

## Biogeographical karyotypic variation of *Rhinophylla fischeriae* (Chiroptera: Phyllostomidae) suggests the occurrence of cryptic species

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**Abstract.** The genus *Rhinophylla* Peters, 1865 (Carolliinae: Phyllostomidae) comprises three species: *R. pumilio* Peters, 1865, *R. fischeriae* Carter, 1966 and *R. alethina* Handley, 1966. Only the first two species have been cytogenetically studied to date. Previous studies on specimens of *Rhinophylla fischeriae* from two populations from East of Andes (Colombia) showed the karyotype with  $2n=34$  and  $FN=56$ . In this paper, we report the results of cytogenetic analysis of six specimens of *Rhinophylla fischeriae* from Brazil. Probably chromosomal differences can be found among the populations because of the geographic distance. Metaphase chromosomes were obtained in the field by direct extraction of bone marrow. The metaphases were analyzed by conventional staining, G- and C-banding, NOR-staining and FISH with telomeric probes. *Rhinophylla fischeriae* has  $2n=38$  and Fundamental Number  $FN=68$ , with small amounts of constitutive heterochromatin in the centromeric regions of the chromosomes and the long arm of pair 16. Fluorescence in situ hybridization using telomeric probes did not show any interstitial sequences. Hybridization with human 18S and 28S rDNA probes and silver staining revealed the presence of Nucleolar Organizer Regions at the long arms of pairs 16 and 18. The pattern of G-banding showed that this population had a huge chromosome variation compared with previous studies on specimens of *Rhinophylla fischeriae*. The chromosomal differences among populations that have been morphologically classified as *R. fischeriae* suggest that this species should be considered a cryptic species complex, and that the populations from different geographical regions analyzed to date should be considered species of this complex, where the chromosomal rearrangements had key importance.

**Key words:** *Rhinophylla fischeriae*, Chiroptera, karyotype, cryptic species, Amazon, biodiversity.

## INTRODUCTION

Phyllostomid bats constitute a complex assemblage of the Neotropical bat fauna with a long history of taxonomic controversies (Wetters et al., 2000; Baker et al., 2003). Brazilian Amazonian rainforest has a rich bat fauna (Handley, 1967; Bernard et al., 2001; Sampaio, 2003); however, the knowledge about this regional fauna is far from satisfactory to understand the complex ecological, geographic and diversity patterns.

There are few cytogenetic studies on bats from Brazilian Amazonia (Rodrigues et al., 2000, 2003; Neves et al., 2000; Ribeiro et al., 2003; Silva et al., 2005; Pieczarka et al., 2005). The results of these studies have shown that the Phyllostomidae has a high intrafamilial karyotypic variation and often new species have been detected first by different karyotypes.

The subfamily Carolliinae encompasses two genera: *Carollia* Gray, 1838 (10 species) and *Rhinophylla* Peters, 1865 (3 species), which are traditionally recognized as monophyletic group. The genus *Rhinophylla* comprises three species: *R. pumilio* Peters, 1865 and *R. fischeriae* Carter, 1966, broadly distributed through the South America, and *R. alethina* Handley, 1966 restricted to west coast of Colombia and Peru (Handley, 1976). This genus is taxonomically stable while *Carollia* has been recently changed by the discovery of new species on the past ten years (Cuartas et al., 2001; Baker et al., 2002; Pacheco et al., 2004; Muñoz et al., 2004 and Solari, Baker, 2006).

Despite of the relative taxonomic stability of *Rhinophylla*, cytogenetic studies have demonstrated karyotypic intraspecific variation in *R. pumilio* with description of four karyomorphs: a)  $2n=36$ , FN=62 from Colombia (Baker, 1971); b)  $2n=34$ , FN=56 from Suriname (Baker, Bickham, 1980); c)  $2n=34$ , FN=64

from Suriname (Honeycutt et al., 1980; Baker et al., 1981); and d)  $2n=26$ , FN=48 from Brazilian Atlantic forest (Toledo, 1973). Karyotype of *R. fischeriae* was described with  $2n=34$  and FN=56 for specimens from Colombia (Baker and Bleier, 1971; Baker et al., 1987). *R. alethina* was not studied to date.

In the present paper, we report a new karyotype for *Rhinophylla fischeriae* from Brazilian Amazonia and discuss the biogeographical karyotypic variation as an evidence of a species complex for this taxon.

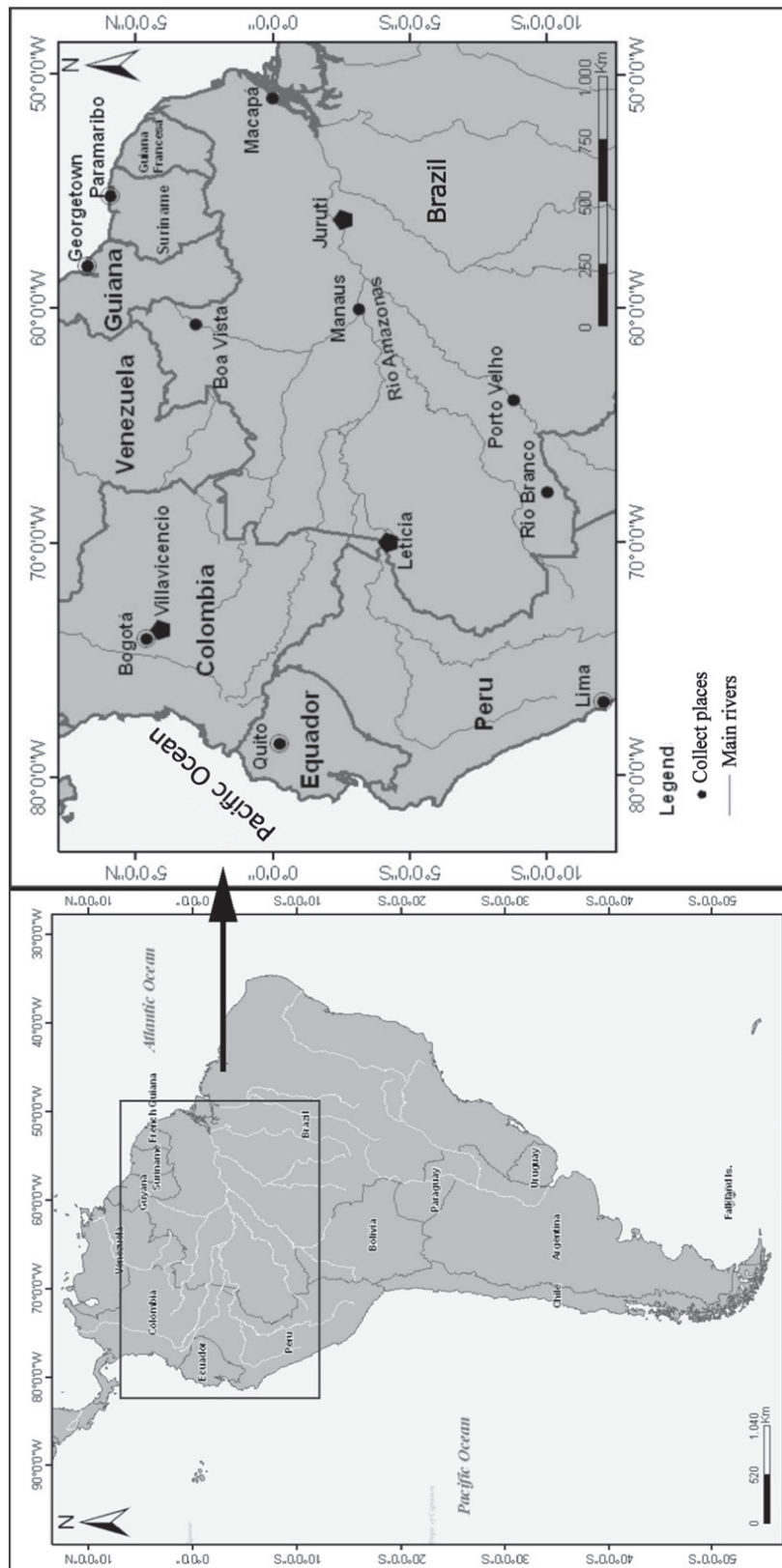
## MATERIAL AND METHODS

### Samples

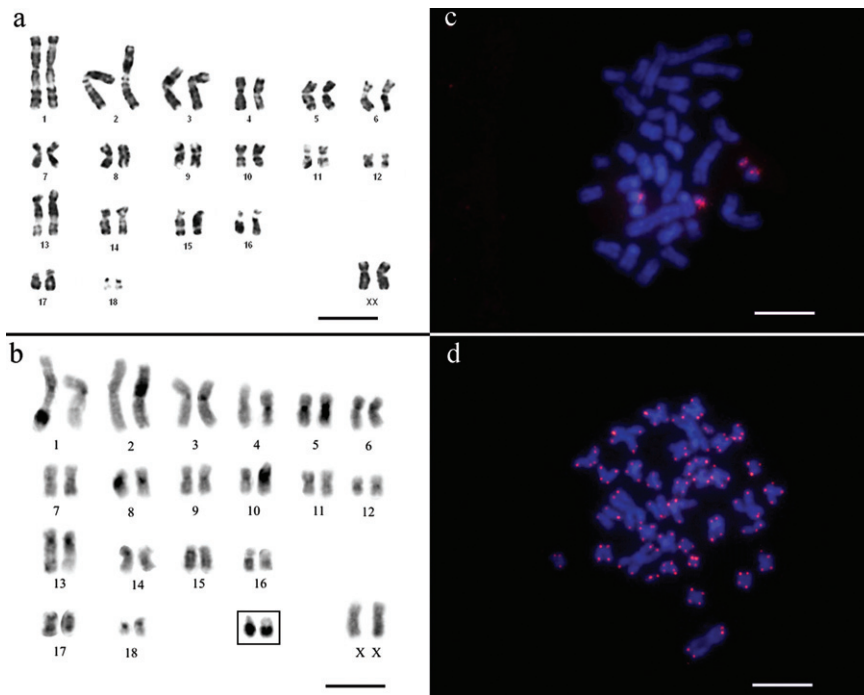
Six specimens (two males, codes LR 765 and LR 855, and four females, codes LR 710, LR 732, LR 763 and LR 818) of *Rhinophylla fischeriae* were obtained for cytogenetic analysis. The bats were collected from natural populations using mist nets, during the expeditions to faunal inventory in the area of bauxite mine of Alcoa Inc. in Juruti, Para state, Brazil ( $02^{\circ}29'38.8''S/56^{\circ}11'27.1''W$ ; Fig. 1). The specimens were identified in the field with the identification key for bats of the Guyanna (Lim, Engstrom, 2001). The identification was confirmed by the presence of diastema between I2 and the superior canines, as well as the hairy edge of interfemoral membrane (Rinehart, Kunz, 2006). Voucher specimens were fixed in formalin 10%, preserved in ethanol 70% and deposited in the mammal collection of the Museu Paraense Emilio Goeldi.

### Chromosome preparations, cell culture and chromosome banding

Metaphase chromosomes were obtained in the field by direct extraction of bone marrow according to Baker et al. (2003). Chromosomal preparations and tissue biopsies were sent to the cytogenetics laboratory at the Universidade Federal do Pará in Belém,



**Fig. 1.** Map of the regions where *Rhinophylla fischeri* with different karyotypes were collected: Villavicencio (2n=34, Baker et al., 1987), Leticia (2n=34, Baker, Bleier, 1971) and Juruti (2n=38, present paper).



**Fig. 2, a-d.** *Rhinophylla fischeriae* metaphases. **a** - G-banded. **b** - C-banded. **c** - FISH with rDNA 28 and 18S probes. **d** - FISH with telomeric probes. Bar = 5 $\mu$ m. (The chromosomal pair 16 from another specimen in the box shows a more intense heterochromatin block in the long arm).

Pará, Brazil. G-banding patterns were obtained by saline solution (2xSSC) incubation and Wright's staining following Verma and Babu (1995). C-banding was processed according to Sumner (1972), and Ag-NOR staining following Howell and Black (1980). The karyotypes were organized according to Baker, Bleier (1971).

#### Fluorescence in situ hybridization (FISH)

FISH with digoxigenin-labeled telomeric probes (All human Telomere probes, Oncor) was performed according to the manufacturer's protocol. To confirm the NOR labelled sites Biotin-dUTP was incorporated into the human 18S and 28S rDNA probes using nick translation. Briefly, the slides were incubated in RNase and pepsin solutions following the procedure described by Martins, Galetti

(1998). The slides were then dehydrated in an ethanol series (70, 90 and 100%), aged in a 65°C incubator for one hour, and denaturated in 70% formamide/2xSSC at 65°C for one minute. Fourteen microliters of the hybridization mixture (50% formamide, 1xSSC, 10% dextran sulfate, 5 mg salmon sperm DNA, and 2 mg mouse Cot-1 DNA) and 1  $\mu$ l of labelled rDNA probe were denaturated at 65°C and dropped onto the denatured chromosome preparations, which were then mounted with 24x24 mm cover slips. In situ hybridization experiments were incubated for 48-72 h at 37°C. The hybridization signal was detected with avidin-Cy3 as described previously (Yang et al., 1995; Pieczarka et al., 2005). The images were captured with an AxioCam Mrm CCD camera, which was controlled by the Zeiss Axiovision 3.0 software. The chro-



mosomes were identified by their morphology and DAPI (4',6-diamidino-2-phenylindole) inverted banding pattern.

## RESULTS

All six specimens of *Rhinophylla fischeriae* from Brazilian Amazonia (Fig. 1) have  $2n=38$  and  $FN=68$  (Fig. 2, a), of which 12 chromosomes pairs are metacentric/submetacentric, four pairs are subtelocentric and two pairs are acrocentric. The X chromosome is submetacentric, while the Y is small and acrocentric. C-banding detected constitutive heterochromatin at the centromeric regions of all chromosomes (Fig. 2, b), and in the long arm of pair 16. Both Ag-NOR staining (not shown) and FISH with 28S and 18S rDNA probes (Fig. 2, c) revealed Nucleolar Organizer Regions (NORs) in the proximal region of the long arm of pairs 16 and 18. FISH with telomeric probe hybridized only at the tips of the chromosomes (Fig. 2, d), without any interstitial telomeric sequence (ITS).

## DISCUSSION

Several studies have confirmed the occurrence of *Rhinophylla fischeriae* in Pará State (Eastern Amazonian region), and the previous reports were consistent with the diagnostic traits for this species (Bernard et al., 2001; Bernard, Fenton, 2007). *Rhinophylla fischeriae* is clearly distinguished from *R. pumilio* by dental and external characters (Rinehart, Kunz, 2006) but, apparently, there is no evident external morphological variation within *R. fischeriae* populations.

The karyotype with  $2n=38$  (24M/SM+8ST+4A) found in *R. fischeriae* from Juruti, Pará state, Brazil (this study – Fig. 1) is clearly different of the *R. fischeriae* from Colômbia (Baker, Bleier, 1971; Baker et al., 1987), with  $2n=34$  (20M/SM+4ST+6A with a

very small pair of chromosomes) previously described by conventional analysis (sample from Leticia) and G-banding (sample from Villavicencio, Baker et al., 1987). Comparative G-banding analysis between the karyotypes of *R. fischeriae* from Villavicencio (Colombia) and from Juruti (Brazil) shows an extensive chromosomal reorganization remaining few chromosomes shared without rearrangement.

The extensive chromosomal divergence between the *R. fischeriae* from different geographical regions suggests that these two cytotypes probably are not part of the same species. This would be an additional cryptic species situation, as already observed in *Carollia brevicauda* Schinz, 1821 and *C. sowelli* Baker, Solari et Hoffmann, 2002 (Baker et al., 2002) and *Carollia castanea* H. Allen, 1890 and *C. benkeithi* Solari et Baker, 2006 (Solari, Baker, 2006). Molecular data would be helpful to reinforce this hypothesis, but they are not available at the moment.

Wright et al. (1999) used data from the Cytochrome-B gene to study the phylogenetic relationships between the genera *Carollia* and *Rhinophylla*. Their results suggest that *R. pumilio* has been separated from *R. fischeriae* for a relatively long time (8-10 million years). In the most parsimonious tree the branch leading to *R. pumilio* and *R. fischeriae* was supported by low bootstrap values and Bremer decay, which was interpreted as a result of intense divergence intra- and inter-species, and may suggest that the nominal taxa *R. pumilio* and/or *R. fischeriae* may encompass more than one species. Our karyotypic results are consistent with these interpretations for *R. fischeriae*.

Based on data here presented the population from Juruti (PA, Brazil) will be named herein as *Rhinophylla fischeriae*. Further, detailed studies using G-banding and chromosome painting, as well as molecular and morphological analyses of all the geographic

variations in this species, will be necessary to fully define the possible new species and populational variants of *R. fischeriae*.

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#### REFERENCES

- Baker R.J., Bleier W.J. 1971.** Karyotypes of bats of the subfamily Carollinae (Mammalia; Phyllostomatidae) and their evolutionary implications // *Experientia*. 27: 220-222.
- Baker R.J., Bickham J.W. 1980.** Karyotypic evolution in bats: evidence of extensive and conservative chromosomal evolution in closely related taxa // *Syst. Zool.* 29: 239-253.
- Baker R.J., Genoways H.H., Seyfarth P.A. 1981.** Results of the Alcoa Foundation-Suriname Expeditions. VI. Additional chromosomal data for bats (Mammalia: Chiroptera) from Suriname // *Ann. Carnegie Mus.* 50: 333-344.
- Baker R.J., Qumsiyeh M.B., Hood C.S. 1987.** Role of chromosomal banding patterns in understanding mammalian evolution., (pp. 67-96) // *Current Mammalogy* (H.H. Genoways, Ed.). Plenum, New York.
- Baker R.J., Solari S., Hoffmann F.G. 2002.** A new Central American species from the *Carollia brevicauda* complex // *Occas. Pap. Mus. Texas Tech. Univ.* 217: 1-11.
- Baker R.J., Hooper S.R., Porter C.A., Van Den Bussche R.A. 2003.** Diversification among New World leaf-nosed bats: an evolutionary hypothesis and classification inferred from digenomic congruence of DNA sequence // *Occasional Papers of Museum Texas Tech University*, 230, 1-32.
- Baker R.J., Hamilton M., Parish D.A. 2003.** Preparations of mammalian karyotypes under field conditions // *Occas. Pap. Mus. Texas Tech. Univ.* 228: 1-7.
- Bernard E., Fenton M.B. 2007.** Bats in a fragmented landscape species composition, diversity and habitat interaction in savannas of Santarém, Central Amazonia, Brazil // *Biol. Conserv.* 34: 332-343.
- Bernard E., Abernaz A.L.K.M., Magnusson W.E. 2001.** Bats species composition in three localities in the Amazon Basin // *Stud. Neotropical Fauna and Environment.* 36: 177-184.
- Cuartas C.A., Muñoz J., González M. 2001.** Una nueva especie de *Carollia* Gray, 1838 (Chiroptera: Phyllostomidae) de Colombia // *Actualidades Biol.* 23: 63-73.
- Handley C.O. Jr. 1967.** Bats of the canopy of an Amazonian forest // *Atlas do Simpósio sobre a Biota Amazônica.* 5: 211-215.
- Handley C.O. Jr. 1976.** Mammals of the Smithsonian Venezuelan project // *Brigham Young Univ. Sci. Bull.* 20: 1-91.
- Honeycutt R.L., Baker R.J., Genoways H.H. 1980.** Results of the Alcoa Foundation-Suriname expeditions. III. Chromosomal data for bats (Mammalia: Chiroptera) from Suriname // *Ann. Carnegie Mus.* 49: 237-250.
- Howell W.M., Black D.A. 1980.** Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: as 1-step method // *Experientia.* 36: 1014-1015.
- Lim B.K., Engstrom M.D. 2001.** Species diversity of bats (Mammalia: Chiroptera) in Iwokrama Forest, Guyana, and Guianan subregion: implication for a conservation // *Biodiversity Conserv.* 10: 613-657.
- Martins C., Galetti P.M., Jr 1998.** Karyotype similarity between two sympatric *Schizodon* fish species (Anostomidae, Characiformes) from Paraguay River basin // *Genet. Mol. Biol.* 21: 355-360.
- Muñoz J., Cuartas C.A., González, M. 2004.** Se describe una nueva especie de murciélago del género *Carollia* Gray, 1838 (Chiroptera: Phyllostomidae) de Colombia // *Actualidades Biol.* 26: 80-90.
- Neves A.C.B., Pieczarka J.C., Barros R.M.S., Marques-Aguiar S., Rodrigues L.R.R., Nagamachi C.Y. 2000.** Cytogenetic studies on *Choeroniscus minor* (Chiroptera, Phyllostomidae) from the Amazon region // *Cytobios.* 105: 91-98.
- Pacheco V., Solari S., Velazco, P.M. 2004.** A new species of *Carollia* (Chiroptera: Phyllostomidae) from the Andes of Peru and Bolivia // *Occas. Pap. Mus.*

- Texas Tech Univ.* 236: 1-16.
- Pieczarka J.C., Nagamachi C.Y., O'Brien P.C.M., Yang F., Rens W., Barros R.M.S., Noronha R.C.R., Rissino J., Oliveira E.H.C., Ferguson-Smith M.A. 2005.** Reciprocal chromosome painting between two South American bats: *Carollia brevicauda* and *Phyllostomus hastatus* (Phyllostomidae, Chiroptera) // *Chromosome Res.* 13: 349-347.
- Rinehart J.B., Kunz T.H. 2006.** *Rhinophylla pumilio* // *Mammalian Species.* 791: 1-5.
- Ribeiro N.A.B., Nagamachi C.Y., Pieczarka J.C., Rissino J.D., Neves A.C.B., Gonçalves A.C.O., Marques-Aguiar S., Barros R.M.S. 2003.** Cytogenetic analysis in species of the Subfamily Glosophaginae (Phyllostomidae-Chiroptera) supports a polyphyletic origin // *Caryologia.* 56: 85-95.
- Rodrigues L.R.R., Barros R.M.S., Marques-Aguiar S., Assis M.F.L., Pieczarka J.C., Nagamachi C.Y. 2000.** Chromosome comparison between two species of *Phyllostomus* (Chiroptera-Phyllostomidae) from Eastern Amazonia, with some phylogenetic insights // *Genet. Mol. Biol.* 3: 593-599.
- Sampaio E.M., Kalko E.K.V., Bernard E., Rodríguez-Herrera B., Handley C.O., Jr. 2003.** A biodiversity assessment of bats (Chiroptera) in a tropical lowland rainforest of central Amazonia, including methodological and conservation considerations // *Stud. Neotropical Fauna and Environment.* 38: 17-31.
- Silva A.M., Marques-Aguiar S., Barros R.M.S., Nagamachi C.Y., Pieczarka J.C. 2005.** Comparative cytogenetics analysis in the species *Uroderma magnirostrum* and *U. bilobatum* (cytotype 2n=42) (Phyllostomidae, Stenodermatinae) in the Brazilian Amazon // *Genet. Mol. Biol.* 28: 248-253.
- Solari S., Baker R.J. 2006.** Mitochondrial DNA sequence, karyotypic, and Morphological variation in the *Carollia castanea* species complex (Chiroptera: Phyllostomidae) with description of the new species // *Occas. Pap. Mus. Texas Tech. Univ.* 254: 1-16.
- Sumner A.T. 1972.** A simple technique for demonstrating centromeric heterochromatin // *Exp. Cell Res.* 75: 304-306.
- Toledo L.A. 1973.** Estudos citogenéticos em morcego brasileiros (Mammalia Chiroptera). Doctorship Thesis, Faculdade de Ciências Médicas e Biológicas de Botucatu, USP, Botucatu, São Paulo, Brasil. 90 p.
- Verma R.S., Babu, A. 1995.** Human chromosomes: Principles and techniques. New York. 419 p.
- Wetterer A.L., Rockman M.V., Simmons N.B. 2000.** Phylogeny of phyllostomid bats (Mammalia: Chiroptera): data from diverse morphological systems, sex chromosomes and restriction sites // *Bull. American Mus. Natur. Hist. New York.* 248: 1-200.
- Wright A.J., Van Den Bussche R.A., Lim B.K., Engstrom M.D., Baker R.J. 1999.** Systematics of the genera *Carollia* and *Rhinophylla* based on the cytochrome-b gene // *J. Mammalogy.* 80: 1202-1213.
- Yang F., Carter N.P., Shi L., Ferguson-Smith M.A. 1995.** A comparative study of karyotypes of muntjacs by chromosome painting // *Chromosoma.* 103: 642-652.

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