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Short Communication

Water Buffalo (*Bubalus bubalis*): Complete Nucleotide Mitochondrial Genome Sequence

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In this work, we report the whole sequence of the water buffalo (*Bubalus bubalis*) mitochondrial genome. The water buffalo mt molecule is 16.355 base pair length and shows a genome organization similar to those reported for other mitochondrial genome. These new data provide an useful tool for many research area, i.e. evolutionary study and identification of food origin.

Keywords: Water buffalo; Mitochondrial genome; Evolution

Database Accession No: AF231028; D82894; AF016397

MAIN TEXT

Availability of mitochondrial genome sequences represents a very important tool for many research area. Mitochondrial DNA or derived amminocidic sequences have been used for studying the phylogenetic correlations between different species or breeds from the same species. These kind of studies have been both reported for water buffalo (*Bubalus bubalis*) using only a small, but significative, part of the mitochondrial genome (Tanaka *et al.*, 1996; Kierstein *et al.*, 2004).

Eucaryotic cells contains only two copies of nuclear genome, in contrast to the mitochondrial genome that are present in many copies in a single cell. This abundance of genetic material represents a very important advantage in studies targeted to the identification of the origin of alimentary product in which the DNA present is often degraded and consequently not suitable for molecular biology analysis. Concerning the water buffalo species, the small part of the mitochondrial genome available as today has been used to design a specific PCR test in order to determine the origin of the mozzarella cheese (Bottero *et al.*, 2002).

Finally mutation in mitochondrial genome sequences have been identified as responsible for genetic defects. In humans, Leigh syndrome has been explained with mutations in the ND5 mitochondrial gene (Sudo *et al.*, 2004).

During the last years there has been a considerable progress in the sequencing of complete mtDNA genomes. Regarding the most important livestock species the complete mitochondrial genome has been published for cattle (Anderson *et al.*, 1982), pig (Lin *et al.*, 1999), sheep (Hiendleder *et al.*, 1998), horse (Xu and Arnason, 1994) and goat (Parma *et al.*, 2003). For those concerning water buffalo (*Bubalus bubalis*) only a very small part of the mitochondrial genome is available. These concerns a partial sequence of t-RNA Phe and 12S ribosomal gene (GenBank AF231028), the cytochrome b gene (D82894) and the D-loop region (AF016397). The number of water buffalo mitochondrial genome base pair available before this report is 2,262.

In this work, we report the first complete nucleotide sequence of water buffalo mtDNA molecule and compare it with those of others species.

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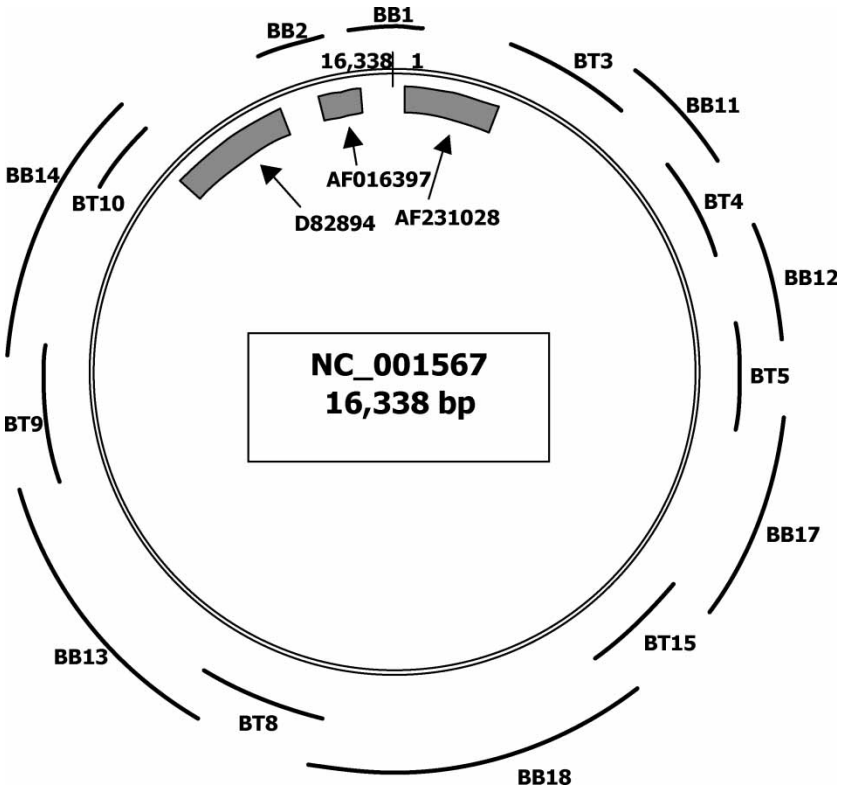

FIGURE 1 Strategy applied for completing the water buffalo mtDNA nucleotide sequence. *Legend* Available sequences are from:NC_01567 (Anderson *et al.*, 1982); AF231028 (Kuznetsov *et al.*, 2001); D82894 (Tanaka *et al.*, 1996); AF016397 (Lau *et al.*, 1997).

Briefly, the available water buffalo sequences (GenBank Accession No: AF231028, D82894 and AF016397), were first aligned with the cattle mtDNA (NC_001567) as shown in Fig. 1; the first base of this genome was placed at the start of t-RNA Phe sequence. The gaps within these three sequences were amplified by PCR using water buffalo specific primers (BB1 and BB2). In order to complete the full sequence between AF231028 and D82894, two set of PCR were designed: the first one was on cattle sequence (BT3, BT4, BT5, BT15, BT8, BT9, BT10). The second one was on water buffalo specific sequence (BB11, BB12, BB17, BB18, BB13, BB14). The sequences of these primers and the amplification conditions are reported in Table I. Genomic DNA was extracted from peripheral blood of one male water buffalo by using standard commercial kit (Quiagen blood kit). PCR amplifications, performed in a Biometra Trio-Thermobloch, were conducted in a 30 ul volume containing 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer and 2 units of AmpliTaq Gold Polymerase (Applied Biosystems). Characteristics of the PCR cycles are

TABLE I Primers and PCR conditions for gaps amplifications

PCRs	Forward primer 5′ → 3′	Reverse primer 5′ → 3′	Amplification profile (35 cycles)
BB1	gatcacgagcttgatcacca	atgcccgctcctcttagttt	96°C 1′–55°C 1′–72°C 1′
BB2	tcacatggattggaggacag	atccttgcctgaagggttg	96°C 1′–55°C 1′–72°C 1′
BT3	ccgtcaccctcctcaagtaa	ggtatccgtttctaaaaggctg	96°C 1′–55°C 1′–72°C 1′
BT4	cgcacgagggttttactgtc	aaggagaggatttgaatctctg	96°C 1′–55°C 1′–72°C 1′
BT5	agtctcgggcttcaacgtag	gggatgccctgtgttacttc	96°C 1′–55°C 1′–72°C 1′
BT15	gaactctgctcggagacgac	gagttggtaagacaattcca	96°C 1′–55°C 1′–72°C 1′
BT8	atcggaggagctacacttgc	aatgcgatgatgacgagta	96°C 1′–55°C 1′–72°C 1′
BT9	tttacacgggaaaatgcact	ggattttccggttgcagcta	96°C 1′–55°C 1′–72°C 1′
BT10	tcactcgcccaaataaaagc	agcagggaggtcaatgaatg	96°C 1′–55°C 1′–72°C 1′
BB11	gctggttgtccagaaaatgaa	cgggaaggtcaatttcactg	96°C 1′–55°C 1′–72°C 1′
BB12	aaggttcgtttgttcaacgatt	tatgggttgtgggatgttcc	96°C 1′–55°C 1′–72°C 1′
BB17	ggccaatggaccgtaataaa	aatcagttatcaaacacctcca	96°C 1′–55°C 1′–72°C 1′
BB18	gccctaggcttcattttcct	ggcttggattatagccactgc	96°C 1′–55°C 1′–72°C 2′
BB13	gctgcctgatattgacactttg	gggcttctattgttagattcac	96°C 1′–55°C 1′–72°C 1′
BB14	caaacacagcagccctacaa	agcagggaggtcaatgaatg	96°C 1′–55°C 1′–72°C 2′

reported in Table I. After purification of the PCR products, sequencing was performed with fluorescent-labeled dideoxynucleotides termination method on Applied Biosystem 3100 ABI PRISM automated DNA sequencer. Specific internal primers were designed for walking at approximately 350 bp intervals. The results of the sequencing were assembled by using the Blast 2 sequences program (Tatusova and Madden 1999). The complete sequence of the water buffalo mtDNA was deposited in the GeneBank database under Accession No. AY488491.

The complete water buffalo (*Bubalus bubalis*) mtDNA is 16.355 bp in length. The length of the genome appears to be highly specific, as no tandem repeats have been found. This is in contrast to those observed in rabbit, horse, harbor seal, sheep and cat, were the length of the mitochondrial genome is highly influenced by the occurrence of a different number of tandem repeat (Arnason and Johnsson 1992; Xu and Arnason 1994; Dufresne *et al.*, 1996; Wood and Phua 1996; Lopez *et al.*, 1996). The composition of the L-strand is: A: 33.1%; T: 26.8%; C: 26.3% and G: 13.8%. As observed in other mammalian species, base A occurs most often and base G the least. This base composition is very similar to those reported for cattle: A: 33.4%; T: 27.2%; C: 25.9% and G: 13.5% (Anderson *et al.*, 1982).

The gene organization of the water buffalo mitochondrial genome is shown in Table II. The limits and nomenclature of each gene was determined by comparison with other known mammalian mtDNAs. Genes responsible for the two rRNA (12S and 16S rRNA), 22 tRNAs, and 13 protein-coding genes (NADH dehydrogenase subunits 1, 2, 3, 4, 4L, 5 and 6, cytochrome c oxidase subunits I, II and III, ATPase subunits 6 and 8 and cytochrome b) are located in the genome following the same order as those reported in the other mammalian species. Among these genes only NADH dehydrogenase subunit 6 and eight tRNAs are encoded in the L-strand while the others are encoded by the H-strand.

Besides providing details of the genome analysis of water buffalo mtDNA, we also examine the possible differences between mtDNA of others

TABLE II Characteristics of the water buffalo mitochondrial genome

Name of gene	Location	Size (bp)	
tRNA-Phe	1–69*	69	H†
12S rRNA	70–1026	957	H
tRNA-Val	1027–1093	67	H
16S rRNA	1094–2662	1569	H
tRNA-Leu (UAA)	2663–2737	75	H
NADH dehydrogenase subunit 1 (*ND1*)	2740–3696	957	H
tRNA-Ile	3696–3764	69	H
tRNA-Gln	3762–3827	66	L
tRNA-Met	3836–3904	69	H
NADH dehydrogenase subunit 2 (*ND2*)	3905–4948	1044	H
tRNA-Trp	4947–5013	67	H
tRNA-Ala	5015–5083	69	L
tRNA-Asn	5083–5157	75	L
tRNA-Cys	5191–5249	59	L
tRNA-Tyr	5251–5318	68	L
Cytocrome c oxidase subunit I (*COI*)	5320–6864	1545	H
tRNA-ser (UGA)	6862–6932	71	L
tRNA-Asp	6937–7005	69	H
Cytocrome c oxidase subunit II (*COII*)	7007–7690	684	H
tRNA-Lys	7694–7764	71	H
ATPase subunit 8 (*ATPase8*)	7767–7967	201	H
ATPase subunit 6 (*ATPase6*)	7928–8608	681	H
Cytocrome c oxidase subunit III (*COIII*)	8608–9388	781	H
tRNA-Gly	9392–9460	69	H
NADH dehydrogenase subunit 3 (*ND3*)	9461–9806	346	H
tRNA-Arg	9808–9876	69	H
NADH dehydrogenase subunit 4L (*ND4L*)	9877–10173	297	H
NADH dehydrogenase subunit 4 (*ND4*)	10167–11544	1378	H
tRNA-His	11545–11615	71	H
tRNA-Ser (AGY)	11616–11674	59	H
tRNA-Leu (UAG)	11677–11746	70	H
NADH dehydrogenase subunit 5 (*ND5*)	11747–13551	1805	H
NADH dehydrogenase subunit 6 (*ND6*)	13551–14078	528	L
tRNA-Glu	14079–14147	69	L
Cytochrome b (*Cytb*)	14152–15291	1140	H
tRNA-Thr	15296–15364	69	H
tRNA-Pro	15364–15429	66	L

*The numbering of positions starts with the 5′ position of tRNA-Phe. †H and L on the size column signify that the indicated gene is transcripted from H-strand or L-strand, respectively.

TABLE III Differences of amino acid sequences between the mitochondrial protein-coding genes of water buffalo and cattle

Gene	Sequence difference %
ND3	2.61
*COII**	3.08
ND1	3.14
COIII	3.46
COI	3.50
ND6	4.00
ND4L	4.08
ND4	5.01
Cytb	5.80
ATPase6	6.68
ND2	8.07
ND5	9.57
ATPase8	12.12
Mean	5.58

*The genes are listed by increasing difference.

specie by comparing the 13 protein-coding genes. The overall differences among these mitochondrial sequences are presented in Table III.

The comparison between individual protein of the water buffalo and the cow shows the amino acid differences ranging from 2.61 in *ND3* to 12.12% in *ATPase8* with a mean value of 5.58%.

As show in Table III the same gene may exhibit similar relative rates of evolution in amino acid sequence from different species pairs. The *COI*, *COII* and *COIII* genes are always between the genes with lowest diversity were the *ATPase8* and *ND5* are the genes with the highest diversity. These data is in agreement with those observed in other specie comparison (Lin *et al.*, 1999).

In the present study, the phylogenetic position of the water buffalo relative to seven other mammalian species was performed based on the concatenated sequences of 13 protein-coding genes encoded on the mt genome. The phylogenetic tree is shown in Fig. 2. The TREECON program version 1.3b (Van de Peer and Wachter, 1994 available free at http://iubio.bio.indiana.edu/soft/molbio/evolve/draw/treecon) was used to reconstruct the phylogenetic tree using the model for amino acid substitution described by Kimura (1983). For robustness of the tree, the analysis was performed using 1000 bootstrap steps. The reliability value of the internal branches of the tree is shown as a percentage. Other data set and reconstruction methods resulted in the same topology.

Moreover, the genetic distance between the eight mammalian species are given in Table IV. Distance values were obtained from the maximun likelihood distances for the PUZZLE program (Schmidt *et al.*, 2002) based on the comparison of the concatenated amino acid sequences of 13 protein-coding mtDNA genes. As model of amino acid substitution the mtREV24 (Adachi and Hasegawa 1996) was used, and as model of rate heterogeneity uniform rate over all sites was taken in account. By applying as reference, a divergence time of 60 million years before present (MYBP) for artidodactyls and cetaceans (Arnason and Gullberg, 1996), the values reported here suggest an evolutionary divergence between water buffalo and cow of 20 MYBF. This data is higher than the 10 MYBF reported for divergence time calculated on *COII* gene only (Janecek *et al.*, 1996) and *SRY* gene (Parma *et al.*, 2004).

As conclusion, in the present work we have completed the sequence of water buffalo mitochondrial genome. Accordingly, the full sequence of water buffalo mtDNA will be useful for further study of the evolution and genetics of the water buffalo species as well as for the documentation of the phylogenetic position of the water buffalo among mammalian orders.

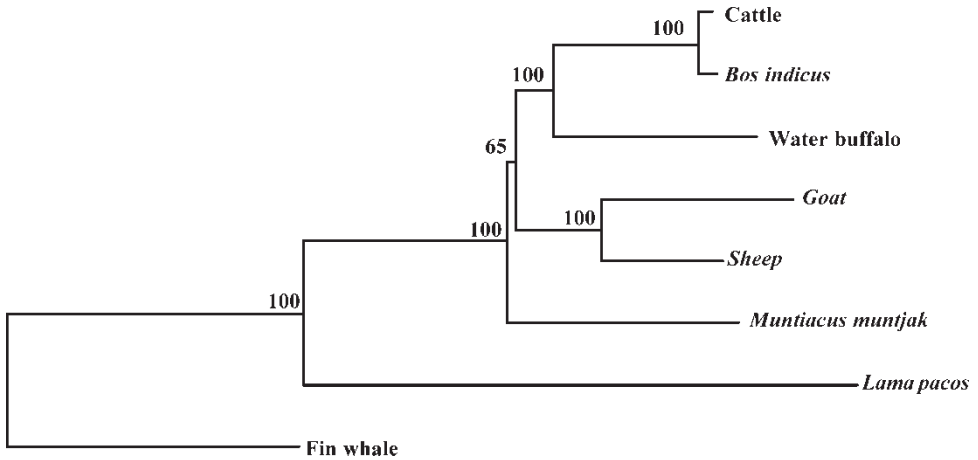

FIGURE 2 Phylogenetic position of the water buffalo relative to seven other mammalian species. *Legend* All mitochondrial sequences are available in the GenBank database: sheep (NC_001941), cow (J01394), fin whale (X61145) and goat (AF533441), *Bos indicus* (AY126697), *Muntiacus muntjak* (AY225986) and *Lama pacos* (Y19184).

TABLE IV Genetic distances based on amino acid differences between eight mammalian species

	Cattle	*Bos indicus*	Water buffalo	Sheep	Goat	*Muntiacus muntjak*	*Lama pacos*	Fin whale
Cattle	0.00000							
Bos indicus	0.00582	0.00000						
Water buffalo	0.05794	0.05683	0.00000					
Sheep	0.06527	0.06352	0.06915	0.0000				
Goat	0.07557	0.07582	0.08052	0.04811	0.0000			
Muntiacus muntjak	0.07143	0.07172	0.07630	0.06891	0.08206	0.0000		
Lama pacos	0.14687	0.14779	0.16139	0.15392	0.16453	0.15554	0.0000	
Fin whale	0.15744	0.15738	0.16288	0.16127	0.016866	0.16225	0.18088	0.0000

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