Cytogenetic Analysis of the Tamaraw (Bubalus mindorensis): A Comparison of RB-Banded Karyotype and Chromosomal Distribution of Centromeric Satellite DNAs, Telomeric Sequence, and 18S-28S rRNA Genes with Domestic Water Buffaloes


The karyotype of the tamaraw (Bubalus mindorensis, 2n = 46) was investigated by RBG-banding technique and compared with those of the river and the swamp cytotypes of domestic water buffalo (B. bubalis). The tamaraw karyotype consisted of 6 submetacentric and 16 acrocentric autosome pairs (NAA = 56), and X and Y chromosomes. The RBG-banded karyotype of the three taxa had a high degree of homology, and the tamaraw karyotype could be explained by a Robertsonian translocation between chromosomes 7 and 15 and by a telomere-centromere tandem fusion between chromosomes 4p and 12 of the standardized river buffalo cytotype (2n = 50, NAA = 58). The buffalo satellite I and II DNAs were localized to the centromeric regions of all the tamaraw chromosomes. The biarmed chromosome 2 of the tamaraw resulting from the fusion between chromosomes 7 and 15 of the standard contained much larger amounts of the satellite I DNA than the other biarmed chromosomes, suggesting that this chromosome was formed by a relatively recent Robertsonian fusion. The (TTAGGG) telomeric sequence was specifically localized to the telomeric region of all the buffalo chromosomes. The 18S + 28S rDNA was localized to the telomeric regions of the chromosomes 5p, 7, 19, 21, and 22 of the tamaraw and of their homologous chromosomes in the river and swamp buffalo cytotypes.

The autosomal number (NAA) in the karyotypes of species in the family of Bovidae is relatively constant at 58 and has been taken as evidence that the primary mechanism of the karyotype evolution in the Bovidae has been Robertsonian translocation (Wurster and Benirschke 1968). This proposal has been tested in numerous bovid species with the advent of the comparative cytogenetic studies, suggesting that bovid chromosomal evolution has proceeded by centric fusion from a primitive karyotype of 58 acrocentric autosomes, a condition seen in domestic cattle and goat (Gallagher and Womack 1992; Gallager and Di Meo 1995). In addition to centric fusions, centromere-to-telomere translocation was documented in the Bovidae (Buckland and Evans 1978; Gallagher and Womack 1992). This kind of translocation is a very rare event in the bovid chromosomal evolution and decreases the NAA.

The genus Bubalus in the tribe of Bovini comprises four extant species: Asiatic bufalo (Bubalus bubalis), lowland anoa (B. depressicornis), mountain anoa (B. quarlesi), and the tamaraw (B. mindorensis) (Groves 1981). The Asiatic buffalo is thought to be the origin of the domestic buffaloes. The domestic buffalo (B. bubalis) has been classified into two types according to broad geographic distribution. The river buffalo is found in areas from India and Egypt to some south and east European countries, and the swamp buffalo is found in Southeast Asia (Mason 1974). Recent systematic studies, however, strongly suggest that the two types of domestic buffaloes differentiated at least at the subspecies level (Barker et al. 1997a,b; Tanaka et al. 1995, 1996). In this study we treat the river buffalo and the swamp buffalo as independent taxa. The karyotypes are 2n = 50, NAA = 58 for the river buffalo and 2n = 48, NAA = 56 for the swamp buffalo (Fischer and Ulbrich 1968). The karyotypes of the two types of buffaloes diverged from each other through a tandem fusion translocation; the swamp buffalo's chromosome 1 cytotype results from a telomere-centromere tandem fusion between the river buffalo chromosome 4p and chromosome 9; that was a loss of the chromosome 9 centromere (Bongso and Hilmi 1982; Di Berardino and Iannuzzi 1981).
The tamaraw (*B. mindorensis*) is the rarest wild buffalo, endemic only to the island of Mindoro in the Philippines. Even though the phylogenetic relationships remain unclear, the result of DNA sequence analysis of the mitochondrial cytochrome *b* gene showed that the tamaraw shared a closer relationship with the swamp buffalo than the other species in *Bubalus* (Tanaka et al. 1996). This result was in agreement with earlier reports containing conventional stained chromosome that the tamaraw has NAA = 56 while the other species in the Bovini have NAA = 58 karyotypes, except for the swamp buffalo which has NAA = 56 (Namikawa et al. 1995; Wurster and Berinschke 1968). In this study we used the RBG-banding technique to analyze the karyotype of the tamaraw and compare the chromosomes of this species with those of the river buffalo and swamp buffalo in order to define whether the tamaraw and the swamp buffalo share the same autosomal translocation.

The centromeric satellite sequences and the telomeric sequence repeat (TTAGGG)$_n$ in the chromosomes are known to be implicated in karyotype evolution and chromosome rearrangement (Meyne et al. 1990; Modi et al. 1996). Tanaka et al. (1999) isolated two types of centromeric satellite sequences from the swamp buffalo that were homologous with bovine satellite I and II DNAs, and described their chromosomal distribution in the river buffalo and the swamp buffalo. The 18S + 28S rRNA genes are located at the nucleolar organizer regions (NORs). The polymorphism of the distribution of the NORs in the chromosomes has been used as an effective cytogenetic marker for differentiation and classification of many mammals (Liu et al. 1995; Suzuki et al. 1990; Zijlstra et al. 1997). In the present study we investigate the chromosomal distribution of the satellite I and II of the buffalo, telomeric sequence, and 18S + 28S rRNA genes in the tamaraw by using direct R-banding fluorescence in situ hybridization (FISH). We compared the results to those of the river buffalo and swamp buffalo in order to provide further information on their chromosomal evolution.

Materials and Methods

Cell Culture and Chromosome Preparation for Replication R-Banding

The R-banded chromosome slides of the river buffalo and the swamp buffalo which were prepared previously by Tanaka et al. (1999) were utilized in this investigation. Blood samples were collected in heparinized vacutainers (10 ml) from a male and a female captive tamaraw at Gene Pool on the island of Mindoro under permit from the Tamaraw Conservation Program. Lymphocyte cultures for replication R-banding were established following methods for human lymphocytes (Takahashi et al. 1990) with slight modification. Mononuclear cells were separated by using Lympho- prep (Nicostron), transferred into culture flasks containing RPMI 1640 supplemented with 20% fetal calf serum, 3 µg/ml concanavalin A (type IV-S, Sigma), 10 µg/ml lipopolysaccharide (Sigma), 2% HA15 (Murex), and 50 µM mercaptoethanol, and were incubated at 37°C in a humidified atmosphere of 5% CO$_2$ in air. Mitogen-stimulated lymphocyte cultures were synchronized by thymidine block (300 µg/ml), and BrdU (25 µg/ml) was incorporated during the late replication stage to obtain differential replication staining after release from excessive thymidine. R-banding was obtained by exposure of chromosome slides to UV light after staining with Hoechst 33258. Chromosome nomenclature followed that of Di Berardino and Iannuzzi (1981) referring to the standard karyotype of the river buffalo (Iannuzzi 1994).

Fluorescence in situ hybridization

For FISH analysis, 6.6 kb mouse 18S + 28S rDNA probe (Kominami et al. 1982), 1.4 kb buffalo satellite I DNA probe, 0.7 kb buffalo satellite II DNA probe (Tanaka et al. 1998), and 0.4 kb telomere probe containing (TTAGGG)$_n$ sequence were used. For the 18S + 28S rDNA probe, the chromosome slides were pretreated with RNase before the probe was applied. Hybridization and detection of fluorescence signals were performed according to Matsuda and Chapman (1995). An amplification method was used for the detection of the telomeric probe. After hybridization and washing, the slides were incubated with goat anti-biotin antibody at 1:500 dilution in 1% BSA in 4× SSC for 1 h at 37°C. The slides were washed with 4× SSC, 4× SSC with 0.05% Nonidet P-40, 4× SSC for 5 min each, and then stained with Fluorolink Cy2-labeled donkey anti-goat-IgG antibody (Amersham) at a 1:500 dilution in 1% BSA in 4× SSC for 1 h at 37°C. After washing as above for 10 min the slides were stained with 0.75 µg/ml propidium iodide. Fluorescence image was photographed with Kodak Ektachrome ISO 100 films using Olympus BX-60 epifluorescence microscope using Olympus filter set U-MWIB (excitation at 460–490 nm), U-MSWG (480–550 nm), and U-MWU (330–385 nm).

Results

Karyotype of Tamaraw

This is the first report on the banded karyotype of the tamaraw and confirms the species diploid number as 2n = 46 (Namikawa et al. 1995). The karyotype (Figure 1) consists 6 submetacentric and 16 acrocentric autosomal pairs (NAA = 56); the X chromosome is the largest acrocentric in the consistent and the Y chromosome is a medium-size acrocentric. A comparison of the RBG-banded chromosomes of the tamaraw to those of river and swamp buffaloes showed the three taxa have a high degree of banding homology (Figure 2). The 2n = 46 (NAA = 56) karyotype of the tamaraw can be cleared from the river buffalo standard (2n = 50, NAA = 58) by a Robertsonian translocation between chromosome 7 and chromosome 15, and a telomere-centromere tandem fusion between the short arm of chromosome 4 and chromosome 12. Table 1 lists the homologous tamaraw and river buffalo autosomes with reference of bovine karyotype (Iannuzzi 1994). As describe above and shown in Figure 2, the karyotype of the
Figure 2. Comparison of the RBG-banded chromosomes of the tamaraw (T), the river buffalo (R), and the swamp buffalo (S).

Table 1. Homologous autosome arms between the tamaraw, river buffalo, swamp buffalo, and cattle

<table>
<thead>
<tr>
<th>Animals</th>
<th>Autosome arms</th>
</tr>
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<tbody>
<tr>
<td>Tamaraw</td>
<td>1p 1q 2p 2q 3p 3q 4p 4q 5p 5q 6p 6q 7 8</td>
</tr>
<tr>
<td>River buffalo</td>
<td>4q 4p + 12 15 7 1p 1q 2p 2q 3p 3q 4p 4q 5p 5q 6q 7 8</td>
</tr>
<tr>
<td>Swamp buffalo</td>
<td>1p 1q - (T9) + 11 14 7 2p 2q 3p 3q 4p 4q 5p 5q 6q 7 8</td>
</tr>
<tr>
<td>Cattle</td>
<td>5 28 + 11 14 4 1 25 2 23 8 19 16 29 3 6</td>
</tr>
<tr>
<td>Tamaraw</td>
<td>9 10 11 12 13 14 15 16 17 18 19 20 21 22</td>
</tr>
<tr>
<td>River buffalo</td>
<td>9 10 11 13 14 16 17 18 19 20 21 22 23 24</td>
</tr>
<tr>
<td>Swamp buffalo</td>
<td>1q 9 10 12 13 15 16 17 18 19 20 21 22 23</td>
</tr>
<tr>
<td>Cattle</td>
<td>7 9 10 12 13 15 17 18 20 21 22 24 26 27</td>
</tr>
</tbody>
</table>

* Data from Iannuzzi (1994).

swamp buffalo diverged from that of the river buffalo by a telomere-centromere tandem fusion between chromosomes 4 and 9 of the river buffalo. Thus the decrease of NAA from 58 to 56 in the karyotype of the tamaraw and the swamp buffalo were not caused by the same autosomal rearrangement.

**FISH Analysis**

Buffalo satellite I DNA. The buffalo satellite I DNA appeared as centromeric heterochromatin blocks in all the chromosomes with the exception of the Y chromosome in the tamaraw, while the intensity of the hybridization signals was quantitatively different among the chromosomes (Figure 3a). The difference in the intensity depended on the morphological difference of the chromosomes, that is, large hybridization signals were found in all the acrocentric autosomes and the X chromosome, while the signals were much weaker in the biarmed autosomes. The considerably weaker signals were detected on the Y chromosome. These extensive variations in the distribution and intensity of the hybridization signals correspond to those described for the river buffalo and the swamp buffalo (Tanaka et al. 1999). The hybridization signals on chromosome 2 of the tamaraw, which is considered to be recently derived through a Robertsonian fusion involving chromosomes 7 and 15 in the river buffalo standard, were much larger than those on the other biarmed chromosomes, though smaller than those on the acrocentric autosomes (Figure 3a). Tanaka et al. (1999) reported an additional hybridization signal with this probe on the interstitial region of the long arm of chromosome 1 of the swamp buffalo as a relic of the telomere-centromere tandem fusion. This was not detected in tamaraw. The hybridization signals on the X chromosomes were quite variable among the three buffalo taxa studied; the river buffalo X chromosome had the largest centromeric block painted by this probe, while those

Figure 3. Partial metaphase chromosomes of tamaraw (a–c) male, (d) female] after direct R-banding FISH with the following probes: (a) buffalo satellite I DNA; (b) buffalo satellite II DNA; (c) (TTAGGG)n telomeric sequence, and (d) 18S + 28S rDNA. The arrowheads point to the hybridization signals and the arrows point to chromosome 1.
of the tamaraw separated into two blocks (Tanaka et al. 1999).

**Buffalo satellite II DNA.** The buffalo satellite II DNA was also localized to the centromeric regions of all the chromosomes in the tamaraw; no interstitial signals were detected. The intensity of the hybridization signals with the buffalo satellite II DNA was relatively weak when compared to those obtained with the buffalo satellite I probe. The only exceptions were the biarmed autosomes and Y chromosome which showed similar intensities with both the satellite I and II probes (Figure 3a,b).

**(TTAGGG)\_n** telomeric sequence. The TTAGGG repeat was detected on the telomeric regions of all the chromosomes (Figure 3c). The intensity of the hybridization signals was relatively constant, and no additional signal was detected on any other regions, that is, interstitial regions nor adjacent to the centromeres of submetacentric chromosomes.

**18S + 28S rDNA.** The hybridization patterns produced by the 18 + 28S rDNA probes in the tamaraw chromosomes is shown in Figure 3d. The 18S + 28S rDNA is localized to the terminal regions of the short arm of chromosomes 5, 7, 19, 21, and 22 of the tamaraw and their banding homologues in the river and the swamp buffaloes. In addition, however, the river buffalo has another hybridization that sits at the terminus of the short arm of chromosome 4 (Table 2). This chromosome with different tandem fusion partners in the evolution of the tamaraw and the swamp buffalo karyotypes, but in both instances we were unable to detect sequences corresponding to the 18S + 28S rDNA FISH probes in the target regions of the two taxa.

**Discussion**

Telomere-centromere tandem fusions are rare events in the Bovidae autosomes evolution and have the potential to be useful phylogenetic markers. At face value we may therefore expect that the tamaraw and the swamp buffalo share the same rearrangement that caused the decrease of NAA from 58 to 56. However, our data show that the decrease of the NAA in the karyotypes of the tamaraw and the swamp buffalo was caused independently by different telomere-centromere fusions (Figure 2). Thus the result of DNA sequence analysis of the mitochondrial cytochrome b gene showing that the tamaraw shared a closer phylogenetic relationship with the swamp buffalo than the river buffalo (Tanaka et al. 1996) was not supported by the present cytogenetic analysis directly.

The satellite DNAs that map to the centromeric heterochromatin blocks in these species as well as the (TTAGGG)\_n telomeric sequence was not detected by the present FISH analysis on the junction region of the telomere-centromere tandem fusions in the long arm of chromosome 1 of the tamaraw. This result strongly suggested that the centromeric region of chromosome 12 and the telomeric region of the short arm of chromosome 4 in the standardized river buffalo cytotype were lost by the tandem fusion, though a very small number of these sequences might have escaped detection as a result of the limited resolution of the technique.

The 18S + 28S rDNA was not detected on the junction of the tandem fusion in the tamaraw as well as the swamp buffalo (Figure 3d, Table 2). The 18S + 28S rDNA on the telomeric region of the short arm of river buffalo chromosome 4 may be the ancestral type, because the presence of the 18S + 28S rDNA was reported on its homologous chromosomal arms (i.e., chromosome 28 in the bovine cytotype) of various species in the Bovidae by Ag-NORs analysis (Di Berardino and Iannuzzi 1981; Mayr et al. 1985). The lack of the 18S + 28S rDNA in the junction of the tandem fusions thus suggests that not only the telomeric repeat sequence but also 18S + 28S rDNA gene clusters in the short arm of chromosome 4 of river buffalo was deleted in the fusion.

The presence of satellite DNAs at centromeric regions coincides with the distribution of constitutive heterochromatin in various species in the Bovidae. This is well illustrated with the bovine satellite I DNA family (1.715 satellite), which is highly conserved and known to be present in high copy numbers in the chromosomes of Bovidae (Modi et al. 1993, 1996). Moreover, bovine satellite I DNA is commonly reduced by Robertsonian fusions and thus biarmed autosomes of Bovidae have smaller amount of this satellite DNA than acrocentrics (Modi et al. 1996). The intensity and the distribution of the buffalo satellite I DNA in the chromosomes of the tamaraw agreed with this general rule (Figure 3a). Burkin et al. (1996) reported that the older Robertsonian fused chromosomes had less satellite I DNA than the later Robertsonian fused chromosomes from the comparative study of sheep chromosomes, suggesting that the reduction of the satellite I DNA correlated with the time after the fusion occurred. We found that chromosome 2 of the tamaraw had obviously larger hybridization signals for the satellite I DNA than the other biarmed autosomes (Figure 3a). Therefore this chromosome may have been formed by a relatively recent centromeric fusion, probably after the lineage of the tamaraw branched from other taxa of *Bubalus*, while the other biarmed autosomes (except the tandem fusions) were formed before the three taxa had diverged from their common ancestor. The substantially larger amounts of satellite I DNA on chromosome 2 of the tamaraw suggested that this chromosome had formed considerably later than the other biarmed autosomes in the karyotype of the tamaraw (Figure 3a).

**References**


Iannuzzi L and Meo GP, 1995. Chromosomal evolution in bovids: a comparison of cattle, sheep and goat...

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**Table 2. Distribution of 18S + 28S rRNA genes detected by FISH analysis**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Chromosome arms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamaraw</td>
<td>5p, 7b, 19, 21v, 22</td>
</tr>
<tr>
<td>River buffalo</td>
<td>3p, 4r, 6o, 21v, 23o, 24r</td>
</tr>
<tr>
<td>Swamp buffalo</td>
<td>4p, 6o, 20, 22i, 23i</td>
</tr>
</tbody>
</table>

The chromosome arms with the same superscripts were homologous.
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