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## Cytogenetic and molecular characterization of *Speothos venaticus* specimens

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**ABSTRACT.** The bush dog (*Speothos venaticus*) is a South American canid, included in the IBAMA (Brazilian Institute of Environment and Renewable Natural Resources) official list of animals threatened with extinction, in the vulnerable category. As a preservation and conservation strategy, specimens kept in captivity by Brazilian Institutions are monitored by a management plan. In order to characterize and analyze the genetic variability of bush dog specimens, a cytogenetic analysis was carried out, and microsatellite data were also obtained through the use of 15 primers, originally developed for the domestic dog (*Canis familiaris*). All tested primers showed transferability and amplified fragment sizes similar to those described for the canine genome. From the total number of primers, eight were tested, and presented two polymorphic regions. Regarding cytogenetic analysis, one of the animals had chromosomal mosaicism, disqualifying it as a reproducer to form stocks. Thus, we concluded that the genetic evaluation of wild animals kept in captivity provides data that can help with the practice of exchange between different institutions, avoiding problems in the reproductive capacity of the breeding stock.

**Key words:** bush dog, canids, chromosome, microsatellite, mosaicism.

**RESUMO.** Caracterização citogenética e molecular de exemplares de *Speothos venaticus*. O cachorro-vinagre (*Speothos venaticus*) é um canídeo sul americano que está na lista oficial do Ibama de animais ameaçados de extinção, na categoria vulnerável. Como estratégia de preservação e conservação, os espécimes mantidos em cativeiro por instituições brasileiras são acompanhados por um plano de manejo. Visando a caracterização genética e posterior análise de variabilidade genética de exemplares de cachorro-vinagre, foi feita a análise citogenética e testou-se a transferabilidade de 15 *primers* de regiões microssatélites desenvolvidos para o cachorro doméstico (*Canis familiaris*) para esta espécie de canídeo. Todos os *primers* testados mostraram transferabilidade, com fragmentos amplificados de tamanhos semelhantes aos descritos para o genoma canino. Do total de *primers*, oito foram testados em 25 animais cativos e, dentre estes, duas regiões apresentaram polimórficas. Em relação à análise citogenética, um dos animais analisados apresentou mosaicismos cromossômico, desqualificando-o para utilização como reprodutor na formação de plantéis. Os demais exemplares apresentaram padrão cariotípico esperado para a espécie. Desta forma, concluiu-se que a avaliação genética de animais silvestres criados em cativeiro fornece dados que podem auxiliar com a prática de intercâmbio entre animais de diferentes instituições, evitando o comprometimento na capacidade reprodutiva do plantel.

**Palavras-chave:** cachorro-vinagre, canídeos, cromossomo, microssatélite, mosaicismo.

### Introduction

The Canidae family is composed of 16 genera and 36 species of wolves, jackals, foxes and dogs (NOWAK, 1999). In Brazil, there are six species: *Speothos venaticus*, *Chrysocyon brachyurus*, *Cerdocyon thous*, *Atelocynus microtis*, *Lycalopex vetulus* and *Pseudalopex gymnocercus*. Among these, *Speothos venaticus* (Lund 1839), commonly known as bush

dog, is highlighted due to the increasing interest in preserving its natural populations, since there are only a few specimens kept in captivity by Brazilian Institutions.

In literature, several studies have reported the use of cytogenetics and molecular genetics for the characterization of species from the Canidae family. Many of these studies aim to contribute with data

about the evolution and phylogeny of this group. However, considering the different canid species occurring in Brazil, studies on genetic variability and chromosomal characterization are scarce.

As regards the species from this family, cytogenetic analyses were performed mainly by Wayne et al. (1987a and b). The authors showed the diploid complements 74, 76 and 74 chromosomes from the three South American canids species - *Speothos venaticus*, *Chrysocyon brachyurus* and *Cerdocyon thous*, respectively. The first two species presented karyotypes composed by acrocentric autosomal chromosomes and, through G-banding analysis it was possible to evidence a higher interspecific homeology. Hatanaka et al. (1998) presented data on chromosomal heteromorphism in a Brazilian species, *Cerdocyon thous*. All studied animals presented a diploid number of 74 chromosomes. Of these, 15 pairs were meta/submetacentric, and the remaining 21, acrocentric. A clear difference in size of the short arm in the large submetacentric pair (no. 3) was detected in three of the four studied animals. Brum-Zorrilla and Langguth (1980) showed *Pseudalopex gymnocercus* karyotype with  $2n = 74$  chromosomes. Pienkowska and Switonski (1998) identified the nucleolus organizer regions in *Canis familiaris* chromosomes.

Besides cytogenetic analysis, molecular genetics techniques were used to determine genetic differences among species, and establish possible phylogenetic relationships. Thus, based on the interest to reproduce specimens in captivity, besides the possibility of specimens relocation among different institutions, along with the probability that specimens kept in captivity are descendants from populations early geographically isolated, the goals of the present study were to analyze the chromosome group of *Speothos venaticus*, in order to obtain cytogenetic data that could be supplemented to the available in literature; to evaluate the transferability of microsatellite markers from the domestic dog (*Canis familiaris*) to the bush dog (*Speothos venaticus*); and to verify, based on the obtained results, the polymorphism degree of the selected loci, the genetic variability and the possibility of a pedigree analysis in these animals.

### Material and methods

Whole blood samples of 5 mL were collected by venipuncture utilizing vacutainer tubes (BD, USA), containing 7.5 mg of EDTA. The sampling included all bush dog (*Speothos venaticus*) specimens kept in captivity by different Brazilian Institutions: Parque Zoológico de Ilha Solteira (1 female), Fundação Zoo-Botânica de Belo Horizonte (1 female), Parque

Zoológico de Americana (4 females and 4 males), Parque Estoril de São Bernardo do Campo (1 female and 1 male), Zoológico da Universidade Federal de Mato Grosso (5 females and 5 males), Fundação Rio-Zoo (3 females and 2 males), Criatório Toca da Raposa (3 females), Parque Zoológico de Ieme (2 males), and Fundação Parque Zoológico de São Paulo (2 males). Peripheral blood samples were also collected from 2 domestic dogs (*Canis familiaris*) as control. For DNA extraction, the kit DNA GenomicPrep Cells and Tissue DNA Isolation (Amersham Biosciences) was used according to the manufacturer's specifications.

### Molecular analysis

Fifteen microsatellite loci, described by Jouquand et al. (2000), were chosen for transferability analysis: Ren02K21, Ren37H09, Ren37A11, Ren41D20, Ren39L15, Ren42N13, Ren44K10, Ren45F03, Ren04M22, Ren05C07, Ren13J22, Ren67C18, Ren48E01, Ren49C08, and Ren50B03.

Amplifications of DNA microsatellite loci from the bush dog were performed through a polymerase chain reaction (PCR). Reactions were carried out in a final volume of 25  $\mu$ L and the amplification mixture consisted of 50 ng genomic DNA (10 ng  $\mu$ L<sup>-1</sup>); 1.0  $\mu$ L each primer (10  $\mu$ M); 13.9  $\mu$ L autoclaved milli-Q water; 1.0  $\mu$ L MgCl<sub>2</sub> (50 mM); 0.5  $\mu$ L mM each dNTP (10 mM); 2.5  $\mu$ L PCR buffer 10X; and 0.1  $\mu$ L Taq DNA polymerase (5U  $\mu$ L<sup>-1</sup>) (Biotools). Thirty-five amplification cycles were performed and annealing temperatures were adjusted, according to each primer.

To verify whether the primer transfer occurred, samples of 5 bush dogs and 2 domestic dogs were tested as positive control. The amplified products were subjected to electrophoresis in 2% agarose gel, for 1 hour and 30 minutes at 100 V. The fragments were visualized by using ethidium bromide staining, and ultraviolet light exposure and photographed by Eagle Eye II (Stratagene).

The microsatellite locus polymorphism was analyzed in silver-stained denaturing polyacrylamide gel 6%. Electrophoresis was carried out at 1,300 V, for two hours.

The amplified product was sequenced with forward and reverse Ren39L15 primers, by using an ABI 377 model automated sequencer, and the kit ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit-Ampli Taq DNA Polymerase, FS (Perkin Elmer), according to the manufacturer's instructions.

### Cytogenetic analysis

From each specimen, 5 mL peripheral blood samples were collected into a sterile syringe heparinized with liquemine (Roche). To obtain metaphasic chromosomes, the technique developed by Moorhead et al. (1960), with modifications, was adopted. The best metaphases were photographed and documented for karyotyping of all the evaluated individuals.

### Results and discussion

All 15 tested primers showed 100% transferability, and the sizes of the amplified DNA fragments from bush dog samples were similar to those described for canine genome. Among these, primers Ren37H09, Ren39L15, Ren42N13, Ren45F03, Ren41D20, Ren44K10, Ren02K21, and Ren37A11 were tested in 25 animals to verify genetic variability, and only the loci Ren44K10 and Ren41D20 showed to be polymorphic. The remaining primers were monomorphic.

The results suggest there is high homology between domestic and bush dog sequences flanking the microsatellite loci. Similar results regarding microsatellite transfer were obtained by Wilton et al. (1999), evaluating the transfer of 14 canine microsatellites in Australian dingoes (*Canis dingo*), and by Roy et al. (1994), who tested 10 canine microsatellites in 3 canid species (*Canis lupus*, *Canis latrans* and *Canis rufus*).

Considering the polymorphism, the results were different from those observed in the studies from Wilton et al. (1999) and Roy et al. (1994), in which 100% of the loci were polymorphic, because although the primers tested in the present study were polymorphic in the domestic dog (*Canis familiaris*), six were monomorphic in the analyzed animals.

Wilton et al. (1999) reported the presence of a smaller number of alleles per locus in the Australian dingo, relative to the dog. Klukowska et al. (2003) also found a higher polymorphism in dogs than in Arctic and red foxes. According to Klukowska et al. (2003), the high polymorphism in dogs is probably due to the different breeds, and the interbreeding among them. Girman et al. (2001) found a smaller number of alleles and lower heterozygosity in African wild dogs in captivity, compared with those of wildlife. For Rosseto (2001), similar PCR products should be carefully compared, since several factors can lead to homoplasy. For example, two products amplified in different species, but with the same size can include mutations, rearrangements

and duplications in the regions flanking or at the microsatellite region. These variations interfere with the use of microsatellites in phylogenetic studies and sometimes can lead to erroneous data interpretation. In this study, the Ren39L15 locus was sequenced in order to confirm the presence of repeat regions. The electrophoretogram analysis indicated a (CA)<sub>17</sub> microsatellite locus, two repeats exceeding the expected pattern for the domestic dog. Thus, although it is a microsatellite locus, this region was monomorphic in the analyzed animals.

Regarding the cytogenetic analysis, in 5 of the 34 analyzed specimens, chromosomal preparations could not be obtained, or the quality from the obtained ones was not suitable for analysis. High-quality metaphase preparations were obtained from 29 specimens, of which 23 presented the same autosomal chromosome group:  $2n = 74$ , in which autosomal chromosomes are acrocentric, and gradually decrease in size from the 1<sup>st</sup> to the 36<sup>th</sup> pair. The size of the smallest autosomal was around 20% from that of the first pair. In all specimens, the X chromosome is submetacentric, and its size is between the first and the second autosomal pair. The Y chromosome is acrocentric, and the smallest of the complement (Figure 1a).

Besides the chromosomal group above-described, two brother animals, from one of the analyzed Institution, evidenced a metacentric Y chromosome (Figure 1b), although it had been the smallest element of the karyotype complement. These were males' offspring of one female of wildlife from the native forest of Mato Grosso State (the coordinates of the capture area were not specified), and were found in the backyard of a house. Such specimens died (parasitosis) some months after the collection of the material for cytogenetic analyses, which made impossible further collections and the establishment of the chromosomal differentiation (chromosomal inversion, translocation) origin.

Chromosomally, the Canidae family is characterized by including species with a large number of acrocentric chromosomes ( $2n > 64$ ), and others with a small number of metacentric ones ( $2n < 50$ ) (WAYNE et al., 1987a and b). In all species, there is heteromorphism between X and Y chromosomes. About the polymorphism observed in Y chromosome, although these data indicate that further chromosomal analyses should be carried out in other specimens from the same region, the hypothesis that populations early isolated from the others could have fixed their own chromosomal forms must be considered. These data must be especially considered in the formation of couples between specimens from different regions.



**Figure 1.** Mitotic metaphase chromosomes of males bush dog (*Speothos venaticus*) visualized by standard Giemsa staining. The Y chromosome is acrocentric and the smallest of the complement (A). The Y chromosome is metacentric at specimens kept in captivity, although these animals correspond to an offspring of a wildlife female (B). The bold arrows indicate the Y chromosomes and the thin arrow indicate the X chromosomes.

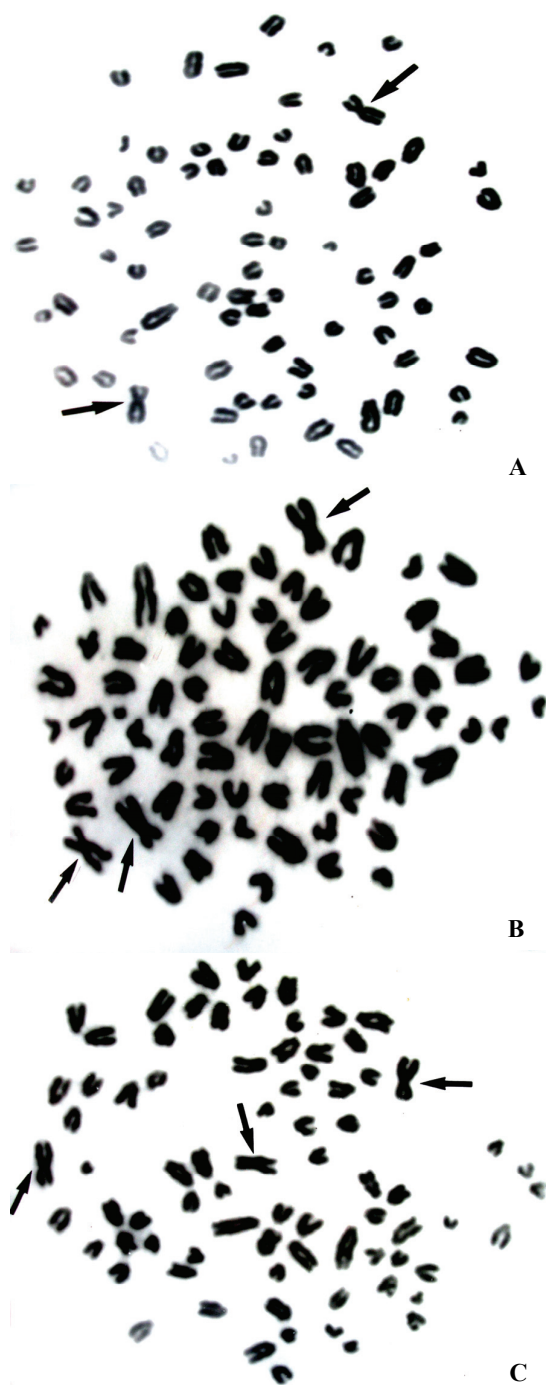
South American canids present as karyotypic pattern, many and small acrocentric chromosomes, whose numbering is difficult, as well the identification of homologous pairs. Their karyotype is very similar to that of the grey wolf (*Canis lupus*), and the latter is regarded as the oldest species, ancestor of the remaining ones. If we consider the *Canis lupus* a karyotype basal for the family, the chromosomal rearrangements that occurred over the group evolution can be suggested. According to Wayne et al. (1987a and b), *Chrysocyon brachyurus* ( $2n = 76$ ), that is considered the oldest South

American canid, lost the autosomal pair number 28 in comparison to *Speothos venaticus* ( $2n = 74$ ). Comparisons of *C. brachyurus* and *Canis lupus* ( $2n = 78$ ) also evidence that the first species lost the chromosome pairs numbers 28 and 22. Similarly to *Speothos venaticus*, the karyotype of *Cerdocyon thous* ( $2n = 74$ ), compared to the one of *C. lupus*, does not present the autosomal pairs 22 and 28, which suggest a chromosomal loss or translocation to other chromosome during speciation. *C. lupus* seems to be the direct ancestor of *C. lupus*, based on the loss or incorporation of the autosomal pair 28. This chromosomal loss/incorporation process continued in the remaining South American canids, relative to the autosomal pair 22. Hatanaka et al. (1998) observed a different karyotype on *Cerdocyon thous*, compared to other canids so far studied, as it presented a higher proportion of meta and submetacentric chromosomes and the highest fundamental number of the Canidae family ( $NF = 106$ ). Moreover, a high proportion of heterochromatin was observed, a feature that can be seen as an important tendency to the stabilization of neo-established chromosomes that were originated by chromosomal fissions.

Of the 29 specimens, from which we obtained high-quality chromosome preparations, one female presented mosaicism. This female, which is kept by an Institution in São Paulo State, was not as prolific as the females kept by other Institutions; however, it had already reproduced, but none of its descendants survived. In this specimen, cells of  $2n = 74$ , XX (Figure 2a);  $2n = 73$ , X0;  $2n = 75$ , XXX;  $2n = 76$ , XXX (Figure 2b) and  $2n = 78$ , XXX (Figure 2c) were identified.

To rule out the possibility that the observed mosaicism was due to the action of a drug and/or agent with which the specimen had contact, or the result of a factor during metaphasic cell formation in vitro, a new collection was performed approximately one year and a half after the first visit to the Institutions. In this occasion, samples were collected from another specimen kept by the same Institution, and which had no relationship with that female. The same cytogenetic results were obtained, characterizing the constitutional mosaicism of the studied female.

Although the mosaicism had been detected in blood cells, the possibility of its extension to other tissues must be considered. In this case, it may be one of the factors responsible for the specimen low prolificacy, due to the formation of chromosomally unbalanced gametes that are not viable to produce a zygote of normal development.



**Figure 2.** Mitotic metaphase chromosomes of a female bush dog (*Speothos venaticus*) that presented mosaicism, visualized by standard Giemsa staining. In this specimen, we identified cells of  $2n = 74, XX$  (A),  $2n = 76, XXX$  (B) and  $2n = 78, XXX$  (C). The arrows indicate the X chromosomes.

### Conclusion

The genetic evaluation of wild animals kept in captivity provides data that can assist with the

exchange practice between different institutions, avoiding problems in the reproductive capacity of the breeding stock.

The microsatellites of the domestic dog were not efficient for genetic analysis in *Speothos venaticus* and, therefore, it would be necessary to develop species-specific primers, in order to evaluate the genetic diversity of the bush dog.

The cytogenetic analysis leads to the detection of polymorphism, and the obtained data can support breeding practices of captive animals.

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