

mtDNA D-loop sequence analysis of Kalang, Krayan, and Thale Noi buffaloes (*Bubalus bubalis*) in Indonesia and Thailand reveal genetic diversity

S. Suhardi^{1, 3*}, P. Sumppunn², and S. Wuthisuthimethavee¹

¹Laboratory of Agricultural Genomics, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat, 80160, Thailand

²Food Technology and Innovation Research Center of Excellence, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat 80160, Thailand

³Department of Animal Science, Agriculture Faculty, Mulawarman University, Samarinda, East Kalimantan, PO BOX 1040, 75123, Indonesia

*Corresponding E-mail: suhardi@faperta.unmul.ac.id

Co-corresponding E-mail: wsuwit@wu.ac.th

Received Desember 01, 2020; Accepted January 29, 2021

ABSTRAK

Kerbau Kalang (KBuf), Krayan (KrBuf), dan Thale Noi (TBuf) merupakan sumberdaya genetik kerbau rawa di Indonesia dan Thailand. Area D-loop DNA mitokondria merupakan material penting untuk inferensi filogenetik dan analisis keragaman genetik. Tujuan dari penelitian ini adalah untuk mengevaluasi keragaman genetik dan untuk merekonstruksi pohon filogenetik dalam bangsa kerbau di Kalimantan, Indonesia, dan Phatthalung, Thailand menggunakan sekuens D-loop DNA mitokondria. Sebanyak 140 ekor kerbau (70 jantan dan 70 betina), dengan masing-masing jumlah sample di provinsi Kalimantan Utara sebanyak 40 ekor, Kalimantan Timur 40 ekor, Kalimantan Selatan 40 ekor dan provinsi Phatthalung, Thailand sebanyak 20 ekor. Sampel DNA diisolasi dari rambut ekor kerbau. Sekuens DNA dirangkai secara manual menggunakan program BioEdit dengan mempertimbangkan celah dan sekuens yang ambigu. Pohon filogenetik kerbau dihasilkan dengan software PHYLIP. Variabel yang diamati meliputi keanekaragaman haplotipe, jarak genetik dan pohon genetik. Total 956 bp mtDNA D-loop yang diamplifikasi dari 2 fragmen set primer 647 bp dan 595 bp menyajikan 24 haplotipe dengan beberapa mutasi yang mencakup transisi (293), transversi (60), delesi (15), dan insersi (20). Pohon neighbor-joining menggunakan model Kimura 2 parameter menunjukkan dua kelompok kerbau lokal antara Kerbau di Kalimantan dan Kerbau di Thailand, sedangkan secara internal Kerbau di Kalimantan (KK dan KKr) menunjukkan empat pola hubungan kekeluargaan. Hasil dari penelitian ini menunjukkan bahwa analisis sekuens DNA kerbau menunjukkan keanekaragaman yang relatif tinggi dan merupakan dasar yang baik untuk melakukan seleksi dan pengembangan pemuliaan kerbau secara modern.

Kata kunci: filogenetik, DNA mitokondria, area D-loop, keragaman genetik, kerbau

ABSTRACT

Kalang (KBuf), Krayan (KrBuf), and Thale Noi buffaloes (TBuf) are swamp buffalo genetic resources in Indonesia and Thailand. The maternally inherited mitochondrial DNA (mtDNA),

particularly D-loop region is an important material for phylogenetic inference and analyzing genetic diversity. Therefore, the objectives of the present study were to identify genetic diversity and to reconstruct the phylogenetic tree within buffalo breeds in Kalimantan, Indonesia, and Phatthalung, Thailand using mtDNA D-loop sequences. A total of 140 buffaloes (70 males and 70 females) were observed including 40 buffaloes from North (NK), 40 from East (EK), and 40 from South Kalimantan (SK) provinces Indonesia and 20 from Phatthalung (PT) province, Thailand. DNA samples were isolated from buffalo tail hairs. DNA sequences were manually assembled using BioEdit program with consideration of gaps and ambiguous sequences. The phylogenetic tree of buffalo was generated by PHYLIP software. The observed variables included haplotype diversity, genetic distance, and genetic tree. The 956 bp of amplified mtDNA D-loop fragment presented a total of 24 haplotypes with several mutations that included transitions (293), transversions (60), deletions (15), and insertions (20). The neighbor-joining tree using the Kimura 2 parameter model demonstrated two local buffalo clusters among buffalo from Kalimantan and Thailand while internally four buffalo relationship patterns observed from buffaloes in Kalimantan Island (KBuf and KrBuf), Indonesia. The Results of the present study demonstrated that the buffaloes sequence analysis revealed relatively high diversity and is a good basis to perform selection and modern buffalo breeding development.

Keywords: buffalo, D-loop region, genetic diversity, mitochondrial DNA, phylogenetic

INTRODUCTION

Kalang buffaloes (KBuf) are important in Indonesia's economy and play a significant role in religion and society (Suhardi *et al.*, 2020; Komariah *et al.*, 2014). Currently, four known buffalo breeds genetically originated from Indonesia, namely: swamp, riverine, spotted Toraja, and Kalang buffaloes (Director General of Livestock Services, 2003). Thale Noi buffalo (TBuf) is a breed originating from the Thale Noi wetlands located in Phatthalung, southern Thailand (FAO, 2020). Unfortunately, the buffalo population in the Southeast Asian region has steadily declined in population due to the long calving interval and low calving rate (Escarcha *et al.*, 2018). Sudden changes in calving rates have been complicated by external and internal factors such as environment and genetics, respectively (El Debaky *et al.*, 2019; Hassan *et al.*, 2018). With this in mind, the potential for commercial rearing of swamp buffaloes through genetic studies has been made possible and to an extent, has become an alternative solution to support the red meat industry of Indonesia (Suhardi *et al.*, 2020). Furthermore, improvement of the genetic potency of a herd through molecular methods may consequently lead to a better quality of traits leading to better yields in terms of livestock (Nguyen *et al.*, 2020).

Variations in genotypic profiles are influenced by constant environmental changes that allow species to adapt for survival (Yusnizar *et al.*, 2015). These variations would also lead to improved hereditary traits in some species

(Hassan *et al.*, 2018). Mitochondrial DNA (mtDNA) D-loop markers are influenced by the movements/genetic drift of the female buffalo and are considered key in understanding the link between current population density and the extent of genetic variation (Ruihua *et al.*, 2018).

The highly variable regions in mtDNA such as the displacement loop (D-loop) have been the focus of genetic variation studies due to its higher mutation rate (Yacoub and Fathi, 2013). Due to this, the mtDNA is considered to be an important material for phylogenetic inference and thus analyzing genetic diversity within the framework of breed conservation, genetic characterization, and genetic resource management (Sayres, 2018). Therefore, to understand the current genetic status of Kalang and Krayan buffaloes, it is essential to observe the buffalo genetic diversity through analysis of the mtDNA D-loop region in buffalo sequences in the three major provinces in Kalimantan, Indonesia, by tracing ancestry, and defining biodiversity for genetic development in the future.

Saputra *et al.* (2020) reported, base on microsatellite marker that Indonesia has two cluster of swamp buffalo, first cluster consisting of Aceh, North Sumatra, and Riau and the second cluster consisting of Banten, Central Java, West Nusa Tenggara, and South Sulawesi. Similarly, Rusdin *et al.* (2020) reported two clusters of eight Indonesian swamp buffalo breeds based on cytochrome b gene marker, the first cluster consisting of Aceh, North Toraja, West Nusa Tenggara, Banten, Kolaka, and Konawe populations, while the second cluster consisting of

Bombana Island, Bombana mainland, Kolaka, and Konawe populations. To date, there are no investigations conducted regarding the genetic diversity of buffalo breeds in Kalimantan using molecular methods. Studies available are focused on epidemiological data of disease rather than genetic variation (Komariah *et al.*, 2014; Natalia *et al.*, 2006). The present study aimed to identify for the first time the genetic diversity and reconstruct the phylogenetic tree within breeds of buffaloes in East, South, and North Kalimantan, Indonesia, coupled with similar analysis conducted on swamp buffaloes from Phatthalung, Thailand using mtDNA D-loop sequences.

MATERIALS AND METHODS

Sample collection and processing

One hundred and forty buffaloes (70 males and 70 females) were selected from the three major provinces in Kalimantan, Indonesia, and one province in Thailand (Figure 1) and studied for genetic diversity using DNA collected from tail hairs. Buffaloes between three to five years of age were qualified and selected as follows: North Kalimantan (NK) (20 males and 20 females

Krayan buffaloes), East Kalimantan (EK) (20 males and 20 females Kalang buffaloes), and South Kalimantan (SK) (20 males and 20 females Kalang buffaloes), provinces Indonesia and Phatthalung (PT) Thailand (10 males and 10 females Thale Noi buffaloes). The study was conducted for twelve months from November 2018 to October 2019. DNA extraction and mtDNA D-loop sequence analysis were performed at the laboratory of Aquaculture Genomics, Walailak University, Thailand.

DNA extraction, amplification, and sequencing

DNA from buffalo tail hair was extracted using previously established methods with slight modification by using guanidine as a buffer (Wuthisuthimethavee, 1999). 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). The oligonucleotide primers were designed using “Primer3plus” software (Primer3plus, 2018). To ensure that all codons in the D-loop region were amplified, two sets of primers were created to avoid missing codons at the beginning and the end of sequence synthesis.

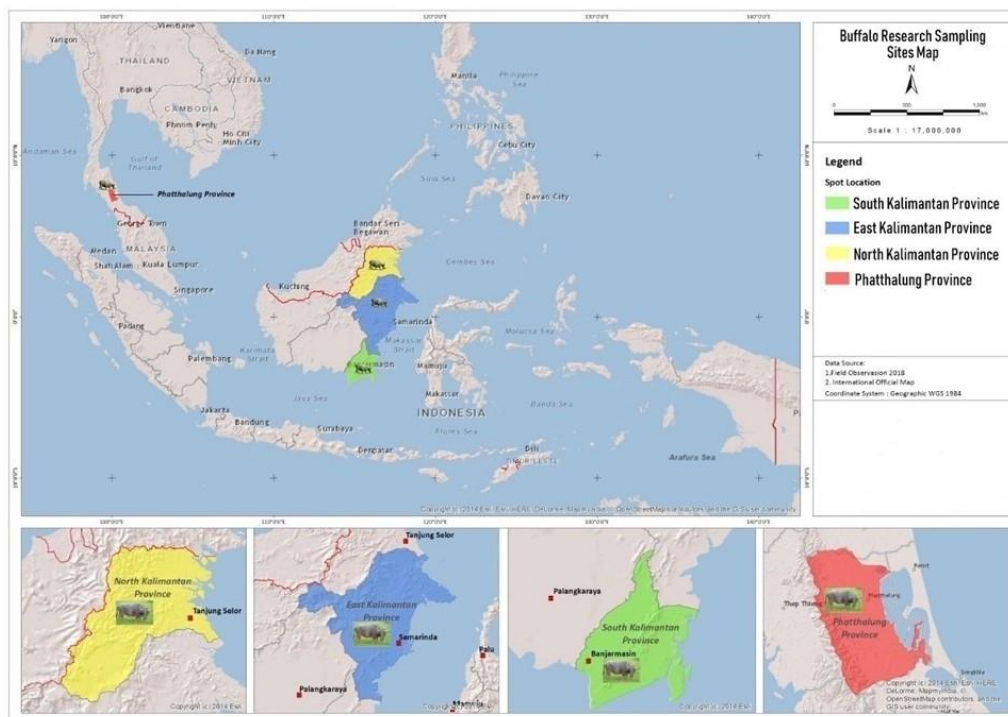


Figure 1. Buffalo sampling sites from the three provinces in Kalimantan Island, Indonesia, and Phatthalung province, Thailand. Krayan buffalo (KrBuf) from North Kalimantan province, Kalang buffalo (KBuf) from South and East Kalimantan provinces, and Thale Noi buffalo (TBuf) from Phatthalung province (ArcGis Online Basemap, 2019).

Polymerase chain reaction (PCR) was carried out using designed primer sets, Forward: (BufD-Lp.AF1) 5'-CAACACCCAAAGCTGAAGTT-3'; Reverse: (BuFD-Lp.AR1) 5'-TACCAAATGCATGACAGCAC-3'; and a second primer set Forward: (BufD-Lp.BF2) 5'-TCATCTAAAATCGCCCACTC-3'; Reverse: (BufD-Lp.BR2) 5'-CGCTCCTCTTAGTCTCGTTG-3' (Figures 2 and 3). Optimized PCR was carried out in a reaction mix containing Hot FIREPol® Blend

United Kingdom).

The mtDNA was purified using GenepHlow™ Gel/PCR Kit (Geneid, New Taipei City, Taiwan) following the manufacturer's protocol. Purified amplicons were sent to a commercial sequencing company for further analysis (BiONEER, Daejeon, South Korea).

Sequence data analysis

Sequences obtained were aligned with the mtDNA of *Bubalus bubalis* isolate YNB26 (GenBank: KX758374.1) and *Bos taurus*

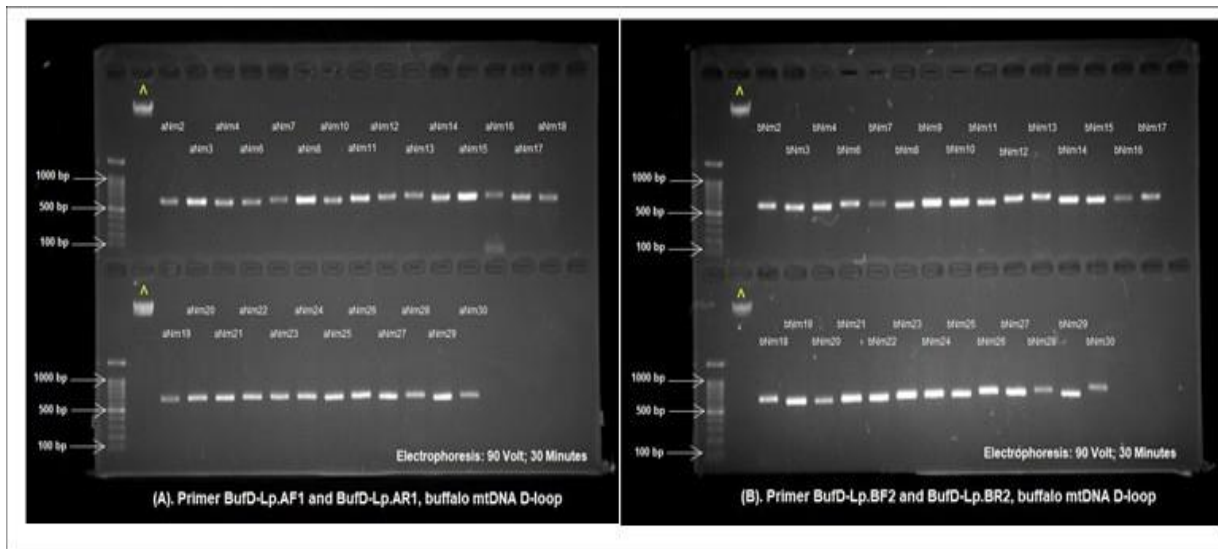


Figure 2. Buffalo sequence amplification through PCR. (A) amplification of buffalo mtDNA D-loop (647 bp) with Primer BufD-Lp.AF1-BufD and Lp.AR1; (B) amplification of buffalo mtDNA D-loop (595 bp) with Primer BufD-Lp.BF2 and BufD-Lp.BR2.

Master Mix (HOT FIREPol® DNA polymerase proofreading enzyme, 2 mM MgCl₂, 200 μM dNTPs of each, BSA) (solis BioDyne, Estonia), 0.3 μM each of the forward and reverse buffalo specific primers, 50 ng of purified DNA, and autoclaved Milli-Q water. Thermal cycling conditions were similar for each primer sets as follows: 95 °C for 15 minutes initial denaturation, 35 cycles of denaturation at 95 °C for 30 seconds, annealing temperature of 60 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes. Amplicons were visualized in 2% agarose gel stained with 6x flouoroDye (Thermo Fisher Scientific, USA), the amplified products were visualized under UV light using a gel documentation system (Syngene,

(GenBank: NC_006853.1) available in the GenBank database. Using BioEdit, the highest percent pairwise identity of the consensus sequence from each species was compared to the percent specimen similarity scores of the consensus sequence from each species within the BOLD-IDS (BOLD Identification system) (Ratnasingham and Hebert, 2007) for genetic diversity evaluation through mutation identification. The observed variables including haplotype diversity, genetic distance, and genetic tree. The neighbor-joining phylogenetic tree was constructed using the Kimura 2 Parameter tree model as suggested by PHYLIP software using 1000 replications (Felsenstein, 1989; Saitou and Nei, 1987).

ATATTAATAACTACTGGTCTTGTAAACCAGAAAAGGAGAAACAACCAACCTCCCCAAGACTCAGGGAAGAGGCTATA
 GCCCACTAC CAACACCCAAAGCTGAAGTTCTATTTAACTACTCCCTGAATACTATTAATATAGCTCCACAAAT
 GCAAAGAGCCTTCTCAGTATCAAATTCCTAACTTGAATAACTTAACTGACTTTACACTCTAGCCTAACA
 TTAGAAATAACTGCAATCATCAACACACCTGACCTCATATGTACAACACACAACATATGGCCTTACCCTCCGAA
 TGGGGGAGGCGTACATAATATTAATGTAACAAGGACATAATATGTATATAGTACATTATATATATACCCCATGC
 ATATAAGCAAGTATATAAGCATGCATGATAGTACATAGTACATTTCGATTGTTAATCGTACATAGCGCATTCAAGT
 CAAATCCGTCCTTGTCAACATGCATATCCCTCCATTAGATCACGAGCTTGACCACCATGCCGCGTGAAACCAGC
 AACCTTCAGACAGGGACCCCTCTTCTCGCTCCGGGCCATACCTTGTGGGGGTAGCTATTTAATGAACTTTAAAC
 AGACATCTGGTTCTTTCTTCAGGGCCATC TCATCTAAAAATCGCCCACTCTTTCCCCTTAAATAAGACATCTCGAT
 GGACTAATGTCTAATCAGCCCATGCTCACACATAACT GTGCTGTCATGCATTGGTATTTTTTTTATTTTGGGGGA
 TGCTTGGACTCAGCTATGGCCGTCAAAGGCCCGACCCGGAGCATGAATTGTAGCTGGACTTAACTGCATCTTGA
 GCACCAGCATAATGGTAGGCATGGGACATTACAGTCAATGGTTACAGGACATAAATATATTTATTTATTTCCCCC
 CTCCTATCAACCCCCCTTAAATACTTACCACCATTTTTAACATGCTTCTCCCTAGATGCTTATTCGAATTTAT
 CACATTTCCAATACTCAAATCAGCACTCAAATAAAGTAACTATATAAGCACCCAATCCATCACTTCACAACACA
 GTTAATGTAGCTTAAACCAAAGCAAGGCATGAAAATGCCTAGATGAGTTCTCCCAACTCCATAAACACATAGG
 TTTGGTCCCAGCCTTCTGTTAACTCTTAATAAACTTACACATGCAAGCATCCGCATCCCGGTGAGAATGCCCT
 AAGT CAACGAGACTAAGAGGAGCGGGCATCAAGCAC

1st fragment 647 bp

F1 (BufD-Lp.AF1) = CAACACCCAAAGCTGAAGTT

R1 (BufD-Lp.AR1) = TACCAAATGCATGACAGCAC

2nd fragment 595 bp

F2 (BufD-Lp.BF2) = TCATCTAAAAATCGCCCACTC

R2 (BufD-Lp.BR2) = CGCTCCTTAGTCTCGTTG

Figure 3. Two different single-round PCRs were used for the detection 956 bp of isolate YNB26 (GenBank: KX758374.1)mitochondrion D-loop region *Bubalus bubalis* (grey highlight) fragments that contained overlapping regions. The amplification using the BufD-Lp.AF1-BufD/ Lp.AR1 primers (647 bp) and the amplicons using BufD-Lp.BF2/BufD-Lp.BR2 primers (595 bp) were designed to overlap in the middle (103 bp).

RESULTS AND DISCUSSIONS

Results

A total of 140 mtDNA D-loop sequences were observed for mutations, including substitutions, deletions, and insertions, and revealed a total of 24 haplotypes. The number of mutations including substitutions (353) was caused by transitions (293), transversions (60), deletions (15), and insertions (20). The analysis of mtDNA D-loop from NK, EK, SK, and PT showed that the average nucleotide composition for T, C, A, G, A+T, and C+G were 27.51, 26.65, 30.95, 14.90, 58.45, and 41.55, respectively (Table 1). The average nucleotide composition between buffalo breeds in the present study as compared to *Bubalus bubalis* was lower compared to *Bos taurus*. It was observed that buffaloes in all the study sites of the present study have a closer genetic relationship with *Bubalus bubalis* compared to *Bos taurus*.

Based on 177 polymorphic sites, 15 deletions and 20 insertions were found in NK,

EK, SK, and PT provinces (Figure 4). Specifically, 11 indels (2 deletions and 9 insertions) in NK, 5 indels (3 deletions and 2 insertions) in EK, 9 indels (6 deletions and 3 insertions) in SK, and 9 indels (4 deletions and 6 insertions) in PT. Further, 92 substitutions were observed in NK (14 transversions and 78 transitions), 80 substitutions in EK (11 transversions and 69 transitions), 93 substitutions in SK (17 transversions and 76 transitions), and 88 substitutions in PT (18 transversions and 70 transitions) (Figure 5).

The difference in the composition of nucleotides between *Bubalus bubalis* and buffalo breeds from NK, EK, SK, and PT provinces can be seen in Table 2. The nucleotide composition of male and female EK buffaloes had the smallest percentage when compared to *Bubalus bubalis* with a difference of 4.18% and 4.29%, respectively. Male EK buffaloes compared to buffaloes from other provinces had different nucleotide base arrangements ranging from 1.25-2.40%, while female EK buffaloes compared to

Table 1. The average nucleotide composition of the buffalo mtDNA D-loop region

Buffalo Region	n	%					
		T	C	A	G	A+T	C+G
Male NK	20	27.39	26.88	31.01	14.72	58.40	41.60
Female NK	20	27.31	26.93	30.73	15.03	58.04	41.96
Male EK	20	27.50	26.55	30.95	15.01	58.45	41.55
Female EK	20	27.47	26.56	30.96	15.01	58.43	41.57
Male SK	20	27.73	26.55	31.02	14.70	58.75	41.25
Female SK	20	27.54	26.60	30.90	14.97	58.43	41.57
Male P	5	27.55	26.47	31.16	14.83	58.71	41.29
Female P	5	27.46	26.50	30.86	15.18	58.32	41.68
<i>Bubalus bubalis</i>	1	28.13	26.22	31.10	14.54	59.24	40.76
<i>Bos taurus</i>	1	28.90	24.51	32.75	13.85	61.65	38.35
Average		27.51	26.65	30.95	14.90	58.45	41.55

A: Adenine; G: Guanine; T: Thymine; C: Cytosine.

NK: North Kalimantan; EK: East Kalimantan; SK: South Kalimantan; PT: Phatthalung.

buffaloes in other provinces ranged from 1.57-2.72%. In contrast, male and female NK buffalo have the largest composition of nucleotides compared to *Bubalus bubalis*, which were 6.28% and 5.65%, respectively. Genetic diversity was observed to start at the initial D-loop nucleotide base sequence starting from site 80 bp to 938 bp.

The largest genetic distance (Kimura 2-parameter distance 0.07) based on the nucleotide sequences of mtDNA D-loop region were between *Bubalus bubalis* and male and female NK buffaloes at 4.39% and 4.14%, respectively. The

smallest genetic distance of 0% was observed between male EK with female EK buffaloes and male EK with female SK buffaloes. These results demonstrated the close relationship between buffalo breeds from EK and SK. Also, genetic distance between *Bubalus bubalis* (GenBank: KX758374.1) compared to male and female EK, male and female SK, male and female PT buffaloes were observed at 3.64%, 3.64%, 3.76%, 3.64%, 3.76%, and 3.52%, respectively (Table 3).

Phylogenetic analysis demonstrated two local buffalo clusters among KBuf, KrBuf, and

84	130	134	151	158	159	160	177	180	184	188	189	199	201	202	203	204	602	794	795	796	797	798	806	824	873	877	936	(bp)	
T	G	A	C	G	T	A	G	T	C	C	C	A	G	C	G	T	-	T	T	-	-	-	C	-	C	T	A	<i>B.b</i>	
C	T	C	A	.	.	.	A	-	T	.	.	G	.	-	.	G	.	.	.	T	T	T	.	C	.	C	.	M-NK	
C	T	C	A	.	.	.	A	C	T	.	.	G	.	-	.	G	.	.	.	T	T	T	-	C	.	C	.	F-NK	
C	T	C	.	-	-	-	A	C	T	.	.	G	-	-	-	-	.	.	.	T	.	.	-	C	T	C	.	M-SK	
C	-	C	A	C	T	.	.	G	.	-	.	G	.	.	.	T	C	.	F-SK
C	T	C	A	C	T	.	-	G	.	-	.	G	.	.	.	T	.	.	-	.	.	C	.	M-EK	
C	T	C	A	C	T	.	.	G	.	-	A	-	.	.	.	T	T	C	-	C	.	-	.	F-EK	
-	T	C	A	C	T	A	A	G	.	-	.	G	T	-	-	G	.	M-PT
-	T	C	A	C	T	A	A	G	.	-	.	G	T	-	-	G	.	F-PT

Note.

(bp) : Base pair; *B.b* : *Bubalus bubalis* (GenBank: KX758374.1); . : Identity; - : Deletion

Figure 4. Distribution of insertions and deletions in buffalo mtDNA D-loop sequences of male North Kalimantan (M-NK), female North Kalimantan (F-NK), male South Kalimantan (M-SK), female South Kalimantan (F-SK), male East Kalimantan (M-EK), female East Kalimantan (F-EK), male Phatthalung (M-PT) and female Phatthalung (F-PT) provinces.

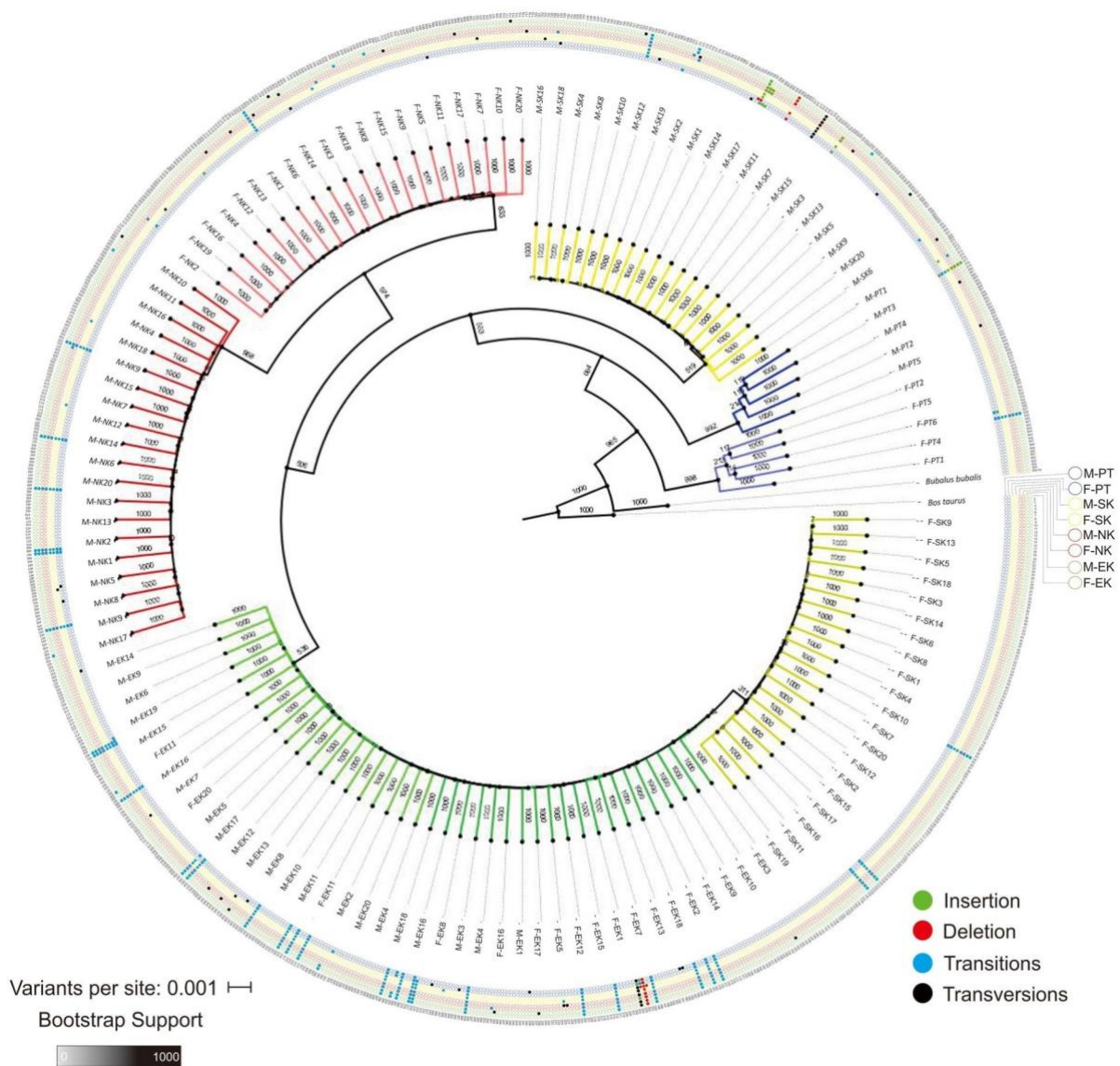


Figure 5. Genetic physical mapping of mtDNA D-loop mutations that occurred through substitutions (transitions and transversions), insertions, and deletions in male and female buffaloes from North, East, and South Kalimantan, Indonesia and Phatthalung, Thailand.

TBuf. In addition, four buffalo patterns of relationship were observed among buffalo breeds from Kalimantan Island (KBuf and K_rBuf), Indonesia. The two local buffalo clusters were the first cluster, the female buffaloes from PT and the second cluster, the male buffaloes from PT, male and female buffaloes from NK, male and female buffaloes from EK, male and female buffaloes from SK (Figure 6). The neighbor-joining tree using the Kimura 2 parameter model showed that male buffaloes from PT were closely related to male and female buffaloes from the three

provinces of Kalimantan Indonesia. The male SK buffalo cluster separated from female SK, male and female EK, and male and female NK buffaloes with a bootstrap value of 70. While female SK buffaloes were found in the same group as male and female EK buffaloes with a bootstrap value of 73. However, male and female NK buffaloes were found in a separate group with a bootstrap value of 97. Overall, based on dendrogram analysis, there were four buffalo sub-clusters in Kalimantan Island, the first sub-cluster consisted of female SK, male EK, and female EK

Table 2. Differences in the composition of buffalo nucleotides

	<i>Bubalus bubalis</i>	Male NK	Female NK	Male EK	Female EK	Male SK	Female SK	Male PT	Female PT
<i>Bubalus bubalis</i>	0								
Male NK	6.28 (60)	0							
Female NK	5.65 (54)	1.46 (14)	0						
Male EK	4.18 (40)	1.67 (16)	1.25 (12)	0					
Female EK	4.29 (41)	1.88 (16)	1.57 (15)	1.04 (10)	0				
Male SK	4.81 (46)	2.40 (23)	1.99 (19)	1.67 (16)	1.99 (19)	0			
Female SK	4.81 (46)	2.30 (22)	1.99 (19)	1.88 (18)	1.88 (18)	2.61 (25)	0		
Male PT	5.44 (52)	2.82 (27)	2.40 (23)	2.40 (23)	2.72 (26)	3.14 (30)	3.03 (29)	0	
Female PT	4.50 (43)	2.61 (25)	2.30 (22)	2.09 (20)	2.40 (23)	3.14 (30)	2.61 (25)	2.51 (24)	0

NK: North Kalimantan; EK: East Kalimantan; SK: South Kalimantan; PT: Phatthalung.

Table 3. Buffalo genetic distance based on the Kimura 2 parameter model

	<i>Bubalus bubalis</i>	Male NK	Female NK	Male EK	Female EK	Male SK	Female SK	Male PT	Female PT
<i>Bubalus bubalis</i>	0.00000								
Male NK	0.04397	0.00000							
Female NK	0.04136	0.00450	0.00000						
Male EK	0.03642	0.00677	0.00449	0.00000					
Female EK	0.03642	0.00677	0.00449	0.00000	0.00000				
Male SK	0.03764	0.00790	0.00562	0.00112	0.00112	0.00000			
Female SK	0.03642	0.00677	0.00449	0.00000	0.00000	0.00112	0.00000		
Male PT	0.03764	0.01021	0.00790	0.00562	0.00562	0.00675	0.00562	0.00000	
Female PT	0.03517	0.01251	0.01019	0.00790	0.00790	0.00604	0.00790	0.01134	0.00000

NK: North Kalimantan; EK: East Kalimantan; SK: South Kalimantan; PT: Phatthalung.

buffaloes, the second sub-cluster only included male NK buffaloes, the third sub-cluster was female NK buffalo, and the fourth sub-cluster only included male SK buffalo. The buffalo clustering from the NK, EK, SK, and PT provinces as a local buffalo group was separated from the Group *Bubalus bubalis* (GenBank: KX758374.1) and *Bos taurus* (Genbank: NC_006853.1) which were out-groups.

Discussion

Variation of nucleotides and haplotypes of the mtDNA D-loop region

The analysis of 140 mtDNA D-loop fragments of KBuf, KrBuf, and TBuf revealed 24 haplotypes which were higher compared to the 15 haplotypes observed from swamp buffaloes from Aceh, Sumatera, Riau, Banten, Central Java, West Nusa Tenggara, South Sulawesi, and riverine buffalo from North Sumatera (Saputra *et al.*, 2013), but lower compared to the 28 different haplotypes resulting from 77 polymorphic sites (Hassan *et al.*, 2009). The results of the present study demonstrated two groups of buffaloes originating from different ancestry in three provinces (NK, SK, and PT) while KBuf from EK originated from different ancestry as well. These genetic findings can be supported by phenotypic characteristics recently observed where male and female buffaloes from EK demonstrated many similarities in qualitative variables such as body appearance, skin color, coat color, horns pattern, chevron, and foot color (Suhardi *et al.*, 2020). Also, this close ancestry can be supported by minimal mutations or genetic differences found in EK buffaloes as having only one transversion (204 bp), one insertion (796 bp), one deletion (806 bp), and one transition (877 bp) (Belfield *et al.*, 2018).

The average value composition of purines and pyrimidines in the present study was 45.84% and 54.16%, respectively. The composition of the 956 bp results of multiple alignments found that the average nucleotide between A+T (58.45%) was more than G+C (41.55%) and influenced the phenotypic character of buffalo breeds (Suhardi *et al.*, 2020). The variations in nucleotide composition in the present study were imparted by mutations in the form of substitutions (transversions and transitions). These results were consistent with reports that the D-loop region accumulates nucleotide substitutions and experiences deletions, insertions, and duplication

more quickly than other regions in mtDNA and is passed to the next generation (Burgstaller *et al.*, 2014). Inheritance of mammalian mtDNA through the mother undergoes no recombination (Nagarajan *et al.*, 2015) but demonstrates a high degree of substitution (Carelli *et al.*, 2015). In mammals, the level of sub-coding (D-loop) substitution was estimated to be 2.8-5 times higher than in other places in the mitochondria and in this region, around the second domain (hypervariable region) or even faster (Carelli *et al.*, 2015).

The nucleotide composition in the buffalo mtDNA D-loop region in the present study demonstrated mutations (Figure 6). Results of the present study demonstrated unique nucleotide arrangements in the mtDNA D-loop region of KBuf and KrBuf which were imparted by deletions, insertions, and substitutions at different sites. Unique nucleotide arrangements were observed at sites 236 to 851 for NK, sites 340 to 796 for EK, and sites 201 to 898 for SK buffaloes while nucleotide sequence variation was observed at sites 188 to 877 from TBuf. These variations in nucleotide sequences in the mtDNA D-loop region have also been reported in other buffalo breeds as follows: river buffalo in Indian (Kumar De *et al.*, 2019; Nagarajan *et al.*, 2015), Thai wild water buffalo (Sarataphan *et al.*, 2017), Chinese swamp buffalo (Lau *et al.*, 1998), swamp buffaloes from Aceh, North Sumatra, Riau, Banten, Central Java, West Nusa Tenggara, South Sulawesi and riverine buffalo from North Sumatera, Indonesia (Saputra *et al.*, 2013). Overall, the results of the present study provided informative polymorphisms for discriminating between different buffalo populations which can be useful for further studies on the evolution and genetics of the KBuf, KrBuf, and TBuf breeds as well as for the documentation of the phylogenetic position of not only buffaloes in Indonesia and Thailand but also among Southeast Asian territories to ensure genetic integrity.

Genetic distance

The close relationship of local buffalo breeds as demonstrated in the present study was possibly influenced by the gene flow due to the enclosed geographical ecology. Male EK buffaloes were observed to have a close genetic distance from female SK buffaloes (0%). This is genetic evidence that supports the historical accounts of field interviews with farmers that originally inhabited SK and later on, migrated to East

Kalimantan along with their livestock decades ago (Wardani, 2007). Environmental conditions, climate, social community, and topography in EK were similar to SK, where land areas are surrounded by swamps and rivers that were optimal habitats for buffalo livestock.

In the present study, male and female KrBuf had the largest genetic distance from *Bubalus bubalis* compared to buffalo breeds from other provinces. The Krayan sub-district area of NK where the buffalo samples were collected is an isolated area, which at present is only accessible via a small aircraft (Cassa, 212) with limited capacity (15 people/flight) and flight schedules of 2-3 times per week. At present, there is no access by land and water to support transportation routes to this province and has influenced the inbreeding of buffalo livestock in the area. The challenges faced in isolated areas are increasingly exacerbated at the genetic level, where an increased effect of genetic drift and the potential for inbreeding causes low genetic variation (Gupta *et al.*, 2015). The application of artificial insemination (AI) programs to buffaloes have not been effectively implemented in this province due to the weaknesses in the service system, human

resources of artificial insemination officers (inseminator), human resources of farmers, and the difficulty of reaching remote areas. In contrast, farmers in cosmopolitan setting are quicker to adopt technological innovations such as artificial insemination because of the availability of resources and less-challenging logistics (Caffaro *et al.*, 2020). The population of the buffalo germplasm is declining rapidly due to the lack of access to proper breeding strategy, making the species very vulnerable to extinction. It is important to evaluate genetic diversity to develop a suitable breeding strategy for the conservation plan of the local buffalo breeds.

Phylogenetic analysis of mtDNA D-loop sequences

The present study demonstrated that the buffalo clustering from NK, EK, SK, and PT were separated from the *Bubalus bubalis* (GenBank: KX758374.1) and *Bos taurus* (Genbank: NC_006853.1) (Figure 6). Genetic cluster analysis demonstrated a closer relationship between male and female EK buffaloes with female SK buffaloes originating from adjacent geographical regions. These findings were

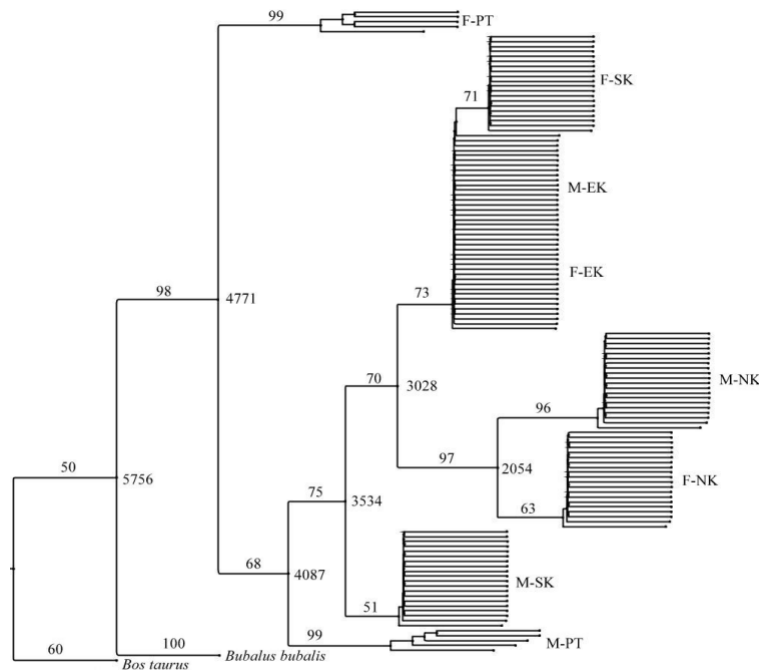


Figure 6. Neighbor-Joining phylogenetic tree of 140 sequences based on 956 nt sequences of buffalo mtDNA D-loop. The distance was computed using the Kimura 2 parameter model using 1000 replicates. M-NK: Male North Kalimantan; F-NK: Female North Kalimantan; M-EK: Male East Kalimantan; F-EK: Female East Kalimantan; M-SK: Male South Kalimantan; F-SK: Female South Kalimantan; M-PT: Male Phatthalung; F-PT: Female Phatthalung; *Bubalus bubalis* (GenBank: KX758374.1); *Bos taurus* (Genbank: NC_006853.1).

consistent with reports that swamp buffaloes in Aceh, Banten, Central Java, West Nusa Tenggara, and South Sulawesi Indonesia are very closely related to swamp buffaloes in China (Saputra *et al.*, 2013). BLAST results of buffalo sequences from the present study demonstrated maternal ancestry from *Bubalus bubalis*. The close relationships between female SK buffaloes with male and female EK buffaloes (genetic distance value 0.00093 and a nucleotide base equation of 98.12%), as being in the same sub-cluster, demonstrated common ancestry and provides further genetic evidence that supports the historical accounts of the buffalo development of the Banjar natives from SK who migrated to EK in 1918 (Wardani, 2007).

Based on the analysis of the mtDNA D-loop region, KBuf, KrBuf, and TBuf kinship to *Bubalus bubalis* provided genetic evidence on the dominant hybridization between females *Bubalus bubalis* and male buffaloes from Kalimantan Island. This indicates that most buffalo genetic material in Kalimantan Island contains genetic material from *Bubalus bubalis*. Sex differences also presented differ greatly in genetic trees compared to differences in the location or origin of buffalo, for example of buffaloes M-PT and F-PT, and M-SK and F-SK (Figure 6). It was showed differences in ancestry between male and female buffaloes even though the buffaloes originate from the same province, due to buffalo migration, genetic drift and the implementation of an artificial insemination program (Sayres, 2018; Kumar *et al.*, 2019). The mtDNA is a marker for maternal genealogy (haploids) which in buffaloes can be used to define the history of herds (Gupta *et al.*, 2015). The results obtained in the present study demonstrated that KBuf, in particular, the males and females from EK and females from SK have a very close relationship. These findings support the hypothesis that domestication of swamp buffaloes in China spread with rice cultivation through two separate routes (a) through Taiwan, to the Philippines, and the eastern islands of Kalimantan and Sulawesi, (b) south through mainland southeast Asia (likely interbreeding with wild buffalo) to peninsular Malaysia and on to the western island of Sumatra and Java (Lau *et al.*, 1998).

The analysis of mtDNA D-loop sequences of buffaloes from NK, EK, SK, and PT provided genetic data that are useful for effective conservation programs of domestic buffaloes. The genetic data is important in facilitating and

maintaining high-quality breeds and managing the genetic resources of biodiversity for buffalo breeding development through selection, pedigree recording, reproductive efficiency, crossbreeding, and genetic modification. Most of the domestic buffalo populations are not unique and might be inter-crossed throughout their geographical distribution. However, the polymorphisms identified here from the hypervariable D-loop region could be linked to the identified haplotypes to discriminate the various buffalo populations. Nevertheless, more molecular studies are required to further improve breed quality and manage the genetic resources of buffalo livestock. To clarify a “unique sequence”, it is necessary to analyze more samples from local swamp buffaloes in other provinces in Indonesia and Thailand.

Since genetic diversity within Kalang, Krayan, and Thale Noi buffaloes was found to be relatively high, this is a good basis to perform selection and breeding development through pedigree recording, reproductive efficiency, crossbreeding, and genetic modification to produce high-quality breeding stock for the buffalo farming industry. The translational application of these findings and perspectives, respective of buffalo breeds, will be beneficial to breeding and effective conservation programs of domestic buffaloes, as well as the maintenance, monitoring, and potential enhancement of the buffalo genetic resource using a sustainable framework for optimal benefits in terms of economy and local biodiversity.

CONCLUSION

The genetic diversity of KBuf and KrBuf in Indonesia and TBuf in Thailand demonstrated high genetic diversity. The phylogenetic analysis of sequences demonstrated two local buffalo clusters among KBuf, KrBuf, and TBuf. There were four patterns of relationship observed among buffalo breeds from Kalimantan, Indonesia. This genetic diversity among local buffalo breeds can be beneficial for the buffalo farming industry through the enhancement of buffalo genetic resources and local biodiversity.

ACKNOWLEDGMENTS

This research was supported by the Graduate Studies Research Fund of Walailak University, Thailand (No. 15/2562) and the Directorate-General for Higher Education, Republic of

Indonesia (Ref. HDE/EDU/1203). We are grateful to The Livestock and Animal Health Services, Provincial Government of North, East and South Kalimantan, Indonesia, and Phatthalung, Thailand for the assistance in literature, data gathering, and conceptualization of real-life scenarios of buffalo farmer concerns. We also thank the anonymous referees for their helpful comments.

REFERENCES

- Belfield, E. J., Z. J. Ding, F. J. C. Jamieson, A. M. Visscher, S. J. Zheng, A. Mithani and N. P. Harberd. 2018. DNA mismatch repair preferentially protects genes from mutation. *Genome Res.* 28(1): 66–74.
- Burgstaller, J. P., I. G. Johnston, N. S. Jones, J. Albrechtova, T. Kolbe, C. Vogl, A. Futschik, C. Mayrhofer, D. Klein, S. Sabitzer, M. Blattner, C. Guilly, J. Poulton, T. Rulicke, J. Pialek, R. Steinborn and G. Brem. 2014. MtDNA segregation in heteroplasmic tissues is common in vivo and modulated by haplotype differences and developmental stage. *Cell Reports.* 7(6): 2031–2041.
- Caffaro, F., M. M. Cremasco, R. Roccato and E. Cavallo. 2020. Drivers of farmers' intention to adopt technological innovations in Italy: The role of information sources, perceived usefulness, and perceived ease of use. *J. Rural Stud.* 76(1): 264-271.
- Carelli, V., A. Maresca, L. Caporali, S. Trifunov, C. Zanna and M. Rugolo. 2015. Mitochondria: biogenesis and mitophagy balance in segregation and clonal expansion of mitochondrial DNA mutations. *Int. J. Biochem. Cell Biol.* 63(1): 21-24.
- Director General of Livestock Services. 2003. National report on animal genetic resources Indonesia. A Strategic Policy Document. FAO.
- El Debaky, H. A., N. A. Kutchy, A. Ul-Husna, R. Indriastuti, S. Akhter, B. Purwantara and E. Memili. 2019. Review: Potential of water buffalo in world agriculture: Challenges and opportunities. *Appl. Anim. Sci.* 35(2): 255-268.
- Escarcha, J. F., J. A. Lassa, E. P. Palacpac and K. K. Zander. 2018. Understanding climate change impacts on water buffalo production through farmers' perceptions. *Journal of Clim. Risk Manag.* 20(1): 50-63.
- FAO. 2020. Agricultural system, Thale Noi Wetland, Phatthalung Province. Ministry of Agriculture and Cooperatives, Thailand. Retrieved from <http://www.fao.org/country-showcase/item-detail/en/c/1276972/> (accessed 15 April 2020).
- Felsenstein, J. 1989. PHYLIP: Phylogeny inference package (Version 3.2). *Cladistics.* 5: 164-166.
- Gupta, A., A. Bhardwaj, Supriya, P. Sharma, Y. Pal, Mamta and S. Kumar. 2015. Mitochondrial DNA- a Tool for Phylogenetic and Biodiversity Search in Equines. *J. Biodivers. Endanger. Species.* S1(6): 1-8.
- Hassan, A. A. M., E. A. Balabel, H. A. S. Oraby and S.A. Darwish. 2018. Buffalo species identification and delineation using genetic barcoding markers. *J. Genet. Eng. Biotechnol.* 16(2): 499-505.
- Komariah., Kartiarso and M. Lita. 2014. Productivity of swamp buffalo in Muara Muntai subdistric, Kutai Kartanegara regency, East Kalimantan. *Bul. Pet.* 38(3): 174-181.
- Kumar De, A., P. Ponraji, D. Malakar, R. Muthiyar, A. Kundu and D. Bhattacharya. 2019. Complete mitogenome sequencing of Andaman buffalo: an endangered germplasm of Andaman and Nicobar Islands, India. *J. Genet.* 98(97): 1-8.
- Lau, C.H., R. D. Drinkwater, K. Yusoff, S. G. Tan, D. J. Hetzel and J. S. Barker. 1998. Genetic diversity of Asian water buffalo (*Bubalus bubalis*): mitochondrial DNA D-loop and cytochrome b sequence variation. *Anim. Genet.* 29(4): 253-264.
- Nagarajan, M., K. Nimisha and S. Kumar. 2015. Mitochondrial DNA variability of domestic river buffalo (*Bubalus bubalis*) populations: Genetic evidence for domestication of river buffalo in Indian subcontinent. *Genome Biol. Evol.* 7(5): 1252-1259.
- Natalia, L., Suhardono and A. Priadi. 2006. Swamp buffalo in South Kalimantan: problem, disease and control. *Wartazoa.* 16(4): 206-215.
- Nguyen, A.H.L., S. Tiawsirisup and M. Kaewthamasorn. 2020. Low level of genetic diversity and high occurrence of vector-borne protozoa in water buffaloes in Thailand based on 18S ribosomal RNA and mitochondrial cytochrome b genes. *Infect. Genet. Evol.* 82(1):104304.
- Primer3plus. 2018. Primer design. Retrieved from <https://primer3plus.com/cgi-bin/dev/primer3plus.cgi>.
- Ratnasingham, S and P. D. N. Hebert. 2007.

- BOLD: The Barcode of Life Data system. *Mol. Ecol. Notes.* 7(3): 355-364.
- Ruihua, Z., J. Ping, S. Chuanbo, S. Deyong, Z. Feng and H. Chaochao. 2018. The analysis of genetic variation in the mitochondrial genome and its application for the identification of *Papilio* species. *Mitochondrial DNA Part B.* 3(2): 687-690.
- Rusdin, M., D. D. Solihin, A. Gunawan, C. Talib and C. Sumantri. 2020. Genetic variation of eight Indonesian swamp-buffalo populations based on cytochrome b gene marker. *Trop. Anim. Sci. J.* 43(1):1-10.
- Saitou, N and M. Nei. 1987. The neighbor-joining method: A new method for reconstruction of phylogenetic trees. *Mol. Biol. Evol.* 4(4): 406-425.
- Saputra, F., Jakaria, A. Anggraeni and C. Sumantri. 2020. Genetic diversity of Indonesian swamp buffalo based on microsatellite markers. *Trop. Anim. Sci. J.* 43(3):191-196.
- Saputra, F., Jakaria and C. Sumantri. 2013. Genetic variation of mtDNA cytochrome oxidase subunit I (COI) in local swamp buffaloes in Indonesia. *Med. Pet.* 36(3): 165-170.
- Sarataphan, N., W. Narongwanichgarn and S. Maneerat. 2013. Phylogenetic analysis of a Thai wild water buffalo (*Bubalus arnee*) through mitochondrial control region. *Int. J. Conserv. Sci.* 8(1): 105-112.
- Sayres, M. A. W. 2018. Genetic diversity on the sex chromosomes. *Genome Biol. Evol.* 10(4): 1064-1078.
- Suhardi., P. Sumppunn, M. Duangjinda and S. Wuthisuthimethavee. 2020. Phenotypic diversity characterization of Kalang and Thale Noi Buffalo (*Bubalus bubalis*) in Indonesia and Thailand: Perspectives for the buffalo breeding development. *Biodiversitas.* 21(1): 5128-5137.
- Wardani. 2007. Madam ka banua urang: beberapa catatan awal tentang migrasi suku Banjar, proses, dan penyebarannya. *Jurnal Kebudayaan.* 14(5): 1- 75.
- Wuthisuthimethavee, S. 1999. DNA marker technologies for biodiversity study in shrimp. Master's Thesis, Kasetsart University, Bangkok. Thailand.
- Yacoub, H.A and M. M. Fathi. 2013. Phylogenetic analysis using D-loop marker of mtDNA of Saudi native chicken strains. *Mitochondrial DNA.* 24(5): 538-551.
- Yusnizar, Y., M. Wilbe, A. O. Herlino, C. Sumantri, R. R. Noor, A. Boediono, L. Andersson and G. Andersson. 2015. Microphthalmia-associated transcription factor mutations are associated with white-spotted coat color in swamp buffalo. *Anim. Genet.* 46(6): 676-682.