Diploid Chromosome Number and Chromosomal Variation in the White Rhinoceros (*Ceratotherium simum*)

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Chromosomal studies were conducted on 38 white rhinoceroses representing both the northern and southern subspecies and one subspecies hybrid. Improvements in tissue culture methods and harvesting techniques have made it possible to obtain a highly repeatable diploid number of 82 chromosomes for both subspecies. Comparison of G-banded karyotypes from the two subspecies failed to indicate a difference in banding pattern, but did reveal size polymorphisms involving short arm additions in five individuals. Chromosomal polymorphism, resulting in three individuals with a diploid number of $2n = 81$, was noted in northern white rhinoceroses.

Chromosomal studies of rhinoceroses have been limited because of difficulties in obtaining samples, in culturing cells, and in producing satisfactory chromosomal preparations. As a result, conflicting findings were reported for the white rhinoceros, *Ceratotherium simum*, with diploid numbers reported to be 82 or 84 in studies involving examination of only one or two animals (Hansen 1976; Heinichen 1967, 1968; Hsu and Benirschke 1973). More detailed and definitive cytogenetic studies of rhinoceros species are desirable for gaining insight into chromosomal evolution in this mammalian family. Chromosomal investigations are also an important component of captive breeding efforts for rhinoceros taxa and for other mammalian taxa, including primates (de Boer 1974), dwarf antelopes (Ryder et al. 1989), and cervids (Shi and Pathak 1981; Wurster and Benirschke 1970).

The white rhinoceros comprises two recognized subspecies. The historic range of the northern white rhinoceros (*C. s. cottoni*) included southern Chad, Central African Republic, southwestern Sudan, northeastern Zaire, and northwestern Uganda. The southern subspecies (*C. s. simum*) occurred in southeastern Angola, southwestern Zambia, Mozambique, Zimbabwe, Botswana, eastern Namibia, and South Africa. Populations of the southern subspecies have increased in size to over 4,500 animals (Gakahu 1993; Ryder 1993) following the population bottleneck in the early 1900s. The northern subspecies, once the most numerous form of white rhinoceros, is now critically endangered. Fewer than 50 individuals comprise the total population that includes the wild population in Parc Nacional de la Garambe in Zaire and the captive population (Smith and Smith 1993). As the population of the northern white rhinoceros declined, the need to gain an understanding of the genetic similarities and differences between the two subspecies for incorporation into conservation planning for African rhinos became apparent.

Materials and Methods

We conducted cytogenetic studies on 38 *C. simum* individuals from nine different institutions; these included nine males and 19 females of the southern subspecies (*C. s. simum*), three males and six females of the northern subspecies (*C. s. cottoni*), and one female subspecies hybrid (see Table 1). Metaphase chromosomes were obtained from either fibroblast or lymphocyte cultures.

We established fibroblast cultures from skin biopsy specimens by tissue dissociation in 0.5% collagenase (Boehringer Mannheim), which usually yields visible fibroblast attachment in a T25 flask in 24-48 h. This represents a considerable improvement over the explant method used previously, which typically required 2-3 weeks before fibroblast growth appeared. We maintained the cultures in a 1:1 mixture of Minimal Essential Medium Alpha (α-MEM) (GIBCO) and Fibroblast Growth Medium (FGM) (Clonetics). The α-MEM was supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% pen-

From the Center for Reproduction of Endangered Species, Zoological Society of San Diego, PO Box 551, San Diego, CA 92112-0551 (Houck and Ryder), Východočeská Zoologická Zahrada ve Dvore Králové n. L., Czech Republic (Váhala), the Kenya Wildlife Service, Nairobi (Kock), and the San Diego Wild Animal Park, Escondido, California (Oosterhuis). We wish to acknowledge the veterinary staffs of the following institutions for their generous help in obtaining samples: Audubon Park Zoo, Busch Gardens, Cincinnati Zoo, Henry Vilas Zoo, Kings Dominion Wild Animal Park, and Knoxville Zoo. Special thanks to Arlene Kumamoto and Petr Špála for their assistance and continuous encouragement during this project. Clonetics Corporation generously provided the necessary growth medium for the fibroblast cultures. All biological samples were collected in compliance with the U.S. Endangered Species Act and the Convention on International Trade in Endangered Species (CITES).

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icillin-streptomycin. The human fibroblast growth factor and insulin found in the FGM medium apparently enhanced the growth and normal cell division of the cultures when compared to cells grown in the supplemented α-MEM alone. We harvested cells 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-min hypotonic incubation at 37°C in 0.075 M KCl.

For lymphocyte cultures, we obtained a cell suspension of autologous plasma and buffy coat (ap/bc) 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 samples over 2 days old did not routinely yield chromosomes. Cultures containing 9.5 ml of α-MEM medium (10% FBS) and 0.5–1.0 ml of ap/bc were supplemented with 0.3-ml pokeweed mitogen (GIBCO) and 6 μg/ml phorbol 12-myristate 13-acetate 4-0 methyl ether (Sigma). Following a 114-h incubation period at 37°C cells were harvested as above.

We examined the chromosomes of all 38 animals by nondifferential Giemsa staining. In addition, five of the specimens were G-banded according to the method of Seabright (1971) and C-banded using the method described by Sumner (1972). All figures presented in this article represent the complete chromosomal complement of a single cell and have not been enhanced.

Results

A summary of the diploid chromosome numbers of all animals included in this study is presented in Table 1. The diploid number was found to be $2n = 82$ in 35 of the 38 animals studied. Karyotypic analyses of three individuals, a male *C. s. cottoni* and his two female offspring, 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 35 *C. simum* individuals examined may be accounted for by a Robertsonian translocation (centromere-centromere) mechanism.

In both subspecies the normal nondifferentially stained karyotype (Figure 1) consisted of 40 telocentric and acrocentric pairs. The X chromosome was identified as the only large submetacentric element; the Y was represented by a small submetacentric chromosome. Resolution of the short arms varied among preparations. In several of the largest chromosomes the short arms were elongated, producing the appearance of bi-armed elements. When we examined karyotypes from the same individual, a chromosome would sometimes appear submetacentric and at other times the same chromosome would appear acrocentric. The chromosomes were aligned according to q-arm length due to p-arm polymorphisms between individual animals.

Comparison of G-banded karyotypes from the two subspecies failed to indicate a difference in banding pattern. However, due to the difficulty in obtaining diagnostic G-bands on small elements, it was not always possible to pair the smaller chromosomes with confidence. Banding patterns of the larger elements were clearly resolved, revealing size polymorphisms involving short arm additions in several individuals (Figure 2).

Table 1. Summary of the diploid chromosome numbers of the 38 *Ceratotherium simum* individuals studied

Species	Studbk no.	Sex	2n	Location	Sire	Dam
C. s. simum	24	F	82	Audubon Zoo, LA	W	W
	45	F	82	Busch Gardens, FL	W	W
	49	F	82	San Diego Wld Anm Pk, CA	W	W
	155	F	82	San Diego Wld Anm Pk, CA	W	W
	156	F	82	San Diego Wld Anm Pk, CA	W	W
	157	F	82	San Diego Wld Anm Pk, CA	W	W
	182	F	82	San Antonio Zoo, TX	W	W
	187	M	82	San Diego Wld Anm Pk, CA	W	W
	417	F	82	Cincinnati Zoo, OH	W	W
	418	F	82	Cincinnati Zoo, OH	W	W
	420	M	82	San Diego Wld Anm Pk, CA	W	W
	452	F	82	Knoxville Zoo, TN	W	W
	579	F	82	Audubon Park Zoo, LA	W	W
	580	M	82	Audubon Park Zoo, LA	W	W
	689	M	82	San Diego Wld Anm Pk, CA	52	157
	696	F	82	Henry Vilas Zoo, WI	W	W
	751	F	82	Kings Dominion Wld Anm Pk, VA	W	W
	824	F	82	San Diego Wld Anm Pk, CA	52	154
	860	F	82	San Diego Wld Anm Pk, CA	420	147
	861	M	82	San Diego Wld Anm Pk, CA	420	155
	862	M	82	San Diego Wld Anm Pk, CA	420	277
	863	F	82	San Diego Wld Anm Pk, CA	420	154
	864	M	82	San Diego Wld Anm Pk, CA	420	159
	908	M	82	San Diego Wld Anm Pk, CA	420	157
	928	F	82	San Diego Wld Anm Pk, CA	420	157
	929	F	82	San Diego Wld Anm Pk, CA	420	159
	930	F	82	San Diego Wld Anm Pk, CA	420	154
	9024	M	82	San Antonio Zoo, TX	180	182
C. s cottoni	28	F	82	San Diego Wld Anm Pk, CA	W	W
	74	M	82	San Diego Wld Anm Pk, CA	W	W
	630	M	82	Dvůr Králové Zoo, Czech Republic	373	351
	351	F	82	Dvůr Králové Zoo, Czech Republic	W	W
	372	M	81	Dvůr Králové Zoo, Czech Republic	W	W
	374	F	82	Dvůr Králové Zoo, Czech Republic	W	W
	376	F	82	Dvůr Králové Zoo, Czech Republic	W	W
	789	F	81	Dvůr Králové Zoo, Czech Republic	372	351
	943	F	81	Dvůr Králové Zoo, Czech Republic	372	351
Subspecies hybrid *C.s.s. × C.s.c.*	476	F	82	Dvůr Králové Zoo, Czech Republic	Unkn	351

One of these length polymorphisms was identified in chromosome 4. G-banding analyses of five individuals (three *C. s. simum* and two *C. s. cottoni*) were consistent for a short arm polymorphism in chromosome 4. Among these five individuals, arm-length polymorphisms were also displayed by at least one individual in one or more of the following chromosomes: CSI 3, CSI 9, CSI 10, CSI 13, CSI 19, and CSI 23 (data not shown).

C-banded karyotypes showed no detectable differences between the two subspecies and revealed that the heterochromatin was largely centromeric and present on all elements. No C-band polymorphisms were observed (Figure 3).

Discussion

The first chromosomal studies of the white rhinoceros were conducted during the infancy of cytogenetic investigations of exotic species and included only one or two animals. The high chromosome number and poor cell growth of *C. simum* made it difficult to accurately determine the dip-

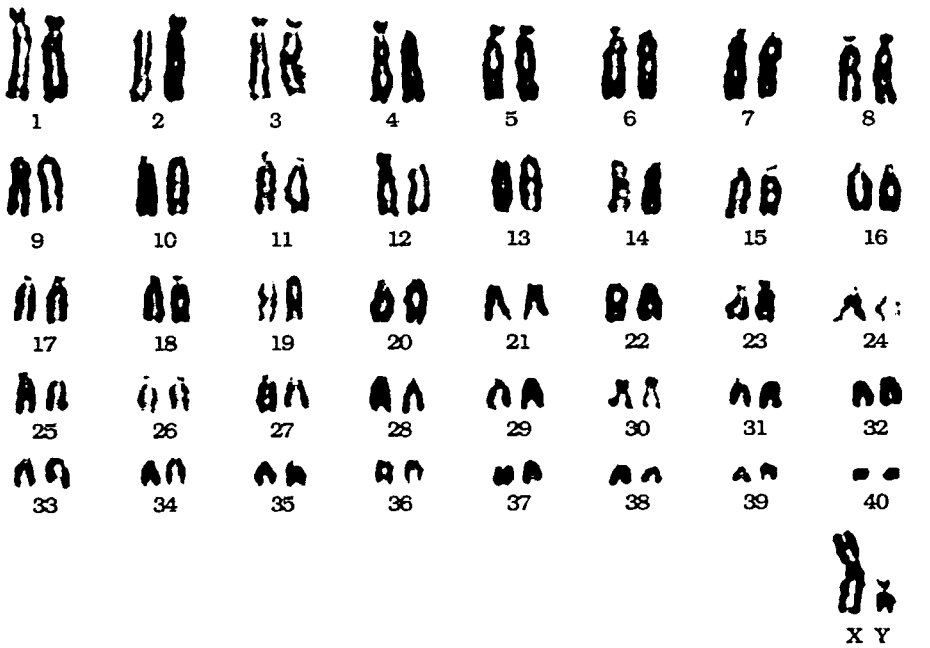

Figure 1. Giemsa-stained karyotype of a male southern white rhinoceros consisting of 40 telocentric and acrocentric pairs. The X chromosome is the only large submetacentric element, and the Y is represented by a small submetacentric chromosome.

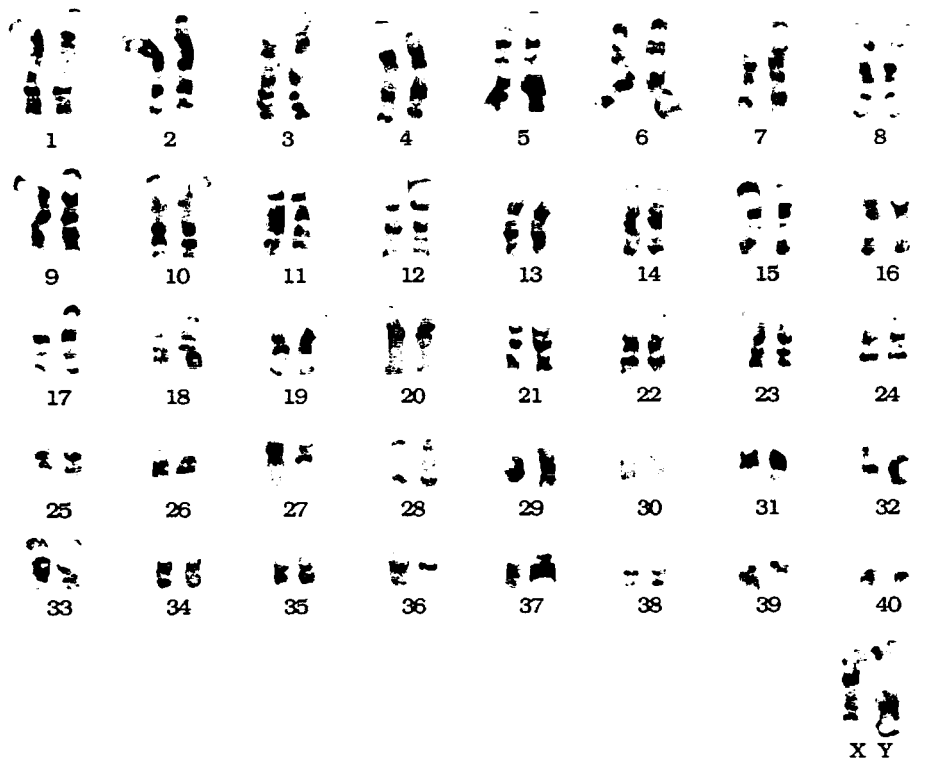

Figure 2. G-banded karyotype of a male northern white rhino showing size polymorphisms in chromosomes 4, 13, 17, and 23.

loid number. Conflicting results were obtained. Heinichen (1967, 1968) first studied the white rhinoceros and reported a diploid number of 82. In 1973 Hsu and Benirschke published the karyotypes of both a male and female white rhino showing $2n = 84$, and Hansen agreed with this number in his report in 1976.

In the study reported here, cell growth was enhanced by using collagenase digestion of skin biopsies when initiating primary cultures. An improvement in chromosomal preparations resulted from the enhanced cell growth that occurred when a growth factor-supplemented medium was used for the rhinoceros cell cultures. The data derived from the karyotypes of 38 animals clearly indicate that the diploid number is $2n = 82$ for both subspecies, with the exception of three related *C. s. cottoni* individuals that have one less chromosome due to a Robertsonian translocation. With prolonged periods of culturing in nonoptimal medium, a chromosomal fission in vitro was observed in some cell lineages. This observation provides a possible explanation for the findings of a higher diploid number in some of the earlier studies.

Of the 28 *C. s. simum* individuals studied, 16 were wild-born, representing 32 haploid complements, assuming all individuals were unrelated. Three captive-born *C. s. simum* individuals, of which one parent was an unsampled wild-born animal, contribute three additional sampled haploid complements. Two wild-born *C. s. simum* parents who were not sampled directly were sampled through two offspring each, corresponding to three additional haploid genomes sampled (2×1.5). One wild-born *C. s. simum* parent was sampled through three of her offspring, which corresponded to 1.75 haploid genomes sampled. Six of the nine *C. s. cottoni* animals were wild-born, thus representing 12 haploid chromosomal complements. One captive-born *C. s. cottoni* individual (of which one parent was an unsampled wildborn) contributed an additional haplotype. The subspecies hybrid contributed one *C. s. simum* haploid complement. Therefore, assuming all wild-born animals were unrelated, a maximum of 40.75 *C. s. simum* and 13 *C. s. cottoni* haploid chromosomal complements were represented.

No difference in G-banding pattern was detected between the two subspecies. Evidence from mtDNA cleavage studies indicates that these two taxa differ by approximately 1.0%–1.4% estimated nucleotide sequence divergence (George et al. 1983, 1993). Variation in mtDNA within populations and between geographical subspecies of African rhinoceroses may be lower than that of other mammals. Mitochondrial DNA variation within populations and between subspecies of black rhinoceros (*Diceros bicornis*) is also apparently low (Ashley et al. 1990; Harley and O'Ryan 1993).

A number of fixed cleavage site differences distinguish the mtDNA of *C. s. simum* and *C. s. cottoni* (George et al. 1993). In spite of the geographical isolation of

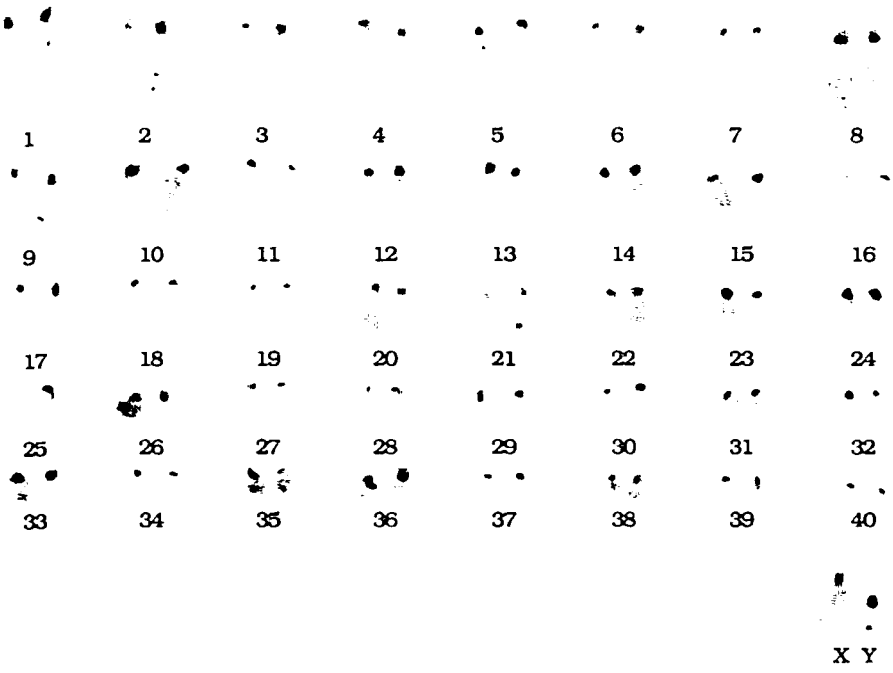

Figure 3. C-banded karyotype of a male southern white rhino.

northern and southern white rhinos and the evolutionary divergence of the mtDNAs of the two taxa, we find no karyotypic difference diagnostic for the two taxa. One adult white rhinoceros individual (a female born in 1977 and still living) is known to have one parent of each of the named subspecies. Although this individual has failed to reproduce, reproduction of pure northern white rhinoceroses in captivity has been low. Consequently, conjecture concerning the fertility of this hybrid is not warranted.

Cytogenetic studies have been conducted on four of the five extant rhinoceros species and indicate that they have remained chromosomally conservative although no G-banding comparisons have been done. Both the greater one-horned rhinoceros, *Rhinoceros unicornis* (Wurster and Benirschke 1968; Houck ML and Ryder OA, unpublished data) and the Sumatran rhinoceros, *Dicerorhinus sumatrensis* (Houck ML and Ryder OA, unpublished data) have diploid numbers of $2n = 82$. The common ancestor of these taxa probably occurred in the late Oligocene to early Miocene (Prothero and Schoch 1989). The black rhinoceros has $2n = 84$ chromosomes (Hungerford et al. 1967; Houck ML and Ryder OA, unpublished data). No cytogenetic studies have been conducted on the rare Javan rhinoceros, *R. sondaicus*. While G-banding comparisons of African and Asian rhinoceroses have not been reported, the similarity in diploid number of taxa separated over such a lengthy period is consistent with a remarkable degree of chromosomal conservatism.

This stands in contrast to another family in the order Perissodactyla—the Equidae. Equids are remarkable for their rapid rate of chromosomal evolution, extant species having diploid numbers between 66 and 32 (Ryder et al. 1978). Equids have a common ancestor in the Pliocene (Evander 1989; Lindsay et al. 1980). Thus, speciation and chromosomal divergence in equids has occurred more recently than among extant rhinocerotids. A molecular explanation of rapid chromosomal divergence in equids has been extended by Wichman et al. (1991). The findings presented here support the notion that equid chromosomal evolution is remarkable within mammals (Ryder et al. 1978; Wichman et al. 1991) and that, in contrast, the related rhinocerotids are chromosomally conservative.

Findings of a relatively low rate of chromosomal evolution in rhinoceroses draw attention to the numerical polymorphism identified in the endangered northern white rhinoceros. We recommend that the incidence of transmission of the rearrangement in captive populations be monitored. Furthermore, it would be useful to determine whether the rearrangement present in the one wild-caught northern white rhinoceros bull and his two daughters also occurs in the wild population surviving in Zaire, provided that appropriate samples can be collected at minimal risk to the rhinos.

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