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REGULAR ARTICLES

Mitochondrial phylogenetic and diversity analysis in Azi-Kheli buffalo

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Abstract

Novelty statement The present study was conducted for the first time in Pakistan to investigate *Cytochrome C Oxidase Subunit 1 (CO1) gene* and full-length *Displacement Loop (D-loop)* region of mitochondrial DNA in Azi-Kheli buffalo breed native to northern hilly areas of Khyber Pakhtunkhwa Province of Pakistan. The present study was designed to investigate phylogeny and diversity in Azi-Kheli buffalo, through two mitochondrial DNA regions, i.e., *Cytochrome C Oxidase Subunit-I (CO1) and Displacement Loop (D-loop)* region. Thirty (30) blood samples were taken from Azi-Kheli pure breed animals from original breeding tract, i.e., Khwazakhela, Swat. Polymerase chain reactions using gene-specific primers were carried out for amplifying 709-bp region of *CO1* gene and 1159-bp region of *D-Loop* for identification, phylogeny, and diversity in Azi-Kheli buffalo, respectively. The sequences of *CO1* gene revealed four (04) haplotypes, whereas *D-loop* sequences revealed five (05) haplotypes. Mean interspecific diversity with related species was 2.56%, and mean intraspecific diversity within Azi-Kheli buffalo was 0.25%, estimated via Kimura-2 parameter. Phylogenetic tree (maximum likelihood) revealed clustering of Azi-Kheli haplotypes with river buffalo and is distinct from swamp buffalo and other related species of genus Bubalus. Mean haplotype and nucleotide diversity of *D-loop* were $Hd = 0.9601 \pm SD = 0.096$ and $\pi = 0.01208 \pm SD = 0.00182$, respectively. Phylogenetic tree (neighbor-joining) revealed two main clades, i.e., river buffalo and swamp buffalo clade. The haplotypes of Azi-Kheli clustered with haplotypes of different river buffalo breeds at different positions. The current study suggests that Azi-Kheli has common origin with other river buffalo breeds; hence, it is river buffalo which harbors high genetic diversity.

Keywords Azi-Kheli buffalo · Mitochondrial DNA · *D-loop* · *CO1* · Phylogenetics · Genetic diversity

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Introduction

There are total five breeds of water buffalo in Pakistan, including *Nili, Ravi, Nili-Ravi, Kundi,* and *Azi-Kheli*. These animals are raised in small herds (1–10 animals/herd) mostly in rural areas (Khan et al., 2007). *Azi-Kheli* buffalo is a native breed of Pakistan propagated in different areas of Swat, Shangla, Malakand, Dir, and Bajur districts in Khyber Pakhtunkhwa (Khan et al., 2013). In a 2006 census report, *Azi-Kheli buffalo* was incorporated for the first time and counted 2.9% of total provincial buffalo population having 107,000 in number with reducing population tendency (Khan, 2003; Khan et al., 2007).

The ancestor of present-day domestic water buffalo is *Bubalus arnee* which is a wild buffalo found in Nepal, Bhutan, Thailand, and few areas of India (Scherf, 2001). It is assumed that domestic water buffalo *Bubalus bubalis* was domesticated as early as 5000 years back in civilization of Indus Valley (Cockrill, 1981) and 7000 years ago in China (Chen and Li, 1989). There are two ideas about domestication of water buffalo, one idea states that both river and swamp buffaloes were independently domesticated while the second idea state that both types of water buffaloes were result of a single domestication event (Kierstein et al., 2004).

In every eukaryotic cell, mitochondria are the most abundant organelles located inside the cytoplasm and termed as power house of the cell (Mandal et al., 2011). Apart from nuclear DNA, mitochondria contain a small size genome which is maternally transmitted, lacks genetic recombination, and is regarded as a vital tool for evaluation of phylogenetic studies in many species or different breeds within the same species (Chen et al., 2013; Zinovkina, 2018). The study of DNA especially mitochondrial genome is used to find out past domestication and diversity of mammalian species (Robinson et al., 2010; Gonzalez-Freire et al., 2015). Little but considerable part of mitochondrial genome studies about phylogeny of domestic water buffalo species (*Bubalus bubalis*) has been reported (Kierstein et al., 2004).

The *COI* gene as a DNA barcoding marker was investigated for the first time as an exact tool in identification and phylogenetic analysis of a species by Hebert et al. (2003). The nucleotide sequence of *CO1* gene within same species is expected to be nearly identical (Hebert et al., 2004). Besides from genes, mitochondrial DNA constitutes (~ 1150 pb) large non-coding region called *D-loop* (Shadel and Clayton, 1997). There are hypervariable regions in the *D-loop* which have high mutation rate as compared to the whole mitochondrial genome. They play a key role in the phylogenetic analysis of eukaryotes (Lang et al., 1999). The data obtained from genetic methodologies like genetic information, evolution, and phylogeny are essential for the conservational management and species monitoring (Schwartz et al., 2007). In Pakistan, phylogenetic analysis using *D-loop* has been attempted in *Kundi* (Hussain et al., 2009), *Nili, Ravi*, and *Nili-Ravi* (Zahoor et al., 2016; Bhatt et al., 2020). Moreover, genetic relationship and diversity analysis using *D-loop* has also been reported in the same breeds with Indian riverine and Chinese swamp buffaloes, sequences retrieved from GenBank (Bhatt et al., 2020). Our project "Characterization of cattle genetic resources of Khyber Pakhtunkhwa through Genetic Markers and Molecular techniques" focused to characterize cattle breeds' native to Khyber Pakhtunkhwa Province of Pakistan. Four buffalo breeds, i.e., Kundi, Nili, Ravi, and Nili-Ravi, being native to Punjab and Sindh provinces of Pakistan were excluded from the current study. *Azi-Kheli* buffalo breed being native to Khyber Pakhtunkhwa Province and mandate of the project was thus selected in the current study to investigate its origin and level of genetic diversity using *CO1* and *D-loop*.

Materials and methods

Sample collection and DNA extraction

For this research, the breeding track of *Azi-Kheli* buffalo—district Swat located Northern belt of Khyber Pakhtunkhwa Province—was visited twice in the start and end of February 2020. Animals having small compact body size, well adopted to mountain slope grazing, typical dominant brown coat color, and sickle shaped horns are typical morphometric description of the *Azi-Kheli* buffalo breed (Khan et al., 2013). A team of experts from LR&DS, Charbagh, District Swat, and *Azi-Kheli* Buffalo Improvement and Development Farm Charbagh, District Swat (34.8346° N, 72.5441° E) was accompanied to ensure sampling from pure *Azi-Kheli* breed. Moreover, history-based pedigree was recorded from the owner of each animal to ensure pure breed and not cross-breed. A total 30 unrelated animals of *Azi-Kheli* buffalo, including bull, cow, and calves, were sampled for blood collection from different herds. The jugular vein of each animal was disinfected and punctured with sterile disposable syringe, and 3 ml of blood was collected in EDTA tubes (REF-XLGA-E3K3, Xinle®, China). DNA was extracted by non-enzymatic salting out method from blood samples as described by Suguna et al., (2014). The research work was conducted at the Genomic Laboratory, Centre of Microbiology and Biotechnology (CMB), Veterinary Research Institute (VRI), Peshawar, from February 2020 to November 2020 (34.0170° N, 71.5699° E).

PCR amplification of CO1 and D-loop

The partial region of *CO1* gene was amplified using: F5′-TCTCAACCAACCATAAAGATATCGG-3′ and R. 5′-TATACTTCAGGGTGTCCGAAGAATCA-3′ primers (accession no. AF547270.1), whereas the whole region of *D-loop* was amplified using F 5′-TAGTGCTAATACCAACGGCC-3′ and R 5′ AGGCATTTTCAGTGCCTTGC-3′ primers (Accession No. AY488491.1); the primers were designed from NCBI data base (www.ncbi.nlm.nih.gov). The designed primers were sent to Macrogen®, Korea, for synthesis. A total of 25-ul PCR reaction was prepared for each sample in a PCR tube by adding 5 ul of master mix (Cat. No.SM213-0250, GeneDireX, Inc.), 1.5 ul of reverse primer, and 1.5 ul of forward primer, 12 ul of PCR water, and 5 ul of template DNA. The reaction was carried out in thermal cycler BIORAD® using the following protocol: 94 °C for 5 min and 34 cycles of 94 °C for 30 s, 58 °C and 59 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min.

Sequencing of CO1 gene and D-loop

The two target regions on isolated DNA were amplified by PCR techniques using specific primers of complete *D-loop* while partial *CO1 gene*. The sequencing reactions of mitochondrial *CO1* gene and *D-loop* were performed through Sanger sequencing method, as described by Sanger et al. (1977). Both the targeted regions were subjected to chain termination PCR and million to billion copies were terminated at random lengths by 5′-ddNTPs. Then, terminated oligonucleotides were separated in gel electrophoresis via supply of electric current. Finally, gel was analyzed and DNA sequence was determined by fluorescence tags through automated Sanger sequencing and results were generated by computer in AB1 format.

Data analysis

The reference sequence of *COI* gene (accession no. AF547270.1) and *D-loop* (accession no. AY488491.1) are downloaded from NCBI GenBank (www.ncbi.nlm.nih.gov). The sequencing results of *COI* and *D-loop* of the current study were compared with its reference sequences, in which single-nucleotide polymorphism (SNP) positions were detected. Every identical sequence was considered as single haplotype Haplotype diversity and nucleotide diversity was also estimated, using DNA SP 6.0 software (Rozas et al., 2017).

Multiple sequence alignment was conducted through ClustalW method in MEGAX software (Kumar et al.2018). Two phylogenetic trees were constructed. The first phylogenetic tree was constructed from haplotypes of *CO1* gene via maximum likelihood method, while the second phylogenetic tree was constructed from haplotypes of *D-loop* via neighbor-joining method using MEGAX software as described by Kumar et al. (2018). The Kimura-2 parameter method was used to estimate genetic distance within *CO1* gene of current study, with other related species using the same software. Similarly, nucleotide composition and nucleotide pair frequencies of *D-loop* nucleotide sequence of the current study was estimated by MEGAX software as described by Kumar et al. (2018).

Results and discussion

Polymorphism (SNP's) in CO1 gene

The sequencing results of *CO1* gene showed four polymorphic sites at positions 119, 231, and 453, 644. The average A/T content % and G/C content % of *COI* gene were 55.3% and 44.7%, respectively. Higher A/T content % (54.2%) and lower G/C content % (44.8%) in *COI* gene of lowland anoa species (genus Bubalus) have also been reported by PRIYONO et al. (2018). Multiple sequence alignment of *COI* gene sequences with reference genome (AF547270.1) revealed four haplotypes, i.e., Azi-Kheli haplotypes one to four, respectively (AZHap1 to AZHap4), as shown in Table 1. These few numbers of haplotypes indicate lesser polymorphism rate in *COI* gene; such few polymorphic sites (4) are comparable with those reported in Egyptian river buffalo (Hassan et al., 2018). *COI* gene showed very low nucleotide variation as compared to other mitochondrial region in Egyptian river buffalo, wherein Hassan et al. (2009) showed 77 variable regions in mt *D-loop*.

Diversity of CO1 gene in Azi-Kheli buffalo from other related species of buffalo

Inter-specific diversity was determined by Kimura-2 parameter method (Table 2). The inter-specific diversity between

Table 1 The identified haplotypes of *CO1* gene with reference sequence to buffalo whole mitochondrial genome via DNA SP 6.0 Software; dots (.) show identical bases with reference sequence. AZ = Azi-Kheli, Hap = haplotype

No	Haplotypes	Variable sites and its positions in gene			
		119	231	453	644
O1	Reference genome (AF547270.1)	G	C	C	A
O2	AZHap1	A	T	T	
03	AZHap2		T	T	G
04	AZHap3	A		T	
05	AZHap4			T	

Table 2 Diversity in Azi-Kheli buffalo and other buffalo types based on K2P method. Bold differentiates Azi-Kheli from Swamp buffalo, African buffalo, and anoa

	Specimen	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	AF547270.1														
2	Azi-Kheli 1	0.004													
3	Azi-Kheli 2	0.004	0.002												
4	Azi-Kheli 3	0.002	0.004	0.001											
5	Azi-Kheli 4	0.001	0.002	0.002	0.001										
6	Swamp buffalo	0.026	0.027	0.024	0.025	0.026									
7	Swamp buffalo	0.026	0.027	0.024	0.025	0.026	0.00								
8	Swamp buffalo	0.026	0.027	0.024	0.025	0.026	0.00	0.00							
9	Land anoa	0.026	0.027	0.024	0.025	0.026	0.022	0.022	0.022						
10	Land anoa	0.026	0.027	0.024	0.025	0.026	0.022	0.022	0.022	0.00					
11	African buffalo	0.116	0.122	0.118	0.116	0.118	0.114	0.114	0.114	0.114	0.114				
12	African buffalo	0.116	0.122	0.118	0.116	0.118	0.114	0.114	0.114	0.114	0.114	0.00			
13	African buffalo	0.118	0.123	0.120	0.118	0.120	0.112	0.112	0.112	0.116	0.116	0.008	0.008		
14	Goat (*Capra hircus*)	0.164	0.170	0.166	0.164	0.166	0.161	0.161	0.161	0.169	0.169	0.148	0.148	0.148	

CO1 gene in Azi-Kheli buffalo and *CO1* gene in closely related species, i.e., swamp buffalo, lowland anoa ranges from 0.024 to 0.027 with an average of 2.56%, while it was 11.8% with African buffalo and 16.6% with domestic goat. However, intra-specific diversity of *CO1* gene within Azi-Kheli buffalo ranges from 0.001 to 0.004 with an average of 0.25%. In a previous study for *CO1* gene sequence, 2.5% sequence diversion is recommended for species identification (Tobe et al., 2010). Another previous study reveals that greater than 2% nucleotide sequence polymorphism of *CO1* is sufficient to identify animal species (Yan et al., 2013). The result of intra-specific diversity of CO1 gene in the current study is higher than that of previously reported studies. In the current study, *CO1* nucleotide sequence successfully differentiated and identified that Azi-Kheli buffalo is a river buffalo (Bubalus bubalis) which is distinct from three related species of buffalo, i.e., swamp buffalo (*Bubalus bubalis carabanesis*), lowland (*Bubalus depressicornis*), and African buffalo (*Syncerus caffer*).

Phylogenetic tree construction through CO1 gene

Phylogenetic tree was determined by maximum likelihood method and Tamura-Nei model from haplotypes of current study and published species (Table 3). The phylogenetic tree revealed four clades. The first clade consisted of Azi-Kheli buffalo haplotypes (AZHAP1 TO AZHAP4) with river buffalo haplotypes, while the second clade consisted of lowland anoa, whereas the third was swamp buffalo clade, and the fourth was African buffalo clade. Being members of Bovidae family, all the clades were rooted by *Capra aegagrus hircus*, as shown in Fig. 1. Similar investigation was done by Hassan et al. (2018) in Egyptian water buffalo which revealed distinct clade clustering of river buffalo, swamp buffalo, and lowland anoa.

Polymorphism (SNP) in D-loop

A total of 28 variable sites were identified in sequencing results of *D-loop*. Such type of high mutation rate has also been reported in *D-loop* by Kierstein et al. (2004) (128 variable regions in 36 haplotypes) in water buffaloes (species). The ratio of transition to transversion in *D-loop* of Azi-Kheli buffalo was 10.7:1 which is bias towards transition. Such bias towards transition has also been reported in previous studies like in Kundi buffalo in which this ratio was 10:1 (Hassan et al., 2009), whereas in Indian river buffaloes, it was 17:1(Kumar et al., 2007), and in Egyptian river buffalo,

Table 3 Specimen with scientific name and NCBI GenBank; accession no. used in construction of phylogenetic tree

No	Specimen	Scientific name	Gene bank accession
1	River buffalo	*Bubalus bubalis*	AF547270
2	Swamp buffalo	*Bubalus bubalis carabanesis*	AY702618
3	Swamp buffalo	*Bubalus bubalis carabanesis*	NC_006295
4	Swamp buffalo	*Bubalus bubalis carabanesis*	KX758296
5	Lowland anoa	*Bubalus depressicornis*	EF536351
6	Lowland anoa	*Bubalus depressicornis*	NC_020615.1
7	African buffalo	*Syncerus caffer*	JQ235540
8	African buffalo	*Syncerus caffer*	JQ235539
9	African buffalo	*Syncerus caffer*	JQ235538
10	Domestic goat	*Capra aegagrus hircus*	GU229278

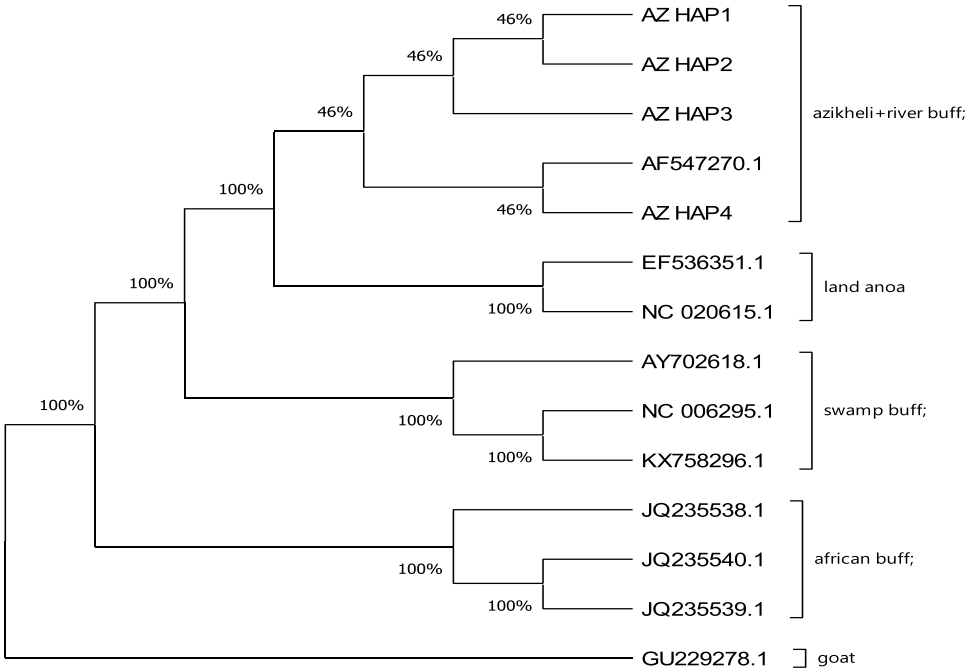

Fig. 1 Azi-Kheli buffalo phylogenetic tree construction by maximum likelihood method and Tamura-Nei model addition of published haplotypes of related species

this ratio was 4.8:1 (Hassan et al., 2009). Average nucleotide frequencies of A/T and G/C contents were 59.9% and 40.1%, respectively. Almost similar results of base compositions with high A/T% have been reported in river buffaloes by Kierstein et al. (2004); i.e., in Mediterranean buffalo, A/T contents was 59.93%; in Murrah buffalo, it was 59.6%; in cattle, it was 61.7%; whereas in swamp buffalo, A/T contents was lower (58.3%).

Multiple sequence alignment of *D-loop* sequences with reference gene showed a total of five haplotypes. Every identical sequence was considered as the same haplotype, as shown in Table 4. The haplotype diversity (Hd) was $Hd = 0.9601 \pm SD = 0.096$ and nucleotide diversity (π) was $\pi = 0.01208 \pm SD = 0.00182$. Almost similar results of haplotype and nucleotide diversity in *D-loop* had been reported in Nili-Ravi buffalo, i.e., $HD = 0.9561 \pm 0.00010$

Table 4 The identified haplotypes of D-loop with reference buffalo mitochondrial genome accession no (AY488491.1); dots (.) show identical bases with reference

Variable sites															
			1	1	1	1	3	3	3	3	3	4	4	5	5
	Specimen	6	1	4	5	6	6	6	7	7	9	0	5	2	3
		1	2	2	2	1	5	8	3	5	5	2	8	8	0
1	Reference AY488491	T	A	A	C	A	G	T	A	C	C	A	G	T	T
2	AZHap1	.	.	.	.	.	.	.	.	.	.	.	A	C	.
3	AZHap2	.	G	G	.	.	A	C		T		G	A	C	.
4	AZHap3	.	G		.	G	.	.	.	.	.	.	A	C	.
5	AZHap4	.	G	G	G	G	A	.	.	T	T	.	.	.	C
6	AZHap5	G	.	.	G	G	A	.	G	T	T	.	.	.	C
Variable sites															
		5	5	5	6	6	6	6	6	6	6	6	7	7	9
	Specimen	5	6	8	0	0	3	3	5	5	6	9	0	7	1
		1	9	6	0	4	3	4	0	9	8	8	4	9	1
1	Reference AY488491	C	C	C	G	A	T	T	C	C	A	C	G	T	G
2	AZHap1	T	.	T	C	.	.	C	.	.	.	T	A	C	.
3	AZHap2	T	.	T	.	.	.	C	.	.	.	.	.	.	A
4	AZHap3	T	.	T	.	G		C	.	.	.	.	.	.	.
5	AZHap4	.	T	T		G	C	C	T	T	G	T	A		
6	AZHap5	.	T	T		G	C	C	T	T	G	T	A		A

Table 5 Specimen with scientific name and NCBI GenBank; accession no. used in construction of *D-loop* phylogenetic tree

No	Specimen	Scientific name	Gene bank accession
1	Jafarabadi buffalo	*Bubalus bubalis*	EF121629 & AF197198
2	Pandharpuri buffalo	*Bubalus bubalis*	EF121700
3	Surti buffalo	*Bubalus bubalis*	EF121716
4	Murrah buffalo	*Bubalus bubalis*	AY195592
5	Egyptian buffalo	*Bubalus bubalis*	EU780708 & EU268885
6	Mediterranean buffalo	*Bubalus bubalis*	AY195595
7	Nili-Ravi buffalo	*Bubalus bubalis*	KM453747, KM453750 & KM453749
8	Kundi buffalo	*Bubalus bubalis*	GQ166748, GQ166747 & GQ166721
9	Swamp buffalo	*Bubalus bubalis carabenesis*	EF053552, EF053549, AF197222, AF127219 & AT195598
10	African buffalo	*Syncerus caffer*	JQ780598
11	Cattle	*Bos taurus*	NC_006853

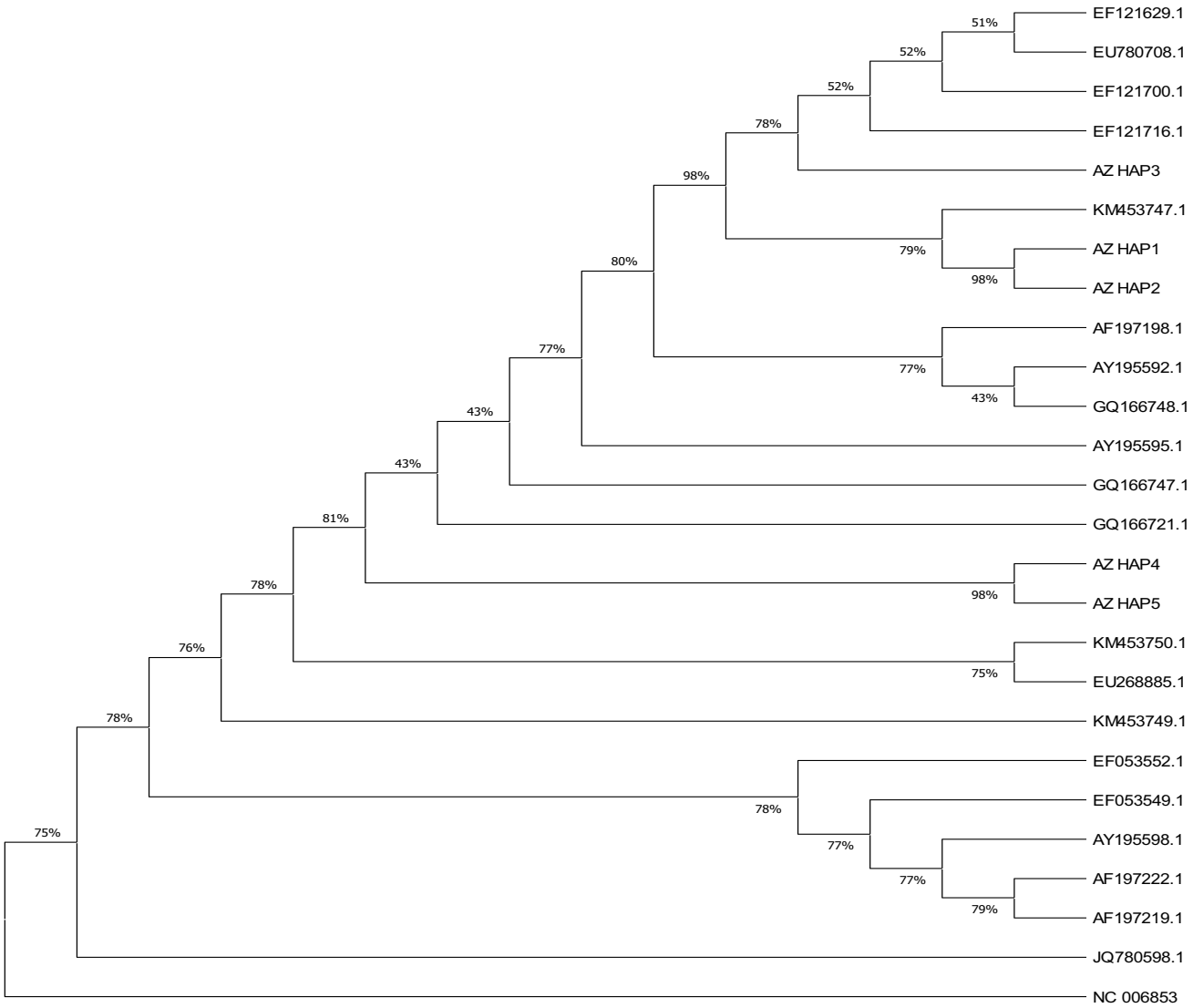

Fig. 2 Neighbor-joining tree constructed from newly found D-loop haplotypes and published D-loop haplotypes of river buffalos, swamp buffalos, African buffalos, and Bos taurus as out group

and $\pi = 0.00988 \pm 0.00060$, respectively; in Kundi buffalo, it was HD = 0.9386 ± 0.00020, $\pi = 0.01043 \pm 0.00083$ (Bhatt et al., 2020), while in swamp buffalo, almost similar haplotype and nucleotide diversities in Xinglong buffalo; i.e., HD = 0.900 ± 0.161, $\pi = 0.00328 \pm 0.00219$, have been reported by Lei Chu-Zhao (2007).

Phylogenetic tree construction through D-loop

Phylogenetic tree was constructed using neighbor-joining method from the haplotypes of Azi-Kheli buffalo *D-loop* and published haplotypes of river buffalo and swamp buffalo as shown in Table 5. The phylogenetic tree showed that newly found haplotypes (AZHAP1 to AZHAP5) in *D-loop* of Azi-Kheli buffalo intermingled with other river buffalo breeds from different geographic regions of the world and formed a clade. The haplotypes of swamp buffalo intermingled in a separate clade that was distinct from river buffalo clade as shown in Fig. 2. The current study supported the clustering pattern of haplotypes, reported in Kundi River buffalo (Hussain et al., 2009) and Egyptian river buffalo, whose haplotypes intermingled with other river buffalo from different geographical regions of the world (Hassan et al., 2009). Similar clustering pattern of haplotypes has also been reported in Indian River buffalo (Kumar et al., 2007). The current study on mitochondrial *D-loop* also showed different origin of swamp and river buffalo.

Conclusions

Azi-Kheli buffalo has high genetic diversity, belongs to river buffalo (*Bubalus bubalis*), and is distinct from swamp buffalo (*Bubalus bubalis carabenesis*) having high divergence of *CO1* gene (2.56%) from recommended threshold of species identification. The phylogenetic results of maximum likelihood tree also confirm presence of Azi-Kheli buffalo within river buffalo clade and distinct from swamp buffalo clade, whereas the phylogenetics results of *D-loop* (neighbor-joining tree) showed common origin of Azi-Kheli buffalo with different breeds of river buffalo of the world.

Author contribution FA, IA, MIA, MHR, and MTZ designed and conceived the study. FA, MHR, and MTZ carried out the research. FA, MTZ, IA, OU, NK, FH, SMS, MTK, and MTZ analyzed the data. FA and MTZ wrote the manuscript. RK, OU, and NK critically reviewed and revised the manuscript.

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Data availability Yes.

Code availability Not applicable.

Declarations

Ethics approval The study does not include any experiment which provides harm(s) to animal health and was according to animal right.

Consent to participate Not applicable.

Consent for publication All the authors of the manuscript agreed to publish the data.

Conflict of interest The authors declare that there is no conflict of interest.

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