Research Article

Genetic Diversity of Native Buffalo Populations in Vietnam Based on Mitochondrial D-Loop Nucleotide Sequence

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Abstract | This study aimed to better understand the genetic diversity of native buffalo populations in different regions of Vietnam based on nucleotide sequence in a displacement loop (D-loop) of mitochondrial DNA (mtDNA). Thirty-one blood samples that were collected from three native buffalo populations such as Bao Yen (BY: 7 samples), LangBiang (LB: 7 samples) and Thanh Chuong (TC: 7 samples) and two imported buffaloes from Thailand (T: 5 samples) and India (Murrah – M: 5 samples) were extracted for total DNA and amplified about 760 bp mtDNA fragment, with 576 bp in the D-loop region, followed by sequencing to analyze genetic diverse indices and genetic distance to construct a phylogenetic tree. Results showed that the fragment of 760 bp was successfully amplified. Variations of nucleotide sequence in 576 bp fragment length from 31 individual buffaloes were analyzed and revealed the nucleotide composition as Adenine (A) = 32.6%, Thymine (T) = 26.8 %, Guanine (G) = 14.7% and Cytosine (C) = 25.9%. A total of 108 nucleotide polymorphic sites and 26 haplotypes were observed. Nucleotide and haplotype diversity index (*π and Hd*) were 0.06267 and 0.987, respectively. Genetic distance among swamp buffalo populations was smaller (ranging 0.050-0.056) than between Murrah and the swamp buffalo populations (ranging 0.091-0.125). Among the three native Vietnamese buffalo groups, the genetic distance between Bao Yen and LangBiang (0.050) buffalo populations was smaller than between Thanh Chuong and Bao Yen (0.055) or LangBiang (0.056). The phylogenetic tree showed that domestic buffalo populations are separated into two clusters that could be distinguished from the Murrah population. In conclusion, the genetic variation on Vietnamese domestic buffaloes is higher than other Asian swamp buffaloes. Most of native buffalo populations cluster in one clade and have a genetic relationship closer to Thai and Philippine swamp buffaloes in maternal origin. Further insights regarding the genetic diversity of Vietnamese native buffaloes will require more in-depth studies.

Keywords | *Bubalus bubalis*, D-loop sequence, Genetic divergence, Mitochondrial DNA, Swamp buffalo

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INTRODUCTION

Buffaloes are raised in many countries in the world and used for multiple purposes including meat, milk, and agronomical works. Buffaloes are divided into two types based on behavior, morphology, ecology, cytology and molecular genetic attributes as river buffalo and swamp buffalo (Cockrill, 1981; Kumar et al., 2007). The swamp buffaloes are found in many countries in Asia spreading from India to China while river buffaloes are widely distributed throughout Eastern Europe, the subcontinent of India, Northern Africa or the Middle-East area (Cockrill, 1981). Vietnamese native swamp buffaloes play an important role in agriculture. They were domesticated in Vietnam in ancient times and became a traditional symbol of Vietnamese culture. In Southeast Asia, buffaloes are kept by smallholders for multiple purposes including draught power, meat, and other by-products (fertilizer, leather). During the past decade, the Vietnamese native swamp buffalo population has declined due to (i) increasing farming mechanization, (ii) low productivity, especially in reproduction. To better understand domestic animal genetic sources, studies of animal husbandry breeds have mainly focused on estimating the relationships among breeds (Barker et al., 1997) to control both preservation and development (Martin-Burriel et al., 1999). Furthermore, understanding of the characterizations of animals such as behaviour, morphology or molecular genetic is one of the critical step for genetic preservation and development procedures (Martin-Burriel et al., 1999). Molecular analysis is an important tool to identify the polymorphism of genome or mtDNA and is now being increasingly used to figure out diversity and evolution (Navani et al., 2001). Genetic divergence profiles are affected by the changes in living environments resulted in allowing species to survive through adaptations (Yusnizar et al., 2015) and can also result in improved genetically transmitted traits (Hassan et al., 2018). The mtDNA has been used to obtain information in order to identify the original species at the molecular levels. The D-loop is a non-coding region, and acts as a promoter for both the heavy and light strands of the mtDNA, and contains essential transcription and replication elements and the evolution in other regions of the mtDNA is lower than in the D-loop (Sharma et al., 2005). Moreover, mtDNA sequences; particularly in the D-loop, have been applied to analyze the phylogeny for over last decades (Moore, 1995). A modification of D-loop sequence was used to identify genetic differences among buffalo types in Southeast Asia or Brazil and Italy (Lau et al., 1998; Kierstein et al., 2004).

The mtDNA markers are impacted through the genetic flow of mammalian females and are acknowledged as key elements to understanding the connection between current genetic structural population and the whole variation of genetic sources (Ruihua et al., 2018). High variation of D-loop region in mtDNA has been the focus of the change in genetic variation research owing to their high mutation rate (Yacoub and Fathi, 2013). Genetic diversity and phylogenetic analyses of swamp buffaloes using variable regions in mtDNA markers have been reported in many previous studies in Asia, and mtDNA is regarded as an important maternal material for analyzing genetic diversity in term of genetic source management, genetic characterization, as well as livestock conservation policies (Lau et al., 1998; Kierstein et al., 2004; Lei et al., 2011; Sayres, 2018; Shaari et al., 2019; Winaya et al., 2019).

In Vietnam, scarce published data exists regarding the genetic diversity of native swamp buffaloes. While, the D-loop sequence in native Vietnam-based buffalo has never been investigated. Therefore, this is the first study in Vietnam aimed to analyze the diversity of genetic and reconstruct a phylogenetic tree for native buffalo breeds.

MATERIALS AND METHODS

Sample collection and DNA extraction

All procedures involved in the handling and caretaking of animals were approved by the Faculty of Animal Science and Veterinary of Nong lam University (NLU-20200106). The buffaloes were handled in accordance with good animal practice and all efforts were made to minimize the stress. The samples are collected at the different households in each sampled area without pedigree information; the age of sampled buffaloes is about more than three years old.

Thirty-one whole blood samples were individually collected from jugular vein and stored at 4 °C in tube with EDTA before transportation to the laboratory, in which 21 samples collected in the three populations of native swamp buffaloes, 7 samples each, as Lang Biang (LB) in Lam Dong Province, Thanh Chuong (TC) in Nghe An Province and Bao Yen (BY) in Lao Cai Province, together with 5 samples each from imported Murrah (M) and Thai (T) swamp buffaloes raised at the Ruminant Research and Development Center, Binh Duong Province (Figure 1). Total DNA extraction was conducted using a GeneJET Whole Blood Genomic DNA Purification Mini Kit according the manufacturer's instructions, extracted DNA were measured OD value using Bio-drop machine (UK) and stored at -80 °C until used.

Primer design

A set of primers (T760) was designed using Primer3 software (Version 4.1.0) based on the sequence from GenBank with access number AY488491.1. The forward primer was 5'- AATACCAACGGCCAGCATAA -3' and the reverse primer was 5'- GAGCATGGGCTGATTAGACA. The

forward primer started from the Cytb region and the reverse primer started in the D-loop region with 760 bp in fragment length, containing 50 bp in Cytb, 134 bp in two tRNAs (Thereonine and Proline) and 567 bp in D-loop.

DNA amplification and sequencing

Amplification of the fragment was done using the Polymerase Chain Reaction (PCR) with the thermocycler machine (MasterCycler Pro S; Eppendorf, Germany). The amplified reaction was performed at a volume of 25 µL, consisting of 2 µL DNA templates (50 ng/µL), 1 µL (10 pM each) primers (Phu Sa, Vietnam), 12.5 µL of My Taq™ Mix 2X (Bioline, UK) and then add the water (ddH_2O) up to 50µL. The PCR process was operated with 35 cycles consisting of (1) 95°C for 4 min, (2) 95°C for 30', (3) 59°C for 30', (4) 72°C for 30', (5) repeated from 2-4 for 35 cycles and (6) 72°C for 5 min. After electrophoresis, the products of PCR were then observed using 1.5% agarose gel (30 min, 100V) with a 100 bp DNA ladder (Thermo). The PCR products were purified and sequenced directly by 1st BASE Sequence Company (Malaysia).

Data analysis

The D-loop sequences were aligned with the selected sequence of D-loop mtDNA from the database in genbank (AY488491.1) using BioEdit (Version 7.2.5), after alignment and adjustment, the nucleotide sequences out of D-loop were subtracted and discarded. The part of sequences with about 576 bp in the D-loop region was used for further analysis. Nucleotide haplotype diversity was calculated using DNA Sequence Polymorphism (Version 6.12.03 x64). The genetic distance and phylogenetic tree using D-loop mtDNA sequences (576 bp) of 21 Vietnamese native swamp buffaloes, 5 Thai swamp buffaloes and 5 Murrah buffaloes were estimated using MEGAX software (Version 10.2.5). Five mtDNA D-loop sequences retrieved from GenBank (MT186741.1; AY488491.1; KU687004.1; FJ873678.1; NC049568.1*)* were used as references for in groups and one for *Bos taurus* (NC006853.1) was applied as the out group to construct the phylogenetic tree.

RESULTS AND DISCUSSION

Nucleotide and haplotype diversity

After sequencing, the results were subjected to alignment and then subtracted the part sequence out of the D-loop, thirty-one sequences with 576 bp in the D-loop region were then used for analysis, and an average nucleotide composition as shown in the Table 1. Average nucleotide compositions for A, T, G and C were 32.63, 26.81, 14.67 and 25.89%. The percentage of A+T was 59.44% and C+G was 40.56%. The trend of low G+C content in this study was also observed and reported in other buffalo breeds in Thailand (Suhardi et al., 2021), Egypt (Youssef et al., 2021) and Pakistan (Babar et al., 2011) or other small ruminants (Ganbold et al., 2020; Nguyen et al., 2022). Beside this, Shaari et al. (2019) found that the A+T content in. The trend of A+T bias in mtDNA D-loop is a strong evidence for nucleotide variations and mutation in D-loop, indicating the base A occurs most often and base G the least in control region of mtDNA of mammalian species (Parma et al ., 2004).

Nucleotide polymorphism was analyzed, with results presented in the Figure 2 and Table 2. A total of 108 polymorphic sites were found, with mutations caused by transition (67), transversion (25), deletion (5) and insertion (8). Two polymorphic sites (247 and 433) were found in all buffaloes. While 24 polymorphic sites were only found in Vietnamese native buffaloes (19; 30; 61; 108; 146; 152; 153; 160; 161; 164; 176; 196; 253; 270; 313; 340; 352; 354; 358; 368; 430; 463; 479; 535), 2 polymorphic sites were found in Thai buffaloes (163; 353) and one site was found only in Murrah buffaloes. Twenty-two polymorphic sites were found in both Vietnamese and Thai buffaloes (19; 30; 61; 108; 146; 152; 153; 161; 164; 176; 196; 253; 270; 313; 340; 352; 354; 358; 368; 430; 479; 535). Suhadi et al. (2021) reported 140 sequences from D-loop elucidated for 24 haplotypes. The sites of mutation by transition and transversion were 293 and 60, respectively and caused by insertions and deletions were 20 and 15. The variations of the fragment sequence in the D-loop of 123 individual buffaloes affirmed 40 singleton sites with 52 haplotypes, and the ratio of transition-to-transversion was a strongly bias toward transition, which is obviously an indicator of mitochondrial D-loop evolution in mammals (Babar et al., 2011). Many studies also stated the trend of strong bias toward transitions is a distinguishing of mitochondrial DNA evolution, as observed in buffaloes (Lau et al., 1998; Kierstein et al., 2004; Lei et al., 2007a; Raungprim et al., 2021), and in other mammal species or chickens (Liu et al., 2004; Chen et al., 2005; Guo et al., 2005; Lai et al., 2006).

Nucleotide diversity (π), haplotype and haplotype diversity (Hd) of three Vietnamese swamp buffalo populations and two types of imported buffaloes were evaluated, and presented in the Table 3. The haplotype diversity (Hd = 1.00) was high in Bao Yen (BY), LangBiang (LB) and Thai (T) buffalo types, then lower (Hd = 0.952) in Thanh Chuong (TC) and lowest (Hd = 0.800) in Murrah buffaloes. The nucleotide diversity (π) was highest recorded in TC (0.05619) and LB (0.05372) buffaloes, with lower values in BY (0.04671) and T (0.01683) and lowest (π = 0.00191) was found in Murrah buffaloes. The diverse values of nucleotide (π) and haplotype (Hd) were 0.06267 and 0.987, respectively. Investigation of 30 individuals from 6 buffalo types in China, the results showed average diversity index

of nucleotide and haplotype were 0.00684 and 0.798, respectively, indicating the plentiful genetic divergence of buffalo populations in China (Lei et al., 2007a,b).

Average haplotype diversity indicated abundant genetic divergence in the three Vietnamese native buffalo groups in this study, comparable to diversity of swamp buffalo genetic in Asian countries (Lau et al., 1998; Yue et al., 2013; Villamor et al., 2021). However, average nucleotide diversity obtained from the current study of Vietnamese native buffaloes was higher (π = 0.062) than reported by previous studies (π = 0.007-0.049) (Lei et al., 2007a, 2011; Yue et al., 2013; Villamor et al., 2021).

Comparing the DNA sequences from 31 individual buffaloes revealed 26 different haplotypes (Table 4) with a haplotype diversity (Hd) of 0.987±0.00015 (Table 3). Two buffaloes shared H-1 (TC1; BY4), three buffaloes shared H-3 (TC3; TC5; LB4), two Murrah buffaloes shared H-24 (M1; M2) and another two shared H-26 (M4; M5). Swamp buffaloes in this study showed higher diversity than Murrah buffaloes. In swamp buffalo, the 7 samples from each native buffalo were placed in different haplotypes, except for the TC group (Table 3). Results were similar in Thai buffaloes, while 5 samples of Murrah buffaloes were placed in three haplotypes. Shaari et al. (2021) found that Murrah buffaloes showed highly potential for variation since all 4 individual samples, which were collected from the same farm, were separated in different sub-clades. In contrast, swamp and crossbred buffaloes showed less diversity as compared to those in Murrah breeds. Borghese and Mazzi (2005) stated that Murrah buffaloes possibly originated from diverse sources after introductions from the subcontinent of India during the last century. Meanwhile, the swamp buffaloes are reported as an indigenous breed not only to Malaysia but also in many countries of Southeast Asia with less imported exotic breeds (Lau et al., 1998; Yue et al., 2013; Shaari et al., 2021). In the current study, sampled Murrah buffaloes were imported from India and reared at RRDC for a prolonged time without any genetic improvement.

Villamor et al. (2021) analyzed 107 sequences from the Philippine Carabao and the results showed that average haplotype and nucleotide diversities in 23 populations of Philippine Carabao were 0.695 and 0.004, respectively. The result in current study indicates that Vietnamese native buffaloes have higher genetic variation than other Asian swamp buffaloes.

Genetic distance and phylogeny analysis

As shown in Table 5, genetic distances were smaller (ranging 0.050-0.056) in among swamp buffalo populations than between Murrah and swamp buffalo populations (ranging 0.091-0.125). Among the Vietnamese native buffalo groups, genetic distances between Bao Yen and LangBiang (0.05) populations were smaller than between Thanh Chuong and Bao Yen or LangBiang (0.054).

The phylogenetic tree in Figure 3 showed that Murrah and swamp buffaloes, including Thai buffalos, formed two clusters, indicating clear maternal divergence. The phylogenetic tree revealed Murrah closer to Indian and Italian buffaloes. By contrast, swamp buffaloes developed into one clade and then separated into two clusters. One cluster consisting of 5 individuals (BY; LB1; LB4; TC3; TC5) was closely related to Chinese swamp buffaloes, while the other consisting of mostly Vietnamese and imported Thai buffaloes was closely related to Thai (KU687004.1) and Philippine (FJ873678.1) swamp buffaloes. Raungprim et al. (2021) analyzed the nucleotide sequence in D-loop of Thai native buffaloes and reported that they segregated into two maternal lineages (A and B), and predominant of lineage A was found. Similarity, Villamor et al. (2021) analyzed the phylogeny of Philippine Carabao buffaloes the results showed that more than 97% of sampled Philippine Cacabao buffaloes belong to maternal lineage A. Beside this, the authors also stated that the current strategic conservation (*in situ* and *ex situ)* and management of swamp buffalo genetic resources are still limited to haplotypes belonged to maternal lineage A, and newly detected individuals from maternal lineage B would be highly considered for new strategic conservation and management program (Villamor et al., 2021)

No general agreement exists on the timing and placement of domestication and migration of buffaloes, especially regarding swamp buffaloes. Scant information is available regarding the historical distribution of swamp buffalo in Asia (Barker et al., 1991; Tulloh and Holmes, 1992). River buffalo domestication most likely existed around 6,300 years ago around of Indian subcontinent (Kumar et al., 2007; Nagarajan et al., 2015), whereas swamp buffaloes were domesticated in Thailand and then dispersed Southwest to Indonesia, and North to Central and Eastern China (Sari et al., 2014; Zhang et al., 2016; Wang et al., 2017; Colli et al., 2018), possibly further spreading to the Philippine islands through Taiwan (Zhang et al., 2011). However, analysis of the whole mitogenome suggests that buffalo domestication in Southeast Asia occurred in around of Indochina border region among Vietnam, Laos and China frontier area (Zhang et al., 2011, 2016; Wang et al., 2017). Additional research is required to determine the relationship and position of Vietnamese native buffaloes against other Asian origins.

Table 1: Distribution of mtDNA D-loop nucleotide composition for each breed and in population based on partialy mtDNA D-loop sequence examed

Buffalo type	N	Percentage (%)					
		A	T	G	C	A+T	G+C
TC	7	32.76	26.86	14.55	25.83	59.62	40.38
BY	7	32.65	26.59	14.84	25.92	59.24	40.76
LB	7	32.65	26.59	14.62	26.14	59.24	40.76
T	5	32.08	26.36	15.19	26.37	58.44	41.56
M	5	32.94	27.82	14.12	25.12	60.76	39.24
Average		32.63	26.81	14.76	25.89	59.44	40.56

TC: Thanh Chuong; BY: Bao Yen; LB: LangBiang; T: Thailand; M: Murrah

Table 2: The parsimony and singleton informative sites, substitution and variable sites based on partialy mtDNA D-loop sequence examed

Items		Number	Substitution	Variable sites
Two variants	Singleton	4	C/T	535
			G/A	273; 504
			A/G	419
	Parsimony	88	C/T	39; 55; 82; 91; 112; 139; 168; 170; 199; 210; 239; 320; 366; 401; 465; 495; 499
			T/C	5; 47; 150; 162; 164; 271; 300; 326; 339; 340; 343; 361; 362; 427; 449; 467; 529
			G/A	105; 114; 138; 146; 147; 161; 213; 247; 256; 308; 354; 369; 415; 416; 461; 521
			A/G	94; 132; 149; 182; 265; 292; 301; 305; 321; 338; 344; 382; 483
			G/C	393; 524
			C/G	16; 507
			A/T	228; 299
			T/A	3; 26; 284
			A/C	21; 77; 126; 133; 270; 509; 514
			C/A	61; 234; 289; 349; 481
			T/G	207; 396; 453
			G/T	142
Three variants	Singleton	0		
	Parsimony	3	T/G/A	192
			T/C/A	522
			A/T/C	450
Insertion		8	G	185; 186; 189
			C	187; 188; 468
			G/A	184
			A/G/T	482
Deletion		5	T	177
			C	194
			A/G/Del A	183; 485
			C/T/Del C	190

Table 3: Values of number of haplotypes, haplotypes diversity (Hd), nucleotide diversity (π) for each breed and population

Buffalo type	Samples	Number of haplotypes	Diversity index (mean ±SE)	
			Haplotype (Hd)	Nucleotide (π)
TC	7	6	0.952±0.00912	0.05619±0.0000755
BY	7	7	1.000±0.00583	0.04671±0.0000823
LB	7	7	1.000±0.00583	0.05372±0.0000771
T	5	5	1.000±0.01600	0.01683±0.0000088
M	5	3	0.800±0.02688	0.00191±0.0000003
Total/Average	31	26	0.987±0.00015	0.06267±0.0000244

TC: Thanh Chuong; BY: Bao Yen; LB: LangBiang; T: Thailand; M: Murrah

Table 4: Distribution of haplotypes and haplotype shared in population examed

Haplotype (H)	Number	Individual buffaloes
H-1	2	TC1, BY1
H-2	1	TC2
H-3	3	TC3, TC5, LB4
H-4	1	TC4
H-5	1	TC6
H-6	1	TC7
H-7	1	BY2
H-8	1	BY3
H-9	1	BY5
H-10	1	BY6
H-11	1	BY7
H-12	1	BY8
H-13	1	LB1
H-14	1	LB2
H-15	1	LB6
H-16	1	LB7
H-17	1	LB9
H-18	1	LB10
H-19	1	T1
H-20	1	T6
H-21	1	T8
H-22	1	T9
H-23	1	T10
H-24	2	M1, M2
H-25	1	M3
H-26	2	M4, M5

TC: Thanh Chuong; BY: Bao Yen; LB: LangBiang; T: Thailand; M: Murrah

Table 5: Genetic distance among buffalo subpopulations

	TC	BY	LB	T	M
TC	0.062	0.008	0.008	0.008	0.017
BY	0.054	0.050	0.008	0.009	0.019
LB	0.054	0.050	0.058	0.008	0.017
T	0.055	0.055	0.056	0.017	0.016

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M	0.111	0.125	0.113	0.091	0.003

Values above the diagonal show the margin of error of genetic distance, with genetic distance values below the diagonal. Values in the diagonal are genetic distance within breeds.TC: Thanh Chuong; BY: Bao Yen; LB: LangBiang; T: Thailand; M: Murrah buffalo.

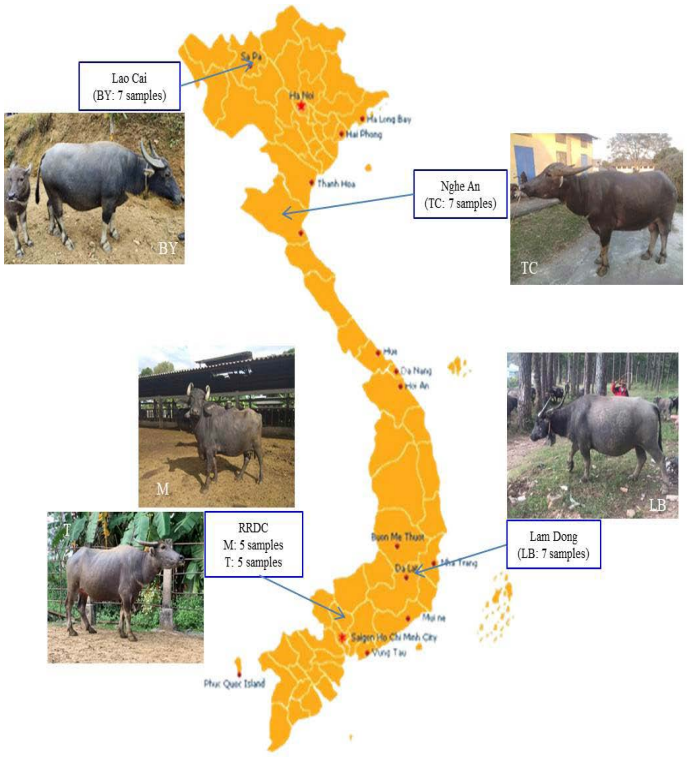

Figure 1: Sampling locations and pictures of the five studied buffalo populations. *BY: Bao Yen; TC: Thanh Chuong; LB: LangBiang; M: Murrah; T: Thailand. RRDC: Ruminant Research and Development Center.*

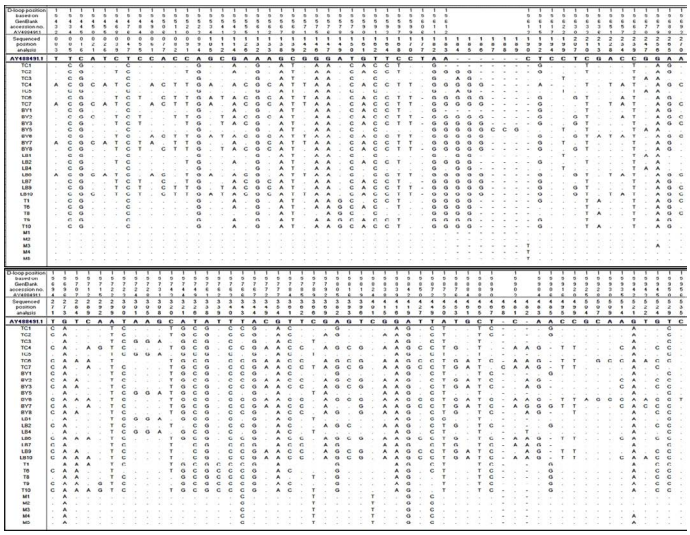

Figure 2: Sequence variation and polymorphic sites observed on the mitochondrial D-loop sequence. *AY488491.1: Ref. from GenBank; TC: Thanh Chuong, BY: Bao Yen, LB: LangBiang, M: Murrah, T: Thailand buffaloes. A: Adenine; T: Thymine; G: Guanine; C: Cytosine; (.): Identity; (-): deletion*

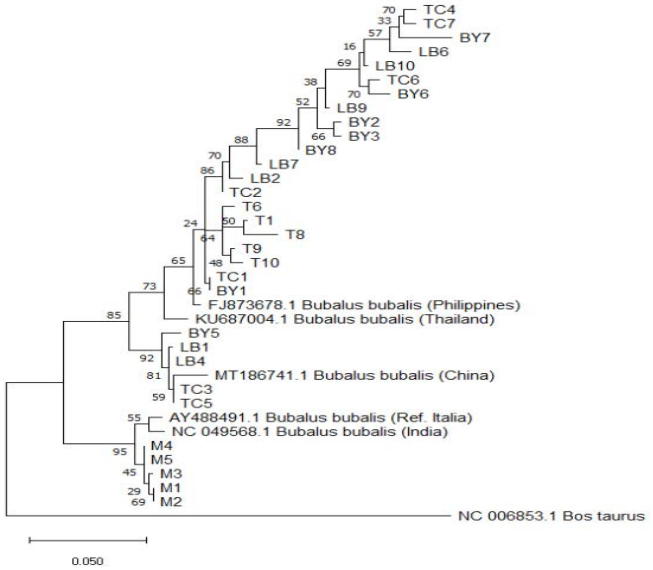

Figure 3: A dendrogram of phylogenetic tree of sampled buffaloes. *TC: Thanh Chuong; BY: Bao Yen; LB: LangBiang; M: Murrah; T: Thailand. Bubalus bubalis (MT186741.1; AY488491.1; KU687004.1; FJ873678.1; AY488491.1); Bos taurus (NC006853.1).*

CONCLUSIONS AND RECOMMENDATIONS

This study, for the first time, reports the genetic diversity of swamp buffalo in three regions of Vietnam using on mtDNA D-loop sequences. The genetic variation in Vietnamese native buffaloes is higher than other Asian swamp buffaloes. Based on phylogenetic tree, the native buffalo populations are separated into two clusters, most of them cluster in one clade and have a genetic relationship closer to Thai and Philippine swamp buffaloes. Further insights regarding the genetic diversity of Vietnamese native buffaloes will require more in-depth studies to gain more the fundamental information to create the policy and strategy for conservation and development in future.

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CONFLICT OF INTEREST

There is no conflict of interest with any finance sources or materials discussed in this manuscript.

NOVELTY STATEMENT

To our knowledge, this is the first study in Vietnam to report the genetic diversity of native buffalo in three representative populations based on mtDNA D-loop, the results will contribute to the scientific literatures on molecular genetic of Vietnamese native buffalo genetic resources and to provide as a useful database to develop the native buffalo conservation and development strategy

AUTHORS CONTRIBUTION

All authors generally contributed to designing the experiments, read and approved the manuscript in each step. NGUYEN, N.T covered all the research, wrote and revised the manuscript. NGUYEN P.K.N and PHAN, T.H. contributed equally to the work on DNA extraction, design primer and amplification of target gene. LE, T.L. and NGUYEN, T.K.L. contributed the work equally on sequence analysis. HOANG, T.T., PHAM, C.T. and NGUYEN C.D. shared the work equally for sample collection. DUONG, N.K. contributed to evaluating the manuscript and checking the plagiarism.

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