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Genetic Kinship and Social Structure in a Herd of Square-Lipped Rhinoceroses (*Ceratotherium simum simum*) at the Zoological Center, Tel Aviv/Ramat-Gan, Israel

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Zoos and zoological parks serve as genetic and demographic reserves for strengthening endangered populations and reestablishing extinct populations in the wild. Knowing the genetic ties within captive populations is a very helpful tool for successful reproductive management. In the present study we addressed kinship relationships and behavior among rhinoceroses (*Ceratotherium simum simum*) raised at the Zoological Center, Tel Aviv/Ramat Gan, Israel, with the hope of identifying reasons for the declining rate of reproduction within the herd. We used the random amplified polymorphic DNA (RAPD) technique to reveal the paternity of the rhinos born at the park. In this way, we identified the paternity of five out of seven young born in the herd, which are currently in Ramat Gan. One male accounted for three (37.5%) births, and two other males accounted for one each. The paternity of the two other animals is unknown and may be of animals that are no longer in the Zoological Center. The genetic determinations were accompanied by behavioral observations, which enabled us to determine the social dynamics in the herd. This study suggests that there are at least three contributing factors to the reproductive decline in the herd: 1) a surplus of males, 2) exclusion of potentially reproductive males from the breeding stock, and 3) specific behavioral and physiological problems in some members of the herd. Zoo Biol 21:561–571, 2002. © 2002 Wiley-Liss, Inc.

Key words: RAPD; parentage; paternity; rhino

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INTRODUCTION

The square-lipped (''white'') rhinoceros (*Ceratotherium simum simum*) and the black rhinoceros (*Diceros bicornis*), are the two African representatives of the family Rhinocerotidae. As with the three Asian members of this family, their dwindling populations are in imminent danger of extinction. The human population explosion, massive destruction of habitat, uncontrolled poaching, and rampant ecological changes on a planetary scale, are some of the reasons for the rapid decline in wild populations we are witnessing today. At the time of this writing, it is estimated that the population of white rhinos is 10,000, of which 700 are in captivity (according to the International Rhino Foundation (IRF), http://www.rhinos-irf.org). The decline in the reproductive rate in captivity, especially in the F_1 generation, has been identified as a major issue affecting the herds held in zoos around the world [Emslie and Brooks, 1999].

At the Zoological Center, Tel Aviv/Ramat-Gan, Israel, a severe decline in reproductive rate was observed over a number of years in the herd of square-lipped rhinoceros. There have been a total of 20 rhino births at the park since 1978, with 16 (80%) born between 1978–1990 and only four (20%) between 1990–1996. None have been born since 1996.

The primary goal of this study was to determine the factors that contributed to the decline in births, with the hope of being able to improve the birth rate in the future. Our study set out to determine the parentage of the rhinos born at the park, and to establish the current social dynamics of the population. Parentage was determined using the random amplified polymorphic DNA (RAPD) technique, which is based on PCR amplification of DNA from all members of the herd.

We began our study assuming that the decline might be due to multiple factors that we could clarify by combining molecular methods and behavioral observations. The ability to determine familial relationships and gather reproductive data among a particular group of individuals is important for their conservation and management [Fowler et al., 1998; Schaffer et al., 1998]. This was particularly relevant in the present study, as this herd is a free-ranging multi-male group for which paternity was unknown. We used the RAPD technique to address paternity connections within the rhino herd. This method has been widely utilized because of its extreme simplicity and rapidity, and because it requires a very small sample of genomic DNA [Neveu et al., 1996; Fowler et al., 1998].

RAPD analysis has been used to assess paternity [Levitan and Grosberg, 1993; Bishop et al., 1996; Hooper and SivaJothy, 1996; Neveu et al., 1996; Billot et al., 1999; Gachot-Neveu et al., 1999], population variability [Gwakisa et al., 1994; Kantanen et al., 1995; Shankaranarayanan et al., 1997], and systematic investigation [Rao et al., 1996]. In this study, we show for the first time that RAPD analysis can be applied for paternity discrimination in *C.s. simum*.

We successfully determined paternity for seven offspring of *C.s. simum*. For each offspring the mother was known, but we had to differentiate between four and nine potential fathers.

The behavioral observations extended over more than 212 hours, between October 1997 and February 2000. The behavioral data was used to establish the social relationships and behavioral dynamics within the rhinoceros herd.

METHODS

Social Group

The rhino herd at the Zoological Center in Tel Aviv-Ramat Gan, Israel, currently consists of a multi-male group of five males and six females. The herd originated as four males and four females that were brought to the park directly from South Africa in 1973 at an estimated age of 2 years. Four of the males and three of the females in the current herd were born at the Zoological Center. The Zoological Center area is approximately 0.7 km^2. This space is shared with large herds of 13 species of ungulates. There are no barriers to separate animals. No rhinos have been introduced, but surplus males born in the group were removed. Since 1978, 20 rhinos have born at the Zoological Center; however, no births have occurred since 1996.

MOLECULAR ANALYSIS

Sample Collection

Five hairs with follicles were plucked from the ear of each rhino and frozen in a sterile tube at –20°C.

DNA Extraction

Genomic DNA was extracted from the hair follicles as described [Ausubel, 1987] with some modifications. Five hair follicles were chopped under sterile conditions and suspended in 1.2 ml 10 mM Tris-Cl, pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and digested with 0.1 mg/ml proteinase K at 50°C overnight. Following phenol extraction and ethanol precipitation, the genomic DNA was suspended in water to a concentration of 50 μg/ml.

RAPD Procedure

The protocol uses a single arbitrary 10-mer oligonucleotide as a primer to scan a genome for small inverted repeats, and amplifies the intervening DNA segments [Williams et al., 1990]. We used primers from the Biotechnology Laboratory, University of British Columbia, Primer Synthesis Project (sets 100/4 and 100/2) for PCR amplification.

Amplifications were performed in volumes of 25 μl containing 18.25 μl sterilized water, 2.5 μl of reaction buffer (50 mM Tris-HCl pH 9.1, 16 mM ammonium sulfate, 3.5 mM $MgCl_2$, 150 μg/μl BSA), 1 of μl dNTP (2.5 mM), 1.25 U of Taq-Zol DNA polymerase (Tal-Ron), 2 μl of UBC-RAPD primer (4 pmol/μl), and 1 μl of genomic DNA (50 ng/μl). The reaction was overlaid with paraffin oil and was amplified in a thermocycler (Minicycler™ PTC-150; MJ Research, Waltham, MA), programmed for 3 min at 94°C, followed by 40 cycles of 94°C for 1 min, 36°C for 1.5 min, and 72°C for 2 min. Amplified products were analyzed on a 2% agarose gel and stained with ethidium bromide. Each reaction was run in duplicate, using DNA from different extractions, to verify the reproducibility of the results. The primers used are listed in Table 1.

TABLE 1. Primers used in the paternity tests of all the 7 individuals examined

Primer name	Primer sequence
UBC116	TACGATGACG
UBC142	ATCTGTTCGG
UBC148	TGTCCACCAG
UBC162	AACTTACCGC
UBC167	CCAATTCACG
UBC179	TCACGTTACG
UBC186	GTGCGTCGCT
UBC190	AGAATCCGCC
UBC306	GTCCTCGTAG
UBC321	ATCTAGGGAC
UBC332	AACGCGTAGA
UBC338	CTGTGGCGGT
UBC350	TGACGCGCTC

Paternity Discrimination

We used the accepted criterion for ascribing paternity to a male candidate if at least three bands of the offspring's RAPD pattern (using three different primers) were found in the pattern of that male, but not in its mother's pattern [Neveu et al., 1996; Billot et al., 1999; Gachot-Neveu et al., 1999].

Behavioral Observations

We concentrated on three major parameters in our observations: 1) the spatial distribution of rhinoceroses in the Zoological Center area; 2) the social preferences of each individual in the herd; and 3) dominance of males as determined by displacement of other males, and backward kicking at marking stations.

Territorial Partitioning

To explore the spatial distribution, we arbitrarily divided the Zoological Center area into four areas (labeled A–D), using the peripheral road as one boundary of each of the areas, and marked the presence of each rhino in these areas.

During observation periods, we documented the location of each individual once per hour. During the course of this study, a dominant male named Shalom was separated from the herd and penned in quarantine. Our observations distinguish between those made before and after Shalom was separated from the herd.

Social Preferences of the Rhinos

The social preferences of a particular rhino were measured by assessing the distances between this individual and another individual. We divided the distances into two categories: 0–10 m, and >10 m. We chose the 10-m limit because it is the minimal distance that a cow in heat will allow a bull to approach her at their first encounter [Nowak and Walker, 1991].

RESULTS

RAPD Paternity Determination

To identify the paternity of as many rhinos born at the Zoological Center as possible, we purified DNA from hair follicles of individual rhinos as described in Materials and Methods. Using random primers, we performed RAPD analysis on these DNA samples, choosing primers for further analysis that gave clear and reproducible banding patterns when run on agarose gels. We identified bands that could be amplified from the DNA of the offspring of a female, but were not from her own DNA. These bands must necessarily originate in the DNA of the father. We then asked which of the potential fathers yielded DNA from which we could uniquely amplify these bands. If three bands that fulfilled the above criteria were identified in the DNA of only one of the potential fathers, we deemed this data sufficient to assign paternity to that male. A potential father was defined as any male who was at least 5 years old on the day of birth of the offspring. We arrived at this number by summing the 3–3.5 years a male spends with his mother, plus 1.5 years of gestation.

In Fig. 1 we present a sample gel of these data using primer UBC332 to determine the paternity of a rhino named Atari. As can be seen in this figure, there is a strongly amplified band which is identified in the PCR products from DNA extracted from the male Rafi, but does not appear in the PCR products of his mother. Two other primers gave similar results (data not shown); thus we determined that Rafi was Atari's father.

Table 2 summarizes the results of the paternity testing. Rafi is the father of at least three offspring (37.5%), Zalman of at least one (12.5%), and Atari of one (12.5%). We were unable to determine the paternity of two offspring, although we know that the fathers are not one of the males currently in the Zoological Center. We were unable to determine paternity for Shalom, as his mother had died several years earlier and a sample of her DNA was unavailable.

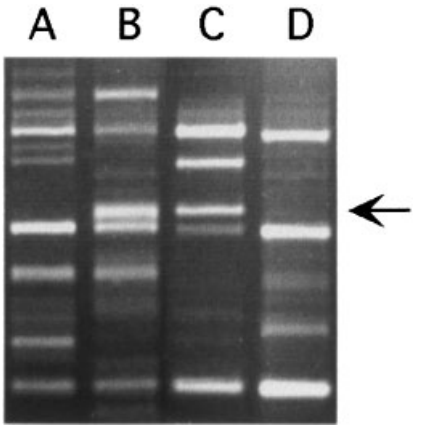

Fig. 1. RAPD patterns with primer UBC332 of DNA from four rhinos displayed on 2% agarose gel. **A:** Ziona. **B:** Ziona's offspring, Atari. **C:** Potential father, Rafi. **D:** Potential father, Zalman. The arrow indicates a differential band that indicates paternity of Rafi.

TABLE 2. Paternity summary

Offspring/studbook number	Mother's name/studbook number	Father's name/studbook number	Current condition of father
Carmi/0945(RAG22)	Carnavela/0641(RAG12)	Atari/0497(RAG10)	In Zoological Center
Zion/0913(RAG19)	Ziona/0241(RAG2)	Not one of the Safari's males	
Maia/0949(RAG24)	Mazal/0242(RAG3)	Not one of the Safari's males	
Carnavela/0641(RAG12)	Mazal/0242(RAG3)	Zalman/0246(RAG7)	Died in 1999
Shalom/0487(RAG9)	Unnamed/0240(RAG1)	Unknown, as DNA sample from mother unavailable	
Atari/0497(RAG10)	Ziona/0241/(RAG2)	Rafi/0245(RAG6)	In Zoological Center
Zafrir/1049(RAG25)	Ziona/0241/(RAG2)	Rafi/0245(RAG6)	In Zoological Center
Kern/1028(RAG27)	Carnavela/0641(RAG12)	Rafi/0245(RAG6)	In Zoological Center

Behavioral Observations

We arbitrarily divided the Zoological Center territory into four areas that we designated A, B, C, and D, respectively. The peripheral road was used as a guide in the partitioning. Food and water were distributed in area B. Our observations are summarized in Figs. 2 and 3.

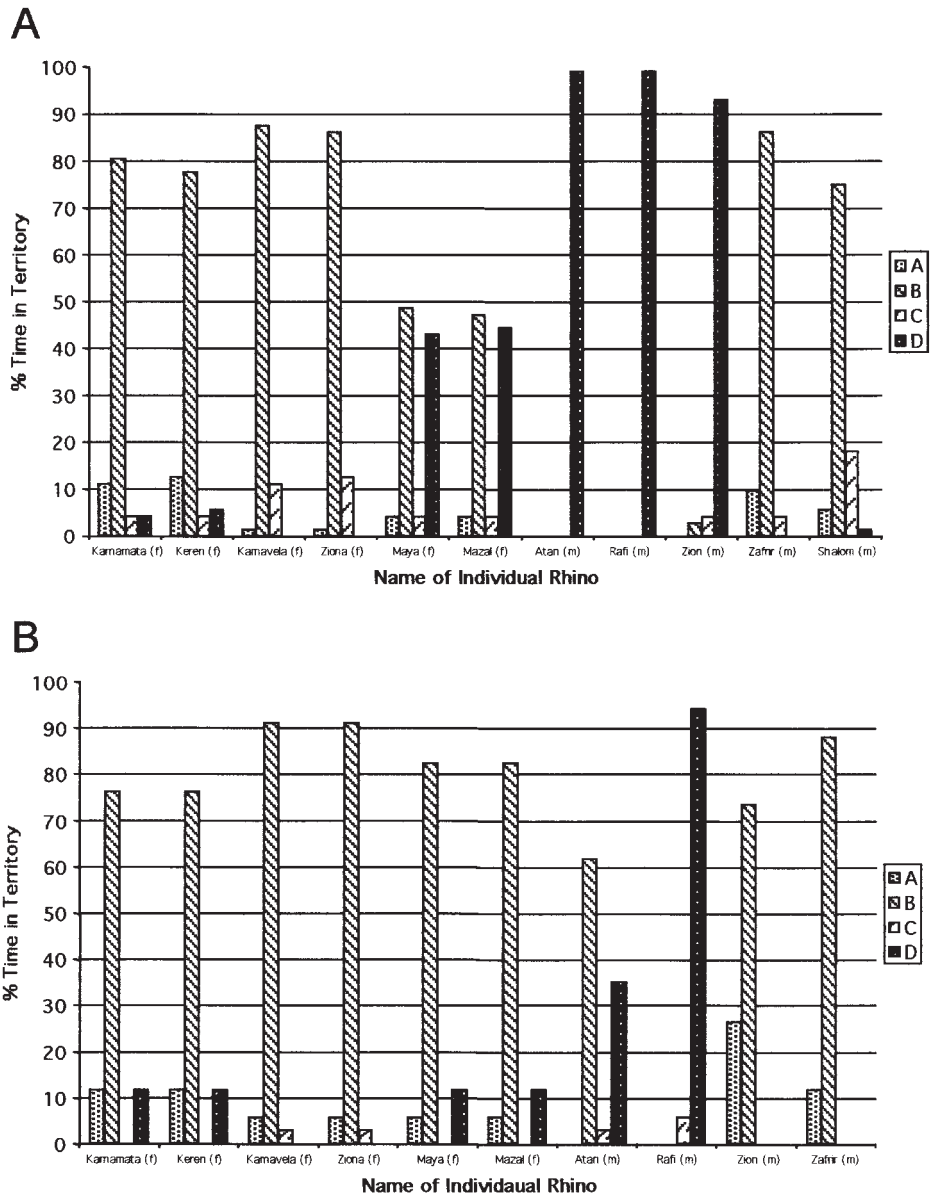

Fig. 2. Percentage of observation time (200 hr) that each rhino was observed in each of the four territories (A–D) of the Zoological Center. For each rhino, the gender is marked (male: m; female: f). **A:** Before separation of Shalom from herd. **B:** After separation of Shalom from herd.

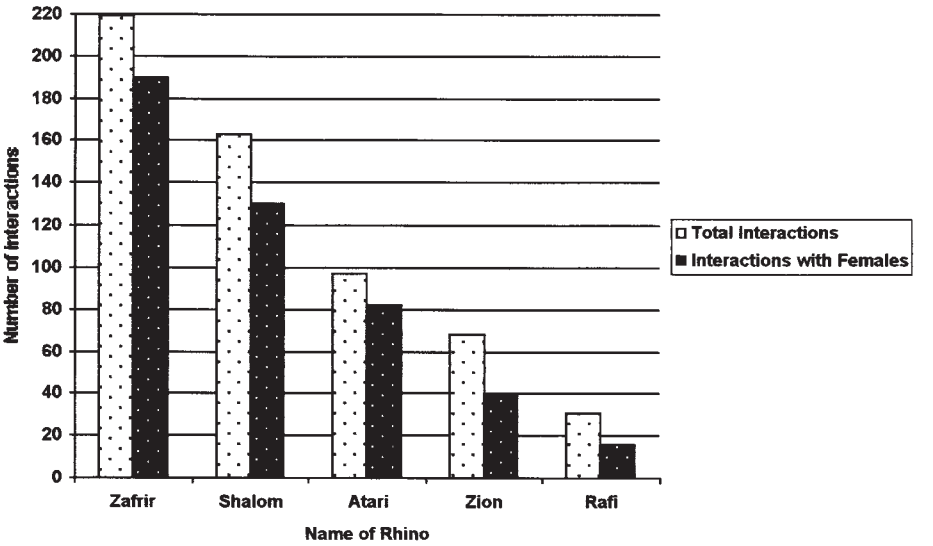

Fig. 3. Number of interactions of male rhinos with all other rhinos, and of males with females. An interaction is registered every time an individual was observed at a distance of <10 m from another individual.

The observations extended over a $2\frac{1}{2}$-year period from October 1997 to February 2000, and are divided into the periods before and after the separation of the dominant male Shalom from the herd. Shalom was separated following the observation that although he attempted to mate frequently, he was unable to achieve an intromission.

Before Shalom's separation from the herd, four females spent almost all their time in area B while two others divided their time equally between areas B and D (Fig. 2A). Following Shalom's separation, all the females spent most of their time in area B (Fig. 2B). The males' use of space changed more drastically. Before Shalom was removed, Shalom and another male, Zafrir, spent almost all their time in area B, while the other three males were observed almost exclusively in area D. After Shalom's separation from the herd, both Zion and Atari moved from area D to area B (Zion 73% and Atari 62% of time), and were often engaged in territorial battles (Figs. 2A and 3). The male Zafrir was observed primarily in area B both before and after Shalom was removed, and was not observed participating in the territorial battles.

We next compared the total interactions of each of the male rhinos with each other and with females. An interaction was scored when two rhinos were within 10 m of each other. Ten meters is the minimal distance that a female in heat will initially allow a male to approach [Nowak and Walker, 1991]. As can be seen in Fig. 3, about 70% of the interactions were between males and females. No significant differences were seen between individual males in this regard. However, there was substantial variation between males in total interactions, with Rafi having the lowest number.

Female–female preferences for consorting were found. Among interactions between females, a founder, nonfertile female (Karnamata), formed a strong bond

with a younger, potentially fertile female (Keren), and physically blocked male courtship. Thus she effectively prevented Keren from copulating.

DISCUSSION

We believe that the reduction in births within a herd of a square-lipped rhinos at the Zoological Center in Israel over the past 10 years is the result of a number of factors. In this study, we identify several of these factors using molecular techniques to determine paternity, and observations to document the social structure of the herd.

Here we demonstrate that RAPD profiling is a repeatable method which can be applied to determination of paternity in captive square-lipped rhinos. The RAPD method is easily performed, is inexpensive, and can be easily accomplished in most laboratories [Scott et al., 1992; Neveu et al., 1996]. We successfully determined paternity for five out of seven offspring of *C.s. simum*. For each offspring, the mother was known, but we had to differentiate between four to six potential fathers. An eighth animal whose mother's DNA was not available could not be analyzed.

Alternative methods to RAPD are microsatellite typing and amplified fragment length polymorphism (AFLP). Neither of these methods has been used to date for *C.s. simum*, though microsatellite typing has been used for *D. bicornis* [Garnier et al., 2001]. An advantage of RAPD over AFLP for a study such as this is that RAPD requires a very small sample size from the animal (e.g., hair roots), whereas AFLP requires a minimum amount of DNA for successful ligation of primers.

The use of space differed between the sexes. The majority of the females preferred to linger in area B. We believe this area was preferred because it has a water source, and normally the bulk of the food is distributed in this territory twice a day.

The removal of the dominant male Shalom was found to be insignificant to the female area preference, but was highly significant to the male area preference. From Fig. 2 we can see that before the removal of Shalom, none of the males (other than Shalom and Zafrir) spent substantial time in territory B, the territory preferred by four of the females. After Shalom was removed, two of the males, Zion and Atari, began spending significantly more time in territory B, closer to the females. Rafi, on the other hand, did not change his behavior, and continued to refrain from association with other rhinos, remaining in territory D.

Our observations showed that Shalom behaved as a dominant male. His dominance prevented Zion and Atari from approaching females, similar to what has been reported previously [Nowak and Walker, 1991; Rachlow et al., 1998]. Although Shalom behaved as a dominant male, our observations showed that he was unable to achieve an intromission, and thus to successfully mate. However, it is likely that he had relatively high levels of testosterone [Rachlow et al., 1998] in his bloodstream, as he aggressively prevented other males from mating. Thus, it is evident that the presence of Shalom was one of the causes for the reproductive decline of the herd.

The male Rafi fathered at least three offspring (37.5% of the total births at the Safari). It is also possible that Rafi fathered Shalom. Since a DNA sample from Shalom's mother was unavailable, we were unable to determine by RAPD analysis who fathered him. Nevertheless, we can deduce from Fig. 3 that during the study

period Rafi had significantly fewer interactions with females than did other males. We note that Rafi rarely spent time in territory B, the territory most preferred by the females. These data support the contention that the exclusion of Rafi from the breeding females (presumably by other males) is another cause of the reproductive decline.

From data presented in Table 2 we also note that some of the males who successfully mated at the Safari are no longer part of the herd, but were sold during 1988–1989. During this period, four adult males were sold without the keepers knowing their hierarchic position in the herd or their reproductive status. We believe that the exclusion of these males from the breeding pool may also have contributed to the declining birth rate at the Zoological Center. Because the sample size of the collection at the Ramat Gan Zoological Center is relatively small, we believe that these data should be extended by performing similar studies in other zoological parks. Comparative studies of several collections would yield a better understanding of the impact of dominance in a rhino herd.

Subsequent to the work presented in the Results, we continued to strive toward an integrated approach for the enhancement of the reproductive performance of our white rhinos. Females were examined using transrectal ultrasound, and male fertility was accessed by sperm counts of electroejaculates. In addition, progesterone levels in females were measured to evaluate estrous cycling [Hermes et al., 2001a, b]. The results of these examinations were that three females had healthy reproductive tracts and were cycling, and that all males were fertile. We concluded, integrating all the data on our collection, that the reproductive failure was a behavioral problem resulting from incomplete dominance of the males, and interference during mating. The final management decision consequent to these studies was to maintain one male with the females and to separate the remainder as a bachelor herd.

CONCLUSIONS

The work presented in this study emphasizes the importance for managers of captive rhino herds to know the genealogy of the members of the herd, and to monitor the social dynamics within the herd. Adjustments of herd membership must consider these factors so that conditions remain as favorable as possible for achieving future pregnancies. The European Endangered Species Program (EEP) recommendation is to initially transfer adult animals between zoos in order to break up sibling relationships and/or overcome mate-choice problems. The effect of the transfers between zoological parks has resulted in a dramatic increase in white rhino births in European zoos in the year 2000 [Tomasova, 2001]. In light of the data presented in this article, it would be most advantageous for zoological parks to maintain tissue samples from all animals in the park for future use.

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