

Phylogenetic Relationship Among All Living Species of the Genus *Bubalus* Based on DNA Sequences of the Cytochrome *b* Gene

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The cytochrome b genes of all living species of Bubalus, including the river type and the swamp type of domestic buffaloes (Bubalus bubalis), were sequenced to clarify their phylogenetic relationships. These sequences were compared together with the African buffalo (Syncerus caffer) and banteng (Bos javanicus) sequences as an outgroup. Phylogenetic trees of Bubalus species based on the DNA sequences of the cytochrome b gene demonstrated that the tamaraw (Bubalus mindorensis), endemic to the Philippines, could be classified into the subgenus Bubalus, not the subgenus Anoa. The divergence time between the lowland anoa (B. depressicornis) and the mountain anoa (B. quarlesi) was estimated at approximately 2.0 million years (Myr), which is almost the same as the coalescence time for the Bubalus sequences. This large genetic distance supports the idea that the lowland anoa and the mountain anoa are different species. An unexpectedly large genetic distance between the river and the swamp type of domestic buffaloes suggests a divergence time of about 1.7 Myr, while the swamp type was noticed to have the closest relationship with the tamaraw (1.5 Myr). This result implies that the two types of domestic buffaloes have differentiated at the full species level.

KEY WORDS: *Bubalus*; tamaraw; anoa; cytochrome *b*; evolution.

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INTRODUCTION

There are four wild species in the genus *Bubalus*: the Asian wild water buffalo, the lowland and the mountain anoas of Sulawesi, and the tamaraw of Mindoro island of the Philippines.

The Asian wild water buffalo (*Bubalus bubalis*) was common on the Indian subcontinent. However, because of recent environmental disruption, they have been decreasing year by year and now survive in only a few regions. This animal is thought to be the origin of domestic buffaloes. The domestic buffalo has been classified into two types according to geographical distribution in general: one is the river-type buffalo, raised in many areas, from Bangladesh, India, and Egypt to some South and East European countries; and the other is the swamp type of Southeast Asia (Mason, 1974). The karyotypes of the two types are different and their diploid chromosome numbers are 48 and 50 for the swamp- and the river-type buffaloes, respectively (Fischer and Ulbrich, 1968). Moreover, distinct genetic differences discriminate the two types in electrophoretic variations of albumin and transferrin (Amano *et al.*, 1980) and in restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) (Amano *et al.*, 1994; Tanaka *et al.*, 1995). However, the scientific name *Bubalus bubalis* has been used for the Asian wild water buffalo as well as for the two types of domestic buffaloes.

The lowland anoa (*Bubalus depressicornis*) and the mountain anoa (*Bubalus quarlesi*) are species endemic to Sulawesi of Indonesia. They are often regarded as the smallest water buffaloes diverged from *Bubalus* species (Klös and Wünschmann, 1972; Walker, 1975). Two forms of the anoas are found all over the Sulawesi: the smaller one in body size (about 70 cm at the shoulder) is generally accepted to be the mountain anoa inhabiting the mountain forest; the larger is the lowland anoa, found mainly in the lowlands (Groves, 1969). However, the distinction between them may not seem very clear-cut to observers and the relationship between them is not well understood. Moreover, the classification or nomenclature of the anoas is different by authors even at a generic or specific level (Dolan, 1965; Groves, 1969; Klös and Wünschmann, 1972; Walker, 1975).

The tamaraw (*Bubalus mindorensis*) is found only on Mindoro island of the Philippines, and it is the rarest bubaline species. The number of tamaraws was claimed to be only about 200 to 300 head (Momongan and Walde, 1993). This animal shows medium morphological characteristics, between those of the Asian water buffaloes and those of the anoas (Nami-kawa *et al.*, 1995).

According to recent literature, *Bubalus* is classified into two subgenera: the subgenus *Bubalus*, including the Asian wild water buffalo and domestic

buffaloes; and the subgenus *Anoa*, including the two species of anoas (see Groves, 1976). Regarding the tamaraw, very little is known about its phylogenetic situation in the *Bubalus* species.

In this study whole cytochrome *b* regions (1140 bp) of mtDNA of all the living species of *Bubalus*, including the two types of domestic buffaloes, were sequenced. Comparison of these sequences provides further information to help understand the phylogenetic relationship among the species of *Bubalus*.

MATERIALS AND METHODS

DNA Sources and Extraction

Table I lists the taxa studied and the tissue from which total cellular DNA was obtained. From RBC samples a total DNA solution was prepared by the following method: wash 1 drop of RBC using 500 μ l of a hypotonic solution (0.2% NaCl, 1 mM EDTA) three times, incubate with 100 μ l of extraction buffer (0.45% Tween 20, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1 mg/ml proteinase K) for 1 hr at 37°C, and then incubate the DNA solution for 10 min at 94°C and immediately cool down with ice. From whole-blood samples, total cellular DNA was isolated using standard techniques (Sambrook *et al.*, 1989). From hair samples, total DNA solution was prepared as described by Morin *et al.* (1994).

Table I. List of Taxa and Source of Samples

Common name	Scientific name	Tissue used	Source
Asian wild water buffalo	<i>Bubalus bubalis</i>	RBC ^{a,b}	Yala National Park, Sri Lanka
Lowland anoa	<i>Bubalus depressicornis</i>	RBC ^b	Ragnan Zoo, Jakarta, Indonesia
Mountain anoa	<i>Bubalus quarlesi</i>	RBC ^b	Ragnan Zoo, Jakarta, Indonesia
Tamaraw	<i>Bubalus mindorensis</i>	Whole blood	Gene Pool of Mindoro, the Philippines
River-type domestic buffalo	<i>Bubalus bubalis</i>	Liver ^c	Dhaka, Bangladesh
Swamp-type domestic buffalo	<i>Bubalus bubalis</i>	Liver ^c	Flores, Indonesia
African buffalo	<i>Syncerus caffer</i>	Hair	Gunma Safari Park, Gunma, Japan
Banteng	<i>Bos javanicus</i>	RBC	Ragnan Zoo, Jakarta, Indonesia

^aRed blood cell (RBC) bed separated from whole blood by centrifugation.

^bOne of the samples used for β -globin analysis (Kakoi *et al.*, 1994).

^cOne of the samples used for RFLP analysis of mtDNA (Tanaka *et al.*, 1995).

Sequencing

The cytochrome *b* region of mtDNA was amplified by the polymerase chain reaction (PCR) on samples using a pair of L14724B and H15915R primers described by Irwin *et al.* (1991). The double-stranded PCR products were directly sequenced with five primers listed in Table II using the Taq Dye Deoxy Terminator Cycle Sequencing Kit and Model 373S DNA Sequencer (Perkin Elmer, USA).

Data Analysis

Raw sequence data were analyzed using the PHYLIP, Version 3.5 (Felsenstein, 1993), program. Since the cytochrome *b* gene is a protein-coding gene, a weighting scheme was employed in analyses of the data set to allow for different evolutionary rates at the three codon positions. The three codon positions were weighted 0.37 (first):1 (second):0.06 (third) (Lento *et al.*, 1995). We also used a transition-to-transversion ratio of 8.0 (Smith and Patton, 1982). Weighted genetic distances were corrected for multiple hits by the two-parameter method of Kimura (Kimura, 1980). Distance trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) based on the weighted genetic distances. The reliability of each interior branch was tested by 1000 bootstrap replications.

RESULTS

Nucleotide Differences

The whole cytochrome *b* regions of mtDNA (1140 bp) for *Bubalus mindorensis*, *B. depressicornis*, *B. quarlesi*, *B. bubalis* (Sri Lankan wild buffalo, river-

Table II. Sequence Information for Oligonucleotide Primers

Name of primer	Sequence	Notes ^a
L14724B	5'-CGAAGCTTGATATGAAAAACCATC-GTTG-3'	a, c, d
MI1	5'-CAAATCCTCACAGGCCTATTCC-TAGC-3'	b, d
L15408	5'-ATAGACAAAATCCCATTC-3'	a, d
H15915R	5'-GGAATTCATCTCTCCCGTTTCAAGAC-3'	a, c, d
MI2	5'-TAGGCGAATAGGAAATATCATTC-GGGTTTGAT-3'	b, d

^a(a) Designed by Irwin *et al.* (1991); (b) designed by Chikuni *et al.* (1994); (c) used in amplification reactions; (d) used in sequencing reactions.

type buffalo, and swamp-type buffalo), *Syncerus caffer*, and *Bos javanicus* were sequenced. These eight nucleotide sequence data will appear in the DDBJ, EMBL, and Gene Bank Nucleotide Sequence Databases with the following accession numbers: D82888 to D82895. The pairwise sequence differences, considering position in codons as well as transition and transversion, are shown in Table III. Only five nucleotide substitutions were found between the Sri Lankan wild buffalo and the river-type buffalo. Furthermore, all of them were transitions at the third positions. We may also find such a small number of substitutions within the river type of domestic buffaloes, because 0.2% sequence divergence was estimated within the river-type buffaloes from RFLP analysis of mtDNA (Tanaka *et al.*, 1995). On the other hand, 28 substitutions were found between the river type and the swamp type of domestic water buffaloes.

Phylogenetic Analysis

Figure 1 is a phylogenetic tree of the eight *Bovinae* sequences (NJ tree) using the pairwise weighted genetic distance in Table IV. Figures above the internal branches are bootstrap probabilities (%) based on 1000 boot-

Table III. Number of Nucleotide Substitutions of Cytochrome *b* Genes Between Bubaline Species

Pairwise comparison	Transition			Transversion		
	First	Second	Third	First	Second	Third
African buffalo vs. tamaraw	12	4	73	2	0	9
African buffalo vs. river-type buffalo	10	2	73	3	0	10
African buffalo vs. Sri Lankan wild	10	2	72	3	0	10
African buffalo vs. swamp-type buffalo	10	2	72	3	0	9
African buffalo vs. mountain anoa	11	4	73	3	0	10
African buffalo vs. lowland anoa	12	3	68	3	0	10
Tamaraw vs. river-type buffalo	4	2	27	1	0	1
Tamaraw vs. Sri Lankan wild	4	2	28	1	0	1
Tamaraw vs. swamp-type buffalo	5	2	20	1	0	0
Tamaraw vs. mountain anoa	6	5	30	1	0	1
Tamaraw vs. lowland anoa	4	3	36	1	0	1
River-type buffalo vs. Sri Lankan wild	0	0	5	0	0	0
River-type buffalo vs. swamp-type buffalo	3	0	24	0	0	1
River-type buffalo vs. mountain anoa	5	3	24	0	0	2
River-type buffalo vs. lowland anoa	4	1	24	0	0	2
Sri Lankan wild vs. swamp-type buffalo	3	0	25	0	0	1
Sri Lankan wild vs. mountain anoa	5	3	25	0	0	2
Sri Lankan wild vs. lowland anoa	4	1	27	0	0	2
Swamp-type buffalo vs. mountain anoa	4	3	30	0	0	1
Swamp-type buffalo vs. lowland anoa	5	1	33	0	0	1
Mountain anoa vs. lowland anoa	7	4	25	0	0	2

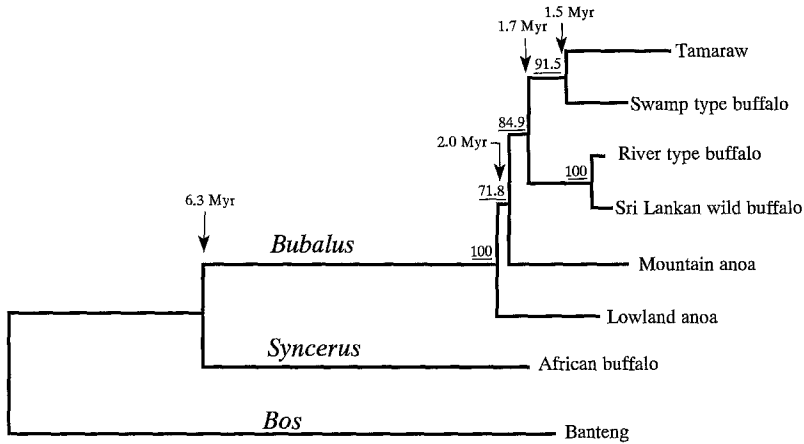


Fig. 1. A phylogenetic tree of the eight *Bovinae* cytochrome *b* sequences constructed using the neighbor-joining method. The underlined numbers above the internal branches are bootstrap probabilities (%) based on 1000 bootstrapped maximum-parsimony trees. Numbers with arrows are estimated divergence times (Myr).

strapped maximum-parsimony trees. Relatively high bootstrap probabilities, 71.8 to 100%, were observed for all the forks of this tree. In Fig. 1, the swamp-type buffalo and the tamaraw made a cluster; then the river-type buffalo and the Sri Lankan wild buffalo were clustered out of them.

Estimation of Divergence Time

According to the fossil record the divergence time between *Bos* and *Syncerus* (or *Bubalus*) is approximately 10 million years (Myr) (Savage and Russell,

Table IV. Weighted Genetic Distance Corrected for Polymorphism for Cytochrome *b* Gene Sequences^a (Above Diagonal) and Estimated Divergence Time (Myr; Below Diagonal)

Animals	LA	MA	T	SB	RB	SWB	AFB	BAN
Lowland anoa	—	0.0354	0.0423	0.0363	0.0286	0.0315	0.1088	0.1700
Mountain anoa	2.0	—	0.0443	0.0363	0.0315	0.0325	0.1138	0.1728
Tamaraw	2.0	2.0	—	0.0256	0.0334	0.0344	0.1108	0.1812
Swamp-type buffalo	2.0	2.0	1.5	—	0.0256	0.0266	0.1071	0.1719
River-type buffalo	2.0	2.0	1.7	1.7	—	0.0044	0.1086	0.1770
Sri Lankan wild buffalo	2.0	2.0	1.7	1.7	0.2	—	0.1074	0.1755
African buffalo	6.3	6.3	6.3	6.3	6.3	6.3	—	0.1673
Banteng	10.0 ^b	10.0 ^b	10.0 ^b	10.0 ^b	10.0 ^b	10.0 ^b	10.0 ^b	—

^aSee text for details.

^bData from fossil record (Savage and Russell, 1983).

1983). Using this divergence time and pairwise weighted genetic distances in Table IV (above), the estimated divergence time was calculated in Table IV (below). The divergence time between *Bubalus* and *Syncerus* was estimated as approximately 6.3 Myr. This value is quite close to that from the fossil record, 5 Myr (Savage and Russell, 1983). The largest divergence within the six *Bubalus* sequences had a mean divergence time of approximately 2.0 Myr. Therefore, the coalescence time for the present *Bubalus* species was estimated as approximately 2.0 Myr.

DISCUSSION

Two Species of Anoa

The divergence time between the lowland anoa and the mountain anoa was estimated as approximately 2.0 Myr. It was almost the same as the coalescence time for the present *Bubalus* species. This large genetic distance is also supported by amino acid sequence differences of the hemoglobin β chains of the two species (Kakoi *et al.*, 1994). However, it was difficult to find a diagnostic character that could decisively classify the two species from chromosomal analysis and electrophoretic analysis of blood protein on the two zoo-living forms of anoas (Schreiber *et al.*, 1993), because of wide variation of chromosome numbers ($2N = 44, 45, 47, 48$) and high polymorphisms at allozyme loci in the two forms. That study disclosed low genetic differentiation between the two forms, in spite of the presence of their high allozyme polymorphisms. To work out these inconsistent results, we set forth two hypotheses. First, the lowland anoa and the mountain anoa are separate species; however, hybridizations of the two species possibly occurred in the history of zoo-living anoas. In this case, investigation of all the zoo-living anoas seems to be necessary to keep the purity of the two species. Second, the lowland anoa and the mountain anoa are now one fused species in Sulawesi island, having had two distinct ancestors, but they are now integrating or integrated on Sulawesi island as discussed by Kakoi *et al.* (1994). The present study was only of single individuals. Therefore, it is difficult to say which view is correct. Anyway, population biological studies on wild-living anoas seem to be essential to clarify the detailed phylogenetic relationship of the two species of anoas.

Taxonomic Position of the Tamaraw

The tamaraw and swamp-type buffalo sequences made a cluster, while the river-type buffalo and the Sri Lankan wild buffalo could be placed out from them (Fig. 1). This root is also supported by karyotypes: the chromosome number of the present *Bubalus* and *Syncerus* species is very variable— $2N =$

44, 45, 47, and 48 for the two species of anoa (Schreiber *et al.*, 1993), $2N = 46$ for the tamaraw (Namikawa *et al.*, 1995), $2N = 48$ for the swamp-type buffalo, $2N = 50$ for the river-type buffalo (Fischer and Ulbrich, 1968), and $2N = 52$ for the African buffalo (Ulbrich and Fischer, 1967). However, the fundamental number (FN) is common to them (FN = 60), except for the swamp-type buffalo and the tamaraw (FN = 58). It is noteworthy that the lineage of the tamaraw is not located at the deepest branch among the *B. bubalis* (Fig. 1). Does this mean uncertainty on the species status of *B. mindorensis*? The divergence time between the tamaraw and the swamp-type buffalo was estimated at approximately 1.5 Myr, 1.7 Myr between the tamaraw (or the swamp-type buffalo) and the river-type buffalo (Table IV). This value is greater than the estimated mean duration of late Cenozoic mammal species (Stanley, 1979). Furthermore, very low sequence divergence was estimated within the swamp-type buffaloes, as well as within the river-type buffaloes, from RFLP analysis of mtDNA of domestic buffaloes (Amano *et al.*, 1994; Tanaka *et al.*, 1995). Thus, even if we accept the branching in Fig. 1, the species status of *B. mindorensis* will not be lost, though we must consider that the river-type buffalo and the swamp-type buffalo are differentiated at the full species level.

From the present result, we can recognize that the tamaraw can be classified in the subgenus *Bubalus*, not in the subgenus *Anoa*. Also, the tamaraw has a relatively closer phylogenetic relationship to the swamp-type buffalo than to the river-type buffalo.

Differentiation Between the River and the Swamp Types of Water Buffalo

As discussed above, karyotypic difference exists between the river-type and the swamp-type buffaloes. This difference is the result of a telomere-centromere tandem fusion between two chromosomes in the river-type buffalo (Di Berardino and Iannuzzi, 1981). This type of fusion occurs rarely but sometimes may be a crucial factor for species differentiation. Natural mating of the two types takes place only if they are associated from calthood to maturity, and the F_1 crossbreeds of both sexes appear to be normal and fertile, but it is not confirmed yet whether the F_1 fertility holds for the F_2 generation (Fischer and Ulbrich, 1968). Although no difference was found in the primary structure of β -globin of the swamp and river types of domestic buffaloes (Kakoi *et al.*, 1994), three amino acid substitutions were found in pancreatic ribonucleases, despite a substitution rate for this enzyme about 40% that of hemoglobin chains in Bovidae (Beintima, 1980). Furthermore, the divergence time between the river-type and the swamp-type buffaloes was estimated as approximately 1.7 Myr. Considering these previous studies and the present results from DNA sequencing of the cytochrome *b* gene, in

our opinion, the river-type buffalo and the swamp-type buffalo are differentiated at the full species level.

This degree of divergence between the two types of domestic buffaloes suggests that domestication of buffalo should have occurred from at least two different populations, because the history of domestic buffaloes is not longer than 5000 years (Zeuner, 1963). This is also supported by the present clearly separated geographical distribution of the two types (Mason, 1974). It is very interesting that quite a close phylogenetic relationship was found between the river-type buffalo and the Sri Lankan wild buffalo. This result strongly suggests that the Asian wild water buffaloes inhabiting the Indian subcontinent or adjacent areas were the source of the river type of domestic buffaloes. This argument, however, relies solely on the genealogy of only one population of Asian wild buffaloes. The Sri Lankan wild buffalo is clustered with the river type of domestic buffalo in the tree in Fig. 1. Thus it is not excluded that a certain degree of gene flow occurred from the river type of domestic buffaloes to the Sri Lankan wild buffalo. In fact, almost all of the domestic buffaloes in Sri Lanka are river-type buffaloes (Amano *et al.*, 1986). Therefore, detailed examination of other populations of Asian wild water buffaloes is necessary to confirm our hypothesis on the domestication process of the river-type buffaloes as well as the swamp-type buffaloes.

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