Vol. 5, N.2: pp. 162-171, May, 2014
ISSN: 2179-4804

Journal of Biotechnology and Biodiversity

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Cytogenetic identification of four generations of crossbred buffaloes maintained in a conservation program in the Marajó island/Brazil

ABSTRACT

The domestic buffaloes were divided in two groups: the river buffalo, which has chromosome number 2n=50 and the swamp buffalo, 2n=48. The crosses between these animals result in F1 specimens with 2n=49 which are viable and fertile. In Brazil the Baio type and the Carabao breed buffalo are found in small number and maintained in genetic conservation programs that aim to keep these groups separated, this way conserving their genetic heritage in situ as well as ex situ. The objective of this study was to evaluate cytogenetically buffaloes from Carabao breed, Baio type and its F1, F2, F3 and F4 crossbreeds kept in a genetic conservation program. The metaphases were obtained from blood culture of 50 animals Carabao breed, 45 Baio type and 10 animals belonging to the crossbreed progeny. The Baio type presented 2n=50 and the Carabao breed 2n=48. In the progeny were observed specimens with chromosome number 2n=48 or 2n=49 with morphological variations in the first pair of chromosomes. The diploid number 2n=49 confirms crossbreeding of the animals from the conservation program. Thus, the exclusion of these animals from the original herds is highly recommended in order to keep separate genotypes for each genetic group.

Key-words: Amazon, biometry, chromosomes, selection.

Identificação citogenética de quatro gerações de búfalos mestiços mantidos em um programa de conservação na ilha de Marajó/Brazil

RESUMO

Os búfalos domésticos são divididos em dois grupos: os búfalos de rio, com número diploide 2n= 50 e os búfalos de pântano, 2n= 48. O cruzamento F1 entre estes resultam em animais com 2n= 49, que são viáveis e férteis. No Brasil os búfalos do tipo Baio e da raça Carabao são mantidos em um programa de conservação genética que visa manter esses grupos separados, conservando seu patrimônio genético *in situ* e *ex situ*. O objetivo deste estudo foi avaliar citogeneticamente búfalos da raça Carabao, tipo Baio e suas crusas F1, F2, F3 e F4 mantidos em um programa de conservação genética. As metáfases foram obtidas a partir de cultura de linfócitos de 50 animais da raça Carabao, 45 do tipo Baio e 10 descendentes de uma fêmea mestiça. Os animais do tipo Baio apresentaram 2n= 50, os da raça Carabao 2n= 48. Para os descendentes foram observados 2n= 48 e 2n= 49, com variações morfológicas no primeiro par. O número diplóides 2n= 49 confirma a presença de animais cruzados no programa de conservação. Assim, a exclusão destes animais e seus descendentes é altamente recomendada dos rebanhos originais a fim de manter os genótipos separados para cada grupo genético.

Palavras-chave: Amazônia, biometria, cromossomos, seleção.

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https://doi.org/10.20873/jbb.uft.cemaf.v5n2.degrandi

INTRODUCTION

The domestic buffaloes or "water buffaloes" belong to the specie *Bubalus bubalis*, which are divided in two subspecies according to cytogenetic characteristics and habitat: the "river buffaloes" (subspecies *bubalis*) have chromosome number of 2n=50 and the "swamp buffaloes" (subspecies *kerebau*) have 2n=48 (Pkhadze 1939, Ulbrich and Fischer 1967). According to studies with G-banding the karyotypic divergence is result of a tandem fusion between chromosomes 4 and 9 of the river buffaloes, reducing the chromosome number to 2n=48 in the swamp buffaloes (Hishinuma *et al*. 1992, Tanaka *et al*. 2000). Besides the reduction of the diploid number other karyotype characteristics can be observed, such as, the large metacentric first chromosome and the loss of one site of nucleolar organizing region (NOR) in swamp buffaloes (Di Berardino and Iannuzzi 1981, Hishinuma *et al*. 1992).

Despite these karyotypic differences, these animals are not isolated reproductively and the F1 crossbreed is viable and fertile, presenting karyotype 2n=49 and producing gametes n=24 and n=25 during meiosis. This fact produced three karyotypic groups (2n=48, 2n=49 and 2n=50) in successive generations F2, F3 and F4 (Fischer and Ulbrich 1968, Harisah *et al*. 1989, Dai *et al*. 1994).

In some countries such, China, crossbreeding between river and swamp buffaloes has been used as an alternative tool for the genetic improvement of livestock (Cruz 2010). However, in Brazil the Baio type buffalo (representatives from river buffaloes) and the Carabao breed (representatives from swamp buffaloes) are found in small number and maintained in genetic conservation programs that aim to keep these groups separated, this way conserving their genetic heritage *in situ* as well as *ex situ* (Egito *et al*. 2002).

In Brazil, there has been a consistent effort to elucidate facts relating to the cytogenetics of domestic buffalo (Marques and Jorge 1991). In view of this, Degrandi and colegues (2010) analyzed the karyotype of 25 Baio type and 30 Carabao breed animals in order to select the breeding stock in the Animal Genetic Resources Conservation National Program. They identified specimens with karyotype 2n=49, suggesting the occurrence of crossbreeding between the Baio type and the Carabao breed in the program. In addition, they observed that these specimens belonged to 26 descendents from the progeny of a crossbreed female. Therefore, cytogenetic analyses were used as a primary tool to select buffaloes, given the need to identify the chromosome number of each specimen in the conservation program.

The aim of this study was to apply classical cytogenetic analyses and chromosome banding to characterize Baio type and Carabao breed buffaloes as well as to identify chromosomal markers for its respective F1, F2, F3 and F4 crossbreeds.

MATERIALS AND METHODS

In this study it were sampled 45 buffaloes from Baio type (*B. bubalis bubalis*) and 50 from Carabao breed (*B. bubalis Kerebau*) belonging to the Animal Genetic Resources Conservation National Program: large species - Code SEG 01.06.01.06.00.04/Animal Network**,** developed at the Eastern Amazon Animal Germplasm Bank (BAGAM) in Salvaterra - Marajó island, part of Eastern Amazon Unit of the Brazilian Agricultural Research Corporation (EMBRAPA) -Belém, Pará - Brazil. In addition, it were sampled 10 animals descending from a female phenotypically identified as Carabao breed but with karyotype 2n=49 (Degrandi *et al*. 2010). In this group were distinguished four generations, F1, F2, F3 and F4, according to genealogical data provided by the conservation program.

Peripheral blood from each specimen was taken (10 ml) using heparin vacuum tubes and stored at 4ºC for transport to the Federal University of Pampa (UNIPAMPA), São Gabriel, Rio Grande do Sul - Brazil. Upon arrival blood cultures were immediately performed in order to obtained the metaphases according to Moorhead *et al*. (1960). The cytogenetic analyses were made with Giemsa stained slides to determine the diploid number. Subsequently, representative methafases were photographed to assemble the individual's karyotypes. In addition, the chromosome biometric analysis were performed using the karyotypes in the Micromeasure 3.1 software (version 3.3 by Colorado State University), where the distance between the centromere and the telomere, for the short (p)

and long (q) arm, was estimated to obtain the centrometric index calculation. The chromosome morphology classification was done according to Guerra (1986). The G- and C-banding techniques were performed according to Seabright (1971) and Sumner (1972), respectively.

RESULTS

All Baio type specimens evaluated was observed the diploid number 2n=50 (Figure 1) while the Carabao breed specimens was 2n=48 (Figure 2).

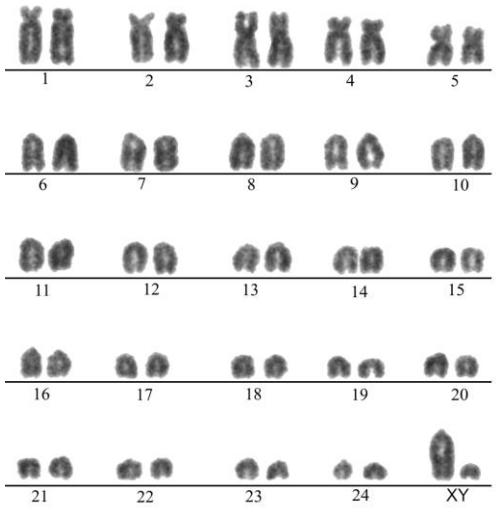

Figure 1- Karyotype a male of the Baio type (*B. bubalis bubalis*) with 2n=50.

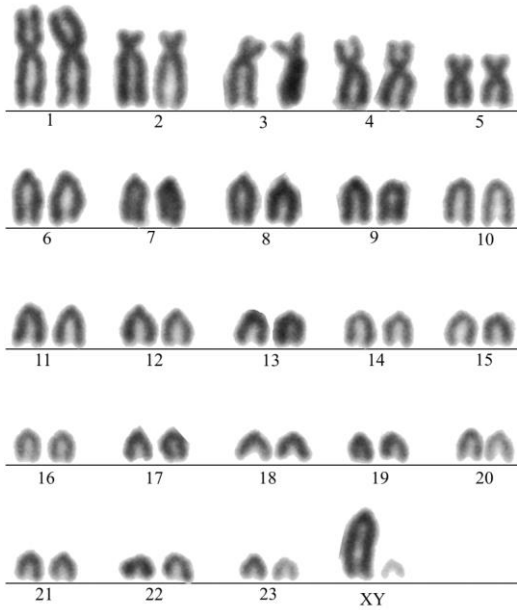

Figure 2- Karyotype a male of the Carabao breed (*B. bubalis kerebau*) with 2n=48.

According to the chromosome biometry data (Table 1), the karyotypic divergence between Baio type and Carabao breed buffaloes is restricted to the morphology of the first chromosome pair observed as metacentric in swamp buffaloes and submetacentric in river buffaloes. The 2nd, 3rd, 4th and 5th pairs are submetacentric and the rest of the chromosome complement is made up of 19 telocentric pairs in river buffalo and 18 telocentric pairs in swamp buffalo. The X and Y sex chromosomes are the largest and the smallest telocentric chromosomes, respectively, within the entire complement.

For the progeny (Table 2) from the female previously identified as F1 2n=49 hybrid (Figure 3), the formation of three different karyotypes was observed: i) 2n=49 karyotype, characterized as presenting a pairing between chromosome 1 from swamp buffalo and 4 and 9 from river buffalo, exactly as was seen in F1 specimens (Figure 3); ii) 2n=48 karyotype (Figure 4) with heteromorphisms in the first chromosome pair; iii) 2n=48 karyotype, typical of the Carabao breed, without morphological variations (Figure 2).

It is important to emphasize that all of the animals belonging to this progeny presented phenotypic characteristics of the Carabao breed and could only be identified using genealogical records and the cytogenetic data from this study.

Table 1: Chromosome biometry of Baio type buffalo (*B. bubalis bubalis*) and Carabao breed (*B. bubalis Kerebau*), showing the averages lengths of the long arm (q), short arm (p), total length (q+p) and the centrometric index (CI) of the autosomes and sex chromosomes and their respective morphology.

	Baio Type					Carabao Breed				
Chromosome Pair	q	p	q + p	CI	Morphology	q	p	q + p	CI	Morphology
1st	22.47	7.73	29.85	0.26	Submetacentric	26.95	19.69	46.64	0.42	Metacentric
2nd	19.40	9.46	28.86	0.33	Submetacentric	24.42	8.83	33.25	0.27	Submetacentric
3rd	17.55	9.85	27.39	0.36	Submetacentric	22.41	9.33	31.74	0.29	Submetacentric
4th	17.47	8.09	25.55	0.31	Submetacentric	20.05	9.58	29.63	0.33	Submetacentric
5th	13.19	8.21	21.40	0.38	Submetacentric	13.17	7.92	21.09	0.38	Submetacentric
6th	20.36	0.00	20.36	0.00	Telocentric	22.76	0.00	22.76	0.00	Telocentric
7th	19.16	0.00	19.16	0.00	Telocentric	20.90	0.00	20.90	0.00	Telocentric
8th	18.61	0.00	18.61	0.00	Telocentric	19.40	0.00	19.40	0.00	Telocentric
9th	18.14	0.00	18.14	0.00	Telocentric	18.97	0.00	18.97	0.00	Telocentric
10th	17.31	0.00	17.31	0.00	Telocentric	18.43	0.00	18.43	0.00	Telocentric
11th	16.68	0.00	16.68	0.00	Telocentric	18.01	0.00	18.01	0.00	Telocentric
12th	16.13	0.00	16.13	0.00	Telocentric	16.41	0.00	16.41	0.00	Telocentric
13th	15.12	0.00	15.12	0.00	Telocentric	15.89	0.00	15.89	0.00	Telocentric
14th	14.59	0.00	14.59	0.00	Telocentric	14.98	0.00	14.98	0.00	Telocentric
15th	14.06	0.00	14.06	0.00	Telocentric	14.28	0.00	14.28	0.00	Telocentric
16th	13.53	0.00	13.53	0.00	Telocentric	13.57	0.00	13.57	0.00	Telocentric
17th	13.10	0.00	13.10	0.00	Telocentric	12.94	0.00	12.94	0.00	Telocentric
18th	12.58	0.00	12.58	0.00	Telocentric	12.20	0.00	12.20	0.00	Telocentric
19th	11.93	0.00	11.93	0.00	Telocentric	11.79	0.00	11.79	0.00	Telocentric
20th	11.38	0.00	11.38	0.00	Telocentric	11.33	0.00	11.33	0.00	Telocentric
21st	10.92	0.00	10.92	0.00	Telocentric	10.47	0.00	10.47	0.00	Telocentric
22nd	10.60	0.00	10.60	0.00	Telocentric	10.01	0.00	10.01	0.00	Telocentric
23rd	9.66	0.00	9.66	0.00	Telocentric	8.69	0.00	8.69	0.00	Telocentric
24th	8.72	0.00	8.72	0.00	Telocentric	-	-	-	-	-
X	26.52	0.00	26.52	0.00	Telocentric	28.73	0.00	28.73	0.00	Telocentric
Y	7.89	0.00	7.89	0.00	Telocentric	7.93	0.00	7.93	0.00	Telocentric

Table 2: Partial genealogy of specimens descendent from a female identified as an F1 hybrid (2n=49) between Carabao breed and Baio type buffalo, showing the variation in diploid number (2n) through generations F2, F3 and F4 and the morphological characteristics of the karyotype.

Generation	Specimen	Sex	Birth Date	2n	Mother	2n-Mother	Father	2n-Father	Morphological characteristics of the karyotype
F1*	02	F	20/03/1991	49	1CA	48	1BA	50	Pairing 1 swamp and 4+9 river in first position
F2	03	F	24/04/2001	48	02	49	5978	48	Typical Carabao karyotype
	04	M	01/10/2004	49	02	49	207	48	Pairing 1 swamp and 4+9 river in first position
	05	M	18/10/2006	48	02	49	207	48	1st pair heteromorphic
	06	M	10/01/2009	48	02	49	246	48	Typical Carabao karyotype
F3	07	F	06/07/2002	49	12	-	5978	48	Pairing 1 swamp and 4+9 river in first position
	08	M	01/06/2005	48	03	48	207	48	Typical Carabao karyotype
	09	M	15/06/2007	48	03	48	207	48	Typical Carabao karyotype
F4	10	F	18/10/2005	48	143	-	207	48	Typical Carabao karyotype
	11	F	15/08/2009	48	07	49	246	48	Typical Carabao karyotype

* = First record evaluated in the group**;** Rg= genealogical register, F= Female, M= Male.

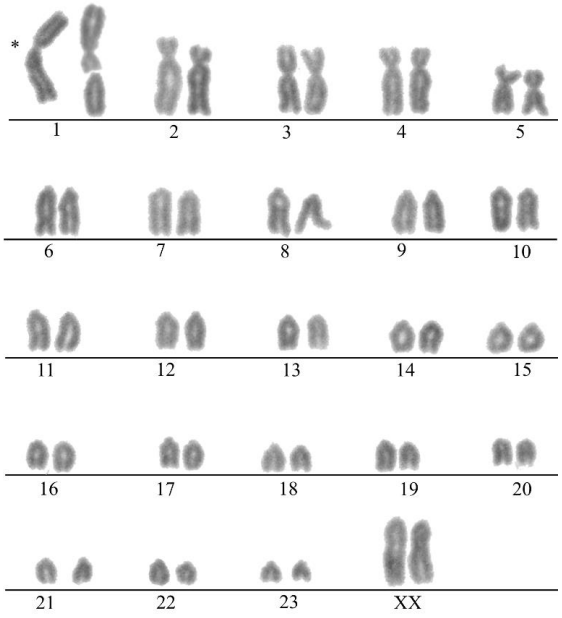

Figure 3- Karyotype of a female F1 crossbreed (Baio type x Carabao breed) 2n=49. * In the first pair, showed chromosomes 1 of the swamp buffaloes and 4 and 9 from river buffaloes.

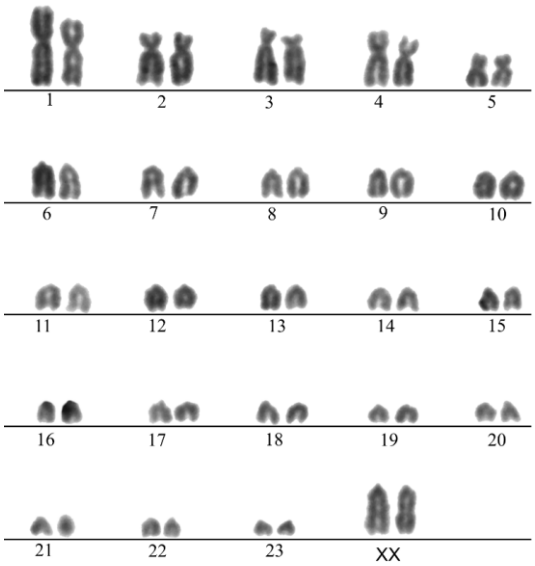

Figure 4- Karyotype of a female F2 specimen with 2n=48 with heteromorphism on the first pair.

The C-banding (Figure 5) revealed the presence of constitutive heterochromatin in centromeric regions across the whole chromosome complement for both river and swamp buffaloes and no differences were observed within this typical marking pattern. In addition, it was observed that the X sex chromosome presented interstitial heterochromatic block on the long arm q and the Y chromosome presented weak coloration, indicating low levels of constitutive heterochromatin.

The G-banding was initially used to carry out the correct identification of homologous pairs and to compare the karyotypes from buffaloes being studied. The G-banding pattern karyotype (Figure 6), where each chromosome pair was assembled using the first chromosome of the Carabao breed and the second member of the pair corresponding to the homologous pair in the Baio type. In addition, the pairing between chromosome 1 from swamp and 4 and 9 from river buffaloes is due to a Tanden fusion evolutive event. Therefore, these specimens presented all homologous chromosomes in the karyotype.

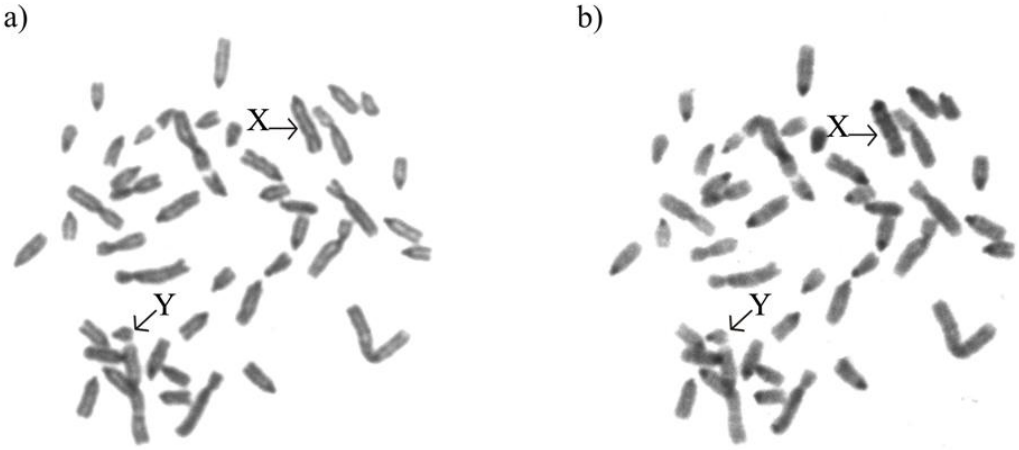

Figure 5- a) Metaphase with conventional staining for a male of the Carabao breed; b) sequential C-banding; the arrows indicate the X and Y sex chromosomes

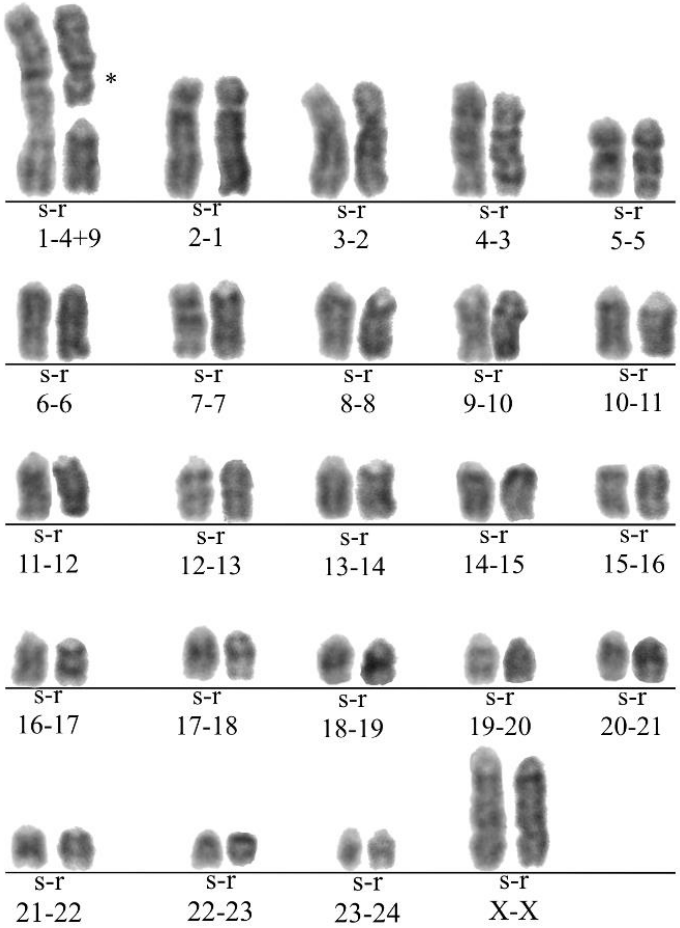

Figure 6- Karyotype showing the G-banding pattern and the homology between the chromosomes of river (Baio type) and swamp buffalo (Carabao breed), where each chromosome pair was assembled using the first chromosome of the Carabao breed and the second chromosome of the Baio type. * Indicates pairing between chromosome 1 in swamp buffalo and chromosomes 4 and 9 in river buffalo

DISCUSSION

The Baio type buffalo presented karyotype 2n=50 (Figure 1), a karyotype characteristic of river buffaloes, while the Carabao breed presented karyotype 2n=48 (FigURE 2), characteristic of swamp buffaloes. Both karyotypes were assembled in order of size and morphology and are in accordance with those described by Pkhadze (1939) and Ulbrich and Fischer (1967) respectively, in their studies with Asiatic buffalo.

The data of chromosome biometry from Baio type and Carabao breed buffaloes (Table 2) indicate that the karyotypic divergence between these is restricted to the first chromosome pair metacentric in swamp buffaloes and submetacentric in the river buffaloes. These divergences are also observed with the G-banding homologies of the whole chromosome complement among buffaloes (Figure 6), it is in accordance with Bernardino and Iannuzzi (1981), Hishinuma et al. (1992) and Tanaka et al. (2000). This homologies had origin in an event of the Tandem fusion between chromosomes 4 and 9 from ancestral karyotype of river buffalo (2n=50), thus reducing the chromosome number in swamp buffalo (2n=48).

Observing the cytogenetic studies carried out in both groups of buffaloes it is possible to identify some consequences related to this Tandem fusion event: the loss of the centromere on chromosome 9; fusion of heterochromatin with euchromatin; loss of an NOR site present on the short arm of the ancestral chromosome 4 from river buffaloes and the formation of a large metacentric chromosome that occupies the first position of the karyotype in swamp buffalo, as observed by Bernardino and Iannuzzi, (1981), Hishinuma et al. (1992) and Tanaka et al. (2000).

The F1 crossbreeding results in the formation of the specimens characterized as presenting karyotype 2n=49 and the pairing between chromosome 1 from swamp buffaloes and 4 and 9 from river buffaloes on the first position of the karyotype (Figure 3). However, the results obtained in this study disagree with those proposed by Fischer and Ulbrich (1968), who reported a karyotype of hybrid specimens with heteromorphism of the first pair and absence of one member homologue at 24.

The F1 crossbreeds specimens presented all of the paired chromosomes during meiosis with the formation of a 1+4+9 trivalent, viable unbalanced gametes n=24 and n=25 are therefore produced, following Mendel's law of segregation and indicating that the gametes produced are not necessarily from pure river or swamp buffaloes (Dai et al. 1994). This is proved by the formation of karyotypes with 2n=49, 50 and 48 in the F2 specimens observed in this study (Table 1), which may or may not present chromosomes 1+4+9 in the first position, as previously observed by Harisah et al. (1989).

It is important to emphasize that the karyotype characteristics of 2n=49 specimens were the same for F2 and F3 and F1 specimens (Figure 3). Therefore the generation identification was possible only from genealogical data records (Table 1). This occurs in the same way for animals identified as having karyotype 2n=48 and not having karyotype characteristics that would enable classification as crossbreed.

The identification of specimens with karyotype 2n=49 confirms the crossbreeding of Baio type and Carabao breed animals in the conservation program. As the main program objective is to maintain both buffaloes groups separated and to avoid crossbreeding between them, it is necessary to exclude not just animals identified as 2n=49 (for F1, F2 and F3 generations), but also their descendents (2n=50 or 2n=48).

Despite the karyotypic differences observed for some animals belonging to the progeny, for others it was not possible to find cytogenetic markers to distinguish crossbreeds from pure breed. It is important to recognize other parameters besides number, morphology and banding of chromosome are necessary, being expected that molecular analyses may provide markers for crossbreeding identification.

This study has broadened the general understanding of the processes involved in crossbreeding between river and swamp buffaloes. The results presented here provide essential parameters to help in the genetic conservation program's future actions, especially those relating to the reproductive management of these animals. Cytogenetic analysis is an excellent tool that can be used for this purpose, enabling the selection of breeding stock that is free of chromosomal alterations.

CONCLUSIONS

The identification of specimens with karyotype 2n=49 confirms the crossbreeding of Baio type and Carabao breed animals in the conservation

program. As the main program objective is to maintain both buffaloes groups separated and to avoid crossbreeding between them, it is necessary to exclude not just animals identified as 2n=49 (for F1, F2 and F3 generations), but also their descendents (2n=50 or 2n=48).

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ACKNOWLEDGMENTS

The authors thanks the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)-Amazônia Oriental by the provision of samples, the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the master's scholarship and the Universidade Federal do Pampa (UNIPAMPA) by structure and financial support.

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