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Genetic Diversity of the Endangered Endemic Anoa (*Bubalus* spp): Implication for Conservation

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ABSTRACT

Anoa is an endemic ungulate in Sulawesi and its status now is endangered because the population continues to decline. Conservation genetics is one of the crucial issues in the anoa conservation strategy and action plan 2013-2022 document, but this genetic data is not yet available. To investigate and provide valuable information for conservation genetics measures, thirteen polymorphic microsatellites were used to analyze 20 adult anoa. Anoa has relatively low genetic diversity within populations (H_O = 0.58), and high genetic differentiation among populations (F_{ST} = 0157). Although the anoa population has a bottleneck signal (T.P.M: 0.019; P0.05), the bottleneck simulation results show that the loss of genetic diversity is being slow over the next 100 years (9.5%). We provide some recommendations for conservation genetics based on the findings in this paper, including monitoring and genetically mapping for other anoa populations due to bottleneck signals, establishing the founder of the *ex-situ* population by examining their genetic diversity status, maintaining and increasing the number of individuals in the *ex-situ* population to genetically safe population size, and managing anoa populations by avoiding inbreeding. *In-situ* and *ex-situ* conservation programs should be combined to maintain the genetic diversity of anoa.

1. Introduction

Anoa is the largest endemic mammal from Sulawesi. Anoa has a morphology that is similar to that of buffalo, but it is smaller, hence it is commonly referred to as miniature buffalo. Anoa is a mammal belonging to Bovidae which is distributed almost throughout Sulawesi (Burton *et al.* 2005). Anoa is divided into two species: lowland anoa (*Bubalus depressicornis*) and mountain anoa (*Bubalus quarlesi*) (Burton *et al.* 2005; Priyono *et al.* 2018, 2020). However, dramatic population declines in these species continue to occur in the wild. Anoa population size has declined dramatically to 90% over the last 16 years, and in some habitats, anoa populations have been assessed to be locally extinct (Burton *et al.* 2005). The anoa population size estimate in Sulawesi was 2,500 individuals in 2005 (Burton *et al.* 2005). However, a recent study found that the illegal hunting of anoa in Sulawesi was extremely high, reaching 283 individuals per year (Rejeki 2018). If this hunting trend continues since 2005, the anoa population was already extinct in their habitat in Sulawesi. The International Union for Conservation of Nature (IUCN) Redlist has categorized anoa as an endangered (Burton *et al.* 2016). Several genetic techniques have succeeded in delivering key insights that critically influence management decisions and provide benefits for the species under research, referred to as the conservation genetics (Frankham *et al.* 2002).

Genetic diversity is a major issue of conservation biology recognized by the IUCN (Frankham *et al.* 2010). Decreases in genetic diversity can reduce evolutionary fitness and trigger a negative feedback loop that leads to decreased population sizes, increasing genetic drift, and inbreeding. Some studies have found that reduced genetic diversity is related to decreased fitness or viability. For example, low heterozygosity has been associated with low juvenile survival, reduced population growth, low body size, reproductive rates, and reduced adult life span (Bradshaw *et al.* 2007; Frankham *et al.* 2002,

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2014; Reed and Frankham 2003). On the other hand, many captive breeding programs have been carried out in order to enhance population size and also save threatened or endangered species from extinction.

Some studies of anoa genetic variation have been published, but tends to focus on taxonomic issues in anoa. These taxonomy debate arose as a result of the diverse variation in the number of chromosomes found in anoa (38, 42, 44, 45, 46, 47, and 48) (Pranadewi 1998; Scheurmann *et al.* 1977; Schreiber *et al.* 1993, 1999). MHC class II RFLPs were polymorphic as well, but there was also variation in anoa populations between two import lines. (Schreiber *et al.* 1993; Schreiber 1992). The use of mitochondrial DNA to investigate genetic diversity in these two anoa morphotypes, lowland anoa and mountain anoa, has been widely reported. The genetic diversity of anoa was studied using mitochondrial DNA cytochrome b, which showed 3.6% sequence divergence between the two types (Kikkawa *et al.* 1997). Recent genetic investigations using DNA barcodes have also confirmed that there are at least two species of anoa in Sulawesi, lowland anoa (*B. depressicornis*) and mountain anoa (*B. quarlesi*) which have genetic distance of 3.4% based on COI (Priyono *et al.* 2018). A recent study that used the complete mitochondrial genome to compare two forms of anoa showed that there was 2.2% genetic variation (Priyono *et al.* 2020). However, because mitochondrial sequences are inherited maternally, species delimitation based only on mitochondrial sequences has been shown to be potentially misleading. Recently, few genetic analyses on anoa species using nuclear microsatellite markers have been conducted (e.g., (Frantz *et al.* 2018), but there is no research that focuses on genetic diversity for conservation genetic purposes, such as determining the level of inbreeding and population structure. The lack of genetic structure and the level of inbreeding information of two of these anoa species also did not allow the complete understanding of their genetic status, as a barometer for evaluating anoa population health and extinction risk.

In this study, we chose 13 pairs of microsatellite markers, then assessed molecular variance among and within populations to determine genetic diversity and population structure, providing critical information for developing an appropriate conservation and management strategy for genetic resources, as well as the deployment of these resources with plans for a future captive breeding strategy.

2. Materials and Methods

2.1. Samples Collection and DNA Extraction

A total of 20 individual anoa samples were obtained from the Anoa Breeding Center, Manado (8 lowland anoa, 1 mountain anoa), Citra Satwa Celebes, South Sulawesi (9 mountain anoa), and two lowland anoa from skull specimens were obtained from residents around the mt Tangkeleboke, Southeast Sulawesi. These blood samples were obtained from previous study (Priyono 2020). Blood was collected from the jugular vein and stored in a 3-ml vacutainer tube containing EDTA. This study has obtained a recommendation and a sampling permit from LIPI with the recommendation letter no. B-242/SKiKH/KS.02.04/XI/2017. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) and preserved at -25°C until further analysis.

2.2. Characterization of Microsatellite Markers

The cross-species microsatellite markers were used from 10 autosomal loci from Bovine, namely CSSM019, CSSM029, BRN, CSSM041, CSSM038, CSSM036, CSSM033, CSRM060, CSSM057, and CSSM032 (Barker *et al.* 1997) and 3 Y-chromosome loci, namely INRA189, BM861, and UM108 (Liu *et al.* 2003) (Table 1). The total volume for PCR amplification mixture was 25 µl consisting of 1x PCR buffer (Promega), 1x GC enhancer, 0.2 mM dNTP (Qiagen), 10pg DNA template, double distilled water (ddH_2O), 0.02 U/µl Taq polymerase (BioLabs, England), 0.03 µM each primer. For all loci, the dye labeling method follows the procedure from Schuelke (2000): forward primer 5 ′ added 18bp oligo of M13 (TGT AAA ACG ACG GCC AGT), and the reverse primer and forward "M13" primer labeled FAM or HEX. The PCR conditions for the microsatellite locus consisted of 94°C (5 minutes), then 30 cycles at 94°C (30s), annealing temperature of each primer (Table 1) (45s), 72°C (45s), followed by 8 cycles of 94 °C (30s), 53° C (45s), 72°C (45s), and final extension at 72°C for 10 minutes. The PCR product was visualized in 1.2% agarose gel. The length of the PCR product is determined based on GS400 (ABI) standard size.

2.3. Data Analysis

The fragment length size that has been obtained from fragment analysis was arranged into sheets according to GenAlex format (Peakall and Smouse

Table 1. Details of Bovine microsatellite locus used in this anoa genetic diversity study

Locus	Repeat unit	Ta (°C)	No. Accesion		Oligonucleotide (5'-3')
CSSM019	$(TG)_{18}$	55	U03794	F	TTGTCAGCAACTTCTTGTATCTTT
				R	TGTTTTAAGCCACCCAATTATTTG
CSSM029	$(AC)_{18}$	55	U03807	F	GCTCCATTATGCACATGCCATGCT
				R	CGTGAGAACCGAAAGCACACATTC
CSSM033	$(GT)_{15}$	65	U03805	F	CACTGTGAATGCATGTGTGTGAGC
				R	CCCATGATAAGAGTGCAGATGACT
CSSM036	$(TG)_{18}$	55	U03827	F	GGATAACTCAACCACACGTCTCTG
				R	AAGAAGTACTGGTTGCCAATCGTG
CSSM038	$(CA)_{24}$	55	U03817	F	TTCATATAAGCAGTTTATAAACGC
				R	ATAGGATCTGGTAACTTACAGATG
CSSM041	$(GT)_{20}$	55	U03816	F	AATTTCAAAGAACCGTTACACAGC
				R	AAGGGACTTGCAGGGACTAAAACA
CSRM060	$(CA)_{17}$	60	AF232758	F	AAGATGTGATCCAAGAGAGAGGCA
				R	AGGACCAGATCGTGAAAGGCATAG
BRN	$(AC)_{23}$	60	X59767	F	CCTCCACACAGGCTTCTCTGACTT
				R	CCTAACTTGCTTGAGTTATTGCCC
CSSM057	$(TG)_{16}$	60	U03840	F	TGTGGTGTTTAACCCTTGTAATCT
				R	GTCGCTGGATAAACAATTTAAAGT
CSSM032	$(CA)_{19}$	55	U03811	F	TTATTTTCAGTGTTTCTAGAAAAC
				R	TATAATATTGCTATCTGGAAATCC
INRA189	$(TG)_{22}$	54	X73941	F	TACACGCATGTCCTTGTTTCGG
				R	CTCTGCATCTGTCCTGGACTGG
BM861	$GT)_6$ (C) $(TG)_9$	56	AF4837	F	TTGAGCCACCTGGAAAGC
				R	CAAGCGGTTGGTTCAGATG
UMN0108	$(TG)_{18}$	60	AF483744	F	GATCCATCCACATTGCTCCA
				R	CCAAGCGTCCATCAATTTAC

2006). Allelic variations of 13 microsatellite loci in anoa were determined by analysis of alleles number per locus (A) and heterozygosity (H). Heterozygosity and allelic frequencies for each population at each locus were calculated from microsatellite data using GENEPOP version 3.1 (Raymond 1995). To evaluate Hardy-Weinberg equilibrium (HWE) and genotyping error, a comparison between heterozygosity observed (Ho) and heterozygosity expected (He) is performed using the test as implemented in the Microchecker program (Van Oosterhout *et al.* 2004). GenePop (Rousset 2008) program uses the Markov Chain method to estimate the probability of significant deviations from the HWE using the following parameters: dememorization = 10,000, batch = 500, and iteration = 1,000. The level of significance is adjusted by the number of simultaneous tests using the sequential Bonferroni procedure (Rice 1989). The genetic structure was analyzed using Principal Coordinate Analysis (PCoA) which was implemented in GenAlex 6.41 program (Peakall and Smouse 2006). STRUCTURE 2,347 program (Pritchard *et al.* 2003) was used to calculate the probability of each individual's genetic cluster membership. Setting in this software uses Markov Chain Monte Carlo simulation (MCMC) to estimate these parameters, with the number of clusters to be tested (K) with provisions 1-7. MCMC simulations were run for 300,000 reps, with burning 100,000. For each K value (number of cluster population). The optimal cluster model was investigated by analyzing L (K) and ΔK probability log plots from Structure analysis data, and implemented in STRUCTURE HARVESTER (Earl 2012).

Anoa populations continue to decline in their natural habitat, we try to detect whether there is a signal that a bottleneck has occurred genetically. Detection of populations experiencing bottlenecks was performed using the test proposed by Cornuet and Luikart (1996) and Piry *et al.* (1998) based on microsatellite allele frequencies. This test is based on the fact that the population has experienced a significant reduction in effective population size, with allelic diversity (e.g., genetic diversity, HE) reducing faster than heterozygosity (e.g., genetic diversity, HE) at the polymorphic locus. Therefore, in populations that have recently experienced bottlenecks, genetic diversity is higher than the heterozygosity equilibrium (Heq) estimated from allele numbers, which is observed on mutations-drift equilibrium assumption (Cornuet and Luikart 1996; Piry *et al.* 1998). We used the program

BOTTLENECK (Cristescu *et al.* 2010) to test for changes in microsatellite allelic frequency distributions in anoa. For each polymorphic locus, 1,000 simulations were undertaken for three microsatellite mutation models (SMM-Stepwise Mutation Model; IAM-Infinite Allele Model; TPM-Two-Phase mutation Model) assuming mutation-drift equilibrium. Genetic diversity is an important indicator of long-term population viability (Soulé and Frankel 1981), this is required in order to develop a biologically "health" conservation strategy. The population bottleneck reduces the population's gene pool because many alleles, or gene variants, that were present in the original population are lost. As a result of the event, the remnant population has relatively low genetic diversity. BottleSIM is a program specifically designed to simulate genetic consequences of bottlenecks and the post-bottleneck population (Kuo and Janzen 2003). The simulation is based on the assumption that anoa lives for 30 years, has sexual maturity at 3 years, and has completely overlapping generations (Jahja 1987).

3. Results

3.1. Genetic Diversity

The thirteen microsatellite loci used in this study were successful in amplifying the locus target, and yielding polymorphic locus. Microchecker program analysis indicated no allelic dropouts or genotyping errors. The number of alleles in the total sample ranges from two (CSSM036) to four (BRN, CSSM036, BM861) (Table 2). The mean of Polymorphic Information Content (PIC) value was 0.625, with values ranging from 0.412 (CSSM060) to 0.737 (CSSM038). All samples have HE and HO values of 0.528 and 0.583, respectively. Interestingly, we found differences in genetic diversity between lowland anoa and mountain anoa. The genetic diversity of mountain anoa (H_o = 0.552) is higher than that of lowland anoa (H_o = 0.503). Based on the F_{ST} (F_{ST}>0.15), we found that anoa populations have a high population genetic differentiation. In this study, the fixation index (F_{IS}) was found positive in the anoa population (lowland anoa, F_{IS}: 0.188; mountain anoa, F_{IS}: 0.115; total population: 0.099).

3.2. Genetics Admixture

Principal coordinate analysis (PCoA) based on Nei's unbiased genetic distance (Figure 1A) generally confirms that lowland and mountain anoa formed two clusters. Mountain anoa composed the first cluster (blue circle), while lowland anoa formed up the second. The highest peak of Delta K (20.5) was found at K = 2 (Figure 1B), indicating that the genetic structure of the anoa population likewise supports the occurrence of two genetic clusters. Bayesian clustering analysis also shows a barplot pattern for two clusters (Figure 1C). Lowland anoa, Cluster I (genetic proportion, q>0.9), and Cluster II formed by mountain anoa (q>0.9). We found that one individual (Isd) have a mixed proportion from both clusters (cluster 1 = 0.45 and cluster II = 0.55).

3.3. Bottleneck Analysis

Bottleneck analysis revealed that two species of anoa departed from neutrality, showing an excess of heterozygosity. The results of the bottleneck test

Table 2. Genetic diversity in anoa based on 13 microsatellite loci. A is the number of alleles, H_O is heterozygosity observed, H_E is heterozygosity expected, and PIC is Polymorphic Information Content

Loci	Lowland (n = 10)			Mountain anoa (n = 10)			Total (n = 10)			PIC
	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	
CSSM019	3	0.600	0.540	4	0.667	0.512	4	0.633	0.526	0.503
CSSM029	4	0.600	0.745	4	0.556	0.599	4	0.578	0.672	0.678
BRN	5	0.300	0.480	4	0.889	0.636	5	0.594	0.558	0.668
CSSM041	4	0.571	0.653	3	0.625	0.461	4	0.598	0.557	0.657
CSSM038	5	0.700	0.695	5	0.556	0.667	5	0.628	0.681	0.737
CSSM036	2	0.111	0.401	3	0.333	0.586	3	0.222	0.494	0.706
CSSM033	4	0.600	0.675	4	0.667	0.667	4	0.633	0.671	0.691
CSRM060	3	0.333	0.549	2	0.600	0.500	3	0.467	0.525	0.412
CSSM057	3	0.556	0.537	3	0.500	0.635	3	0.528	0.586	0.521
CSSM032	3	0.444	0.568	3	0.333	0.549	3	0.389	0.559	0.562
INRA189	5	0.444	0.519	4	0.500	0.615	5	0.472	0.567	0.642
BM861	4	0.778	0.660	3	0.556	0.586	4	0.667	0.623	0.719
UM0108	3	0.500	0.505	4	0.400	0.615	4	0.450	0.560	0.633
Mean	4	0.503	0.579	4	0.552	0.587	3	0.528	0.583	0.625
FIS		0.188			0.115			0.099		
F_{ST} (Total)								0.157		

revealed a strong signal that a bottleneck event had occurred recently in the anoa population. This result was demonstrated by Wilcoxon sign test using T.P.M mutation model. P values for all models were significant for all species (T.P.M: 0.019; P<0.05). Simulation of genetic diversity loss in anoa populations was implemented when bottleneck signals were detected. Based on current heterozygosity variables and generations time in anoa, this data was utilized to simulate the loss of genetic diversity in bottlenecked anoa populations over the next 100 years. The simulation results reveal a small loss of genetic diversity, which is around 9.55% lower than current genetic diversity (H_0) (Figure 2).

4. Discussion

Anoa population showed moderate genetic diversity (H_0 = 0.528), but slightly lower than some other endangered Bovidae species, such as black muntjack (H_0 = 0.704) (Ni *et al.* 2009), Arabian oryx (H_0 = 0.601) (Arif *et al.* 2010), banteng (H_0 = 0.67) (Bradshaw *et al.* 2007). However, caution should be used when interpreting heterozygosity because these studies used different numbers and

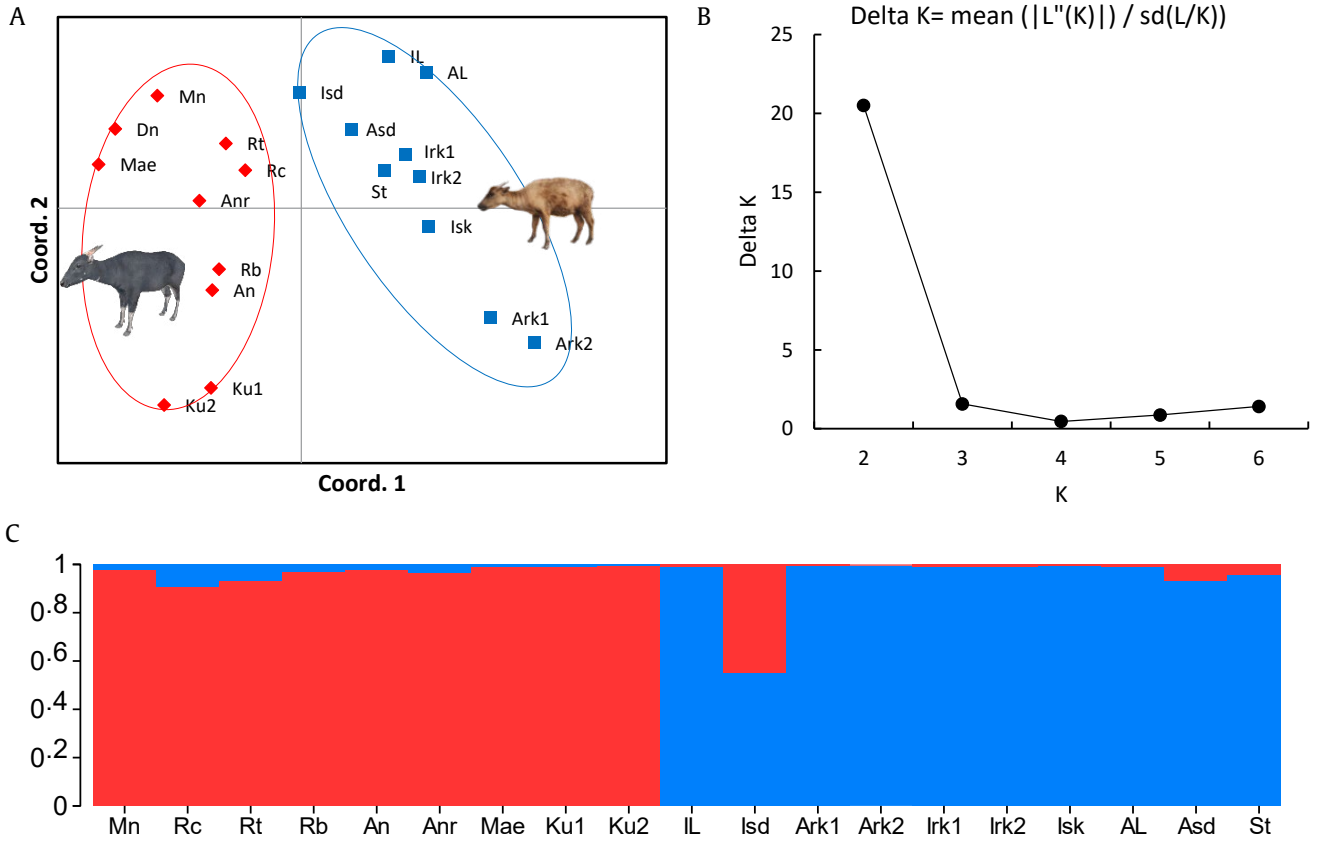

Figure 1. Genetic structure and admixture analysis in anoa based on 13 microsatellite loci. (A) Principal coordinate analysis (PCoA) based on genetic distance anoa. Individuals in a circle represent a cluster, (B) the Delta K calculated by the Evanno method, (C) barplot of genetic admixture population in anoa population

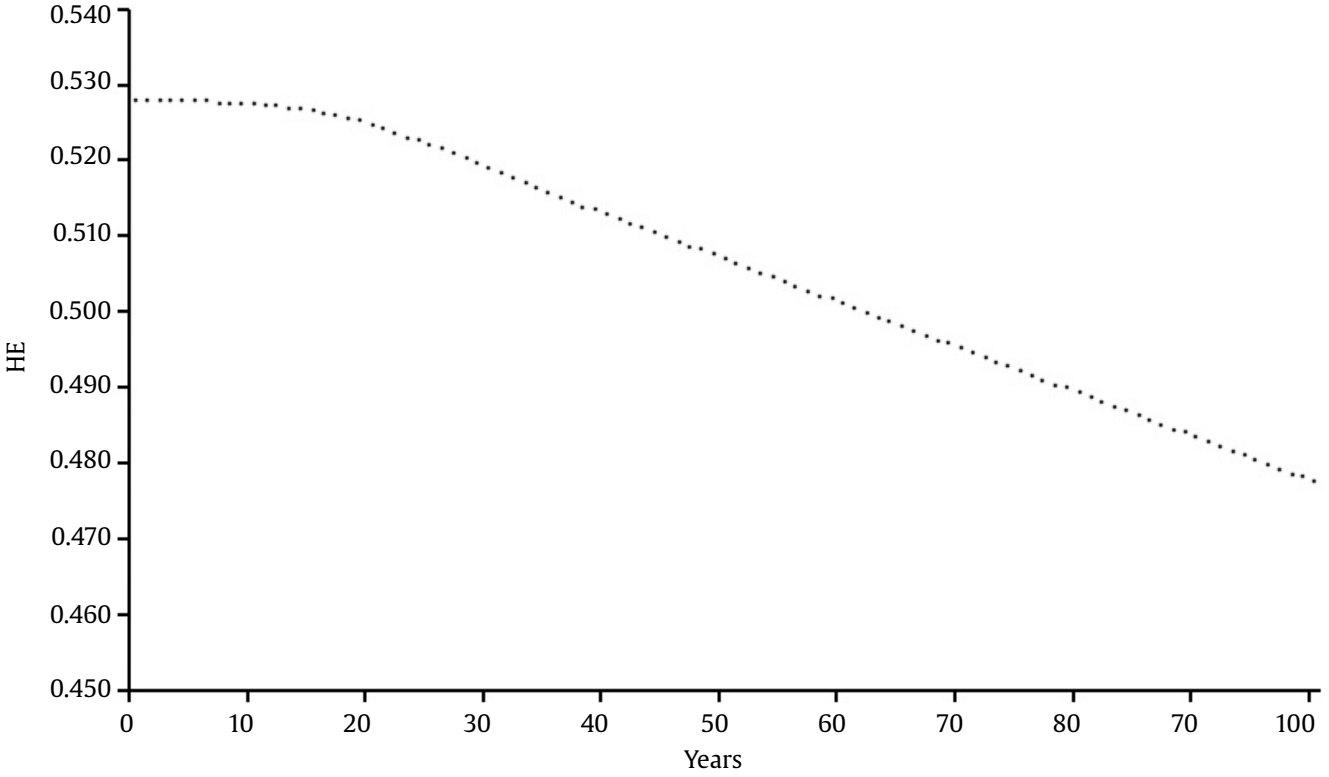

Figure 2. Simulation of genetic diversity loss (as measured by the H_0 of 13 polymorphic loci) in a bottlenecked anoa population. Simulations were carried out assuming a 30 year life span, sexual maturity at 3 years and completely overlapping generations in anoa (Jahja 1987) with 1000 iterations

combinations of microsatellite loci, allowing direct comparisons of genetic diversity impractical. Most populations with a large number of individuals have a high genetic diversity. On the other hand, small populations, island populations, and endangered species, usually have low genetic diversity (Frankham 2010), as in anoa populations. However, several factors can contribute to low genetic diversity in anoa populations. The samples used in this study were collected from captivity. Considering the small size of most captive populations, it is unsurprising that evidence suggests captive populations have lower genetic diversity than wild populations. The *ex-situ* anoa population in Indonesia was distributed over 10 conservation institutions with a total of 32 individuals as of February 2022 (Andrewsa *et al.* 2022), with the anoa breeding center, Manado, having the largest number of anoa individuals (n = 10). As per the 100/1,000 rule (Frankham *et al.* 2014), this number is too small to avoid inbreeding and maintain genetic diversity in captivity. Maintaining genetic diversity in a small, closed captive population is critical, as lower genetic diversity in a small population often leads to decreased fitness across the genome (Hansson and Westerberg 2002).

Further research is also necessary to map the genetic diversity of wild anoa populations with larger sample sizes and compare it with the captive population. On the other hand, the genetic diversity of wild populations has been shown to differ from captive populations (Bailey *et al.* 2007; Dantas *et al.* 2013; Jiang *et al.* 2005; Miller *et al.* 2014). Unfortunately, due to continued habitat loss and poaching, the population size of anoa in their natural habitat is estimated to be rapidly declining (Arini *et al.* 2020). Drastic population decline can lead to genetic drift caused by bottlenecks. Since bottlenecks can increase demographic stochastic, inbreeding depression, loss of genetic diversity, and deleterious allele fixation, and therefore increase the risk of population extinction, detecting populations that have experienced severe reductions in size or bottlenecks is critical (England *et al.* 2003; Whitehouse and Harley 2001).

A bottleneck signal in the anoa population has been detected in this study based on the excess heterozygosity test in BOTTLENECK (T.P.M: 0.019; $P<0.05$). Cornuet and Luikart (1996) developed the heterozygosity excess test, which assesses whether the population in issue deviates from mutation-drift

equilibrium and is effective for identifying historical population bottlenecks. Anoa population size has declined drastically to 90% in the last 16 years, and in some habitats, anoa populations have been determined to be locally extinct (Burton *et al.* 2005). As a result of bottleneck events, the remaining anoa population is considered to have low genetic diversity. This result is consistent with Schreiber *et al.* (1993) observations, which revealed much variation in the genetic distance in anoa individuals. Because of the effect of bottlenecks and incestuous breeding, Schreiber *et al.* (1993) cautioned that the taxonomic validity of these genetic distances was questionable. Another negative effect of bottlenecked populations has been shown in many studies (Leberg 1992; Marsden *et al.* 2016; Nei *et al.* 1975; Price and Hadfield 2014) as a consequence, knowledge of genetic diversity changes is critical for optimal conservation management in anoa populations.

The fluctuations of genetic diversity in bottleneck populations are essential to determine the level of extinction risk and appropriate conservation management strategies for endangered species. We found that after 100 years of simulation using BOTTLESIM, the loss of genetic diversity in the anoa population is relatively low (9.5%). Although the rate of genetic diversity loss in anoa is relatively slow, this does not imply that the species is immune to the process when the population number decreases. Several factors can contribute to the slow decline of genetic diversity. The long life span of anoa (approximately 20-30 years; Jahja 1987) has been hypothesized to have contributed to preventing genetic drift and acting as an intrinsic buffer against rapid loss of genetic diversity during bottlenecked events. Bottleneck simulations of genetic diversity in long-lived animals such as the black rhinoceros (Swart *et al.* 1994), and greater one-horned rhinoceros (Dinerstein and McCracken 1990), and white-tailed eagle (Hailer *et al.* 2006) also have been performed. Furthermore, because anoa is an island species, they may have a smaller effective population size than related mainland species (e.g., wild buffalo), resulting in a faster rate of molecular change (Woolfit *et al.* 2009). Higher molecular evolution rates for island species offer the potential for evolutionary radiation factors, which are thought to occur in many Sulawesi species, including anoa. Recovery programs for bottleneck species are critical to ensuring that anoa has a low risk of extinction. A population size of 300 is required to maintain 90% of the observed number of alleles during 200 years, as recommended by Soule and Simberloff (1986). Nevertheless, species recovery programs should not be entirely focused on increasing population size without appropriate management principles to minimize inbreeding and conserve genetic diversity. In addition, other factors, such as geographical isolation and fragmented distribution, should be considered appropriately. According to theoretical and empirical investigations, habitat fragmentation can reduce populations' neutral and adaptive genetic diversity by reducing effective population size and inter-population connectivity, (Johansson *et al.* 2007; Nei *et al.* 1975).

The population fixation analysis shows the issue of genetically fragmented populations in anoa (F_{ST}>0.15). Frankham *et al.* (2010) explain that if the population has F_{st}>0.15, it can indicate significant population differentiation. This differentiation may be illustrated by the current condition of anoa habitats in Sulawesi. As anoa's natural habitat in Sulawesi, forests continue to be deforested and fragmented, (Cannon *et al.* 2007; Supriatna *et al.* 2020). Forest deforestation and habitat fragmentation has been widely reported as a major factor in genetic population differentiation (e.g., Macdonald *et al.* 2018; Woltmann *et al.* 2012). Following habitat fragmentation, small populations and reduced genetic diversity result in genetic drift, increased inbreeding risks, lower evolutionary potential, and, as a result, a higher risk of extinction (Dixo *et al.* 2009; Johansson *et al.* 2007; Schlaepfer *et al.* 2018). As a result of habitat fragmentation, numerous species in Sulawesi, such as primates (Evans *et al.* 2003; Supriatna *et al.* 2020) and toads (Evans *et al.* 2003), are suffering gene flow issues. For measuring gene flow and describing the natural selection history and genetic relationships among anoa populations, data on population structure is important.

We employed Bayesian clustering, embedded in STRUCTURE program, to determine the gene flow and the population structure in the anoa population. The anoa population was clustered into 2 genetic clusters at K = 2. The result from STRUCTURE, in consonance with earlier studies (Priyono *et al.* 2018, 2020), clearly shows the genetic separation of mountain and lowland anoa. A plausible reason for the separation of lowland from mountain anoa population could be due to divergence events in their biogeographical history. Recent genetic research shows that the clade variation of the anoa population is caused by geological events that occurred on Sulawesi in their biogeographic history (Frantz *et al.* 2018). These results indicate that each anoa cluster is unique and has undergone for long

adaptation process in its habitat, which may have made up the gene pool of the current population. So, for managerial purposes, it is crucial to maintain and conserve Sulawesi's lowland and mountain forest landscape as their habitat, which would reduce the risk of extinction.

The result from STRUCTURE analysis also reveals that an anoa individual (Isd) has a genetic admixture with both lowland and mountain anoa. The narrow areas of Sulawesi lead to several contact zones where anoa species may hybridize. As a consequence of hybridization, this mixture was possible. Many researchers have recognized the hybridization of many species in Sulawesi (Bynum *et al.* 1997; Groves 1980; Ito *et al.* 2015; Supriatna 1996). Some chromosomal studies reveal the possibility of introgressive hybridization between two anoa species. The number of chromosomes found in lowland anoa was 2n = 48 (NF = 60) and that number was same as lowland anoa individual at the San Dieg Zoo (Hsu and Benirschke 1974; Low and Benirschke 1973) and Leipzig Zoo (Schreiber *et al.* 1998). However, an anoa from cross-breeding between captives was found 2n = 47 at the Leipzig zoo (Schreiber *et al.* 1993). Scheurmann *et al.* (1977) examined the number of mountain anoa chromosomes and obtained 2n = 45. Sugiri *et al.* (1996) also obtained chromosome variant 2n = 44 from anoa on Nokilaki Mount, Central Sulawesi. Chromosome variations were also found in the Ragunan Zoo, even with extreme numbers, 2n = 38, 42, 45, and 46 (Pranadewi 1998). These chromosomal variations caused taxonomic issues in anoa. Recently, molecular approaches have succeeded in solving taxonomic issues in anoa (Priyono *et al.* 2018, 2020). The findings are congruent with the results of this study which indicates that at least two anoa cluster that exists in Sulawesi.

4.1. Implication for Conservation

Conservation genetics has been recognized as key in biodiversity conservation efforts. Various conservation efforts have been declared to save the anoa population. The anoa conservation action plan strategy for 2013-2022 as stated in the Regulation of the Minister of Forestry of the Republic of Indonesia (no: P.54/Menhut-II/2013), mentions several sections related to genetic issues. Genetic issues in this document are categorized based on several conservation programs, including genetic mapping and minimizing genetic diversity losses. The document also specifies that a minimum of 90% genetic diversity in 100 next years must be maintained. The simulation results show a 9.55% reduction in genetic diversity in the anoa population over the next 100 years. A decline of less than 10% is nevertheless sufficient to sustain a species' evolutionary adaptability capacity (Frankham *et al.* 2010). The genetic diversity data in this study is the first step toward mapping the genetic diversity of the anoa population. We provide some recommendations for conservation genetics in the anoa population based on the findings in this paper. There are four recommendations, including 1) monitoring and determining the genetic effects of declining wild anoa populations and genetically mapping the anoa population structure in the Sulawesi landscape. In the case of anoa, conservation must consider the existence of different groups and their different habitats (lowland and mountain).

The population genetics structure of anoa suggests that there are two clusters. Thus, they were classified as different populations, indicating strong adaptive differentiation and the recommendation to manage the two populations as separate units for conservation purposes. In light of current circumstances, which encompass rapid decline in population size and the severe endangerment of their natural habitats, in situ and ex situ conservation programs are essential. Policies and interventions to protect natural habitats may be sufficient to maintain the size of the population. In addition to in-situ conservation, it is strongly recommended that each group's anoa populations be established for *ex-situ*, 2) Establishing the founder for the *ex-situ* program by examining their genetic diversity status, 3) maintaining or increasing the number of individuals in the *ex-situ* population to genetically safe population size, 4) managing *ex-situ* populations from generation to generation by avoiding inbreeding through kinship analysis. To summarize, *in-situ* and *ex-situ* conservation programs should be combined to protect the genetic diversity of anoa. Furthermore, genetic studies with broader samples and genomic approaches are needed to enrich genetic information in the endangered anoa populations.

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