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Short Communication: Mitochondrial cytochrome b gene analysis reveals lineage history of Indonesian *Bubalus bubalis*

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Abstract. *Sukri A, Wangiyana IGAS, Jannah H, Hadiprayitno G, Kholik, Drudi F. 2025. Short Communication: Mitochondrial cytochrome b gene analysis reveals lineage history of Indonesian* Bubalus bubalis. *Biodiversitas 26: 4654-4661.* Swamp buffalo, *Bubalus bubalis*, is one of the livestock that strongly influences Indonesian social culture. Phylogeography analysis to predict the lineage history of this species is essential for developing this species, especially for breeding purposes. Thus, this study aims to analyze the lineage history and phylogenetic relationship of Indonesian *B. bubalis* using the cytochrome b gene of mitochondrial DNA. Blood samples of *B. bubalis* were taken from 7 sampling regions in Indonesia: Aceh (3 sites), Riau (1 site), Blitar (2 sites), Kalimantan (3 sites), Lombok (3 sites), Madiun (2 sites), and Tana Toraja (3 sites). DNA extraction is conducted from the blood sample, followed by PCR (Polymerase Chain Reaction) amplification using forward primer L14841 and reverse primer H155149, followed by sequencing and reference search using MegaBLAST (Basic Local Alignment Search Tool). The phylogenetic relationship, based on Neighbor Joining and Maximum Likelihood trees, shows that Indonesian *B. bubalis* from the Western Indonesia group (Aceh, Riau, Madiun, and Blitar) has a close relation with *B. bubalis* from India, Bangladesh, Pakistan, and Nepal. Meanwhile, Indonesian *B. bubalis* from Central Indonesia (Kalimantan, Lombok, and Tator) has a close relation with *B. bubalis* from China, Japan, Laos, Vietnam and Thailand. It could be concluded that Indonesian *B. bubalis* exhibit two distinct maternal lineages, indicating dual domestication routes: the South Asia lineage with a domestication center in India and spread out to Western Indonesia (Aceh, Riau, Blitar) and the East Asia lineage with a domestication center in China and spread out to Central Indonesia (Kalimantan and Lombok). Lineage analysis of Indonesian *B. bubalis* can have a significant impact on biodiversity, enriching worldwide genetic mapping and providing conservation data for breeding this species.

Keywords: *Bubalus bubalis*, cytochrome b, Indonesian buffalo, phylogeography, sequence database

INTRODUCTION

Swamp buffalo (*Bubalus bubalis*) is one of the essential livestock resources in Indonesia. This livestock has the potential to contribute to a farming business that supports small and marginal farmers by providing quality meat and milk (Rianto et al. 2025). This species also significantly influences social culture due to its close relationship with tradition in particular ethnic groups in Indonesia (Usman et al. 2017). The close relatives of *B. bubalis* to Indonesian society indicate that this species requires further development (Komariah et al. 2020; Prihandini et al. 2023).

The development of *B. bubalis* should be prioritized for the excellence of this species in the Indonesian region. One unique characteristic of Indonesian *B. bubalis* is the diversity (Suhardi et al. 2020). FAO Domestic Animal Diversity Information System lists 11 breeds of Indonesian indigenous buffalo, namely: Gayo, Java, Kalang South Kalimantan, Kalang East Kalimantan, Kuntu, Moa, Murah, Simeulue, West Sumatra, North Sumatra, Sumbawa and Toraja (Prihandini et al. 2023). Genetic diversity is the main factor responsible for the characteristics of *B. bubalis* in Indonesia, which is essential to further analysis for this species' development, especially for breeding purposes (Maulana et al. 2023).

Although *B. bubalis* is an essential livestock species in Indonesia, the consideration of its conservation status through genetic diversity exploration is rarely conducted. Several countries with excellent resources of buffalo livestock have been concerned with the conservation status of their buffalo species for sustainable husbandry management. Most of this country is distributed in the Asia region, including Nepal (Khulal et al. 2021), Sri Lanka (Vidana-Pathiranage et al. 2023), India (Bora et al. 2024), and Malaysia (Khalex et al. 2021). The effort to develop comprehensive mapping for the conservation status of *B. bubalis* in Indonesia has just begun in Jambi (Syarifuddin

et al. 2024). However, this conservation study needs more diversity data about the genetic variation of this species from other regions in Indonesia, which remains underexplored.

Genetic diversity study of *B. bubalis* can be conducted using various molecular markers depending on the purpose of the analysis (Dzitsiuk et al. 2020; Vohra et al. 2021). The cytochrome b sequence of mitochondrial DNA has been widely used for studying genetic diversity in *B. bubalis* worldwide, as well as in Indonesia (Santhosh et al. 2025). Haplotype diversity analysis using the cytochrome b sequence has revealed that *B. bubalis* in Indonesia exhibits a variety of haplotypes, ranging from 10 to 16 (Sukri et al. 2014; Rusdin et al. 2020). Phylogenetic analysis using cytochrome b sequence also revealed that Indonesian *B. bubalis* can be divided into two main clusters, namely the western part of Indonesia cluster (Sumatra and Java) and the center part of Indonesia cluster (Kalimantan, Bali, Nusa Tenggara Barat, and Sulawesi) (Amin et al. 2016; Winaya et al. 2019). This result shows the proper genetic diversity mapping of *B. bubalis* based on the Indonesian local buffalo cytochrome b sequence. However, there are still very limited studies that involve the cytochrome b sequence of *B. bubalis* from worldwide to be compared with the Indonesian *B. bubalis* sequence for phylogeography analysis.

Phylogeography analysis can reveal the historical lineage of Indonesian *B. bubalis*, which has rarely been conducted. It is estimated that the domestication history of *B. bubalis* began in China and India, spreading worldwide (Yue et al. 2013; Mishra et al. 2015). However, there is still a missing analysis that can link the sequence database of *B. bubalis* in Asia and Indonesia, which can provide a prediction of how this species spread to this country (Minervino et al. 2020). This issue must be addressed to understand better the driving factors responsible for the diversity of Indonesian *B. bubalis*. Furthermore, by comparing the cytochrome b database from other countries, the genetic mapping position of Indonesian *B. bubalis* worldwide can be determined (Zhang et al. 2020). Thus, this study aims to analyze the lineage history and phylogenetic relationship of Indonesian *B. bubalis* using the cytochrome B gene of mitochondrial DNA.

MATERIALS AND METHODS

Study area

Blood samples from *Bubalus bubalis* were collected in 7 sampling regions in Indonesia: Aceh, Riau, Blitar, Kalimantan, Lombok, Madiun, and Tana Toraja. Each region has 1 to 3 sampling sites with detail: 3 sampling sites of Aceh (Aceh1, Aceh2, and Aceh 3), only 1 sampling site of Riau, 2 sampling sites of Blitar (Blitar1 and Blitar2), 3 sampling sites of Kalimantan (Kalimantan1, Kalimantan2, and Kalimantan3), 3 sampling sites of Lombok (Lombok1, Lombok2, and Lombok3), 2 sampling sites of Madiun (Madiun1 and Madiun2) and 3 sampling sites of Tana Toraja (Tator1, Tator2, and Tator3). The sampling site was chosen based on the number of available livestock centers in each location. Fifteen individuals of *B. bubalis* were selected from each sampling site for the blood sampling. The blood (5 mL) was taken from the jugular vein of *B. bubalus* using a venoject and vacuum tube. The amount of 0.1 g of Ethylenediaminetetraacetic Acid (EDTA) was added to the sample to prevent blood clotting (Chauhan et al. 2024). The blood samples were kept at 4°C for further analysis.

Ethical approval

Blood samples were collected by a qualified veterinarian from the Faculty of Veterinary Medicine, Universitas Pendidikan Mandalika, Indonesia. The blood samples were taken using proper techniques and adequate restraint to minimize pain, stress, and distress to the animal. The study was ethically approved by the Ethics Commission of the Veterinary Faculty of Universitas Pendidikan Mandalika.

Procedures

DNA extraction

DNA genome extraction from a blood sample was carried out using the Nucleospin Quickpure^{R} Blood Kit with protocol according to the manufacturer's recommendation. The DNA genome was inspected by gel electrophoresis with an agarose concentration of 0.8% (w/v). Genomic DNA concentration and purity were analyzed using absorbance measurements of wavelengths 260 nm, 280 nm, and 230 nm (Lucena-Aguilar et al. 2016).

PCR amplification

Amplification of partial mitochondrial DNA cytochrome b gene from the DNA genome was conducted using forward primer L14841 (`AAA AAG CTT CCA TCC AAC ATC TCA GCA T`) and reverse primer H155149 (`AAA CTG CAG CCC CTC AGA ATG ATA TTT G`). A total of 25 µL reaction mixture (KAPA Biosystems) containing: 2.5 µL DNA template, 2.5 µL forward primer, 2.5 µL reverse primer, 12.5 µL PCR mix, and 5 µL dH_2O was used for the PCR reaction. PCR amplification was conducted using 30 cycles with the following profile: pre-denaturation at 93°C for 30 s, denaturation at 93°C for 1 min, annealing at 50°C for 1 min, elongation at 72°C for 5 min, and post-elongation at 40°C.

Sequencing and database reference searching

The PCR amplicon was purified and then sequenced using one forward primer (`AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA`) with the single Taq Dye Deoxy Terminator Cycle Sequencing Kit and 373s DNA Sequencer (PerkinElmer, USA). Raw sequence data in "fastq" format were evaluated for quality control, including several metric parameters, per-base sequence quality, per-sequence quality scores, GC content and bias checks, sequence length distribution, sequence duplication levels, and contamination identification. The sequence length of 330 bp was obtained after trimming, filtering and post-alignment process using BioEdit Sequence Alignment Editor (Sukri et al. 2014).

The FASTA file from the editing process was used for database reference searching. Database reference searching is carried out using the Basic Local Alignment Search Tool

(BLAST) from the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Query sequence input was conducted using blastn with the standard nucleotide database searching option. Program selection was optimized for highly similar sequences (MegaBLAST). All reference sequences were saved as FASTA files (Chen et al. 2015).

Data analysis

The Indonesian *B. bubalis* sequence and reference sequence were analysed to identify regions of similarity and variability for finding homologous positions. This analysis involved a multiple alignment sequence process conducted using ClustalX 2.1. (Ferrari and Patrizio 2021). The aligned sequences were then used for reconstructing a phylogenetic tree with the *Bos javanicus* (FJ556566.1) sequence as the outgroup.

Phylogenetic tree reconstruction is conducted using MEGA 5.1 with two different algorithms (Caspermeyer 2018). The first algorithm is distance-based neighbour-joining. The second algorithm is character-based Maximum Likelihood (Rusinko and McPartlon 2017; Sagulenko et al. 2018). Kimura 2-Parameter was used as an evolution model with 1000 bootstrap replications. The Gamma distribution was chosen as the rate of site evolution approaching. Phylogenetic trees were used to predict the lineage history of Indonesian *B. bubalis*. Alignment sequence databases were also analyzed for the divergence value using Phydit version 3.1.

RESULTS AND DISCUSSION

Sequence reference from the gene bank database

Fifty-five *Bubalus bubalis* cytochrome b sequences from 11 countries were selected based on a MegaBLAST query from NCBI website (https://www.ncbi.nlm.nih.gov/). MegaBLAST, used in this research, is one of the most popular and fundamental alignment tools for reference sequence searching (Chen et al. 2015). All selected reference sequences have a minimum 98% query cover with the sequence of Indonesian *B. bubalis* from Aceh, Riau, Blitar, Kalimantan, Lombok, Madiun, Tana Toraja. Sequence references of *B. bubalis* were predominantly from Asian countries (Table 1). This result confirmed that Asia is the center of *B. bubalis* domestication, with a long history as the origin of this species' ancestor (Zhang et al. 2020). One exclusion from the reference searching is the sequence from Romania (Europe). However, this sequence is still included in phylogenetic tree reconstruction.

Table 1. Reference sequence of *Bubalus bubalis* based on MegaBLAST query from the NCBI database

No	Accession number	Country	Reference	No	Accession number	Country	Reference
1	MT182605.1	Lao	Sun et al. 2020	29	MH718883.1	Nepal	Kandel et al. 2019
2	MT182604.1	Lao		30	MH718882.1	Nepal	
3	MT182603.1	Lao		31	MH718881.1	Nepal	
4	MT182602.1	Lao		32	MT182515.1	Thailand	Sun et al. 2020
5	MT182601.1	Lao		33	MT182514.1	Thailand	
6	LC605626.1	Iraq	Owaid et al. 2022	34	MT182513.1	Thailand	
7	LC605625.1	Iraq		35	MT182512.1	Thailand	
8	LC605624.1	Iraq		36	MT182511.1	Thailand	
9	LC605623.1	Iraq		37	JF946525.1	Pakistan	Saif et al. 2019
10	LC605622.1	Iraq		38	JF946524.1	Pakistan	
11	LC605621.1	Iraq		39	JF946523.1	Pakistan	
12	MT182588.1	Vietnam	Sun et al. 2020	40	JF946522.1	Pakistan	
13	MT182587.1	Vietnam		41	JF946521.1	Pakistan	
14	MT182586.1	Vietnam		42	EF409942.1	India	Kumar et al. 2007
15	MT182585.1	Vietnam		43	EF409941.1	India	
16	MT182584.1	Vietnam		44	EF409940.1	India	
17	D88637.1	Japan	Kikkawa 1997	45	EF409939.1	India	
18	D88635.1	Japan		46	KR009166.1	Bangladesh	Zhang et al. 2016
19	D88633.1	Japan		47	KR009165.1	Bangladesh	
20	D88634.1	Japan		48	KR009164.1	Bangladesh	
21	D88632.1	Japan		49	KR009163.1	Bangladesh	
22	MT182536.1	China	Sun et al. 2020	50	KR009162.1	Bangladesh	
23	MT182535.1	China		51	JQ241283.1	Romania	Coroian et al. 2015
24	MT182534.1	China		52	JQ241282.1	Romania	
25	MT182533.1	China		53	JQ241281.1	Romania	
26	MT182532.1	China		54	JQ241280.1	Romania	
27	MH718885.1	Nepal	Kandel et al. 2019	55	JQ241279.1	Romania	
28	MH718884.1	Nepal					

Phylogenetic analysis

Phylogenetic analysis is a highly reliable and important bioinformatics tool in the era of molecular biology. The essence of phylogenetic analysis lies in the algorithm used as a statistical approach to solve the problem (Roy et al. 2014; Wangiyana et al. 2024). Generally, two common approaches to phylogenetic analysis were used. The first approach is an algorithm based on the evolutionary distance between the organism taxonomic unit. The second approach is algorithm-based to search the likelihoods of the evolutionary tree according to the evolutionary process model and the data set (Horiike 2016; Wangiyana et al. 2021). This research employed distance-based and character-based algorithms as statistical approaches to determine the most accurate evolutionary history of the Indonesian *B. bubalis*. These two algorithms are the Neighbor Joining and Maximum Likelihood methods.

Neighbor Joining was first described by Saito and Nei in 1987 as a distance-based phylogenetic algorithm. This algorithm is also considered the most popular distance-based algorithm for generating phylogenetic trees (Bogusz and Whelan 2017; Rusinko and McPartlon 2017). The neighbor-joining tree in this research reveals two lineages of Indonesian *B. bubalis* with distinct ancestors (Figure 1A). First lineage is Middle-East and South Asia lineage, which consists of 8 clades, including: Nepal-India clade, Bangladesh Clade, Iraq Clade, Pakistan Clade, Aceh Clade, Blitar Clade, Madiun Clade, and Tator Clade. Neighbor Joining tree suggests that the 4 Indonesian *B. bubalis* clades (Aceh, Blitar, Madiun and Tator) have an ancestor from South Asia (India). The second lineage is East Asia-Southeast Asia, consisting of 7 Clades: Thailand Clade, Japan Clade, China Clade, Vietnam Clade, Lao Clade, Kalimantan Clade, and Lombok Clade. Neighbor Joining tree suggests that 2 Indonesian *B. bubalis* clades have ancestors from East Asia (China). This result differs slightly from a similar phylogenetic study using the Neighbor Joining algorithm, in which Tator joins the Kalimantan and Lombok Clade (Winaya et al. 2019). This indication shows that different sequence references can slightly impact different phylogenetic tree topologies, even using the same algorithm (Gonnet 2012; Sukri et al. 2022).

Maximum Likelihood was first described by Felsenstein in 1981 as a character-based phylogenetic algorithm. This algorithm searches for the best evolutionary tree with an optimum heuristic approach (Sagulenko et al. 2018; Zhou et al. 2018). The Maximum Likelihood tree in this study has a topology similar to that of the Neighbor Joining tree (Figure 1.B). Two lineages, including those from the Middle East-South Asia and East-Southeast Asia regions, were also identified in the Maximum Likelihood tree. However, the status of the Tator Clade was slightly different in the Maximum Likelihood Tree. Tator Clade is joining the East-Southeast Asia lineage in the Maximum Likelihood Tree. This result indicates that different topologies can be caused by different algorithmic approaches (Waese et al. 2017). Thus, algorithm selection must be considered and adjusted to the primary purpose of the phylogenetic tree reconstruction (Brazeau et al. 2019).

The divergence value supports the phylogenetic analysis by indicating the similarity of *B. bubalis* cytochrome b sequences (Table 2). Based on sequence similarity, the Indonesian *B. bubalis* clade can be classified into two primary groups. The first group is Western Indonesia, including Aceh, Blitar, Madiun, and Riau. The second group is Central Indonesia, including Kalimantan, Lombok, and Tator. Based on the sequence similarity, Western Indonesian *B. bubalis* can be clustered with *B. bubalis* from India, Nepal, Pakistan, Bangladesh, and Iraq. This cluster group forms the Middle East-South Asia lineage of *B. bubalis*. Meanwhile, Central Indonesian *B. bubalis* can be clustered with *B. bubalis* from China, Japan, Laos, Vietnam, and Thailand. This cluster group forms the East-Southeast Asia lineage of *B. bubalis*. The similarity of sequence that determines the divergence value is affected by the number of different nucleotides and the total number of nucleotides between two comparable sequences after the multiple alignment process. Thus, the length of the sequence can be an essential parameter that determines the genetic distance in the analysis. Since the length of the sequence is determined by the primer, which serves as a molecular marker, it is crucial to select the appropriate primer for determining the divergence value (Ravenscroft et al. 2015).

Lineage history reconstruction

Lineage history shows the evolution history map prediction of Indonesian *B. bubalis*, which can be estimated based on the phylogenetic tree (Figure 2) and divergence value analysis (Table 2). Indonesian *B. bubalis* forms Sumatra Island and Java Island, and was estimated to come from South Asia, the center of domestication in India. This lineage also spread to the Middle East and the European region. On the other Hand, Indonesia *B. bubalis* from Kalimantan and Lombok were estimated to come from East Asia, with the center of domestication in China. This lineage spread to the south to Indonesia and the east to Japan. This lineage history supports the prediction that the center of domestication of *B. bubalis* in Asia is in China and India (Zhang et al. 2020). The lineage of *B. bubalis* from China was reported to spread out to Southeast Asia. (Zhang et al. 2016). Meanwhile, the lineage of *B. bubalis* from India has been reported to have spread to Southeast Asia, as well as to North Africa and Europe (Mishra et al. 2015). This lineage prediction can be used to analyze the possible migration of *B. bubalis* in Indonesia. The migration of this species in Indonesia tends to occur in two patterns. The first is migration between Sumatra Island and Java Island. The second is migration between Kalimantan, Sulawesi, and the Lesser Sunda Islands. This migration data can lead to a better understanding of the influence of geographical location on the genetic variation of *B. bubalis* in Indonesia (Sukri et al. 2014). This information can also be helpful for the domestication strategy of buffalo in Indonesia for sustainable livestock management.

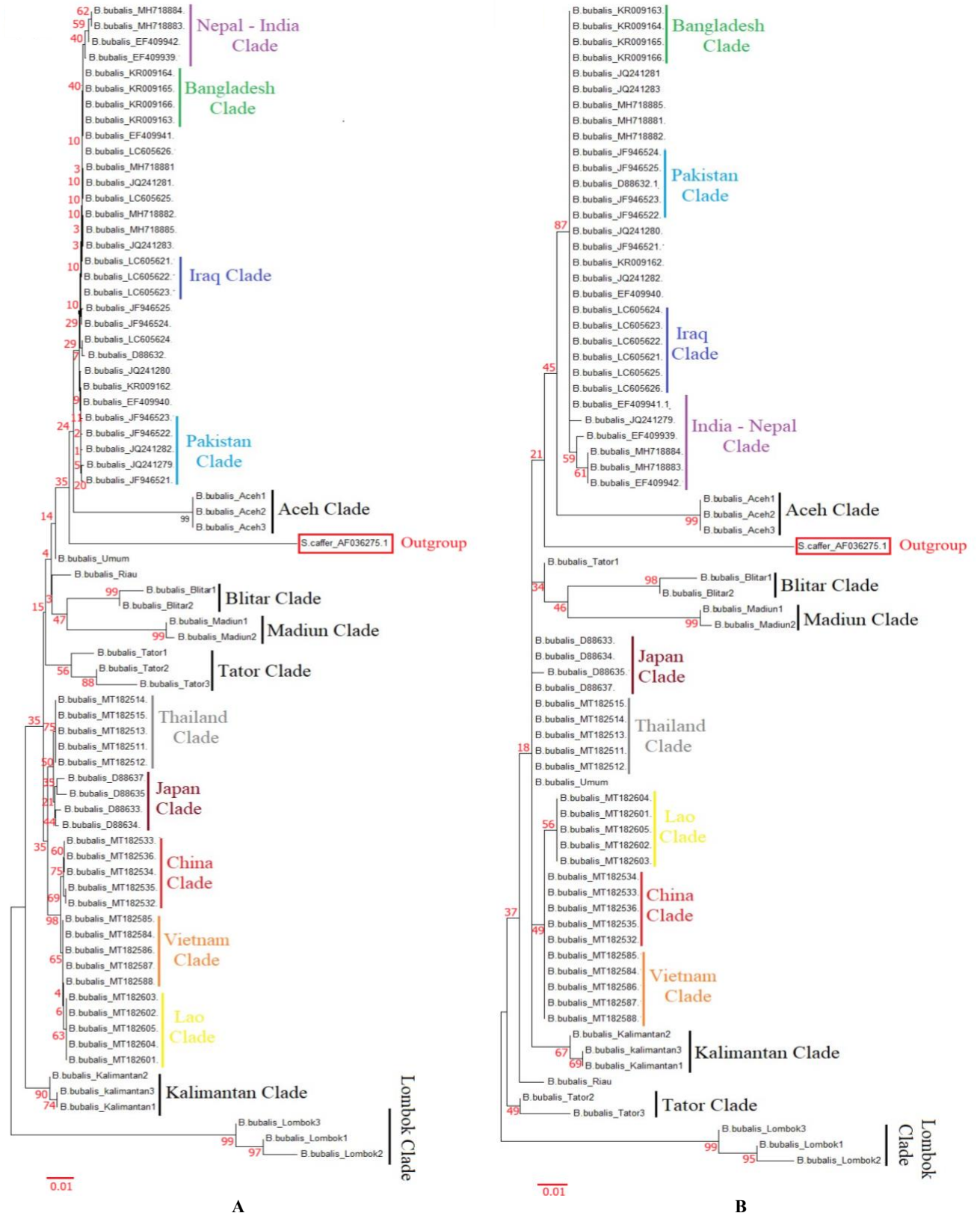

Figure 1. Phylogenetic Tree Based on Different Algorithms showing *Bubalus bubalis* clade from each regional origin. A. Neighbor Joining Tree (Left), B. Maximum Likelihood Tree (Right). Red number in the node of each branch shows the bootstrap value. Red bar in the bottom left shows the divergence scale. The clades colored black (Aceh Clade, Blitar Clade, Madiun Clade, Kalimantan Clade, and Lombok Clade) were established based on the DNA isolation and sequence analysis performed in this study. Meanwhile, the clades colored other than black (India-Nepal Clade, Bangladesh Clade, Pakistan Clade, Iraq Clade, Japan Clade, Thailand Clade, Lao Clade, China Clade, and Vietnam Clade) were established based on sequence reference searching in the NCBI database

Table 2. Divergence value based on sequence similarity of Indonesian *Bubalus bubalis* with reference

	Iraq#	Romania#	Bangladesh#	Pakistan#	Nepal#	India#	Aceh*	Blitar*	Madiun*	Riau*	Laos#	China#	Vietnam#	Umum*	Thailand#	Japan#	Tator*	Lombok*	Kalimantan*
Iraq#	---	0/583	0/583	1/583	0/234	1/583	13/261	14/261	16/261	6/261	15/583	13/583	14/583	3/261	12/583	14/583	7/261	32/261	12/330
Romania#	100	---	1/1140	2/1137	2/401	4/1125	16/330	15/330	18/330	7/330	31/1140	30/1140	30/1140	4/330	27/1140	32/1140	12/330	38/330	11/330
Bangladesh#	100	99.91	---	1/1137	1/401	3/1125	15/330	14/330	17/330	6/330	30/1140	29/1140	29/1140	3/330	26/1140	31/1140	11/330	37/330	12/330
Pakistan#	99.83	99.82	99.91	---	1/401	4/1122	16/330	15/330	18/330	7/330	30/1137	29/1137	29/1137	4/330	27/1137	32/1137	12/330	38/330	9/303
Nepal#	100	99.5	99.75	99.75	---	1/386	14/303	14/303	17/303	6/303	7/401	7/401	6/401	3/303	5/401	6/401	10/303	30/303	12/330
India#	99.83	99.64	99.73	99.64	99.74	---	16/330	15/330	18/330	7/330	28/1125	27/1125	27/1125	4/330	24/1125	29/1125	12/330	38/330	35/329
Aceh*	95.02	95.15	95.45	95.15	95.38	95.15	---	23/330	27/330	17/330	18/330	18/330	17/330	16/330	16/330	16/330	24/330	49/330	9/261
Blitar*	94.64	95.45	95.76	95.45	95.38	95.45	93.03	---	21/330	14/330	15/330	15/330	14/330	13/330	13/330	14/330	18/330	43/330	20/330
Madiun*	93.87	94.55	94.85	94.55	94.39	94.55	91.82	93.64	---	15/330	15/330	16/330	15/330	14/330	14/330	15/330	21/330	45/330	9/330
Riau*	97.7	97.88	98.18	97.88	98.02	97.88	94.85	95.76	95.45	---	5/330	5/330	4/330	3/330	3/330	4/330	9/330	35/330	10/330
Laos#	97.43	97.28	97.37	97.36	98.25	97.51	94.55	95.45	95.45	98.48	---	3/1140	1/1140	2/330	10/1140	17/1140	10/330	36/330	10/330
China#	97.77	97.37	97.46	97.45	98.25	97.6	94.55	95.45	95.15	98.48	99.74	---	2/1140	2/330	9/1140	16/1140	10/330	36/330	9/330
Vietnam#	97.6	97.37	97.46	97.45	98.5	97.6	94.85	95.76	95.45	98.79	99.91	99.82	---	1/330	9/1140	16/1140	9/330	35/330	8/330
Umum*	98.85	98.79	99.09	98.79	99.01	98.79	95.15	96.06	95.76	99.09	99.39	99.39	99.7	---	0/330	1/330	8/330	34/330	8/330
Thailand#	97.94	97.63	97.72	97.63	98.75	97.87	95.15	96.06	95.76	99.09	99.12	99.21	99.21	100	---	7/1140	8/330	34/330	7/330
Japan#	97.6	97.19	97.28	97.19	98.5	97.42	95.15	95.76	95.45	98.79	98.51	98.6	98.6	99.7	99.39	---	9/330	33/330	13/330
Tator*	97.32	96.36	96.67	96.36	96.7	96.36	92.73	94.55	93.64	97.27	96.97	96.97	97.27	97.58	97.58	97.27	---	34/330	33/330
Lombok*	87.74	88.48	88.79	88.48	90.1	88.48	85.15	86.97	86.36	89.39	89.09	89.09	89.39	89.7	89.7	90	89.7	---	33/330
Kalimantan*	96.36	96.67	96.36	97.03	96.36	89.36	96.55	93.94	97.27	96.97	96.97	97.27	97.58	97.58	97.88	96.06	90	90	---

Note: Red color in the table indicates Middle East-South Asia lineage and blue color in the table indicates East-Southeast Asia lineage. Bottom triangle of the matrix shows the similarity percentage value of the sequence. Upper triangle of the matrix shows the number of different nucleotides per total number of nucleotides in the sequence. Symbol * indicates sequences obtained from the DNA isolation and sequencing performed in this study. Symbol # indicates sequences obtained from the search in the NCBI database

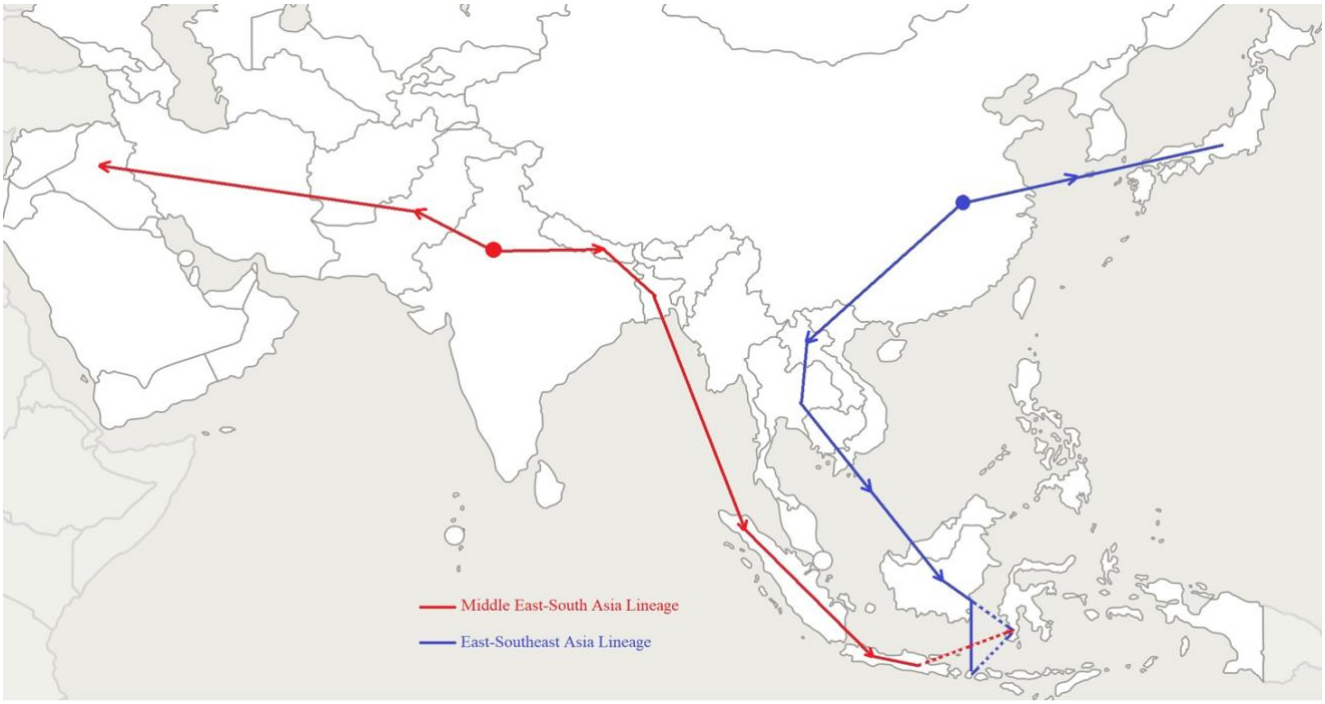

Figure 2. Lineage history prediction map of Indonesian *Bubalus bubalis*

Phylogenetic study of Indonesian *B. bubalis* shows that the Tator Clade, which represents the Sulawesi part, has been clustered in the same clade with the Lombok Clade (Lesser Sunda) and the Kalimantan Clade (Winaya et al. 2019). However, in this study, a different algorithm was used to reconstruct the phylogenetic tree with slightly different topology in the Tator clade. Different clade statuses of the Tator Clade in the Neighbor Joining tree and the Maximum Likelihood tree have caused the lineage history of Indonesian *B. bubalis* from Sulawesi to remain unclear. This clade can be a part of the Middle East-South Asia and East-South East Asia lineage, which is shown as a dotted line in the lineage history map (Figure 2). Thus, more data about Indonesian *B. bubalis* from Sulawesi is needed to reveal this clade's evolution history. Nevertheless, this study can still fill the research gap left by the previous study, which predicted that the ancestor of Indonesian *B. bubalis* originated solely from the China region (Prihandini et al. 2023).

Missing clades from other regions could be added for further study of the lineage history of *B. bubalis*. Two potential regions that should be added are Malaysia and the Philippines. Malaysia Clade can bridge the possible intersection point between the South Asia Lineage (India) and the East Asia Lineage (China) before entering the Indonesian region. Meanwhile, the Philippine Clade could give better insight into the East Asia lineage prediction before entering the Kalimantan or Sulawesi region in Indonesia. However, this study's algorithm query is insufficient to search for a suitable sequence reference that covers these two regions. Further studies should conduct more optimizations in the algorithm query setting. Direct sequencing of *B. bubalis* from Malaysia and the Philippines can also be conducted to provide a reference sequence for these two regions. This effort can enrich the worldwide database of *B. bubalis* as a valuable data source for more accurate lineage history prediction (Rehman et al. 2021; Kumari et al. 2025).

In conclusion, a comprehensive analysis of the cytochrome b mitochondrial sequence, incorporating sequence references from several Asian countries based on MegaBLAST NCBI and phylogenetic analysis using the Neighbor-Joining and Maximum Likelihood algorithms, reveals that Indonesian *B. bubalis* exhibits two distinct maternal lineages, indicating dual domestication routes. The first lineage is the South Asia lineage, which has a domestication center in India that spread out to Sumatra and Java. The second lineage is the East Asia lineage, which originated in China and spread to Kalimantan and the Lesser Sunda Islands. This finding supports the proposal of two main clades in Indonesian *B. bubalis*, namely the Western Indonesia Clade (Sumatra and Java) and the Central Indonesia Clade (Kalimantan, Sulawesi and Lesser Sunda). This finding can be helpful for the genetic resource management and domestication strategies in Indonesia. A lineage map based on the genetic diversity of *B. bubalis* in Indonesia can be estimated, providing helpful information for breeding strategies. Furthermore, the livestock management of this species can also be done effectively without increasing the risk of genetic diversity loss. An integrative genetic diversity study among the cross-breeding of different clade groups of Indonesian *B. bubalis* can provide a better understanding of the breeding strategies of this species.

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