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Primates karyological diagnosis and management programs applications

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Abstract

Background Captive primates are often maintained in groups without geographic origin or genetic heritage information. This could lead to an incorrect assignment of species, which could result in an inadequate management of the colonies.
Methods We present a cytogenetic protocol adapted to be successfully used in an accurate taxonomic diagnosis of non-human primates (Platyrrhini), including lymphocyte culture, G- and C-banding, meiosis, and fluorescent *in situ* hybridization technique (FISH).
Results Using classical cytogenetic diagnosis, the species status was determined in 541 Platyrrhini individuals. Of these, 99 were previously erroneously sexed or assigned to a different species using only morphological characteristics.
Conclusions The cytogenetic results highlight the relevance of the genetic characterization of primates both in captivity and in the wild. These techniques had been used in our research group for more than 30 years in different research projects, not only for characterizing hundreds of primates, but also different for topics regarding primates genomes and evolution.

Introduction

After the birth of human cytogenetics during the early 50s [76], several researchers started to apply these recently developed techniques in non-human primates karyological studies [6, 13, 14, 77, among others]. At that time, the only possible observations were the determination of chromosome number and a simple description of chromosome morphology based on size and centromeric index. In the early 70s, the first chromosome banding technique, Q-banding, was developed [11, 12]. Later, other banding techniques were established: G-banding [26, 66], R-banding [18], and C-banding [74, 78]. These chromosome banding techniques allowed a precise identification of each chromosome pair by revealing a pattern of light and dark transverse bands that is specific to each chromosome. The comparison of banding patterns allows detecting karyotype differences among species, such as inversions, translocations, deletions, duplications, fusions/fissions, as well as differences in the size of heterochromatin blocks. When Finaz et al. [25] showed the remarkable similarity of gene content in homeologous chromosomes of man (*Homo sapiens*) and chimp (*Pan troglodytes*) by comparing their karyotypes, the value of these techniques was highlighted.

At the same time, germ cell analysis techniques began to be applied in non-human primates for the analysis of their meiotic cycle and their sex chromosome systems (sexual systems) [19]. Meiotic characterization becomes even more important in this order of mammals, as the presence of multiple sex chromosome systems was described in several genera of non-human primates [4, 36, 37, 49, 50, 53, 61, 71]. Analysis in somatic cells does not have enough resolution power, since even with a good G-banding pattern, a multiple sex chromosome system could be confused with an

XY sexual system [61]. Only the karyological study of germ cells allows the identification and confirmation of the sex chromosome system. Germ cell analysis is important also in species with XY sexual systems, to analyze the differences of meiotic behavior among non-human primates and humans. It was traditionally considered that *Cebus* possessed a '*human-like*' XY system [50, 68], but when further meiotic cytomolecular analysis was performed, a significally differential behavior from the human XY bivalent was observed [30, 31].

Additionally, meiotic analysis can be useful to analyze the reproductive effect of chromosomal aberrations, such as translocations, inversions, sex chromosome abnormalities, among others, in an individual [7, 40, 67].

Classical cytogenetic techniques will continue to be the main tool for basic cytogenetic analysis; however, molecular cytogenetic techniques are increasingly becoming more important. The application of the fluorescent *in situ* hybridization technique (FISH) with specific probes for whole chromosomes (chromosome painting) has proven to be a fast and reliable method for establishing chromosomal homologies among different taxa [69], as well as for an unambiguous identification of chromosomes and chromosome regions involved in chromosomal abnormalities [34].

All these techniques, originally developed for humans or mammals other than primates, had to be adapted in order to be successfully used in the study of non-human primates [3, 9, 22, 42, 62]. However, a standardized protocol specific for non-human primates was never published in a methodological format. Instead, every primate karyotype described to date was published with independence of the method used. Moreover, not all the species of primates analyzed in morphology, behavior or geographic distribution studies had been karyologically characterized.

The large number of primate specimens currently kept in zoos, breeding stations, and primate centers is a valuable resource for *in situ* conservation. However, this value can only be ensured if animals are correctly identified and if they are genetically representative of their free-ranging conspecific ones. Furthermore, all animals used in research must be well defined in terms of their genetic constitution through genetic monitoring that ensures reproducibility and scientific validated results [5, 79]. Genetic evaluation of captive colonies is essential to establish a proper experimental design, taking into account that an incorrect diagnosis increases the variance of research data [65]. Even more, it adds new biologic information for management programs in zoos and other institutions. In this context, cytogenetic characterizations became useful to reinforce or correct the traditional phenotypic assignment of species, using variables such as chromosome markers that are not subject to environmental changes or health status of the animals, such as pelage coloration. In this regard, the presence of several chromosome markers allows identifying a particular karyotype that does not always have a phenotypic consequence.

Our goals in this contribution are two: first, to present an optimized cytogenetic protocol for use in an accurate taxonomic diagnosis of non-human primates. Second, to exhibit its applications in the characterization of hundreds of these primates, in particular neotropical primates (Platyrrhini), for management in the wild and Zoos, but also in studies comprising topics such as genome dynamics, phylogenetics, primate colony reproduction, and genome structure among others [i.e., 43, 48, 49, 54, 73, 75].

Materials and methods

Humane care guidelines

All the research reported in this manuscript met the appropriate national and institutional guidelines for the legal acquisition and use of laboratory animals and authorized study of wild animals. The authors also adhered to the guide for care and use of experimental animals as promulgated by the American Society of Primatologists (ASP) Principles for the Ethical Treatment of non-human primates.

Biologic material

Peripheral blood samples from 541 individuals of different neotropical primates species have been collected since 1982, both in the wild and in different institutions from Argentina and other Latin American countries (Table 1). Routinely, adult animals from both sexes are handled and anesthetized according to the procedures applied in each institution or according to the veterinarian in charge of the sampling in the wild, preferably after the specimen's fasting. Blood samples are obtained by venipuncture using a sterile Vaccutainer® or a disposable syringe, previously coating the tube/syringe′s walls with a light coat of sodic heparin (Phada Pharma, Buenos Aires, Argentina). The volume of blood to be extracted depends on the animal's weight, usually ranging from 0.5 to 5 ml. The samples are generally preserved at room temperature and immediately transferred to our laboratory in Buenos Aires, Argentina, to be processed. In the case of the samples obtained in Chile, Colombia, Mexico, and Paraguay, the lymphocyte cultures were performed in laboratory facilities

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Table 1 Primate specimens analyzed since 1982 both from the wild ([1]) and institutions of Argentina and other South American countries

Species	Procedence	Males	Females	2n	BG-G-W	BC	Meiosis	FISH
Alouatta caraya	ECAS, Buenos Aires	4	0	✓	✓	✓	✗	✗
	Corrientes Zoo, Argentina	3	3	✓	✓	✓	✓	✓
	Mendoza Zoo, Argentina	3	3	✓	✓	✓	✗	✗
	Buenos Aires Zoo, Argentina	9	8	✓	✓	✓	✗	✗
	Rosario Zoo, Argentina	2	1	✓	✓	✓	✗	✗
	La Plata Zoo, Argentina	3	0	✓	✓	✓	✗	✗
	CRMAN, Argentina	6	6	✓	✓	✓	✓	✓
	Roque Saenz Peña Zoo, Argentina	2	3	✓	✓	✓	✗	✗
	Córdoba Zoo, Argentina	0	1	✓	✓	✓	✓	✓
	Itatí, Corrientes, Argentina[1]	3	1	✓	✓	✓	✗	✗
	Brasilera Island, Chaco, Argentina[1]	35	39	✓	✓	✓	✗	✗
	El Puma, Misiones, Argentina	2	1	✓	✓	✓	✗	✗
	EBCO (formerly CAPRIM), Corrientes, Argentina	3	2	✓	✓	✓	✗	✗
	Loreto, Corrientes, Argentina[1]	1	1	✓	✓	✓	✗	✗
	San Cayetano, Corrientes, Argentina[1]	11	14	✓	✓	✓	✗	✗
	Pontón, Corrientes, Argentina[1]	9	8	✓	✓	✓	✗	✗
	Puerto Bermejo, Chaco, Argentina[1]	3	4	✓	✓	✓	✗	✗
	Modesto Island, Yaciretá, Paraguay[1]	8	5	✓	✓	✓	✗	✗
	Ezeiza Flora and Fauna S.A., Argentina	6	2	✓	✓	✓	✗	✗
		Total 218						
Alouatta guariba	Güira-Oga, Misiones, Argentina	1	0	✓	✓	✓	✗	✗
		Total 1						
Alouatta palliata	Aragón Zoo, Mexico City, Mexico	1	0	✓	✓	✓	✓	✓
	Chapultepec Zoo, Mexico City, Mexico	0	1	✓	✓	✓	✗	✓
	Catemaco, Mexico	3	1	✓	✓	✓	✓	✗
		Total 6						
Alouatta pigra	Campeche, Mexico[1]	4	4	✓	✓	✓	✓	✓
	Aragón Zoo, Mexico City, Mexico	2	1	✓	✓	✓	✓	✓
		Total 11						
Ateles belzebuth	Buenos Aires Zoo, Argentina	1	1	✓	✓	✓	✗	✗
		Total 2						
Ateles chamek	Buenos Aires Zoo, Argentina	4	2	✓	✓	✓	✗	✗
	Córdoba Zoo, Argentina	1	0	✓	✓	✓	✗	✗
	La Esmeralda Farm, Argentina	1	3	✓	✓	✓	✗	✗
		Total 11						
Ateles geoffroyi	Buenos Aires Zoo, Argentina	0	1	✓	✓	✓	✗	✗
	Catemaco, Mexico	5	5	✓	✓	✓	✓	✗
		Total 11						
Ateles paniscus	Buenos Aires Zoo, Argentina	1	0	✓	✓	✓	✗	✗
	La Plata Zoo, Argentina	1	0	✓	✓	✓	✗	✗
		Total 2						
Aotus azarae	Córdoba Zoo, Argentina	2	2	✓	✓	✓	✗	✓
	Gran Guardia, Formosa, Argentina	1	7	✓	✓	✓	✗	✓
	Roque Saenz Peña Zoo, Argentina	0	2	✓	✓	✓	✗	✗
		Total 14						
Callithirix jacchus	La Plata Zoo, Argentina	2	4	✓	✓	✗	✗	✗
		Total 6						
Cebus albifrons	URRAS, Bogotá, Colombia	0	3	✓	✓	✗	✗	✗
	Chapultepec Zoo, Mexico City, Mexico	0	2	✓	✓	✓	✗	✗
		Total 5						
Cebus libidinosus	ECAS, Buenos Aires, Argentina	2	10	✓	✓	✓	✗	✗
	CEMIC, Buenos Aires, Argentina	3	0	✓	✓	✓	✗	✗
	EBCO (formerly CAPRIM), Corrientes, Argentina	12	9	✓	✓	✓	✓	✓

(continued)

Table 1 (continued)

Species	Procedence	Males	Females	2n	BG-G-W	BC	Meiosis	FISH
	Buenos Aires Zoo, Argentina	24	11	✓	✓	✓	✗	✗
	Corrientes Zoo, Argentina	2	2	✓	✓	✓	✗	✗
	La Plata Zoo, Argentina	1	2	✓	✓	✓	✗	✗
	Ezeiza Flora and Fauna S.A., Argentina	4	2	✓	✓	✓	✗	✗
	Córdoba Zoo, Argentina	5	2	✓	✓	✓	✓	✓
	El Puma, Misiones, Argentina	15	10	✓	✓	✓	✗	✗
	PUCCHI Breeding Center, Santiago, Chile	4	8	✓	✓	✓	✗	✗
	URRAS, Bogotá, Colombia	0	1	✓	✓	✓	✗	✗
	Roque Saenz Peña Zoo, Argentina	3	4	✓	✓	✓	✗	✗
	IICS, Paraguay	10	7	✓	✓	✓	✗	✗
		Total 153						
Cebus nigritus	ECAS, Buenos Aires, Argentina	1	0	✓	✓	✓	✗	✗
	El Puma, Misiones, Argentina	15	6	✓	✓	✓	✗	✗
	Córdoba Zoo, Argentina	2	0	✓	✓	✓	✓	✓
	Buenos Aires Zoo, Argentina	1	0	✓	✓	✓	✗	✗
	Iguazú Nacional Park, Misiones, Argentina[1]	1	0	✓	✓	✓	✗	✗
		Total 26						
Saimiri b. boliviensis	EBCO (formerly CAPRIM), Corrientes, Argentina	24	25	✓	✓	✓	✓	✓
	Roque Saenz Peña Zoo, Argentina	6	1	✓	✓	✓	✗	✗
	Buenos Aires Zoo, Argentina	0	6	✓	✓	✓	✗	✗
	Córdoba Zoo, Argentina	0	2	✓	✓	✓	✗	✗
	Mendoza Zoo, Argentina	0	3	✓	✓	✓	✗	✗
	La Plata Zoo, Argentina	3	5	✓	✓	✓	✗	✗
		Total 75						

FISH, fluorescent *in situ* hybridization technique; BG-G-W, G-banding either by Wright or by Trypsin; BC, C-banding; ✓, analysis performed; ✗, not performed/without results.

provided by the collaborating institutions in those countries. The resulting pellets were transported with a refrigerant gel pack to our laboratory in Buenos Aires for analysis. The work which took place in Argentina, Chile, Colombia, and Mexico was done in accordance with the laws of these countries, and the samples were transported after obtaining the appropriate legal permissions.

Lymphocyte culture and banding techniques protocols

Throughout the years, different reagents and suppliers have been used for protocol optimization. Nowadays, lymphocyte cultures are grown for 72 hours at 37°C as follows (modified from [9]): 1 ml of whole blood is added under sterile conditions to a culture glass bottle containing 7.5 ml of F10 medium (GIBCO BRL, Grand Island, NY, USA), 1.5 ml of fetal calf serum (BIOSER, Barcelona, Spain), 0.2 ml of penicillin–streptomycin antibiotic (GIBCO BRL), and 0.2 ml of phytohemagglutinin (GIBCO BRL). Each culture is conducted in duplicate. After 72 hours at 37°C in an incubator, 0.1 ml of colchicine (100 μg/ml; GIBCO BRL) is added to each bottle under sterile conditions and incubated at 37°C during 40 minutes. After that, the content of each bottle is transferred to a 15-ml conic tube and centrifuged for 10 minutes at 1500 rpm (or 269 *g*). The supernatants are discarded, and 8 ml of hypotonic solution (KCl 0.075 M) is added to each tube, gently resuspending the solution. The tubes are then incubated at 37°C for 30 minutes. Hypotonic treatment is stopped with three drops of cold Carnoy's fixative solution (methanol–acetic acid in a 3:1 proportion) followed by centrifugation (269 *g*) of all tubes for 10 minutes. The supernatant is discarded, and 5–7 ml of cold Carnoy's solution is added to each tube. The tubes are incubated at 4°C for 30 minutes. The fixation step is followed by the washing of the pellets. The washes consist in centrifuging at 269 *g* for 10 minutes, discarding the supernatant, and adding new Carnoy's fixative solution. This step is repeated until the supernatant after centrifugation is translucid.

Metaphase spreads are prepared: an aliquot of cellular solution is dripped on a slide covered by a layer of steam. At least 50 metaphases from each specimen are analyzed to confirm the diploid number (2n). Moreover, if the specimens represent a species that has not been previously karyologically characterized, 100 metaphases from each animal should be analyzed to determine the diploid number. G-banding by Trypsin, G-banding by

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Wright, and C-banding techniques are performed with protocols optimized by us. At least 10 metaphases of each banding procedure are photographed for classical cytogenetic characterization.

G-banding by trypsin and Giemsa (modified from [66])
The 1% trypsin solution enzyme (Trypsin, Difco, Franklin Lakes, NJ, USA; 1:250) is prepared in bidistilled water. This solution is agitated for 2 hours and then filtered using cellulose filter paper (Whatman Grade 1: 11 μm). Once prepared, it is kept at −20°C until use, in 5 ml aliquots. Before performing the protocol, the trypsin solution is thawed and 1 ml of the 1% trypsin solution is diluted in 9 ml of a $NaCl/Na_2HPO_4$ buffer (NaCl 0.9%/Na_2HPO_4 0.2N in 1:1 proportion). This 0.1% trypsin solution is then stabilized at 37°C for 10 minutes. Chromosome preparations should have been aged at room temperature. The amount of time is different for each preparation. The optimal moment to perform the banding protocol is achieved when, observed under phase contrast microscopy, the chromosomes reach a dark coloration. The first step of the protocol is the incubation of the preparations in the 0.1% trypsin solution at 37°C. The starting time is 1 s per each day of aging. After this incubation, the preparations are rinsed in a 9 g/500 ml NaCl solution and stained with Giemsa stain solution (10% Giemsa in Sorensen buffer) for 5 minutes. Afterward, the preparations are washed in distilled water, air-dried, and observed under the microscope.

G-banding by Wright's stain (modified from [27])
The chromosome preparations should have been aged at room temperature (see G-banding by trypsin technique). The chromosome preparations are submerged in a Koplin yard containing a 2× SSC solution at 65°C, starting with an 8 s incubation for a 7–day-old slide. The staining solution is prepared mixing Sorensen's buffer (9% KH_2PO_4:19% Na_2HPO_4 in 2:1 proportion) and Wright's stain solution in a 3:1 proportion. After 2× SSC incubation, the slides are washed in distilled water and then submerged in the staining solution for 2 minutes 30 s. Afterward, they are rinsed with distilled water, air-dried, and observed under the microscope.

C-banding (modified from [74]):
The chromosome preparations should have been aged for at least 5 days at room temperature or, alternatively, 1 hour at 70°C. The preparations are incubated in HCl 0.2N at room temperature for 20 minutes, rinsed in distilled water, and air-dried. Then, they are submerged in a 5% $Ba(OH)_2$ solution at 50°C for a time that depends on the aging of the preparation (between 15 s for a 5-day-old slide to 1 minute 30 s). The slides are washed in distilled water and incubated at 2× SSC at 60°C for 1 hour. Finally, the slides are washed again in distilled water and stained in 5% Giemsa for 10 minutes. After staining, the slides are washed with distilled water, air-dried, and observed under the microscope.

Species diagnosis
The karyograms obtained are compared with the karyotype established for the species whenever published. These are listed in Table 2.

Meiosis: sexual system confirmation (modified from [22])

To confirm the sex chromosome system observed by mitotic studies, a testicular biopsy is taken from each adult male specimen under the effect of anesthesia. The material should be taken from the anterior surface of the testis and assuring that the epididymis is posterior, so as not to pierce it. Our 20 years of experience in meiotic analysis, both in captivity as well as in the wild, has showed us that the procedure, if performed by trained veterinarians, has no impact on the specimen fertility or reproductive behavior. A small portion of seminiferous tubules (approximately 6 mm^3) is extracted and placed in a 15-ml conic tube with physiological solution at room temperature. The biopsy material should be processed in a period no longer than three hours after extraction. The testicular tissue is placed in hypotonic solution (1% sodium citrate) and incubated in this solution for 20–40 minutes at room temperature, while gently macerating the tubules. The resulting suspension is then transferred to a centrifuge tube of 15 ml and

Table 2 Karyotype descriptions used as reference in the cytogenetic diagnosis

Species	2n	References
Alouatta caraya	♀♂52	[49]
Alouatta guariba clamitans	♀46 ♂45	[58]
Alouatta palliata	♀53 ♂54	[38]
Alouatta pigra	♀♂58	[71]
Ateles chamek	♀♂34	[8]
Ateles belzebuth	♀♂34	[14]
Ateles geoffroyi	♀♂34	[21]
Ateles paniscus	♀♂32	[60]
Callithrix jacchus	♀♂46	[16]
Cebus albifrons	♀♂52–54	[29, 35]
Cebus capucinus	♀♂54	[17]
Cebus libidinosus	♀♂54	[39]
Cebus nigritus	♀♂54	[46]
Saimiri b. boliviensis	♀♂44	[28]
Aotus azarae	♀50 ♂49	[44]

centrifuged 5 minutes at 500 rpm (or 30 *g*). Once the supernatant is discarded, the cell pellet is resuspended in an amount of Carnoy's fixative solution that doubles the size of the pellet and stored at 4°C. Three or four drops of the cell suspension are dripped on a clean microscope slide covered with a layer of steam and dried in hot air stream. Most of the preparations are stained with 5% Giemsa for assessment of the stages of meiosis. The remaining preparations are aged from 4 to 7 days at room temperature for C-banding technique to detect the heterochromatic centromeres in metaphase I and therefore confirm the number of chromosomes involved in the sex chromosome system.

Fluorescence *in situ* hybridization (FISH)

The FISH procedure is conducted using whole chromosome painting (WCP) probes. When performing a two probe FISH, 0.5 μl of probe 1 and 0.5 μl of probe 2 are dissolved in 2.5 μl of hybridization mixture. This hybridization mixture contains 30% formamide, 30% polyethylene glycol, 10% 20× SSC, 28% NaI, and 2% Tween. If only 1 probe is tested, 0.5 μl of this probe and 0.5 μl of miliQ water are dissolved in 2.5 μl of the previously described hybridization mix. The resulting mixture is denatured at 70°C for 7 minutes and then kept at −20°C for a few min (no more than 10) before hybridization. The chromosome preparation is denatured in either of two forms: (i) 0.1 M NaOH/70% ethanol at room temperature for 2–4 minutes, followed by dehydration in an ethanol series (70, 90 and 100% sequentially, 2 minutes each), or (ii) 70% formamide/2× SSC at 62°–68°C for 2 minutes, followed by dehydration in ice-cold ethanol (70, 90, and 100% sequentially for 1 minute each). The probe mixture is then dripped on the slide, and a coverslip is placed over that region and sealed with synthetic glue. Hybridization is then conducted in a wet chamber at 37°C overnight. The synthetic glue is carefully removed, and the coverslip is removed placing the preparation in a koplin jar with 2× SSC at room temperature for a few minutes. Post-hybridization washes consist in placing the preparations in a koplin jar with 0.4× SSC/0.3% Tween at 70°C for 2 minutes and then place them in another koplin with 2× SSC/0.1% Tween at room temperature for 2 minutes. Slides are then counterstained with DAPI (Sigma). The DAPI solution is prepared by diluting 5 μl of a DAPI stock solution (20 μg/ml in ultrapure water) in 1 ml of antifade solution (50 mg p-phenylenediamine dihydrochloride, Sigma, in 5 ml of PBS). A 15 μl drop of this mixture is then placed over the preparation and covered with a coverslip. The slides are analyzed under a fluorescence microscope. For every experiment, a hybridization control is used. *Homo sapiens* X chromosome is the most frequently used control probe in our research group, taking into account previous data about the conservation of this chromosome in mammals [52, 57]. However, depending on the target DNA and the specific probes used, some modifications may be applied.

Results and discussion

Species diagnosis

Until today, we have described and established both the karyotype and the presence of different chromosomal rearrangements and polymorphisms in various species of neotropical primates belonging to Atelidae and Cebidae families. We studied species with geographic distribution in Argentina and other countries in Central and South America (Table 1), and also some African and Asian primates (data not shown).

Using classical cytogenetic diagnosis, species status was determined in 541 Platyrrhini individuals: 184 *Cebus* sp., 236 *Alouatta* sp., 14 *Aotus* sp., 75 *Saimiri* sp., 26 *Ateles* sp., 6 *Callithrix* sp. In some cases, the karyological analysis allowed the reassignment of several individuals that were previously assigned as belonging to a different species using traditional phenotypic diagnosis. The results of these re-characterizations are listed in Table 3, and here, we describe some examples to illustrate the applications of the genetic characterizations:

Two *Cebus* sp. specimens housed at the Chapultepec Zoo, in Mexico DF, were diagnosed as *Cebus*

Table 3 Species status of the primate specimens before and after the karyological analysis

Before	After	References
2 Cebus capucinus	*2 C. albifrons*	This contribution
12 *Cebus apella*	*8 Cebus libidinosus* + 2 *Cebus nigritus* + 1 *C. queirozi* + 1 *C. nigritus* × *C. libidinosus* hybrid	[55] [23]
1 *Cebus* sp.	*1 C. nigritus*	This contribution
10 *Saimiri sciureus*	10 *Saimiri b. boliviensis*	[47]
23 *S. sciureus*	23 *S. b. boliviensis*	[51]
4 *S. sciureus*	4 *S. b. boliviensis*	[28]
8 *S. sciureus*	8 *S. b. boliviensis*	[70, 72]
1 *Ateles belzebuth*	1 *Ateles chamek*	[54]
5 *Ateles paniscus*	5 *Ateles chamek*	[54]
1 Ateles sp.	*1 Ateles geoffroyi*	[54]
Sex reassignation		
1♂ *Alouatta caraya*	1♀*Alouatta caraya*	[24]
1♂ *Aotus azarae*	1♀*Aotus azarae*	This contribution

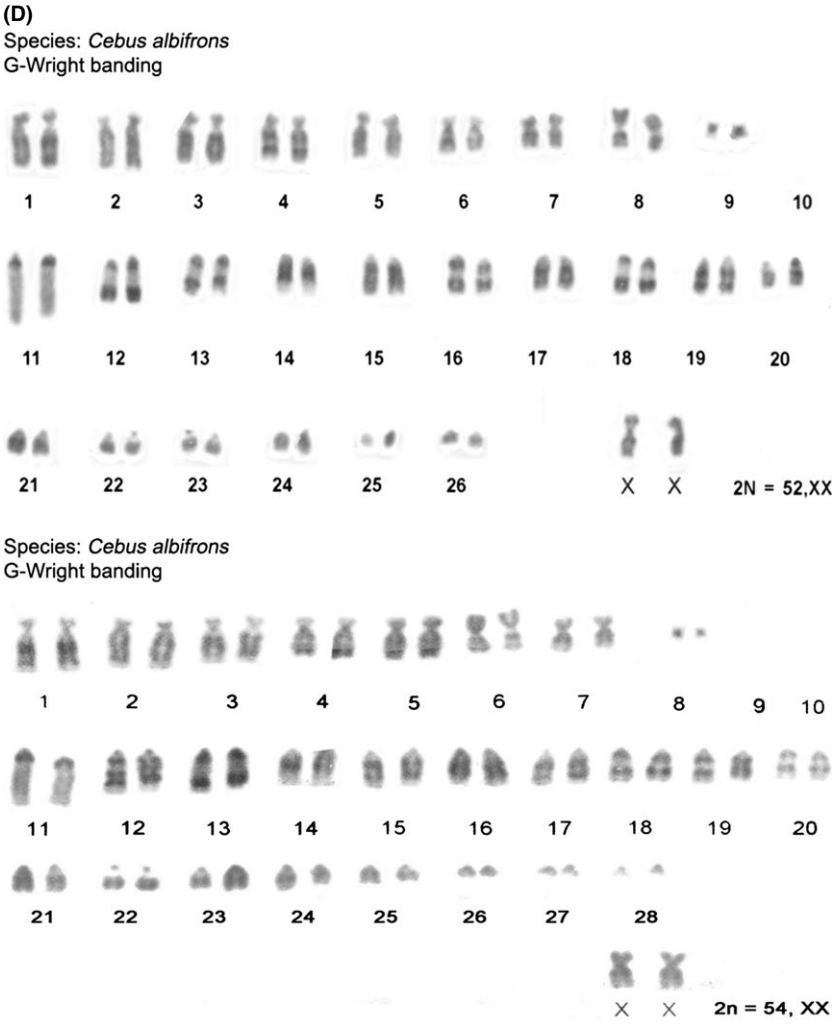

Fig. 1 **(A)** External phenotype of the two *Cebus albifrons* females. **(B)** External phenotype of *Cebus capucinus* (photograph from [63]). **(C)** External phenotype of *C. albifrons* (photograph from [63]). **(D)** G-Wright karyotypes of both females. Top: 2n = 52, XX. Below: 2n = 54, XX.

capucinus (Fig. 1A) using traditional morphological characters such as pelage and coat coloration. These individuals came to the zoo from wildlife trafficking, and their pelage was in poor conditions. Their initial morphological diagnosis was probably performed considering that *C. capucinus's* natural geographic distribution includes Mexico [64]. *C. capucinus* have a white to yellowish throat, head, and shoulders. Their back and tail are black, and the hair on the crown forms a V (Fig. 1B, [10]). *Cebus albifrons*, however, shows a pelage coloration pattern that varies from light to dark brown. They have a dark brown wedge-shaped cap, yellowish underparts, and a tail that is dark at the base and light yellow at the tip (Fig. 1C, [10]). Although the karyotype of *Cebus* sp. appears highly conserved throughout the genus, there are some peculiarities that distinguish species from each other. Karyologically, the main difference between *C. albifrons* (CAL) and *C. capucinus* (CCA) is located in chromosome number (2n): CCA has 54 chromosomes and CAL has both 54 and 52 chromosomes, depending on the geographic origin of the population. Also, among those CAL having 54 chromosomes, the difference is observed in the characteristic G-banding pattern shown in Fig. 1D. The karyological characterization showed that instead of *C. capucinus*, these animals were *C. albifrons,* due to correspondence of the animal's karyotype with the described in the literature for this species (Table 2; Fig. 1D), instead of the one described for *C. capucinus* (Table 2). Taking this into account, it was possible to note that the pelage coloration pattern was in fact consistent with *C. albifrons*.

Some years ago, one *Cebus nigritus* male was found by a local researcher while studying a troop of individuals in the species natural area at Parque Nacional Iguazú, Misiones, Argentina. This animal did not present the typical *nigritus* phenotype: instead of very dark brown to black pelage and two elongated lateral frontal tufts or ridges on the crown (Fig. 2A; [33]), this specimen had a light brown pelage coloration pattern and no tufts (Fig. 2B). The researcher sent us a blood sample asking for an assessment of the species status of that male. The cytogenetic characterization by C-banding technique showed the absence of the extracentromeric heterochromatic block in chromosome #11, a feature characteristic of *C. nigritus* (Table 2; Fig. 2C). The other *Cebus* species with geographic distribution in Argentina, *C. libidinosus,* has this extracentromeric heterochromatic block in chromosome #11 (Table 2), thus allowing the distinction between these species. The confusing phenotype of the referred *Cebus* from Misiones, Argentina, was solved after the cytogenetic characterization, thus allowing the incorporation of the animal into one of the troops. As those groups of *C. nigritus* are currently under vigilance for demographic, ecological, and conservation studies, the correct diagnosis of the individual guaranteed that this inclusion would not prejudice or affect the continuity of the work due to the mixture of species.

Another particular example was the one of the squirrel monkeys (genus *Saimiri*). Seventy five *Saimiri* sp. were historically assigned in Argentinean Zoos and breeding centers to *S. sciureus* using traditional morphological parameters, such as pelage coloration pattern and the pattern of the periocular mask [32]. But the pelage coloration pattern is a polymorphic character, and it is usually influenced by the health status of the animals, making it difficult for the untrained eye to correctly distinguish these differences. The different species of *Saimiri* share a chromosome number of 2N = 44, XX/XY. However, the number of chromosome arms (fundamental number, FN) of their karyotypes is different owing to pericentric inversions, which cause variations in the biarm/acrocentrics ratio [41]. The cytogenetic characterization conducted by us confirmed that all analyzed specimens were *Saimiri boliviensis boliviensis*, as they possess a 15 biarm/6 acrocentrics ratio and a G-banding pattern in agreement with the one described for this species (Table 2). On the contrary, *S. sciureus* possesses a 14 biarm/7 acrocentrics ratio, therefore allowing a proper cytogenetic distinction of these two species [41].

Other remarkable example is the case of one howler monkey in the Mendoza Zoo, Argentina, that was erroneously sexed as male being actually female (Table 3). In *Alouatta caraya*, adult males present black pelage coloration, while the females present a golden coloration in their fur. The infants are born with golden coloration, which darkens in males as they age [10]. One specimen was assigned in the Zoo as a juvenile male, as it had dark golden pelage coloration. In this species, sometimes, the high thickness of the vulva in some animals can be confused by the untrained eye as a pair of small testicles if a thorough examination of the specimen is not performed. The cytogenetic characterization allowed determining that this specimen was actually a female (2n = 52, XX), with a darker pelage coloration pattern, thus allowing to correct the sex assignation of the *A. caraya* specimen.

A similar case occurred with an owl monkey (*Aotus azarae*) specimen from Córdoba Zoo, Argentina. This animal was born in the Zoo, and it was sexed as male.

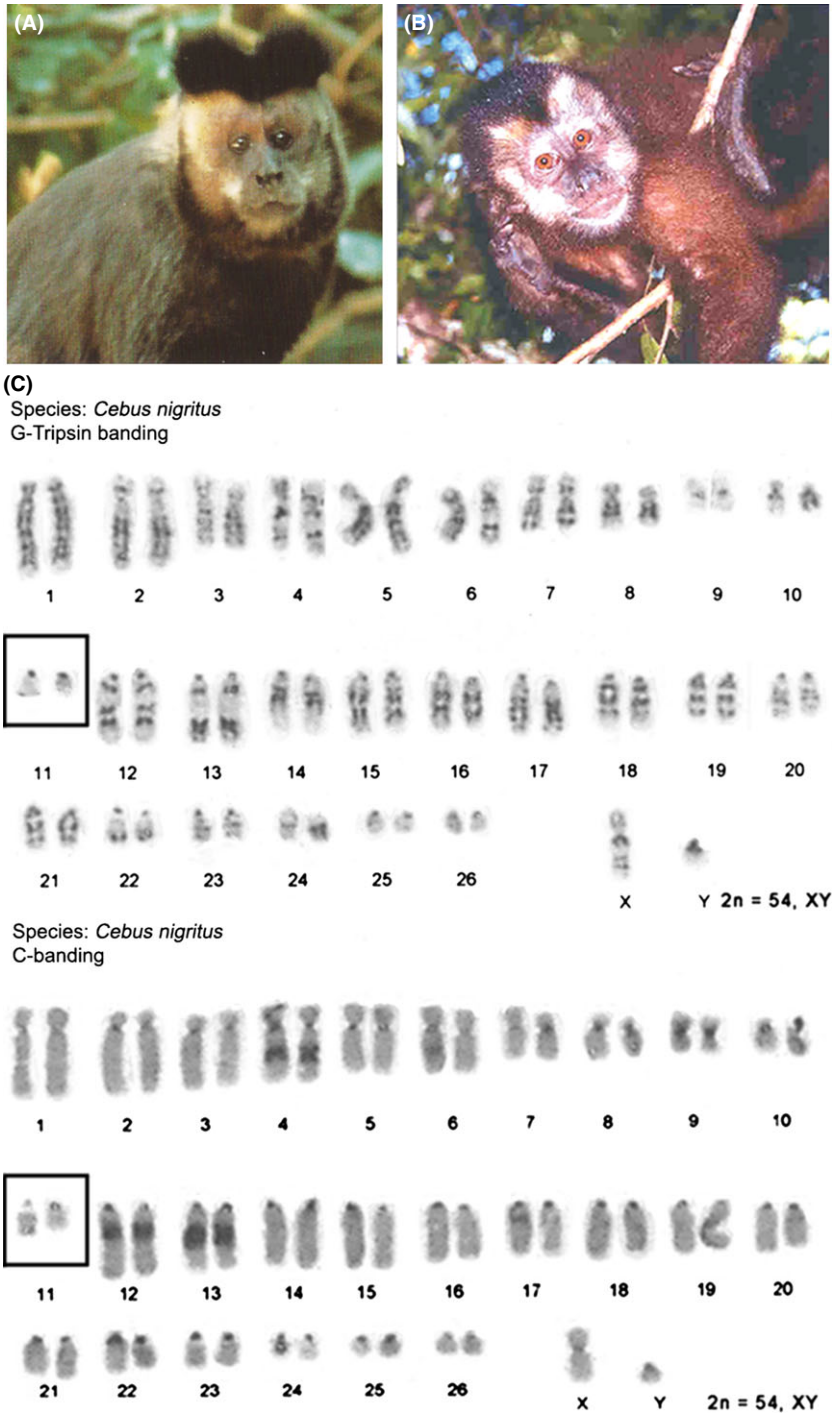

Fig. 2 **(A)** External phenotype of *Cebus nigritus* (photograph from [63]). **(B)** External phenotype of the analyzed male. **(C)** G- and C-banding karyotypes of the analyzed specimens. Box: chromosomal pair #11 without the characteristic extracentromeric heterochromatic block.

Gender identification is difficult in owl monkeys, given the thickness of their pelage and the fact that the testicles are sometimes undescended. Cytogenetic characterization allowed to unequivocally assigning this specimen as a female (2n = 50, XX).

The use of the techniques that combine G–C-banding and FISH protocols is an accurate cytotaxonomic diagnostic methodology that can be applied for improvement of captive programs as well as *in situ* or *ex situ* colony management. One case where the

employment of FISH aided in a proper genetic characterization was the colony in Chile housing 85 *'Cebus apella'* individuals. Twelve animals were chosen for cytogenetic characterization for two particular reasons: (i) all of them showed different phenotypes despite being listed as *C. apella* and (ii) the reproduction patterns observed (focus of the institution's studies) were bizarre in those animals. In this case, a probe of the extracentromeric heterochromatic block in *Cebus* chromosome #11 (11qHe+), generated by chromosome microdissection in our laboratory [56], was employed in conjunction with human chromosome #21 probe (21HSA), due to the known homeology with euchromatic #11q of *Cebus* [55]. The combining of the C–G-banding techniques with the FISH protocol allows us to conclude that these individuals actually belonged to at least three different species other than *apella*. Some phenotypes showed correspondence to the confirmed species karyotype, some others not. Even more, there was a hybrid animal too, who had a good reproductive profile compared to the remaining animals (Fig. 3). As a result of the corrected species status diagnosed with our cytogenetic characterization, the hybrid and her progeny (two generations) were separated from the reproduction program of the institution [55].

As referred in the introduction, the analysis by G-banding pattern alone cannot confirm the sex chromosome system. An example of this is *A. caraya*, where earlier mitotic studies had described XY sexual systems [20, 45, 51]. Later, it was discovered that it possesses a $X_1X_2Y_1Y_2$ multiple sex chromosome system in males [49, 50, 61]. Meiotic analysis in howlers became particularly relevant not only for the description of these sexual systems, but because there are confirmed cases of hybridization in *Alouatta*, such as the hybrids in the wild between *Alouatta pigra* (2N = 58, $X_1X_1X_2X_2/X_1X_2Y_1Y_2$) and *Alouatta palliata* (males 2n = 53, X_1X_2Y and females 2n = 54, $X_1X_1X_2X_2$), species with differences not only in diploid number but also in their sexual system [15]. Other case are the specimens with mixed pelage coloration pattern product of crosses between *A. caraya* and *Alouatta guariba clamitans* (males with 2N = 45, $X_1X_2X_3Y_1Y_2$ and females 2N = 46, $X_1X_1X_2X_2X_3X_3$) found in the wild, both in Brazil and in Argentina [1, 2]. The existence of these hybrids, observed in captivity as well as in the wild, emphasizes the importance of meiotic studies to analyze the reproductive potential of the putative hybrids. The methodology described in this contribution is a simple, inexpensive, and fast method to characterize the meiotic cycle and the sex chromosome system of primate species. New methods for germ cell study are currently being employed, involving cytomolecular techniques both in spermatocytes (e.g., immunofluorescence with antibodies for proteins involved in the synaptonemal complex formation and the recombination process [30, 31]) and in spermatozoa (e.g., array-comparative genomic hybridization on single sperm cells [59]). These techniques, still poorly used or yet not adapted to non-human primates, will provide new information regarding the meiotic process in primates.

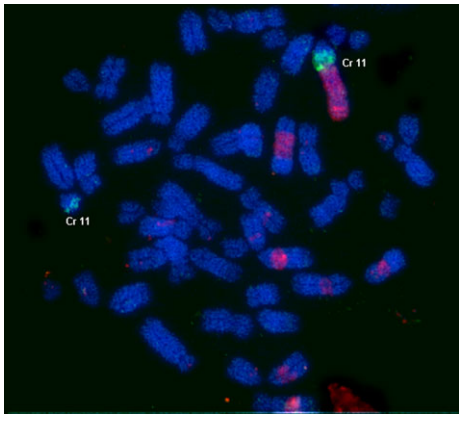

Fig. 3 Fluorescent *in situ* hybridization technique (FISH) of a hybrid *Cebus* specimen analyzed with the 11qHe+ probe (red) and the 21HSA (green).

Taking into account the examples described in this contribution, and considering that chromosomes are the vehicle of genetic information, cytogenetics brings a useful tool to address evolutionary, speciogenic, and taxonomic issues. In the case of neotropical primates, chromosome characterization together with phenotypic, ecological, and ethological descriptions ensures a more effective methodology for species diagnosis. The cytogenetic findings described along this study highlight the relevance of the genetic characterization of primates both in captivity and in the wild.

Cytogenetic data used in the context of a multidisciplinary research approach can orient the management of the groups in captivity, help monitoring overall genetic variation, as well as accompanying the progress of maintenance and care of new specimens born in captivity.

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