From the Center for Reproduction of Endangered Species, Zoological Society of San Diego, California and the Department of Pathology, University of California, San Diego

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CYTOGENETICS OF THE RHINOCEROTIDAE

By Marlys L. Houck, O. A. Ryder, Arlene T. Kumamoto and K. Benirschke

Introduction

The family Rhinocerotidae is comprised of five extant species. *Ceratotherium simum* (white rhinoceros) and *Diceros bicornis* (black rhinoceros) are found in Africa, while *Dicerorhinus sumatrensis* (Sumatran rhinoceros), *Rhinoceros unicornis* (Indian rhinoceros) and *Rhinoceros sondaicus* (Javan rhinoceros) are found in Asia. Rhinoceros populations in both Africa and Asia have declined rapidly in recent decades due to loss of habitat and poaching for the horn (PENNY, 1988). The *1994 IUCN Red List of Threatened Animals*, (GROOMBRIDGE, 1993), lists four of the species as endangered and *C. simum* as vulnerable.

In spite of intensive conservation interest in all five species of the Rhinocerotidae, little is known about the cytogenetics of this family. Although several cytogenetic studies have been conducted on three of the five species, the sample sizes have been small and the results conflicting. The diploid number of two of the species, *D. sumatrensis* and *R. sondaicus*, has not been previously documented. Early reports on the chromosomes of *C. simum* gave conflicting diploid numbers of 82 or 84 based on studies involving only a few specimens (HANSEN, 1976; HEINICHEN, 1967, 1970; HSU and BENIRSCHKE, 1973). The diploid number of *D. bicornis* was reported as 2n = 84 by HUNGERFORD et al (1967) and HEINICHEN (1970), and at that time was the highest diploid number known for mammals. *R. unicornis* was documented as 2n = 82 by WURSTER and BENIRSCHKE (1968) and HSU and BENIRSCHKE (1973). More recently, preliminary information on the chromosomes of *C. simum*, *D. bicornis*, *D. sumatrensis* and *R. unicornis* was reported by RYDER et al (1987).

Little data exists on chromosome banding in rhinoceros species. Initially, one partially Q-banded karyotype of *C. simum* was presented by HANSEN (1976). Recently, the first comprehensive chromosome banding studies of *Ceratotherium* were published including the first chromosomal studies of the northern white rhinoceros (*C. s. cottoni*) (HOUCK et al, 1993). Here, we present for the first time the diploid chromosome number of *D. sumatrensis* as well as the first chromosomal banding studies of *D. bicornis* , *R. unicornis* and *D. sumatrensis*.

Materials and methods

Cytogenetic studies were conducted on 113 rhinoceroses. These included 25 male and 35 female *Diceros bicornis*, 12 male and 27 female *C. simum*, five male and three female *R. unicornis* , and one male and five female *D. sumatrensis*. A summary of the studbook numbers and institutions of the animals included in this study is presented in Table 1. All samples were acquired from captive populations with the exception of 11 *D. bicornis* samples which were obtained from wild populations in Zimbabwe. Metaphase chromosomes were obtained from fibroblast and/or lymphocyte cultures.

Skin biopsies from 11 of the *D. bicornis* individuals were obtained under field conditions in Zimbabwe during the rhinoceros dehorning project conducted by the Zimbabwe Department of National Parks and Wildlife Management. Small epidermal skin samples were taken from the axillary region of anesthetized animals. Field conditions require extreme caution in specimen handling to avoid contamination. The samples were minced into 1 mm^3 fragments and placed in cryovials containing 1 ml of freeze medium. The freeze medium consisted of supplemented α-MEM (described below) with 10% dimethyl sulfoxide. The cryovials were placed into a primed liquid nitrogen dry shipper and transported to the laboratory in San Diego. To initiate fibroblast cultures the samples were quick-thawed at 37°C and digested in collagenase.

Table 1: Location and studbook numbers of the four rhinoceros species included in this study

INSTITUTION	STUDBOOK NUMBER			
	C. simum	*D. bicornis*	*D. sumatrensis*	*R. unicornis*
Atlanta Zoo, GA		38 335		
Audubon Park Zoo, LA	24 579 580			
Busch Gardens, FL	45	343		
Cincinnati Zoo, OH	417 418	180		
Dallas Zoo, TX		67		
Denver Zoo, CO		161 163 328 332 432 *a		
Detroit Zoo, MI		55 281 409		
Dvur Kralove Zoo, Czech Republic	351 372 374			
Fossil Rim Wldlf Cent., TX		470		
Granby Zoo, Canada		293		
Henry Vilas Zoo, WI	696			
Kansas City Zoo, MO		360		
Kings Dominion WAP, VA	751			
Knoxville Zoo, TN	452			
Köln Zoo, Germany				84
Lee Richardson Zoo, KS		124 125		
Los Angeles Zoo, CA		267 333 334 336		
Melaka Zoo, Malaysia			1 19 *b	
Miami MetroZoo, FL		52		*c
Milwaukee Zoo, WI				14
Oklahoma City Zoo, OK		196 330		
Port Lympne Zoo Park, UK			10	
Riverbanks Zoo, SC		381 383		
San Antonio Zoo, TX	182 897			
San Diego Wild Animal Park, CA	28 49 74	110 179 239 302 427 435		9 78 143 *d *e
San Diego Zoo, CA		78 188 390 392 473 SD1	28 33	
San Francisco Zoo, CA		74 351		
Sedgwick County Zoo, KS		53 192		
St. Louis Zoo, MO		121 212 251 353		
White Oak Plantation, FL		471		
Zimbabwe		*f		

*a Male, born Nov. 93, studbook number not assigned
*b Female, "Tengagoh", studbook number unknown
*c Female, stillborn 4/91
*d Male, stillborn 2/92
*e Male, stillborn 11/92
*f Eleven samples were collected in July 1993 during the Zimbabwe Department of National Parks and Wildlife Management rhinoceros dehorning project.

Fibroblast cultures were established from skin biopsies by tissue dissociation in 0.5% collagenase (Boehringer Mannheim). This technique usually yields fibroblast attachment in a T25 flask within 24-48 hours. This represents a considerable improvement over the previously used explant method which typically required two - three weeks before fibroblast growth appeared. Cultures were maintained in a 1:1 mixture of Minimal Essential Medium Alpha (α-MEM; GIBCO BRL) and Fibroblast Growth Medium (FGM; Clonetics). The α-MEM was supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin-streptomycin. The human

fibroblast growth factor and insulin in the FGM medium is believed to have enhanced the growth and normal cell division of the cultures over that of cells grown in supplemented α-MEM alone. Cells were harvested using actinomycin D (Cosmegen, Merck Sharp and Dohme) and Velban (Eli Lily and Company) following the method described by YU et al. (1981) with the modification of a 30 minute hypotonic incubation at 37°C in 0.075 M KCl. For lymphocyte cultures a cell suspension of autologous plasma and buffy coat (ap/bc) was obtained from 10 ml of heparinized blood according to the method described by RYBAK et al. (1982). Lymphocyte cultures were initiated as soon as possible after sampling since the mitotic index at harvest decreased substantially with the age of the sample. Cultures initiated from blood samples over two days old did not usually yield chromosomes. Cultures containing 9.5 ml of supplemented α-MEM medium and 0.5 - 1.0 ml of ap/bc were incubated with 0.3 ml pokeweed mitogen (Gibco BRL) and 6 µg/ml phorbol 12-myristate 13-acetate 4-0 methyl ether (Sigma) co-mitogen. Following a 114 hour incubation period at 37°C cells were harvested as above.
The chromosomes of all 113 animals were examined by non-differential staining using Giemsa. C-banding followed the method described by SUMNER (1972), and G-banding was done according to the method of SEABRIGHT (1971). Chromosomes were aligned in karyotypes by decreasing size, with submetacentric pairs presented first. Because of the difficulty in obtaining diagnostic G-bands, there was no attempt to standardize similar chromosomal pairs among the four species. High resolution banding attempts were unsuccessful due to problems obtaining banding-quality metaphases when dealing with such a high number of mainly acrocentric chromosomes.

Results and discussion

Non-differentially stained karyotypes were completed on all 113 samples. The modal diploid chromosome number for the four species examined was 2n = 82 for *C. simum*, *D. sumatrensis* and *R. unicornis* and 2n = 84 for *D. bicornis*. Nothing is known about the chromosomes of the rare Javan rhinoceros, *R. sondaicus*. A comparison of modal diploid numbers, heterochromatic regions, and morphology of the autosomes and sex chromosomes of the four species are shown in Table 2.

Table 2: Summary of modal diploid numbers, morphology and heterochromatin of the four rhinoceros species studied

	n	MODAL DIPLOID #	AUTOSOMES	X	Y	HETEROCHROMATIC REGIONS
Ceratotherium simum White rhinoceros	36	82	80 t and a	large sm	small sm ?	centromeres some interstitial bands
Diceros bicornis Black rhinoceros	57	84	82 sm, t and a with variable p-arms	large sm	small m or sm ?	centromeres some interstitial bands p-arms
Dicerorhinus sumatrensis Sumatran rhinoceros	6	82	80 a	large sm	small a ?	most centromeres some interstitial bands q terminal end of X
Rhinoceros unicornis Indian rhinoceros	8	82	16 to 20 sm all others a	large sm	small a ?	centromeres some interstitial bands p-arms

a = acrocentric, t = telocentric, sm = submetacentric, m = metacentric

Although detailed G-band comparisons among the four species were not conducted, the dissimilar morphology and lack of G-banding similarities found among the largest chromosomal pairs in each species suggest that the differences in diploid number involve more than a mere fission event of one bi-armed chromosomal pair. Morphological and molecular analyses place the two African rhinoceros species as each others closest relatives with the Asian rhinos as a sister group (GROVES 1983; PROTHERO, 1986; AMATO, et al., 1993; but see MORALES and MELNICK, 1994). Accordingly, the change in diploid number within African rhinoceroses would most parsimoniously be considered to have taken place after the divergence of *Diceros* and *Ceratotherium* from a common ancestor possessing 2n=82, an apparently ancestral condition in the Rhinocerotidae.

Black rhinoceros

Karyotype analyses of *D. bicornis* revealed 82 submetacentric, telocentric and acrocentric autosomes and the two sex chromosomes. The number of bi-armed chromosomes and size of the short arms (p-arms) varied among individuals. These results are consistent with previously published chromosomal data (HEINICHEN, 1970; HUNGERFORD, 1967). C-banding showed that the p-arms were largely heterochromatic. Centromeric heterochromatin was present on all elements, and interstitial C-bands were observed on one or two of the autosomes. G-banding revealed size polymorphisms involving short arm additions in many chromosomal pairs (Figure 1). A bimodal distribution of the number of autosomes with heterochromatic p-arms differentiated the eastern and southern populations of black rhinoceroses. These data, which are consistent with recent limitation of gene flow between these regional populations, will be presented elsewhere.

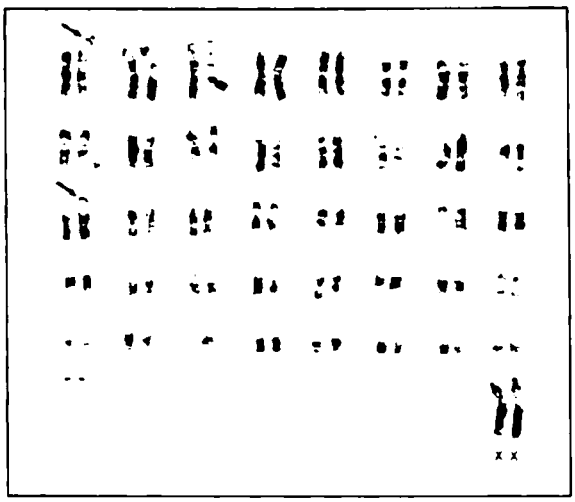

Fig. 1: G-banded karyotype of a female black rhinoceros, 2n = 84. Arrows indicate p-arm polymorphisms.

White rhinoceros

The normal karyotype of *C. simum* consists of 80 telocentric and acrocentric autosomes and the two sex chromosomes. Centromeric heterochromatin was present on all elements (HOUCK et al, 1993). These findings are in agreement with the diploid number of 82 reported by HEINICHEN (1967,1970). The conflicting reports of 2n = 84 (HANSEN, 1976; HSU and BENIRSCHKE, 1973) can be attributed to their small sample sizes and the difficulty of scoring the high number of very small acrocentric elements.

Indian Rhinoceros

The autosomes of *R. unicornis* consisted of 16 - 20 submetacentric chromosomes and the remaining elements were acrocentric. The diploid number was 82, which is consistent with previously published karyotypes for this species (WURSTER and BENIRSCHKE, 1968; HSU and BENIRSCHKE, 1973). As in *D. bicornis*, G-banding revealed short arm polymorphisms which made it difficult to determine a consistent number of bi-armed pairs (Figure 2). C-banded karyotypes showed that the short arms were largely heterochromatic and all elements contained centromeric heterochromatin. One or two autosomal elements also had regions of interstitial heterochromatin (Figure 3).

Sumatran rhinoceros

The normal karyotype of *D. sumatrensis* consisted of 80 acrocentric autosomes of decreasing size and the two sex chromosomes. C-banding revealed centromeric heterochromatin on most elements as well as interstitial bands on one or two autosomal pairs and a large block of heterochromatin near the terminal end of the long arm (q-arm) of the X chromosome (Figure 4). A size polymorphism in the G-band negative area of the second largest chromosome pair was observed in one of the two individuals that were G-banded. The karyotype of the animal with the normal pair 2 is shown here (Figure 5).

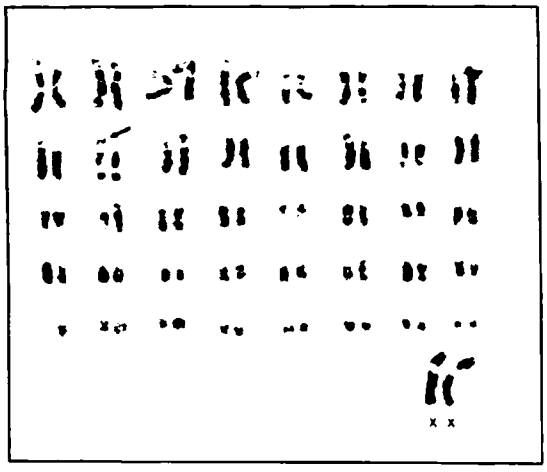

Fig. 3: C-banded karyotype of a male Indian rhinoceros, 2n = 82.

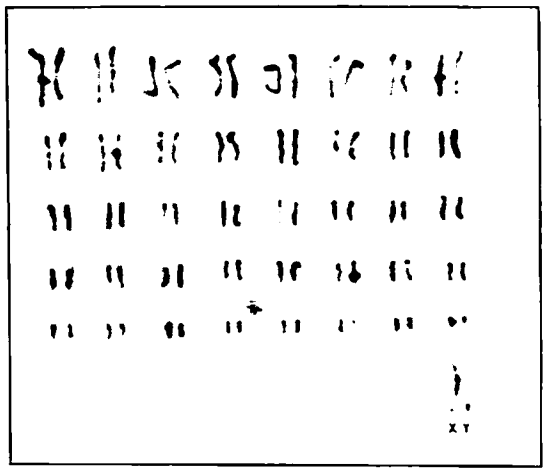

Fig. 2: G-banded karyotype of a female Indian rhinoceros, 2n = 82. Arrow indicates p-arm polymorphism.

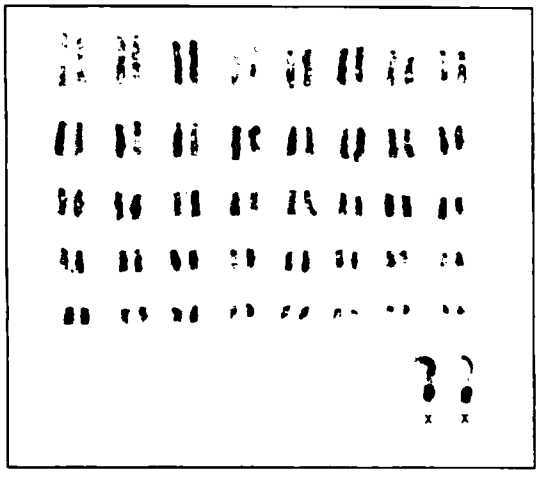

Fig. 4: C-banded karyotype of a female Sumatran rhinoceros, 2n = 82, showing the addition of heterochromatin near the q terminal ends of the X chromosomes.

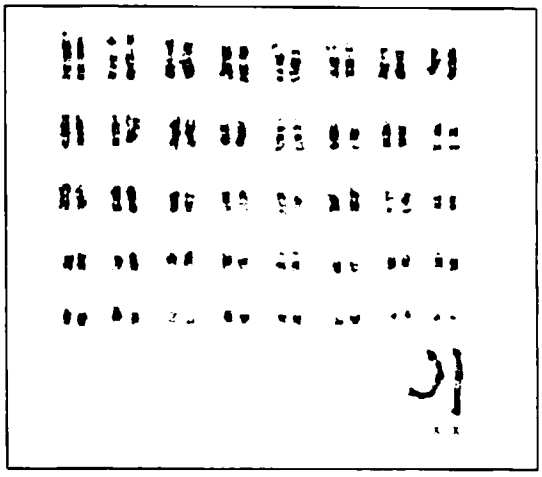

Fig. 5: G-banded karyotype of a female Sumatran rhinoceros, 2n = 82.

The X chromosomes of *C. simum*, *D. bicornis* and *R. unicornis* were identical. *D. sumatrensis*, however, had additional heterochromatic material near the Xq terminal end. In all four species the X was a large submetacentric element having the largest p-arm of any of the elements. With an arm ratio of 1.4, the X chromosomes in *D. bicornis* and *R. unicornis* were easily distinguished from large submetacentric autosomes which consistently had arm ratios of 2. In *D. sumatrensis*, the arm ratio of the X chromosome was 2.6, but it was distinct in this species because it was the only submetacentric element in the karyotype. Except for *D. sumatrensis*, the G-band pattern of the rhinoceros X chromosome was similar to the submetacentric form of the "original or standard type" found in other mammalian species including humans (OHNO, 1967; PATHAK and STOCK, 1974). The banding pattern of the X chromosome of *D. sumatrensis* was similar to that of the other three rhinoceros species with the addition of several bands at the distal portion of the q-arm where the constitutive heterochromatin was observed. Using the standardized banding nomenclature for the human X chromosome, the insertion occurs at Xq27 (PARIS CONFERENCE, 1972). The distinct G- and C-banding patterns found in the X chromosome of *D. sumatrensis* have also been observed in the mountain tapir, *Tapirus pinchaque* (M.L. Houck, unpublished data). Because of the difficulty in obtaining diagnostic G-bands on small elements it was not possible to distinguish the Y chromosome with confidence in any of the four species. The Y chromosome is probably represented by a small metacentric or submetacentric element in *C. simum* and *D. bicornis* and a small acrocentric in *D. sumatrensis* and *R. unicornis*. It was not heterochromatic.

Several abnormal karyotypes were found during this study and are summarized in Table 3. None of these animals had congenital anomalies. Karyotypic analyses of three *C. s cottoni* individuals, a male (#372), and his two female offspring (#789 & #943), revealed a diploid number of 2n = 81 in which two fewer acrocentric chromosomes and one additional metacentric chromosome were observed. The difference between the karyotypes of these individuals and the other 36 specimens of *C. simum* examined can be accounted for by a Robertsonian translocation (centromere-centomere) mechanism (HOUCK et al, 1993).

Table 3: Abnormal chromosomal complements

	STDBK #	2n	SEX	ABNORMALITY
Ceratotherium simum	372	81	M	Robertsonian translocation
	789	81	F	Robertsonian translocation
	943	81	F	Robertsonian translocation
Diceros bicornis	188	84	F	Heterochromatic arm addition
	293	84	F	Heterochromatic arm addition
	360	85	F	Trisomy X

One female *D. bicornis* individual, (#360), was found to have a diploid number of 2n = 85 because of an additional large submetacentric chromosome. Analysis of the G-bands revealed a trisomy X chromosomal complement Presumably the additional X did not have any deleterious effects because of X inactivation (LYON, 1962). This animal died at age 2.5 years. Autopsy findings gave no indications of anomalies or untoward effects of trisomy X. Diffuse encephalopathy was deemed to be the result of an unknown toxin or nutritional deficiency (Eric Miller, personal communication). The dam, (#267), of this individual was sampled and revealed a normal 2n = 84 karyotype. The sire was not sampled directly, but karyotype analyses of his paternal grandparents, (#124 and #125), revealed normal chromosomal complements.

Two other female *D. bicornis* individuals, that were wild-caught and presumably unrelated, had normal diploid numbers, but showed very large heterochromatic areas in the p-arm of one chromosome. In one animal, (#293), the additional heterochromatin resulted in a submetacentric element similar in size to the X chromosomes. Due to the quality of the G-banding it was not possible to determine which chromosome was affected. In the other animal, (#188), a metacentric chromosome was created by the addition of a large heterochromatic arm. G-banding showed a metacentric chromosome in which the q-arm paired with that of a subtelocentric autosome.

This study illustrates the importance of chromosomal analyses in support of animal health and conservation management programs. Further comparative studies need to be conducted on chromosomal polymorphisms which may distinguish different geographic populations of *D. bicornis*. In addition, the numerical polymorphism noted in the critically endangered northern white rhinoceros (*C. s. cottoni*) should be monitored through additional study of captive specimens and, where feasible, of wild populations.

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Summary

Cytogenetics of the Rhinocerotidae

Cytogenetic studies were conducted on 113 rhinoceroses, representing four of the five extant species. Karyotype analyses revealed a modal diploid number of 82 chromosomes for Ceratotherium simum, Rhinoceros unicornis, Dicerorhinus sumatrensis and 84 chromosomes for Diceros bicornis. Comparison of the autosomes of the four species revealed differences in centromere location, ranging from all acrocentric elements in

D. sumatrensis to mostly submetacentric elements in D. bicornis. The X chromosome was identified as a large submetacentric element in all four species. The X chromosome of D. sumatrensis was distinct from the other three species because of an addition of heterochromatic material in the long arm. Abnormal chromosomal complements were identified in three C. simum and three D. bicornis including one trisomy X individual. The diploid number of D. sumatrensis is presented for the first time, as well as the first banding studies of D. sumatrensis , R. unicornis and D. bicornis.

Zusammenfassung

CYTOGENETIK DER RHINOCEROTIDAE

Wir berichten über cytogenetische Untersuchungen an 113 Nashörnern von vier der fünf bestehenden Nashornarten. Die Chromosomenzahl ("diploide Zahl", "2n") beträgt 82 Chromosomen in den folgenden drei Arten: Ceratotherium simum, Rhinoceros unicornis und Dicerorhinus sumatrensis. Hingegen hat Diceros bicornis 84 Chromosomen. Wenn man die Autosomen dieser Arten vergleicht, so findet man eine unterschiedliche Lokalisation der Zentromeren, von ausschließlich akrozentrischen Autosomen in D. sumatrensis zu hauptsächlich submetazentrischen Elementen im D. bicornis. Das X-Chromosom ist ein großes submetazentrisches Element in allen Arten. Das X-Chromosom von D. sumatrensis unterscheidet sich allerdings von den anderen Arten durch ein zusätzliches Stück von Heterochromatin im langen Arm. Chromosomenabnormalitäten wurden in je drei Nashörnern von D. simum und von D. bicornis gefunden, einschließlich einer Trisomie des X-Chromosoms. Dieser Bericht stellt die erste Beschreibung der diploiden Chromosomenzahl des D. sumatrensis dar; ebenso präsentieren wir die ersten gebandeten Chromosomen von D. sumatrensis , R. unicornis und von D. bicornis.

Résumé

La cytogénétique des rhinocérotidés

Nous avons effectué des examens cytogénétiques sur 113 rhincoéros ayant représenté quatre des cinq espèces existantes. Le nombre de chromosomes ("chiffre diploïde", "2n") se chiffre à 82 chromosomes pour les trois espèces qui suivent : Ceratotherium simum, Rhinoceros unicornis et Dicerorhinus sumatrensis. Diceros bicornis par contre, a 84 chromosomes. La comparaison des autosomes de ces espèces révèle une localisation différente des centromères, à partir d'autosomes exclusivement acrocentriques dans l'espèce D. sumatrensis jusqu'aux éléments essentiellement submétacentriques dans D. bicornis.
Le chromosome X est un grand élément submétacentrique dans toutes les espèces. Cependant, le chromosome X de D.sumatrensis se distingue des autres espèces par un élément additionnel d'hétérochromatine au bras long. Nous avons observé des anomalies de chromosomes dans trois spécimens de C. simum et dans trois de D. bicornis ainsi qu'une trisomie du chromosome X. Ce rapport présente pour la première fois une description du chiffre diploïde des chromosomes de D. sumatrensis et fait état des bandes chromosomales de D. sumatrensis, de R. unicornis et de D. bicornis.

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Address of authors: Marlys L. H o u c k
Center for Reproduction of Endangered Species
Zoological Society of San Diego
P. O. Box 551
San Diego, C.A. 92112-0551 (USA)