Semen Collection, Sperm Assessment and Cryo-Preservation in African Rhinoceroses

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The captive white rhinoceros population currently faces a demographic crisis. As a consequence substantial knowledge on reproductive biology of the female white rhinoceros has been gathered over the past years. However, little emphasis has been put on the evaluation of male fertility as a possible contributing factor to the low rate of reproduction. In the present study the reproductive fitness of ten male white and one black rhinoceros was evaluated by ultrasonography and semen assessment. Semen collection was obtained by manual stimulation (n=2) and electro-stimulation (n=9). Based on 39 semen assessment results seven males were identified as reliable semen donors. Preserved semen samples remained viable for up to four days. Cryopreserved samples showed post thaw motility suitable for assisted reproduction. Reproductive assessment provided accurate information on the breeding potential of male white rhinoceros with an implication on management decisions.

AFLP as an Economical Method to Correct Field Observations for Genetic Wildlife Management in Rhinoceros

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Introduction

Due to habitat loss and poaching the numbers of rhinoceros declined severely over the last decades. Intensive protection measures and the translocation of animals to reserves and private game farms managed to stop this decline in the African rhinoceros and led especially in the white rhinoceros even to increasing numbers (Emslie & Brooks 1999). But saving them from habitat loss and poaching is not enough. On a long term basis a specific breeding program has to be applied as well. This would also include shifting of animals between different locations (Mace & Lande 1991), as small and isolated populations can rapidly lose genetic variability and with it their capacity for genetic adaptation (Schreiber et al. 1995; Parker & White 1997). They become more vulnerable to changes in their environment (Foose 1991) and the fertility and viability of these animals can be reduced (Baur et al. 1995). To set up such a breeding management, prior information about the genetic population structure is required for translocations to prevent inbreeding (Moehlmann 1996).

Lacking DNA sequence information in the rhinoceros for a molecular genetic approach, a suitable PCR method had to be found to generate genetic markers from the uncharacterised genome. Therefore the sequence independent DNA fingerprinting method termed "Amplified Fragment Length Polymorphism (AFLP)" was established for the rhinoceros to determine paternity and genetic variability for four different species of rhinoceros: the great Indian one-horned rhinoceros (*Rhinoceros unicornis*), the black rhinoceros (*Diceros bicornis michaeli*), the northern white rhinoceros (*Ceratoterium simum cottoni*) and the southern white rhinoceros (*Ceratotherium simum simum*).

The degree of inbreeding is mainly determined by the number of reproducing males in one area (PARKER & WAITE 1997). Within a separate study on the breeding patterns of white rhinoceros on a game farm in South Africa, genetic analyses was used to determine the proportion of reproducing males within this population and to increase the database on secured parentage.

Method

The study involved blood and tissue samples of 69 southern white rhinoceros from different European zoological parks. For these animals an international studbook exists and their relationship is mainly known.

Further, the study contained 57 southern white rhinoceros from a game farm in South Africa. These samples could be obtained while the animals were immobilised for management purposes.

Of the rarest subspecies, the northern white rhinoceros, 5 samples could be collected. For comparison samples of 20 black rhinoceros and 6 great Indian one-horned rhinoceros were used.

Genomic DNA was digested with 2 restriction enzymes, EcoRI and TaqI. The restriction fragments were selectively amplified in a PCR reaction and analysed on a polyacrylamide gel on a LICOR DNA Sequencer. An external standard was used to determine the size of the fragments in basepairs (bp).

Polymorphisms were detected as the presence or absence of an amplified restriction fragment.

Heterozygosity was determined after Nei (1978) as: $h = 1 - S\, x_i^2$ where x_i is the frequency of allel i. Values between 0 and 0.5 are reached, with 0 indicating the least genetic diversity with a monomorph locus and 0.5 indicating an equilibrium of allel distribution. For paternity testing the combined exclusion rate after Jamieson & Taylor (1997) was used.

Data on territorial status of males and behavioural observations on females were obtained during a two year study on free living white rhinoceros in South Africa. Positions of individually known males and females were either obtained during tracking of certain animals or by patrolling the area with a car. All positions were mapped with a GPS and analysed using Map Info 5.0.

Results

The method AFLP was established for the rhinoceros. A set of 64 AFLP primer combinations was tested and 12 primer combinations were selected for further investigation. They produced an average of 60-80 bands per PCR reaction and animal in a range of 50 to 510/800 basepairs. Only polymorphic bands which could be clearly identified were scored.

For 125 southern white rhino 71 polymorphic loci were analysed. Referring to the total number of scored bands this corresponds to 3,5% polymorphic bands. For the northern white rhino only 37 polymorphisms could be detected (3,9% polymorphic bands).

The fingerprint banding pattern between these two subspecies was similar, but they could be clearly identified as two different species by single extra or absent bands.

For the black rhinoceros a total of 106 bands could be scored showing with 4,7% the biggest number of polymorphic bands.

Fifty-four polymorphic bands could be analysed in the great Indian one horned rhino (2,5% polymorphic bands).

The different species showed for every primer combination their own banding patterns and could be clearly identified.

With an average heterozygosity of 0.36 for all polymorphic loci detected by dominant AFLP markers, all rhino species showed still a high level of genetic variability in their populations.

Table 1: Overview of the number of samples and scored markers used in this study comprising the southern white rhinoceros (Css), northern white rhinoceros (Csc), black rhinoceros (Db) and great Indian one-horned rhinoceros (Ru); percentage of polymorphic bands in proportion to the total number of bands; average frequencies of recessive allel (q); average heterozygosity(h) and combined exclusion probability (P) for all 5 rhinoceros populations:

Population	number of individuals N / markers	Polymorphic bands in proportion total no. bands	Recessive Frequency q Ó	Heterozygosity h Ó	combined exclusion probability P
Css	125 / 71	3,5%	0.60	0.36	0.96
Csc	5 / 37	3,9%	0.65	0.40	0.90
Db	20 / 106	4,7%	0.68	0.31	0.99
Ru	6 / 54	2,5%	0.65	0.38	0.95

Parentage testing

AFLP usually generates dominant, recessive markers. With a band present it can not be distinguished between homozygous (1 1) and heterozygous (0 1) animals, so only recessive loci (0 0) can be taken into account for parentage testing. A recessive locus (0 0) in both parents has to show also (0 0) in the offspring. For AFLP markers parentage testing is only possible when blood or tissue samples of both probable parents is available. Compared with co-dominant markers (e.g. Microsatellites), recessive markers have a lower information content. For the statistic analysis this loss of information can be made up by increasing the number of samples or the number of amplified markers (Sharbel 1999).

An exclusion probability between 90 and 99% was reached which is in line with parentage testing in farm animals. The AFLP markers were validated for parentage testing with 27 offspring of known decendency and both parents present from European Zoological Parks.

For 14 calves of a game farm in South Africa with a known mother the most probable sire of 5 bulls could be determined.

As table 2 shows could four of five sires be excluded with two bands or more for six juveniles (212, 228, 241, 252, 257, 259) and for an other six calves (209, 214, 222, 236, 249, 262) by one band. Only two of 14 animals show two possible sires.

The results revealed, that all adult territorial males on the farm had reproduced. There was no clear preference of females for particular males. Due to the small sample size of 14 juveniles, it is too early to draw any conclusions about mate choice in white rhinoceros. Females were found in the territories of several males but were more often seen in the territory of the sire of her juvenile (fig. 1). In one case, the parentage test showed, that of two males which were together with a receptive female, the male courting less intensively sired her offspring. This shows that behaviour observations by its own are not sufficient. Genetic analyses in combination with direct observations are necessary.

Table 2: Number of loci for 14 calves which exclude paternity of each of 5 possible white rhinoceros bulls on a game farm in South Africa:

Calves	Sire 1	Sire 2	Sire 3	Sire 4	Sire 5
Lab. number	220	229	240	242	247
252	0	3	4	3	3
257	0	5	4	5	4
259	0	5	3	4	3
218	2	0	1	2	0
246	2	0	1	2	0
222	4	1	0	5	3
249	1	1	0	1	1
214	5	1	0	3	1
228	3	2	2	0	3
241	5	5	3	0	4
236	1	2	1	0	2
209	1	2	2	2	0
212	7	2	5	5	0
262	3	2	2	1	0

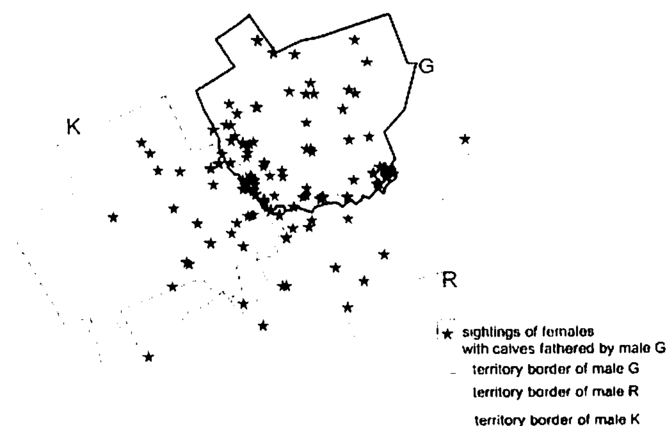

Fig. 1.: Territories of 3 adult male white rhinoceros (male K, G and R). The stars indicate the position of sightings of females with calves which were proved to be fathered by male G. The border line of each territory is drawn around all sightings of the males, only for better illustration of the area.

Conclusions

AFLP provides a powerful and cost-effective DNA fingerprinting technique. Different authors describe AFLP as a very useful method to determine genetic diversity and relationship (Ajmore-Marsan et al., 1997; Greef et al., 1997; Vos & Kuiper, 1997; Mueller et al., 1999; Sharbel, 1999). Large quantities of polymorphisms can be generated throughout

the entire genome without the need of developing costly sequence based markers (Zabeau & Vos, 1993). Every individual shows its specific band patterns (Hill et al., 1996). Thus it can be also used to collect genetic data from other endangered wildlife species, but one has to bear in mind that blood or tissue samples are needed for this method.

Within the study it could be differentiated between rhino species as well as between the two subspecies northern and southern white rhinoceros. The mean heterozygosity of 0.36 for the southern white rhinoceros and a value of 0.40 for the northern white rhino found in this study still suggest high levels of genetic variation. The relative low number of polymorphic loci found in the northern white rhino could be due to the small number of sampled animals.

A slightly higher percentage of the polymorphic bands ratio (3,9%) in the northern white rhinoceros could indicate a decimation in recent times, while the southern white rhino with a percentage of 3,5% polymorphic bands went through a genetic bottleneck already beginning of the 20th century.

While a study of Merenlender et al. (1989) based on allozymic loci found extremely small amounts of intraspecific variations, Stratil et al. (1990) reports also of surprisingly high levels of variations in serum proteins in the same animals of the species northern white rhinoceros.

The black rhinoceros showed the biggest amount of polymorphic bands even though only 20 animals where analysed. With a mean heterozygosity of 0.31 it shows a lower value than the other rhinoceros populations. This might indicate a reduction in numbers of former large populations. But there is no evidence of depauperation. This is consistent with reports of Swart et al. (1994 and 1997) based on electrophoretic analysis of serum and red blood cell protein-encoding loci.

For the great Indian one-horned rhinoceros only a percentage of 2,5% polymorphic bands was found, but a heterozygosity of 0.38 is consistent with findings of Dinerstein et al. (1990) based on protein electrophoresis which suggest, that they also still carry high levels of genetic variation.

These findings of high heterozygosity in the rhinoceros seem to be in contrast with reports for other species that have experienced near extinction like the cheetah (O'Brian et al., 1985). We conclude that high variation persists as the genetic bottleneck occurred only recently and the average generation time is long. Up to now bottleneck effect on genetic variation seems to be small. So if the different rhinoceros populations are treated as a meta-population, and a good breeding management is applied, the different species could still preserve high proportions of genetic variation.

With this study it was demonstrated that AFLP could be a valuable tool for parentage analysis in rhinoceros and other wild animals where little information about DNA sequences are available. It can help to increase the database on breeding pattern and mate choice and with it breeding management in wildlife.

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