COMPARISON OF MORPHOLOGICAL CHARACTERISTICS AND MATERNAL GENETIC LINEAGES IN THAI DWARF AND SWAMP BUFFALOES (*BUBALUS B. CARABANENSIS*)

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ABSTRACT

The objectives of this study was to compare morphological characteristics and to establish evolutionary relationship of 14 Thai dwarf buffaloes and 21 swamp buffaloes based on mitochondrial DNA (mtDNA) D-loop variations. Morphological characteristics could be constructed phylomorphologic tree and showed clearly classified between dwarf and swamp buffaloes. Most of morphologic traits were highly significant difference adult contest swamp buffaloes ($P<0.01$) from 9 contest swamp buffaloes. Only morphological traits of length between eye, length between base of horn, horn length and horn width were not significant difference ($P>0.05$). Average shoulder height of Thai dwarf buffaloes were 108.33±2.08 cm, whereas average shoulder height of contest swamp buffaloes were 156.78±4.21 cm. Thai dwarf buffaloes would be defined as disproportional dwarfism. Sequences of mtDNA D-loop (374 bp) of dwarf and normal swamp buffaloes with reference sequences showed 84 polymorphic sites and defined as 12 and 11 haplotypes, respectively. It was noticed that transversion in Thai dwarf buffaloes occurred twice time of normal swamp buffaloes. Phylogenetic tree showed 2 clades of water buffaloes. Swamp buffaloes were classified into lineage A and lineage B, in which lineage A was more predominant than lineage B. Median joining network showed 2 clades of river and swamp buffaloes. Swamp buffalo (SaenCP) in lineage A1 seem to be ancestral node of some Thai swamp buffaloes, Chinese swamp buffaloes and Philippines carabao. Most of dwarf swamp buffaloes were in lineage A2 and dwarf swamp buffalo with no horn, black color- coat was latter evolution.

Keywords: *Bubalus bubalis*, buffaloes, morphological characteristics, maternal lineages, Thai dwarf buffaloes, swamp buffaloes

INTRODUCTION

Asian domestic buffaloes are commonly classified in two main subspecies, river buffalo and swamp buffalo. Morphology, behavior and number of chromosomes are their distinctive characteristics. The river buffalo distributed in the Indian subcontinent, South Asia and the Mediterranean area (Italy, Egypt and the Balkans), and occasionally in Australia and South America,

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but the swamp buffalo distributed in Northeast India, China (Southern regions and Yangtze valley) and southeast Asia. Ancestor of river and swamp buffaloes supposed descending from the wild Asian buffalo (*Bubalus arnee*), which had a extensively distribution in eastern Indian, Sri Lanka and Southeast Asia (Cockrill, 1981, Lau *et al*., 1998).

Dwarf breed of buffaloes in the world had been recognized to Tamaraw (*Bubalus mindorensis*) in Phillipines and Anoa (*Bubalus quarlesi* and *Bubalus depressicornis*) in Indonesia and Kuttanad buffaloes in India. The tamaraw is smaller compared to the water buffalo (*Bubalus bubalis*) with a compact body, short and thicker neck, 100 to 105 cm of shoulder height, 180 to300 kg of body weight. The anoa have many morphological characteristics and are consider to be most closely related to the water buffalo, which was confirmed through DNA analysis. The Kuttanad buffaloe has an average shoulder height of 109.02±0.78 cm, 150 to 200 kg of body weight. Dwarf buffaloes in Thailand have no morphological information.

The D-loop region of mitochondrial DNA (mtDNA) had been used to distinguish genetic differentiation between the swamp and river buffalo in Southeast Asia (Lau *et al*., 1998), Brazil and Italy (Kierstein *et al*., 2004). However, the D-loop sequence of dwarf buffalo in Thailand had not been investigated. We aimed to study morphological traits and maternal lineages of 21 normal swamp buffaloes and 14 dwarf swamp buffaloes.

MATERIALS AND METHODS

Animals for morphological traits

A total of 559 adult (>3 years) swamp buffaloes were used to study morphological traits. data obtained from 13 location sites, including 12 provinces, Nakhonpanom (NP), Ubolratchathani (UB), Srisaket (SK), Khonkaen (KK), Loei (LO), Bungkan (BK), Phuket (PK), Maehongson (MS), Lumpang (LP), Nan (NA), Payao (PY), Mahasarakam (MK) and buffalo contest at Naraesuan university (NU) on 13 to 14 May 1917. Twenty sites of the body were measured in centimeter, including shoulder height (A), pelvic height (B), heart girth (C), body length (D), pelvic length (E), girth height (F), girth width, pelvic width, fore leg height (G), hind leg height (H), head length (I), length between eye (J), length between base of horn (K), horn length (M) and horn width (N), tail length (O) and tail with hair length. Morphological parameters were analyzed polymorphic information content (PIC) by CERVUS 3.0.7 program (Marshall *et al*., 1998). Phylomorphologic tree based on morphological parameters was constructed by POPTREE2 program (Takezaki *et al*., 2010).

Animals for maternal lineages

Nine frozen semen of swamp buffaloes, donated from Charoen Phokaphan (CP) company for nationwide used artificial insemination and a tissue sample of dead wild buffalo from Huay Khakhaeng wildlife sanctuary were used in this study. Hair follicles of 14 Thai dwarf buffaloes from Mahasarakam (MK) and 21 normal swamp buffaloes from Phuket (PK), Lumpang (LP), Nan (NA), Payao (PY) were used to study maternal lineages, Frozen semen and tissue samples were extracted genomic DNA by adapted protocol using the QIAmp DNA Mini Kit (QIAGEN, U.S.A.). Hair follicles were extracted genomic DNA by Chelex-100 (Walsh *et al*., 2013) and performed to analyze D-loop mtDNA sequences. The D-loop region was amplified by polymerase chain

reaction (PCR) using 2 primers considered from a published water buffalo sequence (GeneBank DQ364160) as following Fw primer: 5'-CTTGCAACTTAACACTGACTTTAC-3' and Rv. primer: 5'- CCATAGCTGAGTCCAGC ATC-3'. The PCR combination contains 1x PCR buffer (50 mM of KCl, 10 mM of Tris-HCl, pH 8.3), 1.5 mM of $MgCl_2$, 200 uM of dNTPs, 0.4 pmole of each primer, 1 U *Taq* polymerase (Ampli Taq GoldTM, Applied Biosystem, USA) and 100 ng of DNA template. The PCR reaction profiles comprised of: stage of denaturation at 94°C for 10 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 1 minute and extension at 72°C for 1 min. and final extension at 72°C for 10 minutes. The PCR yields were initially electrophoresed at 150 Volts for 30 minutes. in 2% agarose gels, and observed under UV light after staining with 0.2 to 0.5 µg/mL of ethidium bromide. The expected sizes of PCR yields were determined comparing to 100 bp DNA size standard. The PCR yields were purified and sequenced using BigDye Terminator Kit (Applied Biosystems, USA) on an ABI PRISM 3010 DNA Sequencer equipped with Sequencing software (Applied Biosystems, USA).

The D-loop sequences were aligned with the following mtDNA D-loop sequences selected in GenBank database (Table 1) using ClustalW via WWW.genome.jp/tools/clustalw. D-loop mtDNA sequences (347 bp) of 14 Thai dwarf and 21 swamp buffaloes, 6 Chinese swamp buffalo sequences, one Philippines carabao and 8 river buffaloes were aligned based on nucleotide position of Haikou buffalo Genbank accession NC006295 by using MEGA6.06 software (Tamura *et al*., 2013). The indistinguishable sequences were considered as the same haplotype. Phylogenetic tree using unweighted pair-group method with the arithmetic mean (UPGMA) method was performed using the function "build" of ETE3 v3.1.1 (Huerta-Cepas *et al*., 2016) on the GenomeNet (https://www.Genome.jp/tools/ete). Median-joining network and nucleotide substitution were generated using the NETWORK 5.0.1.1 program using weighting transversions/transitions parameter (Bandelt *et al*., 1999).

RESULTS AND DISCUSSION

Morphological parameters analysis of 559 swamp buffaloes showed high (0.928) polymorphic information content (PIC) higher than 0.5. It indicated that 20 morphological parameters were high heterozygosity and able to continually manipulate phylomorphological tree. Morphological characteristics of Thai swamp buffaloes were divided into 2 clades based on F_{st} (corrected) UPGMA method. The first clade consists of 2 clusters which the first cluster were normal swamp buffaloes from 11 provinces and buffalo contest at NU and the second cluster were dwarf buffaloes from Mahasarakam (MK). Morphological characteristics of grand champion swamp buffalo at NU buffalo contest were obviously located in clade 2 (Figure 2).

It was noticed that morphological characteristics of swamp buffaloes from Phuket (PK) were more closed to 9 buffaloes at buffalo contest at NU than other swamp buffaloes from various sites. Because buffaloes from PK were selected morphological traits for fighting buffaloes, whereas other swamp buffaloes in various sites obtained from villages.

Average parameter values in morphologic traits of 3 dwarf and 9 adult contest swamp buffaloes was showed in Table 2. Most of morphologic traits in dwarf buffaloes were highly

significant difference (P<0.01) from contest swamp buffaloes. Only morphological traits of length between eye (J), length between base of horn (K), horn length (M) and horn width (N) were not significant difference (P>0.05) from adult swamp buffaloes. It was noticed that head length (I) of dwarf buffaloes were shorter than (P<0.01) contest swamp buffaloes. Average shoulder height of contest swamp buffaloes were 156.78±4.21 cm. Average shoulder height of Thai dwarf buffaloes were 108.33±2.08 cm, whereas the tamaraw and Kuttanad buffaloes had an average shoulder height of 100 to 105 cm. and 109.02±0.78 cm, respectively.

Dwarfism is short statue that results from a genetic or medical condition. Dwarfism is generally defined as an adult height. Disproportional dwarfism is characterized by either short limbs or a short chest (Pauli *et el*., 2012). The most common and recognizable form of dwarfism in human is the genetic disorder achondroplasia. Achondroplasia is caused by a mutation in the fibroblast growth factor receptor3 (FGFR3) gene that results in its protein being overactive (Horton *et al*., 2007). Disproportional dwarfism has been reported in many cattle breeds including Dexter, Holstein, Aberdeen Angus, Hereford, Japanese brown and shorthorn breeds. A directed genome scan was performed with a pedigree consisting of 26 individuals of dwarfism in Angus. Haplotype analysis indicated that the mutation is located on chromosome 6. Dwarfism genes are fine mapping the critical region (Mishra *et al*., 2004).

In this study, mtDNA D-loop sequences (374 bp) of dwarf (yellow box) and normal (blue box) swamp buffaloes with reference sequences (Haikou NC006295) showed 84 polymorphic sites and classified into 22 and 11 haplotypes, respectively as shown in Figure 3.

The mtDNA D-loop sequences reported in this paper have been deposited in the GenBank database (accession no. KC817489-KC817497, KU687004, MF806037 - MF806059). Among these polymorphic sites, there were 29, 39 and 31 transitions (Ts) in dwarf, normal and river buffaloes, respectively whereas were 20, 10 and 3 transversions (Tv) in dwarf, normal and river buffaloes, respectively as showed in Table 3. It was noticed that transversion (Tv) in Thai dwarf buffaloes occurred twice time of normal swamp buffaloes. In contrast, strong bias towards transitions is a characteristic of mtDNA evolution and has been observed not only in buffaloes (Lau *et al*., 1998; Kierstein *et al*., 2004; Lei *et al*., 2007), but also in other mammalian species and chickens (Liu *et al*., 2004; 2006; Chen *et al*., 2005; Guo *et al*., 2005; Lai *et al*., 2006).

Transversion (Tv), in molecular biology refers to a point mutation in DNA in which a single (two ring) purine (A or G) is changed for a (one ring) pyrimidine (T or C) or vice versa. It is generally accepted that high mutation rates of mtDNA are caused by a lack of protective histone, inefficient DNA repair system, and continuous expose to mutagenic effects of oxygen radicles generated by oxidative phosphorylation (Wallace, 1992a, 1992b). The D-loop region of mtDNA contains essential transcription and replication elements, and mutations in this region may serve as a potential sensor for cellular DNA damage and a marker for human cancer development. Several mtDNA mutations in the mtDNA D-loop have been reported in the human breast cancer tissue (Richard *et al*., 2000)

In Figure 4, the phylogenetic tree by UPGMA method revealed two nodes being designated 1 and 2 in which node 1 was consist of swamp and river buffaloes. The node 2 was 5 Thai dwarf buffaloes (MK1, MK10, MK17, MK19,

Table 1. D-loop mtDNA sequences of swamp buffaloes and river buffaloes available in GenBank were used in this study.

Buffalo type	Breed/Isolates	Country	GenBank Accession	Reference
Swamp	Anhui	China	EF053535	*
	Dechang	China	EF053642	*
	Fuling	China	EF053547	*
	Haikou	China	NC006295	**
	Jianghan	China	EF053550	**
	Yunnan	China	EF053552	**
	Carabao	Philippines	FJ873678	***
River Murrah	Murrah	India	AF197216	****
	Murrah	India	AF197213	****
	Murrah	India	AF1972154/	****
	Mediterranean	Italy	AF197208	****
	Mediterranean	Italy	AF197202	****
	Mediterranean	Italy	AF197203	****
	Jafarabad	Brazil	AF197198	****
	Kundi	Pakistan	GQ166748	*****

*Lai *et al*., 2006; **Qian *et al*., 2004; ***Del Barrio *et al*., 2009;

**** Kierstein, *et al*., 2004; *****Hussain *et al*., 2009.

Table 2. Morphological parameters of 3 dwarf and 9 adult contest swamp buffaloes.

Buffaloes	**n**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**
Dwarf	3	108.33	110.67	188.33	146.00	39.33	64.00	64.67	66.00	45.33	21.67	22.67	33.67	52.00	69.00	15.33
		±2.08	±2.08	±8.50	±4.58	±1.53	±5.29	±7.51	±4.58	±1.15	±0.58	±1.15	±6.66	±5.57	±3.61	±1.53
Normal	9	156.78	144.00	252.00	178.56	51.22	93.33	75.00	77.89	56.78	22.44	24.44	42.00	64.22	64.44	46.89
		±4.21	±3.50	±8.83	±10.56	±4.35	±4.12	±5.70	±3.72	±2.33	±1.88	±2.70	±2.92	±14.07	±5.46	±12.31
P		<0.05	<0.01	<0.01	<0.01	<0.01	<0.01	<0.05	<0.01	<0.01	>0.05	>0.05	<0.05	>0.05	>0.05	<0.01

Table 3. Nucleotide mutation of 84 polymorphic sites in D-loop mtDNA sequences according to Figure 2.

Buffalo type		Transition (Ts)	Transversion (Tv)
Swamp	Dwarf	29	20
	Normal	30	10
River		31	3

Figure 1. Measurement sites of swamp buffaloes in dwarf and normal swamp buffaloes.

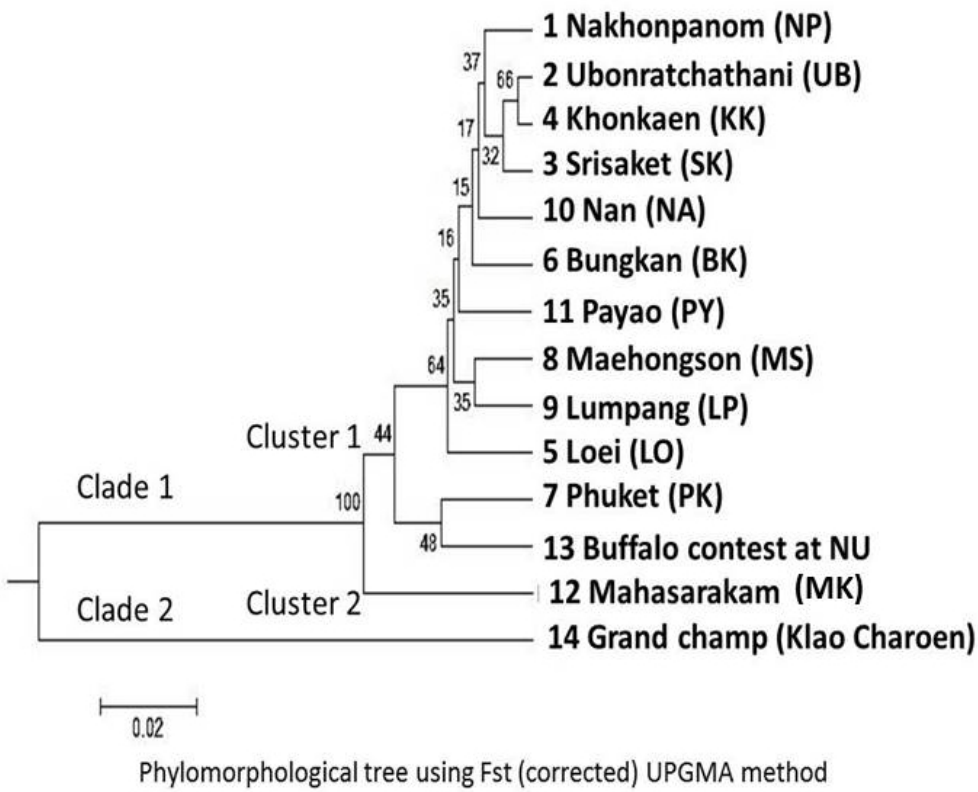

Figure 2. Phylomorphologic tree by F_{st} (corrected) UPGMA method of swamp buffaloes.

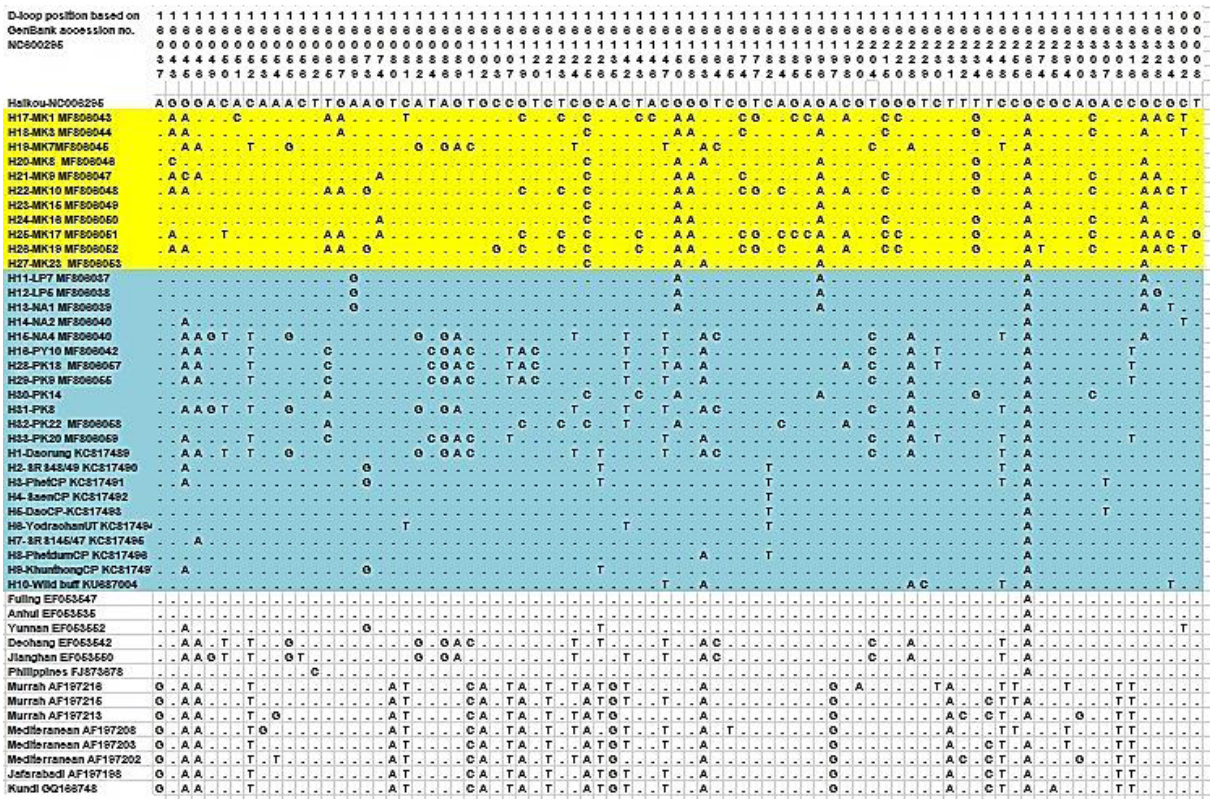

Figure 3. D-loop mtDNA sequences with 84 polymorphic sites of dwarf (yellow box) and normal swamp buffaloes (blue box) were aligned based on nucleotide position of Haikou buffalo Genbank accession NC006295 togather with 6 Chinese swamp buffalo, one Philippines carabao and 8 river buffalo sequences available in Genbank.

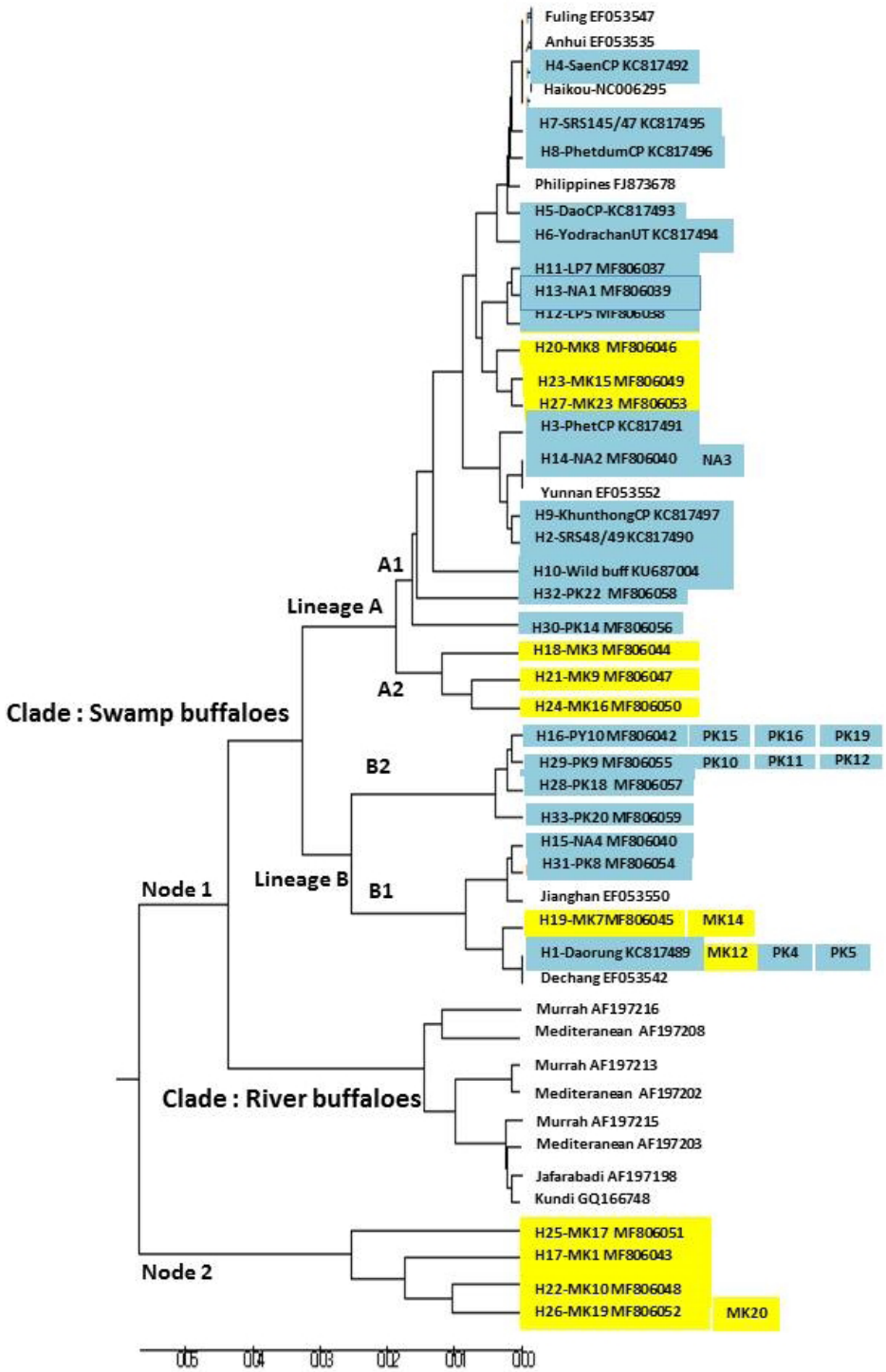

Figure 4. Phylogenetic tree using UPGMA method of 14 dwarf (yellow box) and 31 swamp buffaloes (blue box), 6 Chinese swamp buffaloes, one Philippines carabao and 8 river buffaloes.

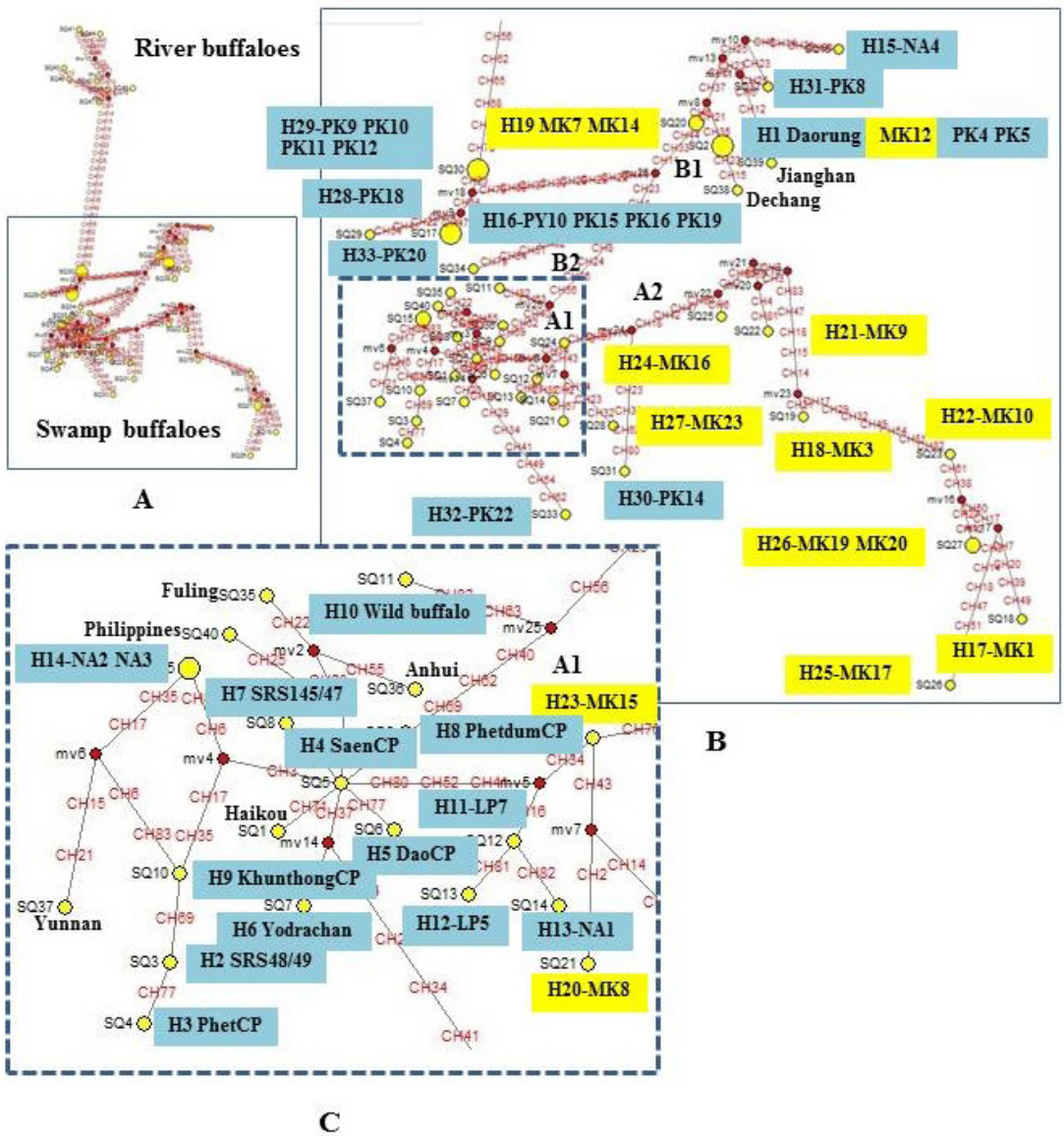

Figure 5. Median joining network using xxx parameter of 14 dwarf (yellow box) and 31 swamp buffaloes (blue box), 6 Chinese swamp buffaloes, one Philippines carabao and 8 river buffaloes.

MK20). The swamp buffaloes were divided into lineage A and lineage B, in which lineage A was more predominant than lineage B. Most of lineage A1 contains wild buffalo, normal Thai, Chinese, Philippines swamp buffaloes, a wild and 3 dwarf swamp buffaloes (MK8, MK15, MK23). Whereas only 3 dwarf swamp buffaloes (MK3, MK9, MK16) located in lineage A2. Other 3 dwarf buffaloes (MK7, MK12, MK14) located in lineage B1, in which one dwarf buffalo (MK12) was belonged to Haplotype 1 of normal swamp buffalo Daorung KC817489) and homology D-loop mtDNA sequences to 2 normal swamp buffaloes (PK4, PK5) and a Chinese swamp buffalo (Dechang EF053542). Only 9 swamp buffaloes from Phuket (PK10-12, PK15-20) and one from Phayao (PY10) located in lineage B2.

In Figure 5, median joining network using weighting transversion/transition parameter of 14 dwarf (yellow box) and 21 swamp buffaloes (blue box), 6 Chinese swamp buffaloes, one Philippines carabao and 8 river buffaloes showed 2 clades of river and swamp buffaloes (Figure 4A). Wild buffalo in lineage A1 had 2 nucleotides mutations and 12 nucleotides mutations of a swamp buffalo (PK20) in lineage B2 from median vector (mv) 25. It was noticed that river buffaloes had been evolved from 4 Phuket swamp buffaloes (PK9-12). Most of dwarf buffaloes (8/14) were belonged to lineage A2 and latest evolution was no horn, black color- coat dwarf buffalo MK17 (Figure 4B). Swamp buffalo (SaenCP) seem to be ancestral node of some Thai swamp buffaloes, Chinese swamp buffaloes (Haikou, Anhui, Fuling, Yunnan) and Philippines carabao (Figure 4C).

CONCLUSION

Thai dwarf buffaloes would be defined as disproportional dwarfism which is characterized by either short limbs or a short chest. Maternal lineages of Thai dwarf buffaloes were located in multiple lineages of swamp buffalo. Most of dwarf buffaloes were belonged to lineage A2. Median joining network revealed that swamp buffalo (SaenCP) in lineage A1 seems to be ancestral node of some Thai swamp buffaloes, Chinese swamp buffaloes (Haikou, Anhui, Fuling, Yunnan) and Philippines carabao. Latest evolution of dwarf buffalo in lineage A2 was no horn, black color-coat dwarf buffalo.

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