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The Phylogeny of Bornean Swamp Buffalo (*Bubalus bubalis*) Analysis Based on D-loop Mitochondrial DNA Sequence Variation

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ABSTRACT

Swamp buffalo (*Bubalus bubalis*) is one of the Indonesian germplasm that adapts well to Borneo Island. This study aimed to determine the genetic diversity, phylogenetic tree, and phylogeographic structure of Bornean swamp buffalo based on control region (D-loop) mitochondrial DNA sequences. A total of 120 animals were collected from three populations in Indonesia, i.e., the North, East, and South of Kalimantan Provinces. Along 1140 bp of D-loop mtDNA gene of Bornean swamp buffaloes was amplified with a design primer of F: 5'-CAA CAC CCA AAG CTG AAG TT-3' and R: 5'-CGC TCC TCT TAG TCT CGT TG-3'. Therefore, the forward and reverse sequencing was performed to visualize the full length of D-loop mtDNA gene sequence (1140 bp). Results showed that a total of 47 haplotypes were detected in the animal study, with haplotype and nucleotide diversities of 0.936 and 0.005, respectively. Consequently, two haplogroups were observed in the animal study, i.e., Haplogroup A (North) and Haplogroup B (East and South). Based on structure analysis, Bornean swamp buffaloes were comparable to filing swamp buffaloes of China based on structure. According to the analysis of molecular (AMOVA), the geographical component contributed over 56.44% of the total mtDNA sequence variations. In conclusion, it was discovered that the haplogroups of buffalo from the East and South populations were identical.

Keywords: Borneo; D-loop mtDNA; haplogroup; haplotype; swamp buffalo

INTRODUCTION

Animal genetic resources are significant genetic components that assist human civilization's need for food security. Swamp buffalo (*Bubalus bubalis*) is one of the major large animals in Indonesia. By 2021, the total buffalo population in Indonesia has reached 1.19 million heads. This number increased by 3.04% compared to the previous year's population of 1.15 million heads (Statistics Indonesia, 2022).

Bornean Swamp Buffalo (*Bubalus bubalis*), commonly known as "Kalang" buffalo in East Kalimantan, is one of the potential farm commodities in terms of supplying meat due to the superior ability of buffaloes to digest roughage compared to the other ruminants (Suhardi *et al.*, 2021). Since 2012, the Kalang buffaloes in East Kalimantan and South Kalimantan Provinces have been decided as two Indonesian native buffaloes through the decision of Indonesian Ministry of Agriculture No: 2843/Kpts/LB.430/8/2012 and 2844/Kpts/LB.430/8/2012, respectively. According to Rusdin *et al.* (2020), buffalo can live in marginal areas with limited availability of good quality nutrient sources. Buffaloes also can proliferate in a wide range of agroecosystems, from moist areas to relatively dry environments. Suhardi *et al.* (2020) stated that Kalang buffalo have an average weight of about 500 kg for adult males, while adult females can weigh up to 400 kg with a carcass percentage of between 43.3% and 50.26%. Kalang buffaloes are maintained with good management and are slaughtered at the age of 16–20 months, which will provide better quality of meat. The costs of Kalang buffalo production are also cheaper than beef production from cattle. Moreover, buffalo meat contains 84 calories and 0.5 g of fat per 100 g of meat, compared to 207 calories and 14 g of fat per 100 g of meat from cattle (Kandeepan *et al.*, 2009).

The genetic characterization in Kalang buffaloes is important to obtain the breed standard of livestock that can influence the economic value. In Indonesia, many studies have been worked to characterize buffaloes based on the mitochondrial DNA of COI (Saputra *et al.*, 2013) and Cyt-b (Rusdin *et al.*, 2020). Although the studies of COI and Cyt-b genes in buffaloes were conducted, the D-loop gene was widely used for genetic characterization in buffaloes (Shaari *et al.*, 2019). Therefore, this study aimed to construct the phylogenetic tree of Bornean swamp buffaloes based on the D-loop gene region. The results of this study are important to char-

acterize Bornean buffaloes for breed standardization purposes.

MATERIALS AND METHODS

Animal and Research Site

One hundred and twenty buffaloes (60 males and 60 females) were selected from three populations in Borneo Island, i.e., the North, East, and South of Kalimantan Provinces, Indonesia. The animal sample in each province consisted of 20 males and 20 females, with an average age of three to five years. The DNA analysis was performed at the Laboratory of Aquaculture Genomics, Wailalak University of Thailand, with an ethical clearance certificate from the National Committee for Research Animal Development-National Research Council of Thailand No: 023/2018.

DNA Extraction

The DNA samples of buffalo were taken from the tail hairs using previously established methods with slight modification by using guanidine as a buffer (Zainabadi *et al.,* 2019). Approximately 20–25 hair strands, two inches from the base of the buffalo tail, were collected using sterile tweezers and placed in zipper-sealed sample bags (Fisher Scientific, Canada).

D-loop mtDNA Region Amplification

The amplification of the D-loop mtDNA region (1140 bp) was performed using a primer pair of forwarding: 5′-CAA CAC CCA AAG CTG AAG TT-3′ and Reverse: 5′-CGC TCC TCT TAG TCT CGT TG-3′. This primer was designed with Primer3plus software (https://www.primer3plus.com) based on the mtDNA of swamp buffalo (GenBank: KX758374.1). The PCR reaction was performed in a total volume of 50 μL containing 25 μL of PCR reaction mix (Hot FIREPol® BLEND, UK), 0.25 μL of each primer, 1 μL of mtDNA template (50 ng/μL), and 23.5 μL of DDW. Therefore, the PCR reaction was performed in a Thermal Cycler machine (Bio-Rad T100, USA) with a PCR program of initial denaturation (95 °C, 15 minutes) followed by 35 cycles of denaturation (95 °C, 30 seconds), primer annealing (60 °C, 30 seconds), extension (72 °C, 30 seconds), and final extension (72 °C, 5 minutes). Thus, the PCR products were held at 4 °C until electrophoresis. Agarose gel (2%) was prepared in 0.5X TBE buffer and the PCR products (3 μL) stained with 6X flouroDye DNA fluorescent loading dye (0.5 μL) (Smobio technology. Inc, Taiwan) were electrophoresed at 100V for 50 minutes with fluoroBand 100 bp fluorescent DNA ladder (Smobio technology. Inc, Taiwan). The amplified products were visualized under UV using a gel documentation system.

Purification and Sequencing

The mtDNA was purified using the GenepHlowTM Gel/PCR Kit (Geneid, New Taipei City, Taiwan) following the manufacturer's protocol. Amount: 30 μL of purified amplicons (30 ng/μL) with 5 μL of each primer were sent to a commercial sequencing company for further analysis (BiONEER, Daejon, South Korea).

Sequence Analysis

The obtained mtDNA sequences were used to compare with the D-loop mtDNA of six Chinese swamp buffalo breeds from GenBank, i.e., Anhui (EF053535.1), Dechang (EF053536.1), Fuling (EF053547.1), Haikou (NC_006295.1), Jianghan (EF053550.1), and Yunnan (EF053552.1). The alignment and phylogenetic tree analyses of the obtained sequences were performed with the sequence references from GenBank using the BioEdit and MEGA-X packages, respectively (Hall, 2011; Hall, 2013). The haplotype and nucleotide diversities in the animal study were calculated using the DNAsp package (Librado & Rozas, 2009). The median-joining network was performed in this study to observe the common ancestor in animal studies based on haplotype using the NETWORK package (Kong *et al.,* 2016). A STRUCTURE package (Earl & vonHoldt, 2012) was computed in this study to observe the genetic admixture in the Bornean and Chinese buffaloes. Therefore, an ARLEQUINE package (Excoffier & Lischer, 2010) was computed in this study to obtain pairwise distances among the population. In addition, the molecular (AMOVA) analysis was performed in this study using the ARLEQUINE package to calculate the contribution of geographical factors to mtDNA sequence variations in the animal study.

RESULTS

The 1140 bp of the D-loop mtDNA region in this animal study was successfully amplified with a primer pair according to the Primer3Plus package (Figure 1). Therefore, a total of 47 haplotypes were observed from 120 D-loop mtDNA sequences, as presented in Table 1. Table 1 shows the highest number of haplotype (k) in the South Asian population (51%) than in the other populations. Therefore, the number of polymorphic sites (P) and haplotype diversity (Hd) values in the South population were higher than in the other populations (Table 2). Thus, the nucleotide diversity (π) in the South and North populations was similar (0.003) and higher than in the Eastern population (0.001).

The Fu's test (Fs) value in the animal study showed a negative value in each population. As a result, the negative Fs value of the buffalo in the South population is higher than in the other populations. The genetic diversity of D-loop mtDNA in some buffalo populations is presented in Table 3. The pairwise distance of buffalo between the South and East populations was relatively close (Table 4). Thus, buffaloes in the northern population were more distant from the south and east populations. Analysis of molecular variation (AMOVA) revealed that about 56% of D-loop mtDNA variation in the animal study was affected by geographical factors and the remaining (44%) was affected by factors within the population (Table 5). Compared to the Chinese swamp buffalo, the Bornean swamp buffalo was grouped into

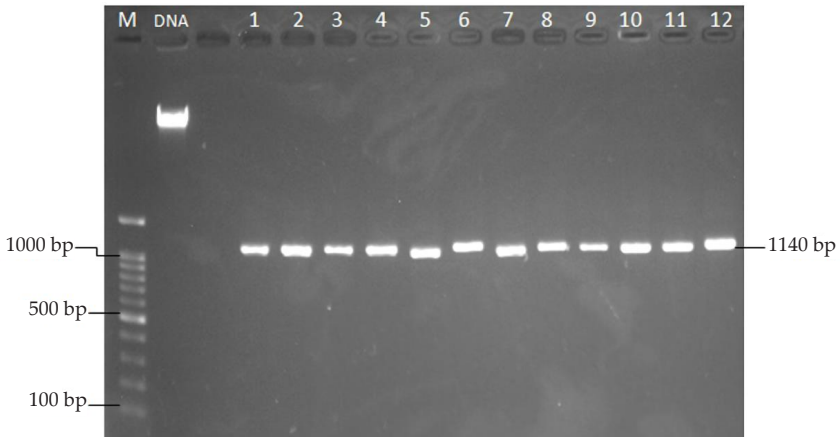

Figure 1. The amplification of D-loop mtDNA region in Bornean swamp buffalo (*Bubalus bubalis*) along 1140 bp. M= DNA ladder 100 bp; Lane 1-12= DNA sample.

Table 1. The number of individu in each haplotype of D-loop mtDNA in Bornean swamp buffaloes (*Bubalus bubalis*) from three different population areas of Borneo (Kalimantan) island

Haplotype	Population area			Total	Haplotype	Population area			Total
	North	East	South			North	East	South	
Hap.1	14	0	0	14	Hap.25	0	0	1	1
Hap.2	1	0	0	1	Hap.26	0	0	1	1
Hap.3	1	0	0	1	Hap.27	0	0	1	1
Hap.4	1	0	0	1	Hap.28	0	0	1	1
Hap.5	2	0	0	2	Hap.29	0	0	1	1
Hap.6	1	0	0	1	Hap.30	0	0	1	1
Hap.7	11	0	0	11	Hap.31	0	0	1	1
Hap.8	1	0	0	1	Hap.32	0	0	1	1
Hap.9	1	0	0	1	Hap.33	0	0	1	1
Hap.10	2	0	0	2	Hap.34	0	0	1	1
Hap.11	3	0	0	3	Hap.35	0	0	1	1
Hap.12	2	0	0	2	Hap.36	0	0	1	1
Hap.13	0	18	5	23	Hap.37	0	0	3	3
Hap.14	0	1	0	1	Hap.38	0	0	3	3
Hap.15	0	4	0	4	Hap.39	0	0	1	1
Hap.16	0	5	0	5	Hap.40	0	0	1	1
Hap.17	0	5	0	5	Hap.41	0	0	1	1
Hap.18	0	1	0	1	Hap.42	0	0	1	1
Hap.19	0	1	0	1	Hap.43	0	0	1	1
Hap.20	0	1	0	1	Hap.44	0	0	1	1
Hap.21	0	1	0	1	Hap.45	0	0	1	1
Hap.22	0	2	0	2	Hap.46	0	0	1	1
Hap.23	0	1	0	1	Hap.47	0	0	1	1
Hap.24	0	0	7	7					

Table 2. Source and genetic diversity of D-loop mtDNA in Bornean swamp buffaloes (*Bubalus bubalis*)

Population area	N	k	P	H_d	π	Fs
Northern Borneo	40	12	14	0.805	0.003	-2.045
Eastern Borneo	40	11	10	0.773	0.001	-6.121
Southern Borneo	40	25	20	0.951	0.003	-23.330
Pool	120	47	77	0.936	0.005	-10.499

Note: N: sample size; k: number of haplotype; P: number of polymophic site; Hd: haplotype diversity; π: nucleotide diversity; Fs: Fu's test.

Table 3. The genetic diversity of D-loop mtDNA in some buffalo populations in the world

Population	N	k	H_d	π	Reference
Southeast Asia, Australia, Brazil, Italy	112	46	0.943	0.021	Petersen *et al.* (2013)
China					
Population 1	119	43	0.786	0.014	Lorenzo *et al.* (2018)
Population 2	152	57	0.833	0.013	Lorenzo *et al.* (2018)
India					
Region 1	51	31	0.953	0.012	Joshi *et al.* (2013)
Region 2	95	47	0.950	0.012	Joshi *et al.* (2013)
Region 3	36	28	0.973	0.019	Joshi *et al.* (2013)
Region 4	35	10	0.877	0.009	Joshi *et al.* (2013)
Region 5	42	17	0.926	0.014	Joshi *et al.* (2013)
Philippine					
Luzon Island	56	12	0.560	0.003	Villamor *et al.* (2021)
Visayas	27	7	0.761	0.004	Villamor *et al.* (2021)
Mindanao	24	6	0.761	0.003	Villamor *et al.* (2021)

Note: N: sample size; k: number of haplotype; Hd: haplotype diversity; π: nucleotide diversity; Fs: Fu's test.

Table 4. Pairwise distances of Bornean swamp buffalo (*Bubalus bubalis*) from three different population areas of Borneo

Population area	1	2	3
Northern Borneo (1)	0		
Eastern Borneo (2)	0.659	0	
Southern Borneo (3)	0.598	0.187	0

Table 5. Analysis of molecular (AMOVA) of D-loop mtDNA in Bornean swamp buffalo (*Bubalus bubalis*)

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage (%) of variation
Among populations	2	119.7	1.47	56.44
Within populations	117	133.23	1.13	43.56
Total	119	252.93	2.60	100.00
Fixation index (F_{st})	0.56			

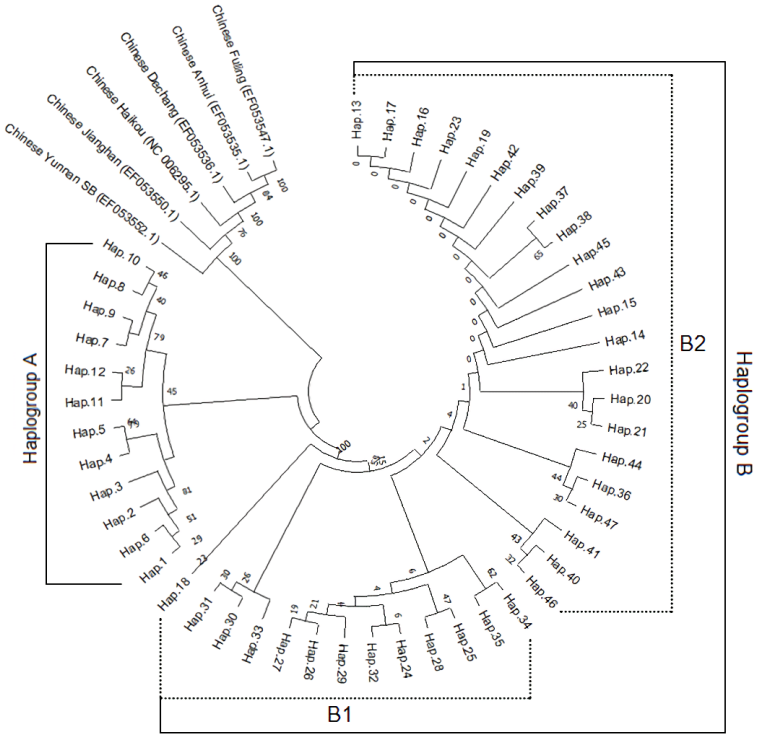

Figure 2. Phylogenetic tree with UPGMA method (1000× bootstrap) of D-loop mtDNA between 47 haplotypes of swamp Bornean and six (6) Chinese swamp buffaloes

a different cluster (Figure 2). In addition, the D-loop mtDNA in animal studies has two haplogroups: Hap-A (North) and Hap-B (South and East). Therefore, Hap-B in animal studies could be separated into Hap-B1 and Hap-B2. Mostly, the frequency of Hap-B2 was higher than Hap-B1 in eastern and southern populations (Figure 3). According to the median-joining network analysis, buffalo in H24 and H13 groups are the common ancestral buffaloes in the South population (Figure 4). Meanwhile, buffalo from the H1 and H7 groups are the common ancestors of buffalo from the north. In addition, buffaloes in the East population have genetic introgression from buffaloes in the Southern population. Furthermore, the Chinese Fuling swamp buffalo (C4) has the highest genetic introgression from Bornean buffalo of any population (Figure 5).

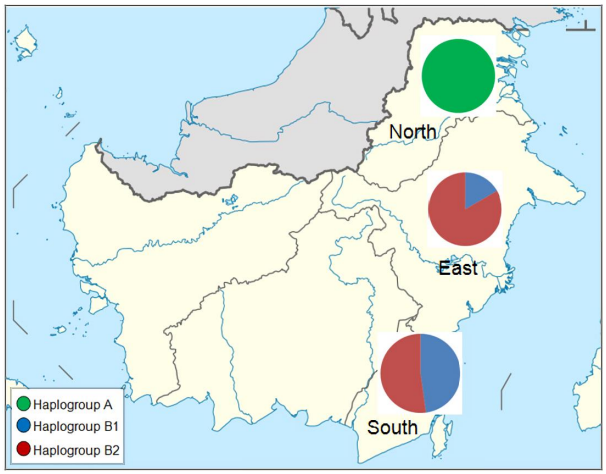

Figure 3. Map showing the frequency of distribution of the haplogroup variants in swamp Bornean buffalo at three different populations areas of Borneo (Kalimantan) island

DISCUSSION

In the current study, a total of 47 haplotypes of D-loop mtDNA were obtained from 120 buffaloes. In Petersen *et al.* (2013) and Lei *et al.* (2007), 46 haplotypes (112 buffaloes) and 43 haplotypes (119 buffaloes), respectively, were obtained and are close to the present study (Table 3). The haplotype diversity of livestock could be caused by random mating, selection, and migration (Falconer, 1989). The Hd value in animal studies in each population was included in the high category. According to Nei & Kumar (2000), the Hd value has two categories low (<0.50) and high (0.51 to 1.00). Meanwhile, the μ value in an animal study in each population was included in the low category. The μ value has three categories, i.e., low (0.01 to 0.04), moderate (0.05 to 0.07), and high (0.08 to 1.00). Negative Fs values in animal studies indicated that Bornean swamp buffalo experienced inbreeding. A negative Fs value indicated that an excessive number of alleles (gene flow) had occurred in Bornean swamp buffalo. Kusumaningrum *et al.* (2020) obtained the negative Fs value (-1.766) in the D-loop mtDNA of Sragen Black cattle. High haplotype diversity will be followed by a high number of polymor-

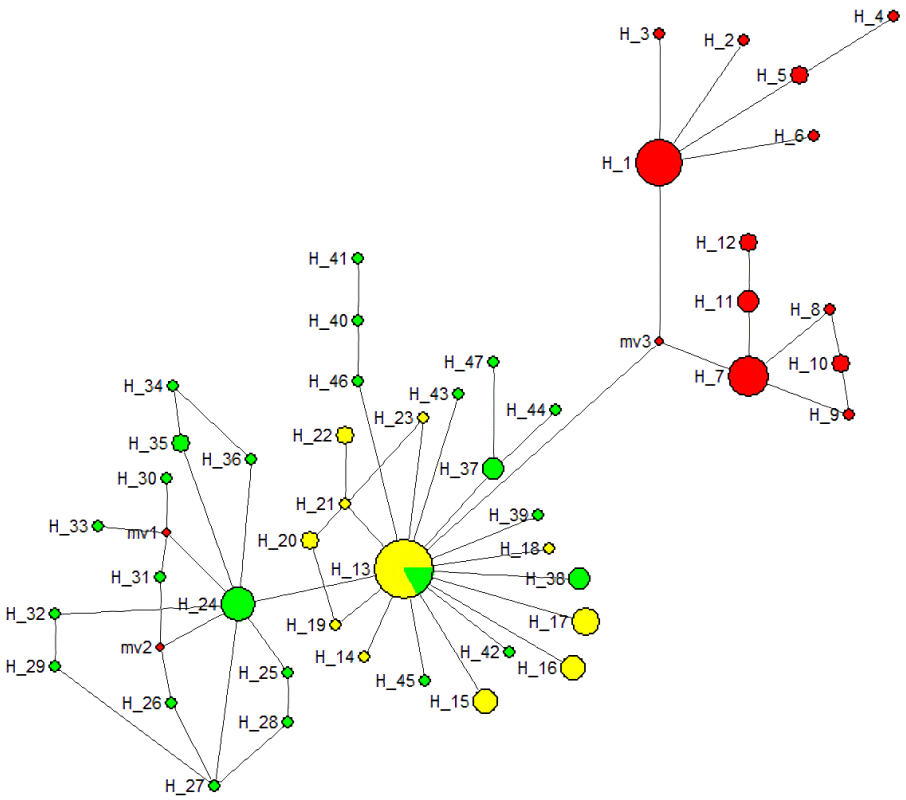

Figure 4. Median-joining network of 47 haplotypes in the D-loop mitochondrial DNA of Bornean swamp buffalo (*Bubalus bubalis*) at Northern (red), Eastern (yellow), and Southern (green) of Borneo island

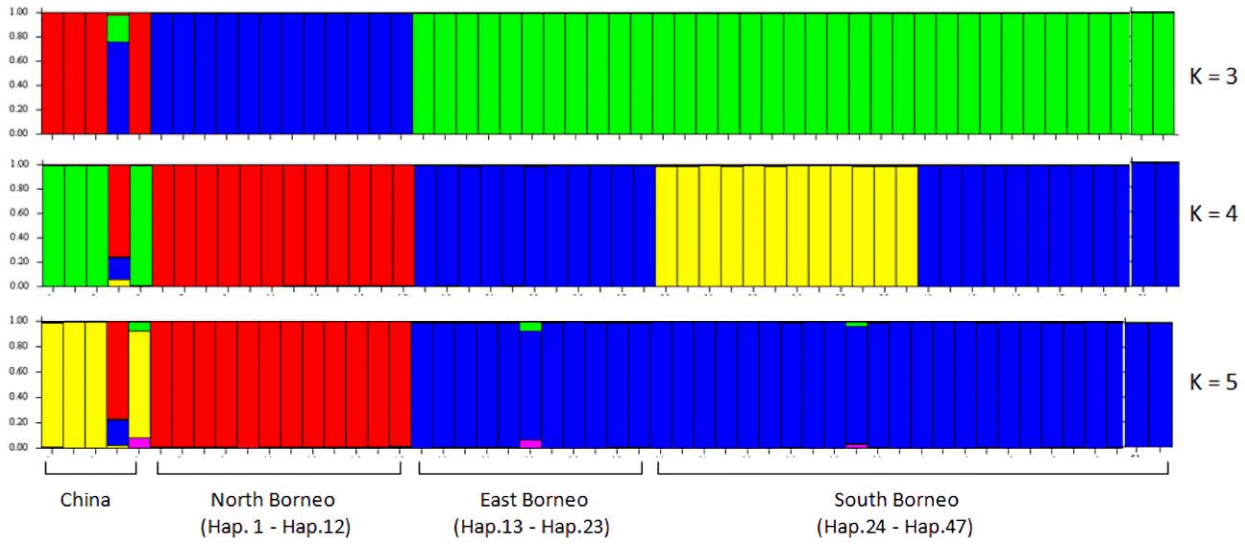

Figure 5. Plot of individual Bornean swamp buffalo (*Bubalus bubalis*) cluster membership coefficients defined by STRUCTURE analysis

phic sites. Hd and μ values are two important indicators for assessing population polymorphism and genetic diversity of mtDNA (Fang *et al.*, 2018; Nabholz *et al.*, 2008; Suhardi *et al.*, 2021b).

According to the pairwise distance, buffalo in the East and South populations were close to each other, which suggests that both populations have similar genetic characteristics. The buffaloes in South and East populations were imminent and caused by an AI program with buffalo bulls from the South population (called Kalang buffalo). Hence, the AI program was successfully spread in the imminent area of the Southern population. So, the genetic flow of buffaloes from southern to eastern populations occurred decades ago (Suhardi *et al.*, 2021a). Hussain *et al.* (2009) reported that the Pakistani Kundi buffalo was grouped into a similar cluster with Indian buffalo breeds (Mohasana, Surti, Jaffarabadi, and Pandharpuri) based on the D-loop mtDNA. Thus, the imminent area of Pakistan and India was caused by the genetic similarity of buffalo in Pakistan and India. Therefore, two haplogroups or two maternal lineages were detected in the animal study. Two maternal lineages of D-loop mtDNA were reported in Chinese, Philippine, and Thai buffaloes (Villamor *et al.*, 2021; Raungprim *et al.*, 2021).

Compared to the Chinese buffalo, the Bornean buffalo was grouped into different clusters. Furthermore, the Chinese Fuling buffalo had a genetic introgression from the Bornean buffalo. This finding is similar to the Pakistani and Indian buffaloes that clustered separately from Chinese buffaloes (Hussain *et al.*, 2009). In this study, about 56% of sequence variation was caused by geographical factors. Zhang *et al.* (2018) reported that about 37.3% of D-loop mtDNA variation was influenced by geographical factors. It can be suggested that the geographical areas in Borneo Island may be due to the many restricted buffalo populations with various haplotype diversities.

CONCLUSION

The haplotype diversity in Bornean swamp buffalo was high, totalling 47 haplotypes. Two haplogroups of Hap-A (North) and Hap-B (East and South) were detected in the D-loop mtDNA of the animal study. Therefore, Bornean buffalo were grouped into different clusters and separated from the Chinese buffalo group. However, the Chinese Fuling buffalo has a genetic introgression from the Bornean buffalo.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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