J. Agric. Sci., Tokyo Univ. Agric., 66 (3), 65–74 (2021)
東京農大農学集報，66（3），65–74（2021）

Genetic Divergence and Phylogenetic Relationship of Philippine Carabao (*Bubalus bubalis*) and Other Swamp Buffalo Populations in Neighboring Countries Revealed by the Mitochondrial DNA D-loop Region

By

Lilian P. VILLAMOR*, Koh NOMURA**, Takashi AMANO*** and Yukimizu TAKAHASHI**†

(Received May 20, 2021/Accepted September 14, 2021)

Summary : The swamp buffalo (*Bubalus bubalis*) provides a major source of draft power in cultivating rice farms and has great potential for meat, milk, and hide sources, particularly to smallhold farmers. To date, there is limited published information on mtDNA D-loop sequence variation, genetic divergence, and genetic relationship between the Philippine carabao and other swamp buffalo populations in Asia. Thus, the objective of the study is to determine the phylogenetic relationship among the swamp buffalo populations with Asiatic origins. Dataset construction and data analyses were performed from 307-bp of 367 mtDNA D-loop sequences of swamp buffaloes from the Philippines and Asian countries which were retrieved from NCBI GenBank. The research findings highlighted the genetic relationship among the modern Asian swamp buffalo populations and could be explained in two points : First, the Philippine carabao (native swamp buffalo), particularly the Visayas and Mindanao populations, had the closest affinity to Taiwan swamp buffalo population based on the low pair-wise distance (FST), and, second, the Chinese swamp buffalo could be the ancestral population of the modern population of the swamp buffaloes in Asia, as inferred by mtDNA haplotype phylogenetic tree. The results of this research agreed with the hypothesis of LAU *et al.* 1998 that after the domestication in the China region, the domesticated swamp buffalo spread with rice farming into Taiwan to the Philippines and the eastern islands of Borneo and Sulawesi. This study revealed that native buffaloes in the Philippines have various genes of buffaloes of which numbers are declining in Southeast Asian countries and showed that it is necessary to conserve and maintain them as valuable genetic resources while utilizing them in the Philippines.

Key words : genetic differentiation, mtDNA D loop, native carabao, phylogenetic relationship

1. Introduction

The domestic swamp buffalo (*Bubalus bubalis*) is widespread and contributes tremendous economic importance to several Asian countries. This species is widely distributed in the Philippines, China, Laos, Thailand, Taiwan, Vietnam, Indonesia, Myanmar, and Malaysia[1–6]. The swamp buffalo provides a major source of draft power in cultivating rice farms and has great potential for meat, milk, and hide sources, particularly to smallhold farmers[3, 7]. In the Philippines, the swamp buffalo is considered the national animal and is locally recognized as the Philippine carabao (PC) or native carabao. The Philippine buffalo population between 1998 and 2018 had decreased by 130, 345 heads and at a rate of −0.216% yearly (Retrieved from http://www.fao.org /faostat/en/#data/QA on 21 January

---

* Department of Agriculture, Philippine Carabao Center-National Headquarters and Gene Pool, Science City of Munoz, Nueva Ecija, 3120 Philippines
** Department of Animal Science, Faculty of Agriculture, Tokyo University of Agriculture
*** Professor Emeritus, Tokyo University of Agriculture
† Corresponding author（E-mail : y2takaha@nodai.ac.jp）

2021)[8]. These animals are non-descript between river or swamp buffalo and commonly raised in the backyard or at commercial farms. Thus, this becomes part of the priority to conserve and manage the water buffaloes, particularly the swamp buffaloes, across the major island groups in the Philippines. The archipelagic features of the Philippines consist of Luzon, Visayas, and Mindanao, which are situated in the northern, central, and southern parts of the country, respectively. Luzon is considered the largest island group, the Visayas is the third major island group, and the Mindanao is the second-largest landmass group of the country in terms of land area (https://www.britanicca.com/place/Philippines).

Previous studies reported the genetic diversity, phylogenetic, and phylogeography analyses of swamp buffaloes in Asia using diverse mitochondrial DNA (mtDNA) markers. For instance, genetic diversity of mitochondrial cytochrome b (cyt b) and mtDNA D-loop genes in Chinese native buffalo reported the two maternal lineages A and B[1, 4, 9, 10]. The closer genetic affinity between the Indonesian and Philippine swamp buffalo groups was affirmed based on the cytochrome b (cyt b) gene partial sequences[5]. In another study, the Malaysian water buffaloes and their crossbreds showed their close relationship to swamp maternal lineage based on the mtDNA D-loop[6].

The PC population genetic divergence and phylogenetic analyses within the major island sub-groups and between the neighboring countries have little published information and are still unidentified. The mtDNA cytochrome c oxidase I (COI) gene was informative to confirm the species identification of buffaloes in Calayan Island in Cagayan as swamp buffaloes and appropriate for selecting this animal for conservation and management in the PC sanctuary[12]. Previous studies showed that mtDNA D-loop sequences of Asian buffaloes, including the Philippine swamp buffaloes, originated from China[9]. The data obtained from other related studies showed the closer affinity of Chinese swamp buffaloes to Philippine swamp buffaloes, which were identified in maternal lineage A[11, 13]. However, the recent findings on the Philippine swamp buffalo mtDNA D-loop sequence variation first reported the detection of the two haplotypes in lineage B, which were traced from the two populations in Luzon and Visayas[14]. All these studies were good baseline information which provided a glimpse of the genetic relationship of swamp buffaloes in Asian countries. However, it is still unresolved to understand the spread of domesticated Chinese swamp buffaloes to the major islands of the Philippines. Thus, the objective of the study is to determine the genetic differentiation and phylogenetic history of the modern Philippines swamp buffaloes with selected swamp buffalo populations in Asia using the mtDNA D-loop sequences.

2. Materials and Methods

2.1 Sample Collection

A total of 376 D-loop sequences of swamp buffaloes from the Philippines and Asian countries were retrieved from NCBI GenBank. The 107 sequences from Philippine carabao previously submitted to NCBI GenBank (MT642457–MT642563) were retrieved and included in the analysis. These samples were grouped within the original areas of the sub-populations in Luzon, Visayas, and Mindanao. The detailed information of the samples and the downstream molecular analysis were described in VILLAMOR *et al.*[14]. A further 269 reference D-loop sequences were also retrieved from NCBI GenBank (Table 1). These were represented by swamp buffaloes from the Asia region, including China, Myanmar, Laos, Thailand, Vietnam, Taiwan, and Malaysia[6, 9, 10, 15, 16]. Additional D-loop sequences from two river buffaloes were included in the analysis to be used as outgroups (AF197209.1 and AF475218.1).

2.2 Dataset construction and Data Analyses

Sequence editing, alignment, and model test of 307 bp of 367 mtDNA D-loop PCR amplified fragments were conducted using MEGA version X[17]. Insertions/deletions in the aligned sequences were excluded in the analyses. The software Arlequin version 3.1[18] was conducted to (1) perform an Analysis of Molecular Variance (AMOVA), (2) calculate the population pair-wise FST, and (3) calculate the population-specific FST indices. The criterion for genetic differentiation (FST) by Wright (1978) was used to define the varying degrees of FST, as low for $FST < 0.05$, moderate for $0.05 < FST < 0.15$, high for $0.15 < FST < 0.25$, and very high for $FST > 0.25$. Inferred phylogenetic tree using Neighbour-joining (NJ) and UPGMA methods were constructed based on population differentiation (FST) using the using MEGA version X[17]. Haplotype diversity (*h*) and nucleotide diversity (*Pi*) were computed using DnaSP v6 program[19]. All the analyses were performed in two levels : the PC sub-populations and the neighboring Asian swamp buffalo populations.

3. Results

3.1 Philippine Carabao populations in Major Island Sub-groups

AMOVA analysis at the PC sub-populations or the island groups showed significant genetic differentiation of 3.10% among the populations and 96.90% within the populations ($P < 0.05$) (Table 2). The significant population-specific FST indices had an average of 0.031 and obtained the highest in Luzon at 0.063, followed by Mindanao at 0.061, and the least in the Visayas at 0.055. The phyloge-

Table 1 Sample information of 269 Asian swamp buffaloes retrieved from NCBI Genbank included in the analysis

NCBI Genbank Accession No.	Country of Origin	References
EF053531.1-EF053552.1	China	Lai *et al*. 2007
DQ364160.1-DQ364189.1; DQ658051.1-DQ658139.1	China	Lei *et al*. 2007
KX758297.1; KX758299.1-KX758301.1; KX758301.1; KX758304.1; KX758310.1; KX758315.1-KX758316.1; KX758323.1-KX758324.1; KX758332.1-KX758336.1; KX758352.1-KX758353.1; KX758355.1-KX758355.1- KX758358.1; KX758362.1-KX758363.1; KX758366.1- KX758369.1; KX758373.1; KX758375.1; KX758378.1; KX758382.1; KX758384.1-KX758389.1; KX758391.1-KX758392.1; KX758394.1; KX758396.1-KX758398.1; KX758402.1	China	Wang *et al*. 2017
MH746466.1-MH746475.1; MH746481.1	Malaysia	Shaari *et al.* 2019
KX758305.1-KX758309.1; KX758314.1; KX758317.1-KX758319.1; KX758325.1-KX758326.1; KX758337.1-KX758340.1; KX758359.1-KX758361.1; KX758371.1; KX758376.1; KX758383.1; KX758390.1; KX758395.1	Laos	Wang et al. 2017
KX758311-KX758313; KX758320-KX758321; KX758349-KX758351; KX758354; KX758364-KX758365; KX758370; KX758372; KX758377; KX758393	Vietnam	Wang *et al*. 2017
KX758322.1; KX758329.1-KX758331.1; KX758342.1-KX758348.1; KX758399.1-KX758401.1	Thailand	Wang *et al*. 2017
KR008940.1-KR008957.1	Taiwan	Zhang et al. 2016
KX758327-KX758328; KX758341	Myanmar	Wang *et al*. 2017

Table 2 AMOVA among Philippine swamp buffalo sub-populations.

Among populations	2	6.518	0.051	3.10
Within populations	104	165.407	1.590	96.90
Total	375	171.925	1.641	

netic relationship using the NJ method based on the genetic distance (FST) showed the separation of populations between the swamp buffaloes in Luzon and those in Visayas and Mindanao (Fig. 1). The result of NJ tree was concordant with the topology and relationship of swamp buffaloes among the major islands obtained using the UPGMA method (data not shown).

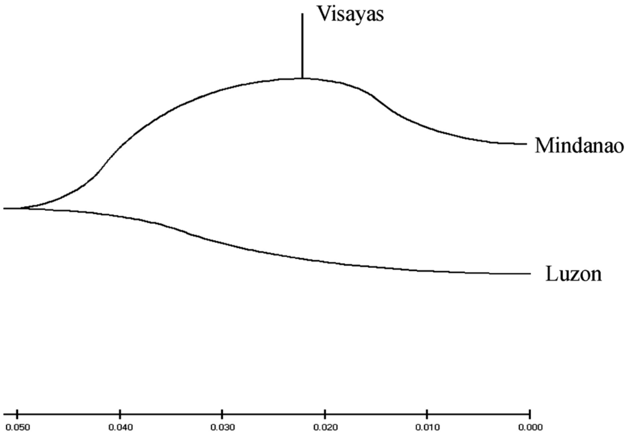

Fig. 1 Unrooted Neighbour-joining tree using the genetic distance (FST) for Philippine swamp buffaloes from major island sub-groups

3.2 Asian Swamp Buffaloes

AMOVA analysis among the swamp buffalo sub-populations in the Asia region showed significant genetic variation of 10.45% among the populations and 89.55% within the populations ($P<0.05$) (Table 3). Population-specific FST indices among countries had an average of 0.101, ranging from the highest 0.122 in Myanmar and Malaysia to the lowest 0.093 in Vietnam. FST index 0.118 in Taiwan was second to the highest and followed by 0.113 in the Philippines. However, the Philippines

Table 3 AMOVA among Asian swamp buffalo populations.

Source of variation	Sum d.f.	Sum of squares	Variance components	Percentage of Variation
Among populations	7	86.465	0.278	10.45
Within populations	368	877.708	2.385	89.55
Total	375	964.173	2.663	

Table 4 Pair-wise distance among Asian swamp buffalo populations.

Population	Philippines	China	Myanmar	Laos	Thailand	Vietnam	Taiwan	Malaysia
Philippines	-							
China	0.104	-						
Myanmar	-0.101	-0.005	-					
Laos	0.190	-0.012	0.029	-				
Thailand	0.254	0.103	0.073	0.066	-			
Vietnam	0.308	0.055	0.106	-0.002	0.051	-		
Taiwan	0.029	0.103	-0.089	0.170	0.249	0.263	-	
Malaysia	0.215	0.096	0.352	0.121	0.232	0.252	0.284	-

Fst P values <0.0500 (Underline)

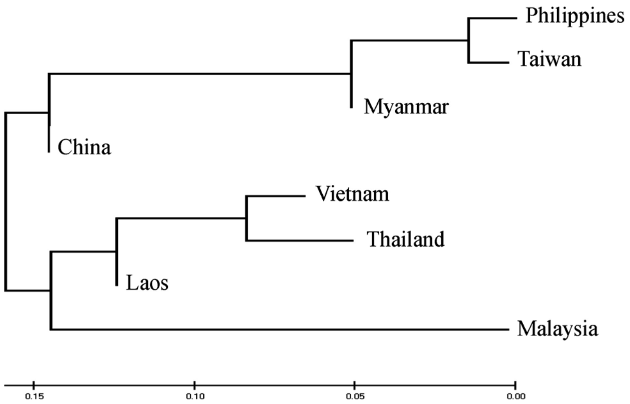

Fig. 2 Neighbour-joining tree for eight Asian swamp buffalo populations, based on average pair-wise distance (FST) between populations.

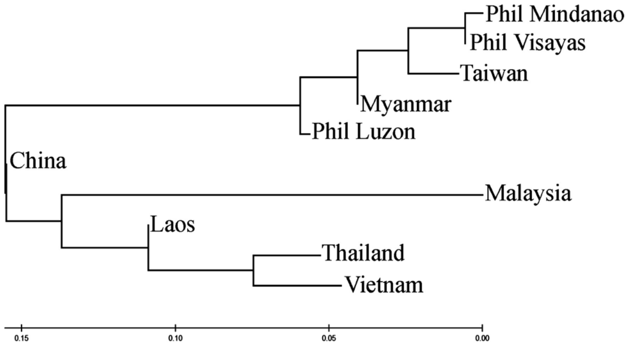

Fig. 3 Neighbour-joining tree for Philippine swamp buffalo from major island sub-groups and Asia region, based on average pair-wise distance (FST) between populations.

indicated higher FST indices than those of 0.103 in Thailand, 0.099 in China, 0.096 in Laos, and 0.092 in Vietnam.

The values of population pair-wise FST ranged from −0.005 to 0.352. Out of 28 iterations, 17 significant populations pair-wise differences (FST, $P < 0.050$) were detected and ranged from relatively low to very high genetic variations (Table 4). The highest significant pair-wise FST value (0.308) was indicated between the Philippines and Vietnam, suggesting that swamp buffaloes living in these countries had very high genetic differentiation. Conversely, the pair-wise FST value (0.029) between the Philippines and Taiwan suggested a relatively low genetic differentiation. The non-significant pair-wise FST values between Laos-China, Laos-Thailand, Vietnam-Thailand, and Vietnam-Laos populations indicated that swamp buffaloes from these countries had the absence of genetic differentiation. However, Myanmar was the only population that revealed non-significant FST values, showing a lack of genetic differentiation among swamp buffaloes from other Asian countries included in the study.

The phylogenetic relationship was analyzed in the NJ method and showed two main clusters. The inferred tree formed Cluster 1 that showed closer affinity among Philippines, Taiwan, Myanmar, and China while Cluster 2 entailed closer affinity among Vietnam, Thailand, Laos, and Malaysia (Fig. 2). The evolutionary history of the Philippines' three major islands, including Luzon, Visayas, and Mindanao, together with other Asian origins, confirmed further the formation of two clusters (Fig. 3). Cluster 1 consisted mostly of the three major islands in the Philippines, Myanmar, and Taiwan, while Cluster 2 was found predominantly from the mainland populations encompassing China, Malaysia, Laos, Thailand, and Vietnam. The topology of the phylogenetic trees was congruent using the NJ (Fig. 3) and UPGMA (data not shown) methods.

3.3 Genetic Diversity in Asian countries

The 376 sequences of swamp buffaloes from the Philippines and neighboring countries revealed 39 haplotypes with 23 polymorphic nucleotide sites (Table 5). Haplotype and nucleotide diversities were highest in Vietnam at 0.810 and 0.010, with the lowest in Malaysia at 0.473 and 0.002, respectively. The Philippines had higher haplotypes and nucleotide diversities than those in Taiwan, Myanmar, and Malaysia. Focusing on the eight Asian swamp populations revealed uneven distribution of haplotypes. China had 24 haplotypes, followed by 17 haplotypes for the Philippines and less than ten haplotypes for other Asian countries. The frequency of unique haplotypes detected 17 in China, 11 in the Philippines, two in Laos, and one in Malaysia (Table 6 & Fig.4).

Table 5 Source and genetic diversity indices of Asian swamp buffaloes in mtDNA D-loop.

Country	No of Sequences	No. of Haplotypes	S	*h*	SD	*Pi*	SD
Overall	376	39	23	0.748	0.022	0.007	0.010
Philippines	107	17	13	0.704	0.041	0.004	0.007
China	185	24	18	0.719	0.033	0.008	0.009
Laos	23	9	12	0.794	0.076	0.009	0.009
Vietnam	15	5	8	0.810	0.059	0.010	0.007
Thailand	14	5	7	0.725	0.104	0.007	0.006
Taiwan	18	5	7	0.660	0.102	0.003	0.006
Myanmar	3	2	2	0.667	0.314	0.004	0.004
Malaysia	11	3	2	0.473	0.162	0.002	0.002

^{S} Polymorphic sites; ^{h}Haplotype diversity; ^{Pi} Nucleotide Diversity; ^{SD} Standard Deviation

Table 6 Sources information on lineages and frequency of haplotype distribution of swamp buffaloes from Asia region.

Lineage	A	A	A	A	A	A	A	A	B	B	B	A	A	B	A	B	B	A	B	A	B	A
Haplotype No.	SW 1	SW 2	SW 3	SW 4	SW 5	SW 6	SW 7	SW 8	SW 9	SW 10	SW 11	SW 12	SW 13	SW 14	SW 15	SW 16	SW 17	SW 18	SW 19	SW 20	SW 21	SW 22
Country																						
No. of Sequences	179	36	7	1	2	23	1	2	1	30	1	2	3	29	7	3	2	1	1	4	1	1
Philippines	54	21	7	1	2	4	1	2	1	2	0	0	0	0	2	0	0	0	0	1	0	0
Taiwan	12	4	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Thailand	0	2	0	0	0	1	0	0	0	3	0	0	0	0	1	0	0	0	0	0	0	0
Myanmar	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vietnam	5	0	0	0	0	3	0	0	0	2	0	0	0	4	0	0	0	0	0	0	0	0
Laos	10	1	0	0	0	3	0	0	0	2	0	0	0	3	0	0	0	0	0	0	0	0
Malaysia	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
China	93	8	0	0	0	10	0	0	0	20	1	2	3	22	4	3	2	1	1	3	1	1

Table 6 Continuation

Lineage	A	A	A	A	B	A	A	B	B	B	C	E	A	A	A	A	A			
Haplotype No.	SW 23	SW 24	SW 25	SW 26	SW 27	SW 28	SW 29	SW 30	SW 31	SW 32	SW 33	SW 34	SW 35	SW 36	SW 37	SW 38	SW 39	A*	B**	C***
Country																				
No. of Sequences	1	1	1	2	1	2	9	2	1	1	2	1	1	2	3	1	8			
Philippines	0	0	0	0	0	2	0	0	0	0	0	0	1	2	3	1	0	11	107	17
Taiwan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	4
Thailand	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	14	5
Myanmar	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2
Vietnam	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	15	5
Laos	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	2	23	9
Malaysia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	1	11	2
China	1	1	1	2	1	0	1	0	0	0	2	1	0	0	0	0	0	17	185	24

* No. of Unique Haplotype, ** No. of sequences, *** No. of Haplotype

3.4 NJ Tree Phylogenetic Relationship

The phylogeny analysis of D-loop sequences obtained from the Philippine swamp buffaloes and retrieved from the NCBI GenBank confirmed the separation between the swamp and riverine groups (Fig. 5). The phylogenetic tree of Asian swamp buffaloes confirmed four maternal lineages, A, B, C, and E (Fig. 5). Moreover, the NJ tree showed delineation between two lineages, A and B, but with low bootstrap support (PB = 14%). The majority of sequences (80%, 300/376) were included in lineage A, and the minority of the sequences were observed in lineage B (19%, 73/376). Few sequences were observed in lineage C (0.5%, 2/376) and lineage E (0.3%, 1/376). Twenty-five of the 39 haplotypes were incorporated in lineage A (PB = 11%), which entailed SW1-SW8, SW12-SW13, SW18, SW20, SW22-SW26, SW28-SW29, SW35-SW39. Among the group of haplotypes, SW39 clustered separately, with meager statistical support (PB = 33%). On the other hand, 12 haplotypes belonged to lineage B (PB = 27%), which showed a closer affinity among haplotypes SW9-SW11, SW14, SW16-17, SW19, SW21, SW27, and SW30-SW32. However, the groupings of the haplotypes (SW) that belonged to maternal lineages A and B were affirmed by the pair-wise difference (FST = 0.532 ; ($P < 0.000$), implying a very high genetic differentiation between the two lineages (data not shown). The groupings of swamp haplotypes were also confirmed by the reference sequences obtained from the NCBI GenBank from the previous

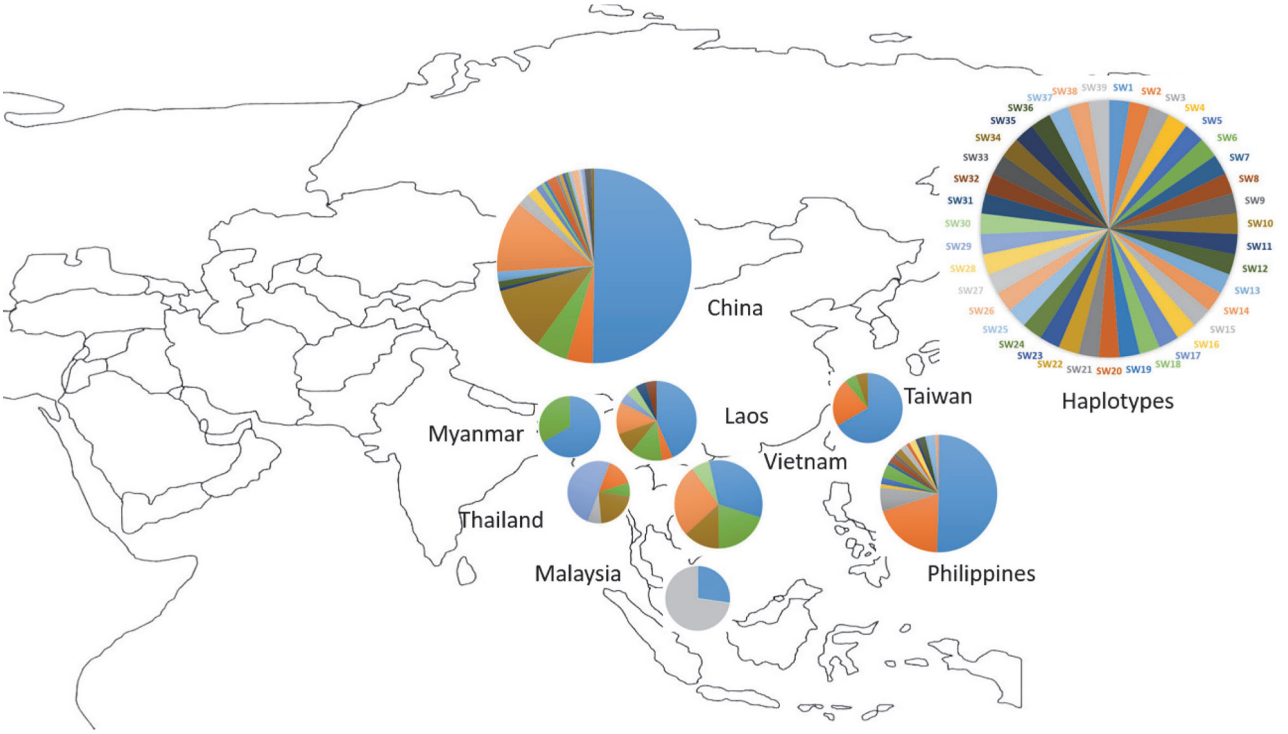

Fig. 4 Map showing the frequency of geographic distribution of the 39 haplotypes from Asian swamp buffaloes. (Asian map retrieved from https://whatsanswer.com/world-map/blank-map-of-asia-printable-outline-map-of-asia/)

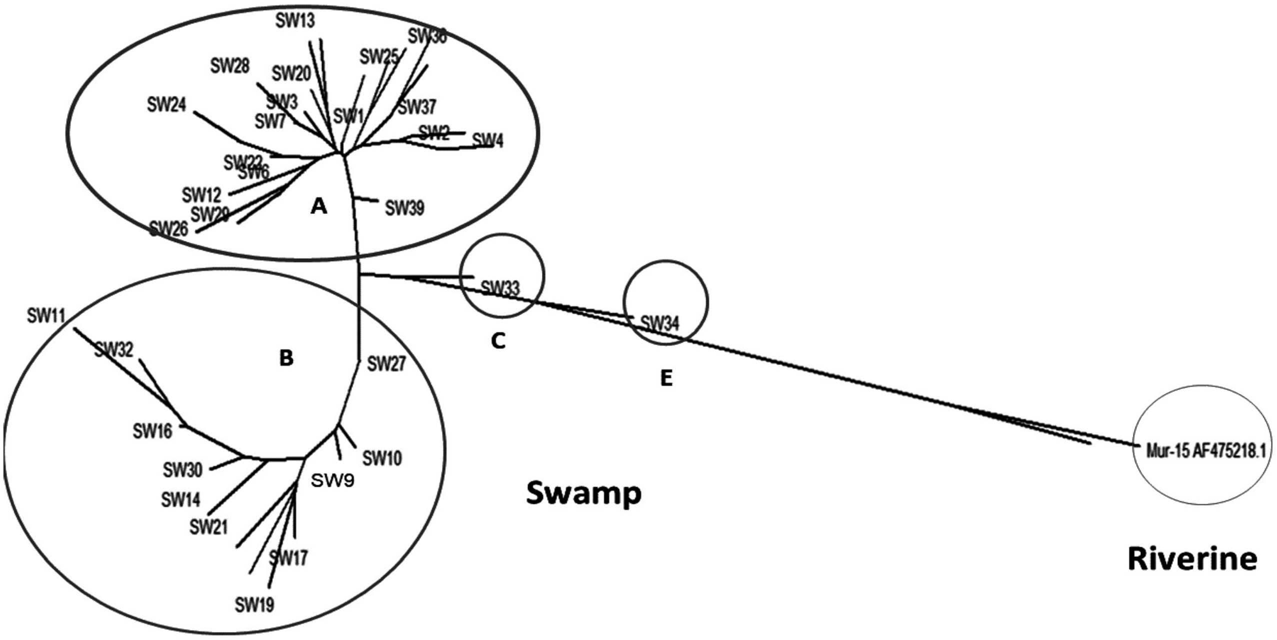

Fig. 5 Neighbour-joining tree for 39 mtDNA D-loop region haplotypes of swamp buffaloes from selected Asia region, with the riverine sequences as outgroups.

studies on Asian swamp buffaloes[11, 14, 15, 16] (Table 1). The other rare haplotypes SW33 in lineage C and SW34 in lineage E were clustered separately from lineages A and B and showed recent divergence from the other swamp haplotypes.

4. Discussion

The molecular variance analysis for mtDNA D-loop sequences among the Philippine swamp buffaloes implied that the genetic variation was attributed by the differences between individuals rather than among populations. The

AMOVA from this study showed reasonable agreement with previous reports on the Philippine carabao haplotype network that little population differentiation existed. The unique haplotypes in the Philippines were also previously reported but they were determined in small numbers[14)]. It would be interesting to include additional samples from other populations of the Philippines in future studies. For instance, Palawan Island in Luzon and Tawi-Tawi Island in Mindanao, which have been the central ports for settlers and serve as the hub of transporting goods and passengers, could be more likely be genetically differentiated. However, the genetic data to support this hypothesis is lacking.

The average population-specific FST index of the modern Philippine carabao populations obtained from this present study indicated low genetic differentiation. This finding was concordant with the topology of inferred phylogenetic relationship of PC's sub-group of major islands obtained from this study (Fig. 1). The low genetic differentiation of PC obtained from this study could be attributed to lack of population pair-wise difference (FST $= -0.014$, $P < 0.050$) between Visayas and Mindanao, indicating the high genetic exchange of swamp buffaloes living in these sub-groups of major islands[14)]. However, a closer look at the population-specific FST indices at the PC sub-populations from this present study indicated moderate genetic variation within the three sub-groups of major islands. Thus, the highest genetic differentiation within Luzon indicated swamp species' restricted gene flow due to geographical features of the island surrounded by the mountainous and coastal areas.

The AMOVA showed higher genetic variation obtained in Asia region swamp buffalo populations than Philippine carabao population, but most of the differences in the former populations could be described by the variation between individuals rather than among populations. While the unique haplotypes were detected in China, Philippines, Laos, and Malaysia, they were found in small numbers and were obtained from the three most numerous haplotypes. This implied that more individuals from Asian regions like Cambodia and Indonesia would be needed to be certain that low population differentiation is not an artefact of sampling from the Asia populations.

The genetic divergence (FST) explained further the genetic differentiation of Asian region. The population pair-wise differentiation elucidated the closer affinity of swamp buffaloes between the Philippines and Taiwan than in China. Moreover, among the Philippines' major islands, there was a very low genetic variation detected between Visayas and Mindanao in the Philippines and Taiwan. This shed light on the closer relationship between the Philippine carabao's modern population in Visayas and Mindanao and Taiwan than in Luzon and China. In contrast, the moderate and significant genetic differentiation between PC in Luzon and Taiwan implied the closer affinity between the two populations compared with China. In this analysis, Taiwan swamp buffaloes were obtained from an old population from the conservation sites[16)]. The relatively low genetic divergence (FST) obtained from this study did not agree with the results obtained by BARKER *et al.*[20)], which stated that one of the dispersal routes of the domestication of swamp buffalo from mainland Asia followed from China through the Philippines to the eastern islands of Borneo and Sulawesi. In fact, in other study, the inferred phylogenetic tree based on mtDNA D-loop sequences of PC from the major island groups of the Philippines was confirmed to be closely related to the Chinese swamp buffaloes[13)]. The hypothesis that swamp buffalo of the Philippines originated from China was further supported by the mtDNA D-loop sequences and mitogenomic data from the previous studies[21–23)]. However, the results of the genetic divergence and phylogenetic relationship of this research indicated otherwise. Our results agreed with the hypothesis by LAU *et al.*[1)] that after domestication in the China region, the domesticated Chinese swamp buffaloes had the first dispersal pathway through Taiwan and the Philippines to the eastern islands of Borneo and Sulawesi[1, 9)] .

In contrast, a lack of genetic differentiation of Myanmar swamp buffaloes among other Asian countries included in this study could indicate a high gene flow of swamp buffaloes from Myanmar to other neighboring countries. This genetic pattern can be partially elucidated by the geographical positioning of Myanmar as a Southeast Asian nation bordered by various countries and linked to historical events of human migration together with their swamp buffaloes. The additional research findings are pertinent to understand the relationship of swamp buffaloes from Myanmar and with other neighboring countries.

The mtDNA haplotype analysis confirmed the previous reports on the two maternal lineages A and B in swamp buffalo haplotypes[1, 9–11)]. YUE *et al.*[11)] previously reported that no sequences in the Philippines and Thailand fell into lineage B. In contrast, the present study highlighted that Philippines, Taiwan, Thailand, Vietnam, and Laos had haplotypes which were incorporated to maternal lineage B. The undetected haplotypes from the previous studies could be attributed to the limited samples and sampling sites from other Asiatic origins since the previous study focused mainly on the mtDNA D-loop sequences of Chinese swamp buffaloes. It would also be worth mentioning that rare haplotypes from Chinese buffaloes were detected and fell to the maternal lineages C and E, which was previously identified by WANG *et al.*[15)]. This study also reported that the Philippine swamp buffaloes predominantly fell in lineage A, very little in lineage B, and none in lineages C and E, which was consistent with the previous study on PC mtDNA genetic diversity[14]. However, three sequences of swamp buffaloes from Northeast Thailand and South Thailand, which were previously reported to belong to lineage D based on the mitogenomes from the study of Wang *et al.* 2017[15], all fell to haplotype SW10 in Lineage B of this study. The low resolution between haplotypes in Lineage D and Lineage B, which failed to delineate, could imply the need for additional representative samples to confirm the relationship of rare haplotypes to predominant haplotypes in maternal lineages A and B.

The predominant haplotype SW1 in lineage A could be considered the ancestral haplotype. In fact, SW1 had the highest frequency of intermixing of the individuals from all Asian countries except Thailand. The present study that indicated one individual or shared by only a small number of samples could be attributed to restricted gene flow among some swamp buffalo sub-populations, including the Philippines, China, Laos, and Malaysia. The presence of unique haplotypes was common in breeds within the mtDNA variation, according to the previous study of Naderi *et al.*[24].

The other pertinent findings from the mtDNA haplotype analysis explained the pattern and frequency of the swamp buffaloes' haplotype distribution across the Asian neighboring populations. The Philippines shared the highest haplotypes with China, followed by Laos. These research findings could be attributed to the bias by number of swamp buffaloes and sampling sites derived particularly from China and Philippines, which could contribute to a higher number of haplotypes and haplotype diversity. However, the inferred phylogenetic tree based on the mtDNA haplotypes showed that the two unique haplotypes SW34 and SW33 from China had the earliest divergence from the clusters of swamp buffaloes and were the closest to the riverine buffaloes. In lineage B, haplotype SW27 had a closer relationship to haplotype SW9, which had an individual source from Capiz in the Philippines' western Visayas. An earlier study of Lau *et al.*[1] indicated a pattern of differentiation in the phylogenetic tree, wherein three haplotypes were detected and traced predominantly in the eastern island populations of the Philippines. From this study, the Philippine swamp populations from the Visayas and Mindanao sub-group of major islands seemed to be closely related to Taiwan swamp buffaloes and suggested an extensive level of gene flow. However, the high genetic exchange of swamp buffaloes between the two major islands could be explained by two perspectives: first, the history of early migration of farmers from the Visayas to Mindanao, who brought with them their native swamp buffaloes for rice cultivation and second, it would be possible that access within and between inter-island transportation could cause genetic exchange of swamp buffaloes among sub-populations in the Philippines. Furthermore, this study supported the genetic analysis that showed a lack of genetic differentiation between Visayas and Mindanao, suggesting that swamp buffaloes are introduced farm animals in Mindanao[14, 25]. Thus, the spread of domesticated Chinese swamp buffaloes from Taiwan to the Philippines would probably be introduced to the Visayas rather than Mindanao Island.

The research findings highlighted the phylogenetic history among the modern Asian swamp buffalo populations and could be explained in two points : (1) Philippine carabao had the closest affinity to Taiwan swamp buffalo population based on the low pair-wise distance (FST) and (2) Chinese swamp buffalo could be the ancestral population of the modern population of the swamp buffaloes in Asia, which was spread to Taiwan then introduced to the Visayas island of the Philippines, as supported by inferred mtDNA haplotypes.

Future studies will compare these Asian swamp buffalo mtDNA D-loop sequences to Asian wild buffalos and would consider the utility of other polymorphic mtDNA markers such as cyt b to gain a better perspective on phylogenetic history of Philippine carabao.

5. Conclusion

The mtDNA D-loop sequences of swamp buffaloes were informative to establish the genetic divergence and elucidate the relationship of swamp buffaloes in eight Asian populations. Low genetic divergence between the PC sub-populations in Visayas and Mindanao and Taiwan shed light on the most likely route of the swamp buffalo's introduction and spread in the Philippines. Also, the dataset supported China as the most likely ancestor of the modern swamp buffalo populations in Asia. This will provide essential insights on phylogenetic relationship of Asian swamp buffaloes which will have implications for the utilization, conservation, and management of the swamp buffalo genetic resources of the swamp buffaloes with Asiatic origins.

Acknowledgement

All the authors would like to extend gratitude to the Philippine Carabao Center National Headquarters and Gene Pool (PCC-NHQGP), the Tokyo University of Agriculture-Southeast Asian Regional Center for Graduate Study and Research in Agriculture (Tokyo NODAI-SEARCA) Scholarship for Dissertation Doctorate Program, Laboratory of Animal Genetics and Breeding in Tokyo

University of Agriculture Atsugi Campus (TUA), and the Department of Agriculture Livestock Biotech Program and the Bureau of Agricultural Research through the DABIOTECHR1506 project for the research funds.

References

1) Lau CH, Drinkwater RD, Yusoff K, Tan SG, Hetzel DJ, Barker JS. (1998) Genetic diversity of Asian water buffalo (Bubalus bubalis) : mitochondrial DNA D-loop and cytochrome b sequence variation. *Anim. Genet.* **29** : 253–264.

2) Kierstein G, Vallinoto M, Silva A, Schneider MP, Iannuzzil, Brenig B. (2004) Analysis of mitochondrial D-loop region casts new light on domestic water buffalo (Bubalus bubalis) phylogeny. *Mol. Phylogenet. Evol.* **30** : 308–324.

3) Cruz LC. (2015) Institutionalization of swamp buffalo development in the Philippines. *Proceedings of International Seminar on Improving Tropical Animal Production for Food Security ; 2015 November 3–5 Southeast Sulawesi, Indonesia* : 15–37.

4) Lei CZ, Zhang CM, Weining S, Campana MG, Bower MA, Zhang XM, Liu L, Lan XY, Chen H. (2011) Genetic diversity of mitochondrial cytochrome b gene in Chinese native buffalo. *Anim. Genet.* **42** : 432–436.

5) Winaya A, Sukri A, Gofur A, Amin M. (2019) The genetic divergence and phylogenetic relationship of Indonesia swamp buffalo (Bubalus bubalis) based on partial sequences of cytochrome b gene of mitochondrial DNA. *Int. J. Eng. Technol.* **8** : 96–100.

6) Shaari NAL, Jaoi-Edward M, Loo SS, Salisi MS, Yusoff R, Ab Ghani NI, Saad MZ, Ahmad H. (2019) Karyotypic and mtDNA based characterization of Malaysian water buffalo. *BMC Genet.* **20** : 37.

7) Villamor LP. (2015) Conserving Animal Genetic Diversity to adapt to Climate Change in the Philippines. In : Learning and Coping with Change : Case Stories of Climate Change Adaptation in Southeast Asia. SEARCA Philippines. 235 p.

8) Food and Agriculture Organization of the United Nations (FAO) FAOSTAT, http://www.fao.org/faostat/en/#data/QA (Last Access January 21 2021)

9) Lei CZ, Zhang W, Chen H, Lu F, Ge QL, Liu RY, Dang RH, Yao YY, Yao LB, Lu ZF, Zhao ZL. (2007a) Two maternal lineages revealed by mitochondrial DNA Dloop sequences in Chinese native water buffaloes (Bubalus Bubalis). *Asian-Aust. J. Anim. Sci.* **20** : 471–476.

10) Lei CZ, Zhang W, Chen H, Lu F, Liu RY, Yang XY, Zhang HC, Liu ZG, Yao LB, Lu ZF, Zhao ZL. (2007b) Independent maternal origin of Chinese swamp buffalo (Bubalus bubalis). *Anim. Genet.* **38** : 97-102.

11) Yue XP, Li R, Xie WM, Xu P, Chang TC, Liu L, Cheng F, Zhang RF, Lan XY, Chen H, Lei CZ. (2013) Phylogeography and domestication of Chinese swamp buffalo. *PLoS One* **8** : e56552.

12) Paraguas AM, Cailipan TPC, Flores EB, Villamor LP. (2018) Morphology and phylogeny of swamp buffaloes (Bubalus bubalis) in Calayan Island, Cagayan. *Philipp. J. Vet. Anim. Sci.* **44** : 59–67.

13) Del Barrio LAM, Dela Vina CB, Yebron JR. MGN, Estrella CAS, Herrera JRV, Del Barrio AN. (2009) Molecular characterization of the Philippine carabao (Bubalus bubalis L.) from the major island groups of the Philippines using molecular cloning and sequence analysis of the D-loop of mitochondrial DNA. [BS thesis]. Los Baños, Laguna : University of the Philippines-Los Baños. 75. (Available at the UPLB library).

14) Villamor LP., TakahashI Y., Nomura K., Amano T. (2021) Genetic Diversity of Philippine Carabao (Bubalus bubalis) using Mitochondrial DNA D-loop Variation : Implications to Conservation and Management. *Philipp. J. Sci.* **150** : 839–848.

15) Wang, S, Chen N, Capodiferro MR, Zhang T, Lancioni H, Zhang H, Miao Y, Chanthakhoun V, Wanapat M, Yindee M, Zhang Y, Lu H, Caporali L, Dang R, Huang Y, Lan X, Plath M, Chen H, Lenstra JA, Achilli A. Lei C. (2017) Whole Mitogenomes Reveal the History of Swamp Buffalo: Initially Shaped by Glacial Periods and Eventually Modelled By Domestication. *Sci. Rep.* **7** : 4708.

16) Zhang Y, Lu Y, Yindee M, Li K-Y, Kuo H-Y, Ju Y-T, Barker JSF. (2016). Strong and Stable Geographic Differentiation of Swamp Buffalo Maternal and Paternal Lineages Indicates Domestication in the China/Indochina Border Region. *Mol. Ecol.* **25** : 1530–1550.

17) Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (2018) MEGA X : Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* **35**: 1547–1549.

18) Excoffier L, Lischer HE. (2010) Arlequin suite ver 3.5 : a new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour* **10** : 564–567.

19) Rozas J, Ferrer-Mata A, Sánchez-Delbarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A. (2017) DnaSP 6 : DNA sequence polymorphism analysis of large datasets. *Mol. Biol. Evol.* **34** : 3299–3302.

20) Barker J.S.F., Moore S.S., Hetzel D.J.S., Evans D., Tan S.G., Byrne K. (1997) Genetic diversity of Asian water buffalo (*Bubalus bubalis*) : microsatellite variation and a comparison with protein-coding loci. *Anim. Genet.* **28** : 103–115.

21) Zhang Y, Vankan D, Zhang Y, Barker JS. (2011) Genetic differentiation of water buffalo (*Bubalus bubalis*) populations in China, Nepal and south-east Asia : inferences on the region of domestication of the swamp buffalo. *Anim. Genet.* **42** : 366–77.

22) Licia C *et al.* (2018) New Insights on Water Buffalo Genomic Diversity and Post-Domestication Migration Routes From Medium Density SNP Chip Data. *Front. Genet.* **9** : Article 53.

23) Zhang K, Lenstra JA, Zhang S, Liu W, Liu J. (2020) Evolution and domestication of the Bovini species. *Anim. Genet.* **51** : 637–657.

24) Naderi S, Rezaei HR, Taberlet P, Zundel S, Rafat SA, Naghash HR, El-Barody MA, Ertugrul O, Pompanon F. (2007) Large-scale mitochondrial DNA analysis of the domestic goat reveals six haplogroups with high diversity. *PLoS One* **2** : e1012

25) Pendleton RL. (1942) Land Utilization and Agriculture of Mindanao, Philippine Islands. *Geogr. Rev.* **32** : 180–210.

ミトコンドリアDNA D-loop領域から明らかにされたフィリピンおよびその周辺諸国スイギュウ集団の遺伝的分化と系統遺伝学的関係

Lilian P. VILLAMOR*・野村こう**・天野　卓***・髙橋幸水**†

（令和3年5月20日受付/令和3年9月14日受理）

要約：沼沢スイギュウ（*Bubalus bubalis*）は，小規模な稲作農家の役畜として大変重要であり，乳，肉および皮も活用されている。これまでにフィリピンとその他周辺諸国スイギュウの遺伝的分化や系統遺伝学的関係は明確にされていない。本研究は，フィリピンで飼養されているスイギュウ集団と中国を含むインドシナ半島集団間の遺伝的分化と系統遺伝学的関係を明らかにすることを目的とした。367頭のミトコンドリアDNA D-loop領域の塩基配列を用い解析を行った。その結果，遺伝的分化の程度を示す指数からフィリピンのVisayasおよびMindanao集団は，台湾集団と近いことが推定された。また，系統樹からもフィリピンおよび台湾集団は近い関係を示し，ミャンマーおよび中国集団と同一のクラスターを形成した。これまでに報告された仮説と一致していることから，中国から台湾，フィリピンへスイギュウが伝わってきたことが推察された。本研究は，頭数が減少しつつある東南アジアのスイギュウの多様な遺伝子をフィリピンスイギュウが保有していることから，国内で利活用しながら貴重な遺伝資源として保全・維持する必要があることを示した。

キーワード：遺伝的分化，ミトコンドリアDNA D-loop，在来スイギュウ，系統遺伝関係

---

[*] Department of Agriculture, Philippine Carabao Center-National Headquarters and Gene Pool, Science City of Munoz, Nueva Ecija, 3120 Philippines

[**] 東京農業大学農学部動物科学科

[***] 東京農業大学名誉教授

[†] Corresponding author（E-mail : y2takaha@nodai.ac.jp）