



Research article

Development of novel primers for species identification and population genetic studies of ancient swamp buffalo remains

Pee Boonleang^a, Jirakit Sangsom^a, Pipad Krajaejun^b, Athiwat Wattanapituksakul^c, Wunrada Surat^{a,*}

^a Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

^b Department of History, Faculty of Liberal Arts, Thammasat University, Pathum Thani 12120, Thailand

^c The Timing and the Cause of the Transition in Domestic Cattle Species in Thailand project, Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

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Abstract

Importance of the work: Ancient domestic buffalo remains have been discovered in China and Thailand, suggesting that they were domesticated in these countries thousands of years ago. However, no genetic information of ancient buffaloes has been used to confirm this.

Objectives: To design primers for amplifying ancient DNA of swamp buffaloes.

Materials and Methods: The 49 complete D-loop sequences of three buffalo species (river, swamp, and wild water) from Asia and other regions were used to analyze polymorphism distributions for identifying suitable regions for species identification and studying the population genetics of the ancient specimens. Then, primers were designed and the sequences within each primer pair were used for phylogenetic analyses. Modern and ancient specimens were used to test the performance of the primers.

Results: The nucleotide polymorphisms of swamp buffaloes, mostly located between the 100th–500th positions of the complete D-loop, were used to design primers within this region. Then, the phylogenetic tree of the complete D-loop and those of sequences within each primer set were compared. Topologies and bootstrap values of the tree from Primer I had the most similarity to that of the complete D-loop tree. In addition, nested polymerase chain reaction (PCR) with Primer I and Primer II could successfully amplify the ancient DNA sample.

Main findings: This was the first report of the successful amplification of ancient DNA from swamp buffalo. Nested PCR with the new primers could be used to amplify ancient DNA samples and their sequences could be further used for species identification and studying the origin of swamp buffaloes.

* Corresponding author.

E-mail address: fsciwrds@ku.ac.th (W. Surat)

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Introduction

Water buffaloes are widely distributed in Asia and some countries in Europe, South America and Africa (Kierstein et al., 2004; Yue et al., 2013; Sun et al., 2019; Youssef et al., 2021). Nowadays, there are three water buffalo species: the wild water buffalo (*Bubalus arnee*) and two domestic water buffalo species—*Bubalus bubalis* (the river buffalo) and *Bubalus carabanensis* (the swamp buffalo) (Yindee, 2010; Zhang et al., 2020; Cailipan et al., 2023). River buffaloes are mostly found in India, where they predominantly contribute to milk production and meat consumption (Kumar and Singh, 2010). Swamp buffaloes are mainly found in southern China and Southeast Asia, including Thailand, where they are mainly used for rice farming (Chantalakhana and Skunmun, 2003).

Swamp buffaloes have been closely associated with people dating back to ancient times, with the origin of the buffalo species having been investigated using archaeological and DNA evidence. Analysis of the morphological characteristics of buffalo remains found along the Yangtze River region suggested that swamp buffaloes in China may have been domesticated 7,000 years ago (Chen and Li, 1989). However, a study of the ancient DNA from buffalo remains along the Wei River valley in Shaanxi province, China dated to 8,000–3,600 years before present (YBP), confirmed that they belonged to *Bubalus mephistopheles*, an extinct wild water buffalo species (Yang et al., 2008). Until now, there has been no published evidence based on ancient DNA supporting the presence of swamp buffaloes in China. In Thailand, buffalo remains have been discovered in many archeological sites in Central and Northeast regions such as the Ban Chiang World Heritage Site in Udon Thani province, the Si Bua Thong archaeological site in Ang Thong province and at Wat Phra Si Rattana Mahathat in Ayutthaya province (Thai Fine Art, 2024). The oldest archaeological evidence showing that domestic buffaloes were present in Thailand is a cave painting of an animal similar to a domestic buffalo (dated approximately 4,000–3,000 YBP) at Khoa Pla Raa in Uthai Thani province (Yindee, 2010). The archaeological evidence showed that buffaloes have been domesticated in China and Thailand for several thousand years. In addition, several genetic studies have indicated that the origin of the swamp buffalo occurred in the region close to the China/Indochina border and possibly occurred in Thailand (Yindee, 2010; Yue et al., 2013; Colli et al., 2018; Zhang et al., 2020). However, there has been no sample of ancient DNA of swamp buffaloes included in any

study. If the DNA from ancient swamp buffaloes could be retrieved, it might help to shed light on the origin of the swamp buffalo.

Based on archaeological evidence, swamp buffaloes have been distributed in Southeast Asia since ancient times; however, the tropical climate in this region can completely destroy the DNA in ancient specimens. Conditions of high temperature and high humidity not only greatly decrease the DNA concentration but also affect the DNA quality, where most of the DNA fragments are shorter than 200 bp (Dabney et al., 2013). Polymerase chain reaction (PCR) products (179 bp and 164 bp) have been successfully amplified from ancient pig and cattle specimens in Thailand (Wannajuk et al., 2013; Siripan et al., 2019; Chittavichai et al., 2021). Hence, it is very important to use primers that can amplify small, fragmented DNA <200 bp in ancient samples. However, the use of a primer pair for amplifying short fragments of extremely damaged DNA specimens cannot produce any PCR product; thus, nested PCR must be used (Katanyuphan, 2024). Recently, short DNA fragments (160–179 bp) of Asian rhinos dated 100 YBP were successfully amplified by nested PCR based on amplifying the 257-bp PCR product in the first round of PCR and then using that as a template for producing smaller fragments in the second round of PCR (Katanyuphan and Surat, 2023; Katanyuphan, 2024). The primers for nested PCR need to be designed for successful amplification in the case of extremely damaged DNA specimens.

The current study aimed to design primers in the D-loop region for nested PCR for species classification and for studying the population genetics of swamp buffaloes. It was expected that the newly designed primers could be used to study the origin of the swamp buffalo, especially from extremely damaged DNA specimens.

Materials and Methods

Data collection and polymorphism detection in D-loop region

In total, 49 complete D-loop sequences of three water buffalo species in the GenBank database were used, consisting of 23 swamp (from China, Laos, India and Thailand), 22 river (from Brazil, Egypt, India and Italy) and four wild water buffaloes (from India), as shown in Table S1. All the complete D-loop sequences of each water buffalo species were separately aligned and then trimmed using the ClustalW and MEGA11 software packages, respectively (Tamura et al., 2021).

Then, the sequences of each buffalo species were analyzed using the DNAsp6 application to generate the distribution of nucleotide polymorphism across the D-loop region (Rozas et al., 2017).

Primer design

After screening for the region containing high nucleotide polymorphisms, a target region was selected for primer design. Two sets of primers were newly designed to cover the most variable region among water buffalo species, with one set of additional primers from Yang et al. (2008) included in further analysis. Subsequently, each primer set was checked for melting temperature, annealing temperature, %GC and self-compatibility using the Oligo Analysis Tool (<https://eurofinsgenomics.eu/en/ecom/tools/oligo-analysis/>). The specificity of each primers was primarily checked by alignment with the reference *B. carabensis* sequence (accession number NC006295) using ClustalOmega (www.ebi.ac.uk/jdispatcher/msa/clustalo).

Phylogenetic analysis

After the completion of the primer design process, all water buffalo sequences were aligned with each primer set and then trimmed using the MEGA11 software. DNAsp6 was used for haplotype analysis to examine polymorphism in the sequences within each primer set.

Selected regions were investigated for their ability to identify buffalo species based on the MEGA11 software that was used to generate neighbor-joining phylogenetic trees with 1,000 replications. Afterward, all trees constructed based on the sequences within three primer pairs were compared to the complete D-loop tree generated with the same set of the sequences.

DNA extraction and polymerase chain reaction amplification for specificity and sensitivity tests

The primers were investigated for their ability to amplify the selected region using DNA from buffalo meat that was extracted using a GF-1 Tissue DNA Extraction Kit (Vivantis; Malaysia) and subsequently used as the DNA template in PCR. In a 50 μ L total volume, the reaction mixture consisted of 5 μ L of 10X PCR buffer, 1.5 μ L 50 mM MgCl₂, 0.2 μ L Platinum™ Taq polymerase (Invitrogen; USA), 0.5 μ L of 10mM of dNTP mixture, 1.25 μ L of each of the forward

and reverse primers, 1 μ L of DNA template, 0.5 μ L of each 10 μ M primer, 0.5 μ L of RSA (Sigma-Aldrich; USA) and distilled water. The PCR conditions were: initial denaturation at 94°C for 30 s, followed by 50 cycles of denaturation at 94°C for 30 s, annealing at 52–55°C for 30 s (Table 1) and extension at 72°C for 40 s, with a final extension at 72°C for 5 min. Then, the PCR product was visualized using gel electrophoresis on a 2% agarose gel stained with Novel Juice (Bio-Helix; Taiwan).

The primer sensitivity was tested using 10-fold dilutions of DNA concentrations at 1 ng/ μ L to 0.01fg/ μ L. The thickness of the PCR products from Primer I was detected using gel electrophoresis on 2% agarose gel.

DNA extraction and nested polymerase chain reaction of ancient DNA

Some remains dated approximately 3,000 YBP of ancient buffalo, characterized by comparative morphological characteristics (Suraprasit et al., 2016; Wattanapitaksakul, 2016), were obtained from the Ban Chiang Museum (Fig. S1). To prevent exogenous DNA contamination, the surface of the specimen was removed using a hand drill and exposed to ultraviolet light in a laminar flow for 30 min. Then, the sample was ground into a fine powder using a sterilized mortar and pestle; 0.25 g of the powder was used for DNA extraction.

The DNA was extracted following the method described by Damgaard et al. (2015). Throughout DNA extraction, blank extraction was processed alongside the ancient specimen to provide a negative control. Then, the ancient DNA and the blank extract were used as templates in nested PCR. The reaction mixtures were the same as mentioned earlier, except that the amount of DNA template was changed to 4 μ L. The first round of PCR used the forward primer of Primer II and the reverse primer of Primer I. The PCR condition was similar to the specificity test, except that the annealing temperature was changed to 53°C. Then, the PCR products from the first round were diluted with sterile water in the ratio of 1:3 and used as DNA templates for the second round of PCR. The forward primer and the reverse primer of Primer II were used. Subsequently, the PCR product was observed using gel electrophoresis and sequenced based on Sanger sequencing. Finally, the species of the ancient specimen was identified using the Blast program.

Results and Discussion

Haplotype analysis and selection of target region

The complete D-loop sequences of each buffalo species were separately analyzed to target a proper region suitable for species identification and diversity analysis (Fig.1). The distribution of polymorphic sites in swamp buffaloes was mostly located at positions 100–500 bp. For the river buffalo, the distribution of polymorphic sites was in the range 200–500 bp. For the wild water buffalo, only one polymorphic site was found at position 480 bp. These results corresponded with the results from Nakamura et al. (2008), which indicated that the positions in the range 100–500 bp in the D-loop region were in hypervariable region I in mammals. Then, the sequences in the range 100–500 bp were selected for designing the primer because they covered most of the polymorphic sites among the three buffalo species.

The locations and the details of three primer sets used in the current study are shown in Fig. 2 and Table 1, respectively. Primer I consisted of a forward primer (F134) and a reverse primer (R424) located at the positions 134–153 bp and 405–424 bp, respectively. Primer II consisted of a forward primer (F240) and a reverse primer (R402) located at 240–264 bp and 378–402 bp, respectively. Primer III consisted of a forward primer (F149) and a reverse primer (R506) located at 149–173 bp and 481–506, respectively. Each primer produced PCR product sizes of 291, 163 and 358 bp for Primers I, II and III, respectively.

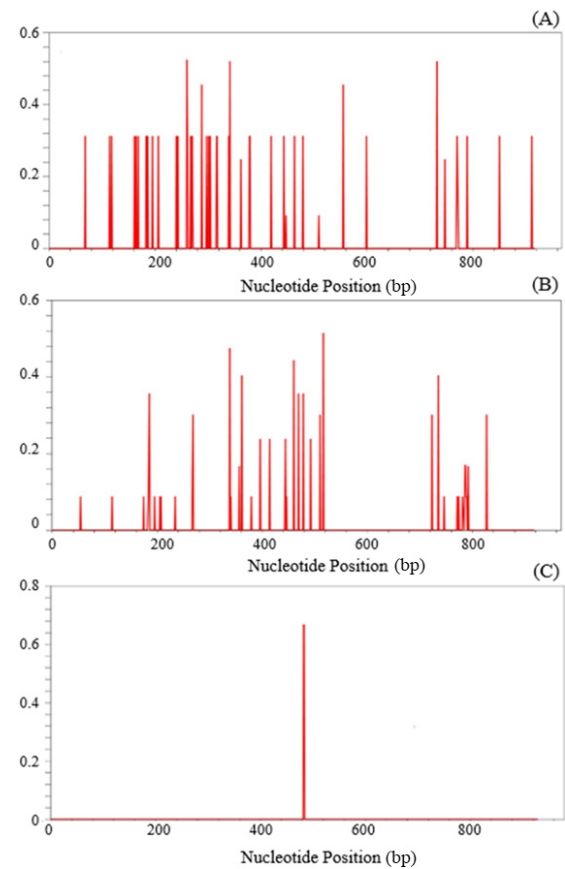


Fig. 1 Distribution of nucleotide diversity (π) across the complete D-loop in three water buffalo species: (A) swamp buffalo (*Bubalus carabanensis*) ($n = 23$); (B) river buffalo (*Bubalus bubalis*) ($n = 22$); (C) wild water buffalo (*Bubalus arnee*) ($n = 4$)

Primer design

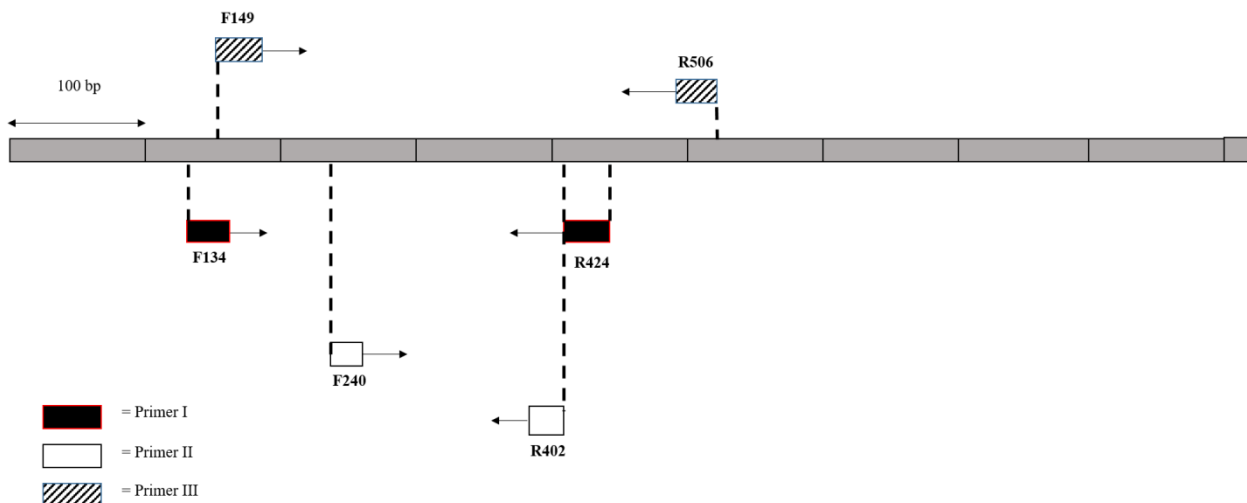


Fig. 2 Primer positions based on the complete D-loop sequence of *Bubalus bubalis* (NC_006295).

Table 1 Sequence details of Primer sets I, II and III

Set	Species	Primer	Sequence (5'–3')	Length	%GC	T _m	IUPAC	Max annealing score opt. ≤ 14	T _a	Product size
I	<i>B. bubalis</i>	F134	Forward:							
	<i>B. carabanensis</i>		CCATCAACACACCTRACCTC	20 bp	50–55	52°C –54°C	R = A or G	4	53°C	291 bp
	<i>B. arnee</i>	R405	Reverse:							
			GAAGGGTTGCTGYTTTCACG	20 bp	50–55	52°C –54°C	Y = T or C	8		
II	<i>B. bubalis</i>	F213	Forward:							
	<i>B. carabanensis</i>		TAGTACATTAAATTATATGCCCCAT	25 bp	28	49°C		10	52°C	163 bp
	<i>B. arnee</i>	R381	Reverse:							
			GCATGGTAAYTAAGCTCGTGATCTA	25 bp	40–44	54°C –56°C	R = A or G	12		
III	<i>B. bubalis</i>	F149	Forward:							
	<i>B. carabanensis</i>		ACCTCAYATGTACRRCAACAACAT	25 bp	32–48	51°C –58°C	R = A or G		56 °C	358 bp
		R481	Reverse:							
			AAGAACCAGATGRCTGTAAAGTTC	25 bp	36–40	53°C –54°C	R = A or G	4		

T_m = melting temperature; T_a = annealing temperature; IUPAC ambiguous base: R = adenine (A) and guanine (G); Y = cytosine (C) and thymine (T)

Polymorphism detection in polymerase chain reaction products

The complete D-loop sequences of all three water buffaloes were aligned with each of the designed primers and the sequences between each primer pair were used for analyzing nucleotide polymorphism (Tables 2–4). The sequences from Primer I had 52 polymorphic sites that were identified into 22 haplotypes (Table 2). Of these 52 sites, 14 were able to separate between the swamp and the river buffalo species, with 12 of them being transitions and 2 being transversions. Additionally, within swamp haplotypes, there were 8 polymorphic sites unique to swamp buffalo haplotypes 6 (1 sample) and 7 (3 samples), with 7 of them being transition and 1 being transversion. The sequences from Primer II had 25 polymorphic sites, categorized into 13 haplotypes (Table 3). Of these 25 sites, 9 were able to separate between the swamp and river species, with 8 of them being transitions and 1 being transversion. Primer II had 4 polymorphic sites that were unique to swamp buffalo haplotypes 5 (1 sample) and 6 (3 samples), with all of them being transitions. The sequences from Primer III had 58 polymorphic sites, separated into 24 haplotypes (Table 4). Of these 58 sites, 13 were able to separate between swamp and river buffaloes, with 9 of them being transitions and 4 of them being transversions. Furthermore, the sequences from Primer III contained 11 polymorphic sites that were unique to swamp buffalo haplotypes 6 (1 sample) and 7 (3 samples), with all of them being transitions. There was no polymorphic site unique to wild water buffaloes, which may have been due to the very low number of DNA sequences of this buffalo species in the database.

Phylogenetic analysis

The ability to classify the water buffalo species of each primer pair was investigated by constructing phylogenetic trees based on the sequences within each primer set using the neighbor-joining method with 1,000 replications compared to the complete D-loop tree (Fig.3). The results indicated that the tree based on sequences from Primer I and Primer III had a topology that was most similar to that of the complete D-loop tree. The Primer I tree clustered swamp buffaloes together, with high bootstrap values of 93, while river buffaloes were clustered together, with bootstrap values of 73. Similarly, the Primer III tree clustered swamp buffaloes together, with high bootstrap values of 99, while river buffaloes were clustered together, with bootstrap values of 37. Since the Primer I and Primer III trees had similar topology and the PCR product of Primer I was shorter than that of Primer III, only Primer I was used in the further experiment. For the Primer II tree, swamp buffaloes were grouped together, with high bootstrap values of 92, while river buffaloes were not well clustered together, being separated into several subclades, with low bootstrap values. Wild water buffalo was clustered in the same clade as river buffalo in all trees. The wild water buffaloes included in the current study were from a wild sanctuary, resulting in the possibility of nearby river buffaloes entering the sanctuary area and interbreeding, leading to the high genetic similarity between the wild and the river buffaloes. This was consistent with Achyut et al. (2011), who reported that the DNA of river buffaloes was integrated into 131 wild water buffaloes in the Koshi Tappu Wildlife Reserve, Nepal.

Table 2 Polymorphic sites within sequences produced by Primer I in three water buffalo species

Species/Haplotype/ Country		Variable sites																									
Number of samples		1	2	8	9	11	23	24	26	30	36	44	45	46	47	48	49	50	57	58	68	70	97	102	105	114	122
S/H1/6 S/H2/7 S/H3/1 S/H4/2 S/H5/6 S/H6/1 S/H7/3	CH ₁ ,IN,LA	A	T	A	A	C	A	C	T	C	G	G	G	G	G	G	G	-	C	A	T	A	T	T	G	A	G
	CH ₂ ,LA,TH	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A
	LA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	
	LA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	
	TH	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	
	CH	.	.	.	.	.	G	.	T	C	.	.	A	A	.	C	.	T	T	.	C	.	C	A	.	A	
	CH ₁ ,IN	.	.	.	.	.	G	.	T	C	.	.	A	A	.	C	.	T	T	.	C	.	C	A	.	A	
R/H8/1 R/H9/4 R/H10/1 R/H11/2 R/H12/1 R/H13/1 R/H14/4 R/H15/2 R/H16/1 R/H17/1 R/H18/2 R/H19/1 R/H20/1 W,R/H21/1,1 W/H22/3	EG	G	C	G	G	T	G	T	T	C	.	-	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A
	BR,EG,IT	G	C	G	G	T	G	T	T	C	.	-	A	A	C	.	T	-	.	.	C	.	.	.	.	G	A
	IT	G	C	G	G	T	G	T	T	C	A	.	A	A	T	.	T	-	.	.	.	G	C	.	.	G	A
	BR,IT	G	C	G	G	T	G	T	T	C	.	.	A	-	T	.	T	-	.	.	C	.	C	.	.	G	A
	BR	G	C	G	G	T	G	T	T	C	.	.	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A
	IN	G	C	G	G	T	G	T	T	C	.	.	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A
	IN	G	C	G	G	T	G	T	T	C	.	A	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A
	IN	G	C	G	G	T	G	T	T	C	.	.	A	A	C	.	T	-	.	.	C	.	C	.	.	G	A
	IN	G	C	G	G	T	G	T	T	C	.	.	A	A	C	.	T	-	.	.	C	.	C	.	.	G	A
	IN	G	C	G	G	T	G	T	T	C	.	.	A	A	C	.	T	-	.	.	C	.	C	.	.	G	A
	IN	G	C	G	G	T	G	T	T	C	.	.	A	A	C	.	T	-	.	.	C	.	C	.	.	G	A
	EG,IN	G	C	G	G	T	G	T	T	C	.	.	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A
	IN	G	C	G	G	T	G	T	T	C	.	.	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A
	IN	G	C	G	G	T	G	T	T	C	.	.	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A
	EG	G	C	G	G	T	G	T	T	C	.	-	A	A	T	.	T	-	.	.	G	C	.	C	.	G	A
	BR,IN	G	C	G	G	T	G	T	T	C	.	A	A	-	C	.	T	-	.	.	C	.	C	.	.	G	A
	IN	G	C	G	G	T	G	T	T	C	.	-	A	A	C	.	T	-	.	.	C	.	C	.	.	G	A
Species/Haplotype/ Country		Variable sites																									
Number of samples		123	129	130	131	132	133	150	157	158	163	166	178	179	184	196	198	201	202	203	212	219	224	240	241	242	
S/H1/6 S/H2/7 S/H3/1 S/H4/2 S/H5/6 S/H6/1 S/H7/3	CH ₁ ,IN,LA	G	C	A	A	A	C	A	T	C	A	G	T	G	C	G	C	C	C	G	C	A	C	C	G	T	C
	CH ₂ ,LA,TH	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
	LA	.	.	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
	LA	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
	TH	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
	CH	A	T	.	.	G	.	.	.	.	G	A	C	A	.	.	.	.	T	.	T	.	.	A	C	.	.
	CH ₁ ,IN	A	T	.	.	G	.	.	.	.	G	A	C	A	.	.	.	T	.	T	.	T	A	C	.	.	.
R/H8/1 R/H9/4 R/H10/1 R/H11/2 R/H12/1 R/H13/1 R/H14/4 R/H15/2 R/H16/1 R/H17/1 R/H18/2 R/H19/1 R/H20/1 W,R/H21/1,1 W/H22/3	EG	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	T	.	A	T	T	G	T	T	A	.	.
	BR,EG,IT	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	A	T	.	A	T	G	T	T	A	.	.
	IT	A	T	G	.	.	.	.	A	T	.	.	C	A	T	A	A	T	T	A	.	G	T	T	A	.	T
	BR,IT	A	T	.	G	.	.	.	A	T	.	.	C	A	T	A	A	T	T	A	T	G	.	.	A	.	.
	BR	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	A	T	T	A	T	G	T	T	A	.	.
	IN	A	T	.	G	.	.	.	A	T	.	.	C	A	T	A	A	T	.	A	T	G	T	T	A	.	.
	IN	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	A	T	.	A	T	G	T	T	A	.	.
	IN	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	A	T	.	A	T	G	T	T	A	.	.
	IN	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	A	T	.	A	T	G	T	T	A	.	.
	IN	A	T	G	.	.	.	.	A	T	.	.	C	A	T	A	A	T	T	A	T	G	T	T	A	.	.
	IN	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	A	T	.	A	T	G	T	T	A	.	.
	IN	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	A	T	.	A	T	G	T	T	A	.	.
	EG,IN	A	T	.	G	.	.	.	.	A	T	.	.	C	A	T	A	T	T	A	T	G	T	T	A	.	.
	IN	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	A	T	T	A	T	G	T	T	A	.	.
	IN	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	A	T	T	A	T	G	T	T	A	.	.
	EG	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	A	T	.	A	T	G	T	T	A	.	.
	W,R/H21/1,1	BR,IN	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	T	.	A	T	G	T	T	A	.	.
W/H22/3	IN	A	T	.	.	.	.	.	A	T	.	.	C	A	T	A	T	.	A	T	G	T	T	A	.	.	

Dot (.) = identical sequence to reference sequence in first line; S = swamp buffalo (*Bubalus carabanensis*); R = river buffalo (*Bubalus bubalis*); W = wild water buffalo (*Bubalus arnee*); BR = Brazil; CH = China; EG = Egypt; IN = India; IT = Italy; LA = Laos; TH = Thailand.

Table 3 Polymorphic sites within sequences produced by Primer II in three water buffalo species

Species/ Haplotype/ Number of samples	Country		Variable sites																			
	2	10	11	17	18	19	20	21	38	45	46	47	51	54	66	67	72	78	83	86	89	90
S/H1/6	A	A	G	C	A	A	A	C	G	T	C	A	A	G	T	G	C	G	C	C	G	T
S/H2/1	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C
S/H3/10	.	G	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
S/H4/1	.	G	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
S/H5/3	.	.	.	A	T	.	G	.	A	.	.	G	G	A	C	A	.	.	.	T	.	.
S/H6/1	.	.	.	A	T	.	G	.	A	.	.	G	G	A	C	A	.	.	.	T	.	.
R/H7/1	G	.	A	T	G	.	.	.	A	A	T	.	.	.	C	A	T	A	T	T	A	C
R/H8/1	G	.	A	T	.	G	.	.	A	A	T	.	.	.	C	A	T	A	T	T	A	C
R/H9/2	G	.	A	T	.	G	.	.	A	A	T	.	.	.	C	A	T	A	T	A	.	G
R/H10/1	G	.	A	T	.	G	.	.	A	A	T	.	.	.	C	A	T	A	T	T	A	.
R/H11/3	G	.	A	T	.	.	.	.	A	A	T	.	.	.	C	A	T	A	T	T	A	.
R/H12/1	G	.	A	T	G	.	.	.	A	A	T	.	.	.	C	A	T	A	T	T	A	.
W,R/H13/4,14	G	.	A	T	.	.	.	.	A	A	T	.	.	.	C	A	T	A	T	T	A	.

Dot (.) = identical sequence to reference sequence in first line; S = Swamp buffalo (*Bubalus carabanensis*); R = river buffalo (*Bubalus bubalis*); W = wild water buffalo (*Bubalus arnee*); BR = Brazil; CH = China; EG = Egypt; IN = India; IT = Italy; LA = Laos; TH = Thailand.

Table 4 Polymorphic sites within sequences produced by Primer III in three water buffalo species

Species/ Haplotype/ Number of samples	Country		Variable sites																			
	4	5	7	11	16	24	25	27	28	29	30	31	38	39	49	51	78	83	86	95	103	104
S/H1/6	A	C	C	T	G	G	G	G	G	G	G	-	C	A	T	A	T	T	G	A	G	G
S/H2/4	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
S/H3/1	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.
S/H4/1	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
S/H5/6	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	A	.
S/H6/1	G	.	T	C	.	.	A	A	.	C	.	T	T	.	C	.	.	C	A	.	A	A
S/H7/3	G	.	T	C	.	.	A	A	.	C	.	T	T	.	C	.	.	C	A	.	A	A
R/H8/1	G	T	T	C	.	-	A	A	C	.	T	-	.	.	C	.	.	.	.	G	A	A
R/H9/3	G	T	T	C	.	A	A	-	T	.	T	-	.	.	C	.	C	.	.	G	A	A
R/H10/1	G	T	T	C	A	.	A	A	T	.	T	-	.	.	.	G	C	.	.	G	A	A
R/H11/1	G	T	T	C	.	.	A	-	T	.	T	-	.	.	C	.	C	.	.	G	A	A
R/H12/1	G	T	T	C	.	.	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A	A
R/H13/1	G	T	T	C	.	.	A	-	T	.	T	-	.	.	C	.	C	.	.	G	A	A
R/H14/1	G	T	T	C	.	.	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A	A
R/H15/4	G	T	T	C	.	A	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A	A
R/H16/2	G	T	T	C	.	.	A	A	C	.	T	-	.	.	C	.	C	.	.	G	A	A
R/H17/1	G	T	T	C	.	A	A	A	C	.	T	-	.	.	C	.	C	.	.	G	A	A
R/H18/2	G	T	T	C	.	.	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A	A
R/H19/1	G	T	T	C	.	.	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A	A
R/H20/1	G	T	T	C	.	.	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A	A
R/H21/1	G	T	T	C	.	-	A	A	T	.	T	-	.	G	C	.	C	.	.	G	A	A
R/H22/1	G	T	T	C	.	-	A	A	T	.	T	-	.	.	C	.	C	.	.	G	A	A
W,R/H23/3,1	G	T	T	C	.	A	A	-	C	.	T	-	.	.	C	.	C	.	.	G	A	A
W/H24/1	G	T	T	C	.	-	A	A	C	.	T	-	.	.	C	.	C	.	.	G	A	A

Dot (.) = identical sequence to reference sequence in first line; S = swamp buffalo (*Bubalus carabanensis*); R = river buffalo (*Bubalus bubalis*); W = wild water buffalo (*Bubalus arnee*); BR = Brazil; CH = China; EG = Egypt; IN = India; IT = Italy; LA = Laos; TH = Thailand.

Table 4 Polymorphic sites within sequences produced by Primer III in three water buffalo species (continued)

Species/Haplotype/ Number of samples	Country	Variable sites																						
		111	112	113	114	131	138	139	140	144	147	159	160	165	177	179	182	183	184	193	200	205	221	222
S/H1/6	CH,IN,LA	A	A	A	C	A	T	C	A	A	G	T	G	C	G	C	C	G	A	C	C	C	G	T
S/H2/4	CH,LA,TH	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
S/H3/1	LA	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
S/H4/1	LA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
S/H5/6	TH	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
S/H6/1	CH	.	.	G	.	.	.	.	G	G	A	C	A	.	.	.	T	.	T	.	.	.	A	C
S/H7/3	CH,IN	.	.	G	.	.	.	.	G	G	A	C	A	.	.	.	T	.	T	.	T	A	C	C
R/H8/1	EG	.	.	.	.	.	A	T	.	.	.	C	A	T	A	T	.	A	T	G	T	A	.	.
R/H9/3	BR,IT	.	.	.	.	.	A	T	.	.	.	C	A	T	A	T	.	A	T	G	T	A	.	.
R/H10/1	IT	G	.	.	.	.	A	T	.	.	.	C	A	T	A	T	T	A	.	G	T	A	.	.
R/H11/1	IT	.	G	.	.	.	A	T	.	.	.	C	A	T	A	T	T	A	.	G	T	A	.	.
R/H12/1	BR	.	.	.	.	.	A	T	.	.	.	C	A	T	A	T	T	A	.	G	.	A	.	.
R/H13/1	BR	.	G	.	.	.	A	T	.	.	.	C	A	T	A	T	T	A	.	G	.	A	.	.
R/H14/1	IN	.	G	.	.	.	A	T	.	.	.	C	A	T	A	T	T	A	.	G	.	A	.	.
R/H15/4	IN	.	.	.	.	.	A	T	.	.	.	C	A	T	A	T	.	A	T	G	T	A	.	.
R/H16/2	IN	.	.	.	.	.	A	T	.	.	.	C	A	T	A	T	.	A	T	G	T	A	.	.
R/H17/1	IN	.	.	.	.	.	A	T	.	.	.	C	A	T	A	T	.	A	T	G	T	A	.	.
R/H18/2	IN	G	.	.	.	.	A	T	.	.	.	C	A	T	A	T	T	A	.	G	T	A	.	.
R/H19/1	IN	.	.	.	.	.	A	T	.	.	.	C	A	T	A	T	T	A	.	G	T	A	.	.
R/H20/1	IN	.	G	.	.	.	A	T	.	.	.	C	A	T	A	T	T	A	.	G	T	A	.	.
R/H21/1	EG	.	.	.	.	.	A	T	.	.	.	C	A	T	A	T	.	A	T	G	T	A	.	.
R/H22/1	EG	.	.	.	.	.	A	T	.	.	.	C	A	T	A	T	.	A	T	G	T	A	.	.
W,R/H23/3,1	BR,IN	.	.	.	.	.	A	T	.	.	.	C	A	T	A	T	.	A	T	G	T	A	.	.
W/H24/1	IN	.	.	.	.	.	A	T	.	.	.	C	A	T	A	T	.	A	T	G	T	A	.	.

Species/Haplotype/ Number of samples	Country	Variable sites													
		223	240	240	255	258	262	286	287	288	291	290	304	306	
S/H1/6	CH,IN,LA	C	C	C	A	G	T	G	T	C	T	T	T	C	
S/H2/4	CH,LA,TH	.	.	.	.	.	.	.	.	.	.	.	.	.	
S/H3/1	LA	.	.	.	.	.	.	.	.	.	.	.	.	.	
S/H4/1	LA	.	.	.	.	.	.	.	.	.	.	.	.	.	
S/H5/6	TH	.	.	.	.	.	.	.	.	.	.	.	.	.	
S/H6/1	CH	.	.	.	.	.	C	A	.	.	.	.	.	.	
S/H7/3	CH,IN	.	.	.	.	.	C	A	.	.	.	.	.	.	
R/H8/1	EG	.	T	T	G	.	.	.	.	.	A	.	C	T	
R/H9/3	BR,IT	.	T	T	G	.	.	.	.	.	A	.	C	T	
R/H10/1	IT	T	T	T	G	.	.	.	C	.	A	.	.	T	
R/H11/1	IT	.	T	T	G	.	.	.	.	.	A	C	.	T	
R/H12/1	BR	.	.	.	G	A	.	.	T	.	A	.	.	T	
R/H13/1	BR	.	T	T	G	.	.	.	.	.	A	.	C	T	
R/H14/1	IN	.	T	T	G	.	.	.	.	.	A	.	.	T	
R/H15/4	IN	.	T	T	G	.	.	.	.	.	A	.	C	T	
R/H16/2	IN	.	T	T	G	.	.	.	.	.	A	.	C	T	
R/H17/1	IN	.	T	T	G	.	.	.	.	.	A	.	C	T	
R/H18/2	IN	.	T	T	G	A	.	.	T	.	A	.	.	T	
R/H19/1	IN	.	.	.	G	.	.	.	.	.	A	.	.	T	
R/H20/1	IN	.	G	.	.	.	.	.	.	.	A	.	.	T	
R/H21/1	EG	.	.	.	.	.	.	.	.	.	A	.	.	T	
R/H22/1	EG	.	.	.	.	.	.	.	.	.	A	.	.	T	
W,R/H23/3,1	BR,IN	.	.	.	.	.	.	.	.	.	A	.	.	T	
W/H24/1	IN	.	.	.	.	.	.	.	.	.	A	.	.	T	

Dot (.) = identical sequence to reference sequence in first line; S = swamp buffalo (*Bubalus carabanensis*); R = river buffalo (*Bubalus bubalis*); W = wild water buffalo (*Bubalus arnee*); BR = Brazil; CH = China; EG = Egypt; IN = India; IT = Italy; LA = Laos; TH = Thailand.

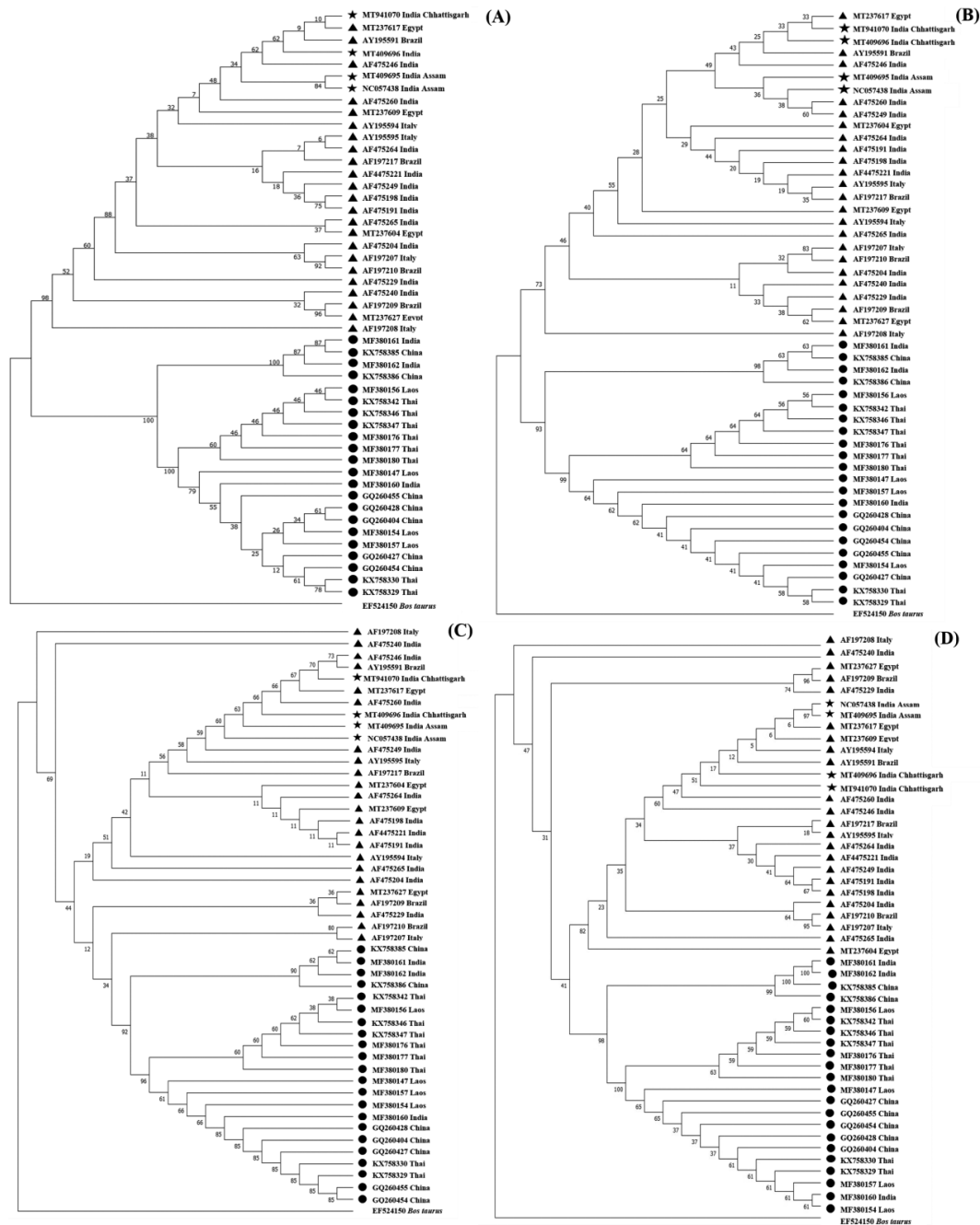


Fig. 3 Neighbor-joining trees reconstructed from: (A) the complete D-loop sequences of water buffaloes; (B) PCR products amplified by Primer I; (C) Primer II; (D) Primer III, where ▲, ● and ★ represent river (*Bubalus bubalis*), swamp (*Bubalus carabanensis*) and wild water buffaloes (*Bubalus arnee*), respectively. *Bos taurus* (EF524150) was included as the outgroup. Numbers at nodes indicate bootstrap values based on 1,000 replications.

The results confirmed that the sequences within Primers I, II and III could be used to separate swamp from other water buffalo species and so could be used to identify swamp buffalo species. In addition, the clustering of swamp buffaloes in all trees was similar and was divided into two clades. The first clade consisted of four swamp buffaloes split equally between two countries (China (KX758385, KX758386) and India

(MF380161, MF380162). The second clade was further divided into two subclades, with one containing swamp buffaloes from two countries (Laos (MF380156) and Thailand (KX758342, KX758346, KX758347, MF380176, MF380177, MF380180) and another subclade, with a different branching pattern for each primer. Hence, these primers could be used for studying the population genetics of water buffalo species.

With the river buffalo, each primer tree established different clustering patterns from the complete D-loop tree. Additionally, in the Primer II tree, there were five sequences of the river buffalo (MT237627 from Egypt, AF197209 and AF197210 from Brazil, AF197207 from Italy and AF475229 from India) that showed a close relationship to the swamp buffalo. However, they were clustered together with the river in subclades; thus, Primer II could be used to identify domestic buffalo species. Since only the first half of the D-loop region was used in the current study, the distribution of nucleotide polymorphism in the river buffalo showed that another region in the D-loop at positions between 700–800 bp had high polymorphism. Hence, this region should be included in any population genetic study of the river buffalo.

Primer specificity and sensitivity tests

The specificity test was based on the DNA extracted from swamp buffalo meat that was used as a DNA template in PCR. Based on the results shown in Fig. 4A, the expected PCR product size of approximately 300 bp was produced in the Primer I assay. Then, it was used as a template for nested PCR with Primer II. As shown in Fig. 4B, the expected product size of approximately 150 bp was amplified. These results indicated that the primers could bind to the specific DNA of the swamp buffalo. Thus, the newly designed nested primers could be used to amplify ancient DNA for studying the origin of the swamp buffalo.

The primer sensitivity test used the 10-fold dilutions of swamp buffalo DNA from concentration levels in the range 1 ng/μL to 0.01 fg/μL as templates (Fig. 5). The minimum concentration of DNA that could be amplified by Primer I was 0.1 pg/μL, compared to Ali et al. (2015) who reported the limitation sensitivity levels in a study using DNA extracted from the meat of mammals were in the range 0.02–0.01 ng. Thus, the current finding indicated that the newly-designed primers had very high sensitivity and the use of the primers might increase the chance of successfully amplifying the D-loop region from extremely damaged DNA specimens.

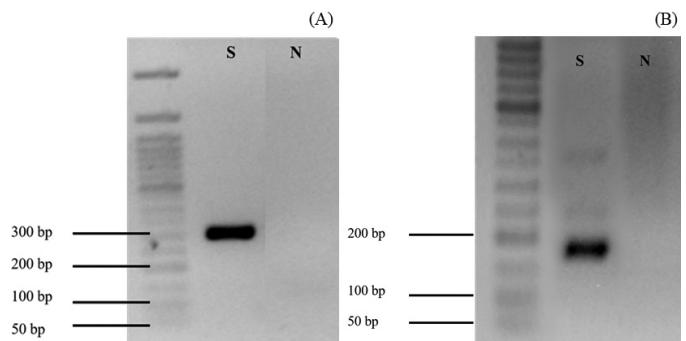


Fig. 4 Agarose gel electrophoresis showing DNA bands for the specificity test of: (A) Primer I; (B) Primer II, where lane S shows PCR products amplified from modern water buffalo DNA and lane N represents the negative control (no DNA template).

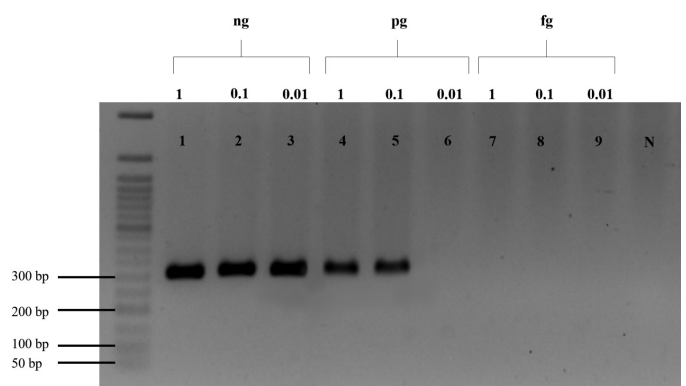


Fig. 5 Agarose gel electrophoresis showing DNA bands from the sensitivity test of Primer I, where lanes 1–9 represent PCR products amplified from 10-fold serial dilutions of modern water buffalo DNA, ranging from 1 ng to 0.01 fg. Lane N represents the negative control (no template DNA).

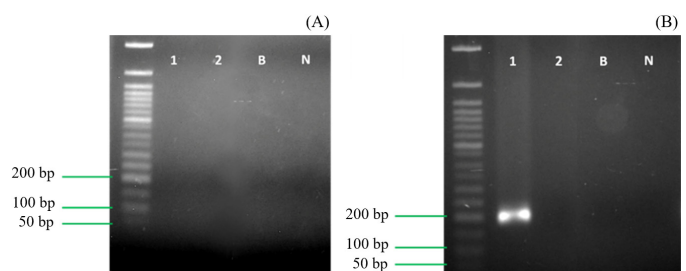


Fig. 6 Agarose gel electrophoresis of: (A) first round and (B) second round of nested polymerized chain reaction (PCR) using ancient water buffalo DNA (~ 3,000 years before present, YBP) as the DNA template. Lanes 1 and 2 represent PCR products from ancient DNA samples, while lanes B and N represent the blank extraction control and the negative control, respectively.

Amplification of ancient DNA using nested polymerase chain reaction

DNA was extracted from an ancient buffalo remain from the Ban Chiang Museum dated approximately 3,000 YBP and it was used as a DNA template in the nested PCR which the expected sizes of the PCR products from the first and second rounds of PCR were 185 and 164 bp, respectively. No PCR product was observed in the first round of PCR; however, PCR products with the expected size of the ancient specimen were observed after performing the second round of PCR (Fig. 6). The PCR product was sequenced using the Sanger method. The sequences from the forward and the reverse primers were pairwise aligned (Fig. S2). The 83-bp overlap sequence of the PCR product was identified to the species level based on the Blast program (Fig. S3). The result confirmed that the Ban Chiang sample belonged to the swamp buffalo species and that it was very closely related to the swamp buffalo samples from Laos (KR008748; Zhang et al., 2016), China (GQ260445; Yue et al., 2013) and Thailand (MT857920; Hongchuta et al., 2021). The current report was the first to document the successful amplification of ancient DNA from the swamp buffalo.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

- Achyut, A., Tej Kumar, S., Ashok, R., et al. 2011. Call to conserve the wild water buffalo (*Bubalus arnee*) in Nepal. *Int. J. Conserv. Sci.* 2: 261–268.
- Ali, M.E., Razzak, M.A., Hamid, S.B.A., Rahman, M.M., Amin, M.A., Rashid, N.R.A., Asing, N. 2015. Multiplex PCR assay for the detection of five meat species forbidden in Islamic foods. *Food Chem.* 177: 214–224. doi.org/10.1016/j.foodchem.2014.12.098
- Cailipan, T.P.C., Paraguas, A.M., Cuanang, A.J.E., et al. 2023. Molecular data and karyotype revealed two distinct species of domesticated water buffaloes in the Philippines. *Philippine J. Sci.* 152: 1861–1872.
- Chen, Y.S., Li, X.H. 1989. New evidence of the origin and domestication of the Chinese swamp buffalo (*Bubalus bubalis*). *Buffalo J.* 5: 51–55.
- Chittavichai, T., Surat, W., Wetchaphan, S., et al. 2021. Origin and distribution of ancient Thai pig lineages. *Int. J. Osteoarchaeol.* 31: 406–416. doi.org/10.1002/oa.2959
- Colli, L., Milanese, M., Vajana, E., et al. 2018. New insights on water buffalo genomic diversity and post-domestication migration routes from medium density SNP chip data. *Front. Genet.* 9: 53. doi.org/10.3389/fgene.2018.00053
- Dabney, J., Meyer, M., Paabo, S. 2013. Ancient DNA damage. *Cold Spring Harb. Perspec. Biol.* 5: a012567. doi.org/10.1101/cshperspect.a012567
- Hongchuta, S., Intapan, P.M., Thanchomnang, T., et al. 2021. Preliminary findings and molecular characterization of thin-walled *Sarcocystis* species in hearts of cattle and buffaloes in Thailand, Lao PDR, and Cambodia. *Parasitol. Res.* 120: 2819–2825. doi.org/10.1007/s00436-021-07241-5
- Katanyuphan, Y., Surat, W. 2023. Primer design for extremely damaged DNA specimens of Asian rhinoceros species. *ANRES.* 57: 885–894. doi.org/10.34044/j.anres.2023.57.5.15
- Katanyuphan, Y. 2024. Development of D-loop primers for studying mitochondrial lineage of ancient rhinoceros in Thailand. M.S. Thesis, Department of Genetics, Kasetsart University, Bangkok, Thailand.
- Kierstein, G., Vallinoto, M., Silva, A., Schneider, M.P., Iannuzzi, L., Brenig, B. 2004. Analysis of mitochondrial D-loop region casts new light on domestic water buffalo (*Bubalus bubalis*) phylogeny. *Molec. Phylogenet. Evol.* 30: 308–324. doi.org/10.1016/s1055-7903(03)00221-5
- Kumar, R., Singh, R. 2010. Buffalo production system in India. In: *Proceedings 9th World Buffalo Congress*. Buenos Aires, Argentina, pp. 32–37.
- Nakamura, H., Muro, T., Imamura, S., Yuasa, I. 2008. Forensic species identification based on size variation of mitochondrial DNA hypervariable regions. *Int. J. Legal Med.* 123: 177–184. doi.org/10.1007/s00414-008-0306-7
- Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E., Sánchez-Gracia, A. 2017. DNASP 6: DNA sequence polymorphism analysis of large data sets. *Mol. Biol. Evol.* 34: 3299–3302. doi.org/10.1093/molbev/msx248
- Siripan, S., Wonnapijit, P., Auetrakulvit, P., Wangthongchaicharoen, N., Surat, W. 2019. Origin of prehistoric cattle excavated from four archaeological sites in central and northeastern Thailand. *Mitochondrial DNA Part A.* 30: 609–617. doi.org/10.1080/24701394.2019.1597072
- Sun, T., Wang, S., Chanthakhoun, V., Dang, R., Huang, Y., Chen, H., Lei, C. 2019. Multiple domestication of swamp buffalo in China and South East Asia. *J. Anim. Breed. Genet.* 137: 331–340. doi.org/10.1111/jbg.12445

- Suraprasit, K., Jaeger, J.J., Chaimanee, Y., Chavasseau, O., Yamee, C., Tian, P., Panha, S. 2016. The middle Pleistocene vertebrate fauna from Khok Sung (Nakhon Ratchasima, Thailand): Biochronological and paleobiogeographical implications. *ZooKeys*. 613: 1–157. doi.org/10.3897/zookeys.613.8309
- Tamura, K., Stecher, G., Kumar, S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* 38: 3022–3027. doi.org/10.1093/molbev/msab120
- Thai Fine Art. 2024. Knowledge. <https://www.fine-arts.go.th/main/departments>, 9 October 2024. [in Thai]
- Wannajuk, M., Sangthong, P., Natapintu, P., et al. 2013. Ancient DNA of pigs in Thailand: Evidence of multiple origins of Thai pigs in the late Neolithic period. *Sci. Asia*. 39: 456–465.
- Wattanapitaksakul, A., 2016. Diversity and diet of middle to late Pleistocene Bovid Assemblages in Thailand. Ph.D. thesis, Maharakarm University, Maha Sarakarm, Thailand.
- Yang, D.Y., Liu, L., Chen, X., Speller, C.F. 2008. Wild or domesticated: DNA analysis of ancient water buffalo remains from north China. *J. Archaeol. Sci.* 35: 2778–2785. doi.org/10.1016/j.jas.2008.05.010
- Yindee, M. 2010. Reproduction and genetic diversity of swamp buffalo. Ph.D. Thesis, Utrecht University. Utrecht, the Netherlands.
- Yue, X., Li, R., Xie, W., et al. 2013. Phylogeography and domestication of Chinese swamp buffalo. *PLoS ONE*. 8: e56552. doi.org/10.1371/journal.pone.0056552
- Youssef, N.A., Curaudeau, M., Nahas, S.M.E., Hassan, A.A.M., Hassanin, A. 2021. Haplotype diversity in the mitochondrial genome of the Egyptian river buffalo (*Bubalus bubalis*). *Mitochondrial DNA Part B*. 6: 145–147. doi.org/10.1080/23802359.2020.1852622
- Zhang, Y., Colli, L., Barker, J.S.F. 2020. Asian water buffalo: Domestication, history and genetics. *Anim. Genet.* 51: 177–191. doi.org/10.1111/age.12911
- Zhang, Y., Lu, Y., Yindee, M., et al. 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. doi.org/10.1111/mec.13518