content (PIC), and heterozygosity. The results showed that based on the INRA032 locus, the Tuban buffalo population had a higher allele frequency range (0.08 to 0.33) than the Pacitan population (0.18 to 0.31). The average PIC value in both populations was 0.39, so it can be concluded that the INRA032 locus is informative enough to detect polymorphisms in both populations. The percentage heterozygosity of the Pacitan buffalo population is 88%, which is higher than the Tuban population at 50%, suggesting that the genetic diversity of the two populations is still quite high despite the decreasing trend in population numbers. The INRA032 locus was shown to be moderately effective for assessing genetic profile in local buffaloes, but its application in future studies will require an increase in the number of samples representing the population and the addition of other microsatellite markers to obtain a more accurate conclusion.

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INTRODUCTION

The need for animal protein consumption is increasing along with the human population size of Indonesia, and one of the sources of animal protein is a large ruminant. One of the large ruminants that are the main source of animal protein in Indonesia is buffalo (*Bubalus bubalis*) (Gunawan & Romjali, 2009; Komariah et al., 2018; Nuraini et al., 2018). The number of buffaloes in East Java Province in 2017-2021 has gradually decreased, to 26.622, 24.364, 23994, 22.975, and 22.970, respectively. The number buffaloes in East Java are very small compared to the number of buffaloes in West Java and Central Java Provinces (Director General of Livestock and Animal Health, 2021). In more detail, from 2014 to 2017, the average number of buffaloes in Pacitan was only 117, while the average number of buffaloes in Tuban was 1.695 (BPS-statistics of East Java, 2018).

In general, buffaloes are kept by farmers in rural areas, with an average of 1 to 2 buffalo per farmer. The decline in buffalo population is due to various factors, such as limited stock of high-quality brooders, low quality feed, inbreeding, and despite

farmers have a lot of local wisdom on production and reproduction, they seem unable to handle large herds of buffalo. The relatively low reproduction rate may be due to more difficult detection of the estrous phase in buffaloes with a longer gestation period compared to cattle (Gunawan & Romjali, 2009). The decline in buffalo population is associated with an increased in the probability of negative impacts due to inbreeding. According to data from BPS-statistics of East Java (2018), the buffalo population in Pacitan has a higher potential for inbreeding compared to the Tuban population because of its smaller population. Based on these problems, assessments of the buffalo genetic profile should be conducted regularly, for example by using the molecular markers of microsatellite.

Microsatellites or short tandem repeats (STRs) are the most polymorphic DNA molecular markers and have a high abundance in eukaryotic genomes. Microsatellites are di-, tri-, or tetra nucleotide of DNA tandem repeats presented in variable copy numbers at each locus and throughout the genome. Microsatellites are one of the popular molecular markers used to estimate genetic diversity in livestock. The development of microsatellite markers in genetics and biotechnology increases opportunities for livestock selection, health improvement, and production (Brenig & Schütz, 2016; Viryanski, 2019).

One of the microsatellite markers that has been widely used in the assessment of genetic diversity in buffalo populations is the INRA032 locus which is located on chromosome 11 (Vaiman et al., 1994). Not only in buffalo, the use of the INRA032 locus in microsatellite-based genetic diversity assessments has also been applied to dairy cattle and horses (Vanessa et al., 2018; Sukri et al., 2022). Several previous researches have used the INRA032 locus in microsatellite-based genetic diversity assessment of buffaloes in Indonesia, specifically in Kudus (Central Java), East Java, and West Nusa Tenggara populations (Sukri, 2014; Amin & Lestari, 2014; Lathifah, 2016). Complementing previous studies, the investigation of the effectiveness of the INRA032 locus for genetic profile assessment in this study focused on local buffalo populations in Pacitan and Tuban Regencies, East Java.

MATERIALS AND METHODS

The total number of buffalo samples in this study was 16, obtained through purposive random sampling, with 8 representing the Pacitan population and 8 representing the Tuban population. Samples were obtained from local farmers in Pacitan and Tuban Regencies. Blood samples were collected through the jugular vein on the neck of the buffalo using a venojack and preserved in EDTA solution and then stored in an ice box until DNA isolation. Furthermore, DNA was isolated from blood using the standard protocols of SDS/proteinase K and phenol/chloroform method (Sambrook et al. 1989). The sequence of microsatellite primer pairs of INRA032 locus, referring to Vaiman et al. (1994) is presented in Table 1.

Table 1. Primer sequences of INRA032 locus

Primer position	Primer sequences (5'-3')	Annealing temperature
Forward	AAACTGTATTCTCTAATAGCAC	57 °C
Reverse	GCAAGACATATCTCCATTCCTTT	

The DNA amplification process through Polymerase Chain Reaction (PCR) was composed of a reaction mixture of 2.5 µl of DNA template (10mg/µl), 12.5 µl of PCR mix (dNTP, buffer, Taq-polymerase, H2O), 2.5 µl of dH2O, 2.5 µl of forward primer, and 2.5 µl of reverse primer. The PCR process was done for 30 cycles with the following steps: predenaturation at 95 °C for 2 minutes, denaturation at 92 °C for 1 minute, annealing at 57°C for 1 minute, first elongation at 72 °C for 1-minute, final elongation at 72 °C for 10 minutes, and the last step at a temperature ranging from 4°C.

After PCR, DNA separation was carried out using 10% of Polyacrylamide Gel Electrophoresis (PAGE), followed by silver staining and electrophoresis at 135 volts with duration of 60 minutes. Electrophoresis results were documented in the form of images and analyzed manually with the help of Gel Analyzer software version 19.1 (www.gelanalyzer.com) by Istvan Lazar Jr., PhD, and Istvan Lazar Sr., PhD, CSc, to increase the accuracy of allele observations. All alleles that appeared were recorded according to size, and then based on the allele size, allele frequency, heterozygosity, and polymorphic information content (PIC), they were calculated using GENPOP software version 3.1 (Liu et al., 2000).

RESULTS AND DISCUSSION

The maintenance of genetic diversity is a vital and critical goal in livestock conservation programs so that livestock populations can adapt to future environmental challenges and respond to long-term selection, both natural and engineered, for certain

traits of interest that are important to the economy and culture. According to Putman & Carbone (2014) the use of highly variable molecular genetic markers, such as microsatellite, is one of the most potential ways to assess the genetic diversity due to their high level of polymorphism, abundance, random distribution across the genome, co-dominant inheritance, and neutrality with respect to selection.

The possibility of detecting polymorphisms is very important when choosing a molecular marker. If a molecular marker is unable to detect genetic differences that exist within a group of individuals, it is considered ineffective. A molecular marker is considered polymorphic if it has at least two alleles and the most frequent allele has a frequency of up to 99% (Shete et al., 2000; Serrote et al., 2020). The result of this study shows that the INRA032 locus is moderately effective in detecting polymorphism between individuals in Pacitan and Tuban buffalo populations.

Allele frequency is the proportion or ratio of all gene copies that comprise a particular gene variant. In other words, allele frequency is the number of copies of a particular allele divided by the total number of copies of alleles at a locus in a population. Allele frequency can be presented as a percentage (Gillespie, 2004). According to the number of allele sizes at the INRA032 locus in the two buffalo populations, the frequency of alleles at the INRA032 locus in each population was calculated. The allele frequency of the INRA032 locus in the Pacitan buffalo population was between 0.18 and 0.31, while in the Tuban buffalo population the allele frequency was between 0.08 and 0.33. Thus, it was known that the allele frequency range of the INRA032 locus in the Tuban buffalo population is higher than that in the Pacitan population. The results of the allele frequency analysis of the Pacitan and Tuban buffalo populations are presented in Table 2. While allele frequencies at the INRA032 locus in buffalo populations in Kudus Regency (Central Java) ranged from 0.08 to 0.591, South Sumatra populations ranged from 0.06 to 0.75, Tana Toraja Regency (South Sulawesi) populations range from 0.36 to 0.64, and Lombok (West Nusa Tenggara) populations range from 0.38 to 0.63 (Lestari, 2013; Afrida et al., 2014; Lathifah, 2016). This indicates that the INRA032 locus has variable allele frequencies, which will be important preliminary information for the detection of intrapopulation genetic diversity in buffalo.

Table 2. Allele frequencies of INRA032 locus in Pacitan and Tuban buffalo populations

Allele size (base pair)	Populations	
	Pacitan (%)	Tuban (%)
100	0.31	0.16
110	0.00	0.16
180	0.25	0.33
220	0.18	0.25
230	0.25	0.00
300	0.00	0.08

The PIC value indicated that the molecular marker used was capable of detecting polymorphisms among individuals in a population, and the higher that capacity, the greater the value. PIC values for co-dominant molecular markers ranged from 0 (monomorphic) to 1 (highly informative, with multiple alleles of equal frequency) (Serrote et al., 2020). The PIC values of the Pacitan and Tuban buffalo populations in this research are presented in Table 3. Following the criteria of Botstein et al. (1980), the microsatellite marker INRA032 locus observed in the study was highly informative in the Tuban buffalo population (PIC > 0.5) and less informative (PIC < 0.25) in the Pacitan population. The mean value of 0.39 for PIC indicated that the INRA032 locus was moderately informative (0.25 < PIC < 0.5) for both populations. Furthermore, higher PIC values indicated the usefulness of this molecular marker in the assessment of genetic diversity in a population (MacHugh et al., 1997) as well as genome mapping studies (Kayang et al., 2002). For comparison, the average PIC value of the INRA 032 locus in the buffalo population in Kudus Regency (Central Java) is 0.42, the South Sumatra population is 0.41, in the Tana Toraja Regency population (South Sulawesi) is 0.35, in the Lombok population (West Nusa Tenggara) is 0.34, and in the Yogyakarta, population is 0.29 (Afrida et al. 2014; Limiansi, 2015; Lathifah, 2016). This indicates that the INRA032 locus is moderately informative for the detection of genetic diversity in buffalo populations in different regions of Indonesia. Nevertheless, based on the analysis, it is recommended to increase the number of samples and add other microsatellite markers to ensure the accuracy of the PIC values.

Table 3. Polymorphism information content (PIC) values in Pacitan and Tuban buffalo populations

Populations		Average
Pacitan	Tuban	
0.12	0.66	0.39

Heterozygosity is the probability of an individual being heterozygous at the location where the molecular marker is used and depends on the number of alleles and their frequency in the population. Heterozygosity values range from 0 (no heterozygosity) to 1 (high number of alleles with equal frequency) (Serrote et al., 2020). The calculated allele frequency values of the INRA032 locus were used to calculate the expected heterozygosity values, while the observed heterozygosity values were also obtained from the observation of heterozygous individuals. The results of the calculation of heterozygosity values (observed heterozygosity and expected heterozygosity) at the INRA032 locus for both populations are presented in Table 4.

Table 4. Heterozygosity of INRA032 locus in Pacitan and Tuban buffalo populations

Pacitan (%)		Tuban (%)	
Ho	**He**	**Ho**	**He**
88	15.7	50	23.4

Notes: Ho = observed heterozygosity, He = expected heterozygosity

If observed heterozygosity (Ho) is higher than expected heterozygosity (He), it indicates that there is no deviation from Hardy-Weinberg equilibrium and low probability of inbreeding (Sharma et al. 2016). In both buffalo populations in this research, Ho value was higher than He value, indicating no deviation from Hardy-Weinberg equilibrium and low probability of inbreeding. Interestingly, the Ho to He value for the Pacitan buffalo population was higher than the Tuban population, even though the Pacitan population is much lower than Tuban population. According to BPS-statistics of East Java (2018), from 2014 to 2017, the average number of buffaloes in Pacitan was only 117, while the average number of buffaloes in Tuban was 1695. Based on this population size, Pacitan buffalo population has higher potential for inbreeding compared to Tuban population, and therefore the heterozygosity of Pacitan population should be lower than Tuban. In fact, Tuban is geographically more accessible than Pacitan, which should increase the potential for outbreeding in the Tuban buffalo population, resulting in higher heterozygosity than Pacitan. The results of this study are in line with the results of Amin (2012), Afrida et al. (2014), and Lathifah (2016), which showed that the application of the INRA032 locus in buffalo populations of Kudus Regency (Central Java), Tana Toraja Regency (South Sulawesi), and Lombok (West Nusa Tenggara) also had higher Ho values than He.

Heterozygosity results in population genetics research are an important and essential part. However, homozygosity is not expected. According to Sharma et al. (2016) if homozygotes are present in a population, various factors can contribute to the occurrence of excess homozygotes. First, the observed locus is under selection. Second, there may be "null alleles" that lead to incorrect observations of excess homozygotes. Third, inbreeding may be common in the observed population. The likelihood of each of these explanations should be assessed from extra data, such as demographic information and population distribution. "Null alleles" are unlikely to completely segregate the locus. Another possibility of the high heterozygosity in the Pacitan and Tuban buffalo populations is the considerable knowledge of local farmers in the mating management of their buffalo herds. Hale et al. (2012) suggest that to improve the accuracy of microsatellite-based genetic studies, a sample size of 25 to 30 samples per population is required. In line with this suggestion, we also recommend this sample size to improve the quality of our future studies.

Information on allelic variation in microsatellite DNA indicating genetic diversity in buffalo is expected to be used as a reference for determining the genetic quality of buffalo through the provision of breeding stock for outbreeding programs or to reduce the effects of inbreeding, so that genetic quality can be maintained or improved (Amin & Lestari, 2014). The combination of the INRA032 locus with other microsatellite loci will improve the quality of information on the genetic diversity of local buffalo, thereby increasing the accuracy of decision-making regarding conservation measures. In addition, based on previous studies, it was also explained that the INRA032 locus is associated with reproductive characteristics (calving traits) and body conformation (hocks, rear leg set, rear view) in dairy cattle (Harder et al. 2006; Buitenhuis et al. 2007; Vanessa et al. 2018). Therefore, future research needs to investigate the relationship between the INRA032 locus and other microsatellite markers to morphological characters associated with superior traits in local buffaloes in Indonesia.

CONCLUSION

In general, the INRA032 locus is suitable for assessing the genetic profile of Pacitan and Tuban

buffalo populations in East Java. The number of alleles at the INRA032 locus in the Pacitan buffalo population is less than that in the Tuban population. This was also the same result for the Polymorphism Information Content (PIC) value of the Pacitan buffalo population, which was lower than that of the Tuban population. However, the average PIC value (0.39) indicated that the INRA032 locus was moderately informative for the assessment of genetic diversity in both populations. The percentage heterozygosity of the Pacitan population (88%) was higher than that of the Tuban population (50%), even though the Pacitan population is smaller than Tuban population. Various factors may influence the difference in heterozygosity between the two populations including artificial insemination program. Future studies need to increase the number of samples and other microsatellite markers to improve the accuracy of local buffalo genetic profile information.

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