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DETERMINATION OF GENETIC VARIATION AND FAMILY RELATIONSHIPS IN RHINOCEROS

Katharina KELLNER, I. MEDUGORAC, M. FÖRSTER*

Address
Institute for Animal Breeding, Ludwig-Maximilians-University Munich (Director: Prof. Dr. M. Förster)
Veterinärstr. 13 D- 80539 München, Germany
*) author for correspondence , tel.: +49(0)89-21802559; fax: +49-(0)89-344925;
e-mail: katharina.kellner@ stud. uni-muenchen.de

Abstract

This work reports on the use of amplified fragment length polymorphism (AFLP) in rhinoceros. The restriction enzymes EcoRI and TaqI were used for the digestion of genomic DNA as suggested for mammals. A set of 64 AFLP primer combinations was analyzed and 12 primer combinations were selected for further investigation. For 125 southern white rhinoceros 71 and for 5 northern white rhinoceros 37 polymorphic bands could be scored. Both white rhino species showed similar DNA fingerprint banding patterns, but they could be differentiated as two different species. For 20 black rhinoceros a total of 106 bands could be scored and for 6 great Indian one-horned rhinos 54 polymorphic loci were analyzed. With an average heterozygosity of 0.36 for all polymorphic loci detected by dominant AFLP markers for the different rhinoceros species, all species seem to show a high level of genetic variability in their populations. For parentage testing a combined exclusion rate between 90 and 99 % was reached. For 13 calves of a game farm with a known mother the most probable sire of 5 possible bulls should be determined.

Zusammenfassung

Diese Arbeit berichtet über den Gebrauch von AFLP (amplified fragment length Diese Arbeit berichtet über den Gebrauch von AFLP (amplified fragment length polymorphism) beim Nashorn. Zur Verdauung genomischer DNA wurden die Restriktionsenzyme EcoRI und TaqI verwendet, so wie dies für Säugetiere vorgeschlagen wird. Es wurden 64 Primerkombinationen getestet und 12 Primerkombinationen für weitere Untersuchungen selektiert. Für 125 südliche weisse Nashörner zeigten sich 71 polymorphe Banden und für 5 nördliche weisse Nashörner konnten 37 polymorphe Banden gezählt werden. Beide Unterarten des weissen Nashornes zeigten ein sehr ähnliches Bandenmuster, sie konnen jedoch klar als Unterarten unterschieden werden. Für 20 schwarze Nashörner zeigten sich insgesamt 106 polymorphe Bande und für 6 indische Nashörner konnten 54 polymorphe Loci festgestellt werden. Mit einem für alle Nashornspezies berechneten durchschnittlichen Heterozygotiegrad von 0,36 für alle polymorphe Loci, die mit Hilfe dominanter AFLP Marker generiert wurden, scheinen alle Populationen einen recht hohen Grad an genetischen Variabilität bewahrt zu haben. Für einen Abstammungsnachweis wurde eine kombinierte Ausschlussrate von 90 bis 99% erreicht. Für 13 Kälber einer Wildtierfarm in Südafrika mit bekannter Mutter, sollte das mögliche Vatertier aus einer Anzahl von 5 in Frage kommenden Bullen herausgefunden werden.

Résumé

Cette communication présente l'utilisation des AFLPs (Amplified Fragment Length Polymorphism) chez le rhinocéros. Les enzymes de restriction, EcoRI et TaqI, furent utilisées pour digérer l'ADN génomique, comme cela est suggéré chez les mammifères. Un total de 64 combinaisons de primersAFLP furent analysées et 12 d'entre elles furent retenues pour poursuivre les analyses. 71 et 5 séquences polymorphiques ont été

identifiées respectivement chez 125 rhinocéros blancs du sud et 37 rhinocéros blancs du nord. Les deux espèces de rhinocéros blanc présentent des profils d'empreintes génétiques comparables, néamoins elles peuvent être clairement differenciées en deux espèces. Chez 20 rhinocéros noirs, un total de 106 bandes a été dénombré, et chez 6 Rhinocéros indiens 54 loci polymorphiques furent analysés. Avec une hétérozygotie moyenne de 0.36 pour tous les loci polymorphiques détectés par des marqueurs AFLP dominants chez les différentes espèces de rhinocéros,toutes ces espèces semblent montrer une grande variabilité génétique au sein de leurs populations. Pour évaluer le degré de parenté entre les individus, un taux d'exclusion combiné compris entre 90 et 99 % a été atteint. Pour 13 jeunes issus d'une ferme d'élevage et de mère connue, le père le plus probable pourra ainsi être déterminé parmi 5 mâles possibles.

Keywords
AFLP marker, dominant marker, Rhinoceros, DNA-fingerprinting, PCR, paternity, population structure

Introduction

Many species are endangered due to habitat loss and poaching. African and Asian rhinoceros (*Ceratoterium simum*, *Diceros bicornis*, *Rhinoceros unicornis*) belong to this category.

Considering the rapid decline of the rhino population, saving them from habitat loss and poaching is not enough. A specific breeding program has to be applied since the remaining small populations are usually prevented from natural migration. Small populations can rapidly lose genetic variability and their capacity for genetic adaptation is reduced. They become more vulnerable to changes in their environment, which can imperil their survival (2, 6). An effective breeding management requires the handling of smaller populations as one large group (meta-population) to reach a maximum genetic diversity and effective population size. This can include wild and zoo populations. But to set up such a breeding management, prior information about the genetic population structure are required to prevent inbreeding (3, 6, 15). As the relationship of wild living animals and even some zoo animals is often not known, an attempt to find a molecular genetic approach was made to determine paternity and genetic variability in the rhinoceros.
In our case, a PCR (Polymerase Chain Reaction) based method was the best way since it requires only a small amount of DNA. Lacking DNA sequence information in the rhinoceros, a suitable PCR method had to be found to generate genetic markers from the uncharacterized genome. Therefore we established the sequence independent DNA fingerprinting method termed "amplified fragment length polymorphism (AFLP)" for the rhinoceros.

As purified, genomic DNA is needed for AFLP reactions, ways of sampling blood and tissue in the rhinoceros had to be found.

Material

The study comprises blood and tissue samples of 69 southern white rhinoceros (*Ceratoterium simum simum*) of different zoos connected to the EEP, for which an international studbook exists. The sample material contains animals of different origin and 4 families, Beekse Bergen, Whipsnade, Hodenhagen and Münster. From a game farm in South Africa an another 56 white rhino samples could be obtained during a marking session for which all animals were immobilized.
Of the rarest subspecies, the northern white rhinoceros (*Ceratoterium simum cottoni*), 5 samples from Dvur Kralove Wild Animal Park could be collected. For comparison samples of 20 eastern black rhinoceros (*Diceros bicornis michaeli*) and 6 great Indian one-horned rhinoceros (*Rhinoceros unicornis*) were collected in different European Zoos (see table 1). Most black rhinoceros were from 2 families, East Berlin Zoo and Dvur Kralove Wild Animal Park.

Sampling and Sample preservation

Blood
Most samples were taken as EDTA blood as described of Walzer (22) under local dermal anesthesia in the ear vein. EDTA tubes were stored refrigerated at - 20 °C until use.

Species/ Subspecies	Abbreviation	Origin	number of samples
Southern white rhinoceros (*Cerathoterium simum simum*)	Css	European Zoos	69
Southern white rhinoceros (*Cerathoterium simum simum*)	Csw	game farm South Afrika	56
Northern white rhinoceros (*Cerathoterium simum cottoni*)	Csc	Dvur Kralove Wild Animal Park	5
Eastern black rhinoceros (*Diceros bicornis michaeli*)	Db	European Zoos	20
Great indian one-horned rhinoceros (*Rhinoceros unicornis*)	Ru	European Zoos	6

Table 1
Overview of sample material used in this study comprising a wild (Csw) and zoo population (Css) of the southern white rhinoceros, northern white rhinoceros (Csc), black rhinoceros (Db) and great indian one-horned rhinoceros (Ru)

Tissue
With a pair of tagging pliers a small piece of tissue is punched out of the ear. This method is very quick and works out very well even for animals which are not accustomed to frequent handling. Tissue samples were stored in 70 % Ethanol or were refrigerated at - 20 °C until use.
The rhinos tolerate both methods very well and they did not seem to be overly disturbed by it.

Method

The AFLP technique applied for animals is rather new as it was originally developed for plant genetics (20). It provides a powerful DNA fingerprinting technique for DNAs of any origin and complexity. Thus it could provide a useful method to collect genetic data not only from the rhinoceros but also from other endangered wildlife species. Different authors describe AFLP as a very useful method to determine genetic diversity and relationship within, as well as between populations (1, 7, 11, 16, 21). Genomic DNA was digested with 2 restriction enzymes, EcoRI and TaqI, and a subset of restriction fragments was produced. Adaptors with known sequences were ligated to each end of the fragments. These fragments were selectively amplified in a PCR reaction using oligonucleotide primers complementary to the sequences of the adaptors. The amplified fragments were analyzed on a polyacrylamide gel on a LICOR DNA Sequencer. An external standard was used to determine the size of the fragments in base-pairs (bp). Polymorphisms are usually detected as the presence or absence of an amplified restriction fragment. It can't be distinguished between heterozygous band presence and homozygous band presence, which means dominant, recessive markers are generated. Every individual shows its specific band patterns (8).

Data analysis

Polymorphic fragments were scored as 1 for presence and 0 for absence of a fragment. Only the mayor fragments were used in the data analysis. We assumec that each band position corresponded to a locus with two alles and that every locus under investigation is in the Hardy Weinberg equilibrium.
Genetic similarities between individuals of the same population and between different populations were estimated using the formula of Nei & Li (13) and Hill et al. (8) as: $Gs(ij) = C(ij)/N(ij)$ where $Gs(ij)$ is the measure of genetic similarity between the i th and j th accession. $C(ij)$ is the number of shared bands in i and j and $N(ij)$ is the total sum of scored bands. Genetic distance between individuals and populations were calculated as $-\ln(Gs)$. Using the unweighed pair group method average (UPGMA) dendrograms were constructed with the program PHYLIP (5).

	Primer combination	Southern White/ Css	Northern White/ Csc	Black/ Db	Indian Ru	Ø of loci / primer
1	N02	10	5	8	10	8.25
2	N03	7	1	14	6	7.00
3	N04	5	3	8	1	4.25
4	N10	6	2	11	2	5.25
5	N25	3	8	9	2	5.50
6	N26	7	1	11	3	5.50
7	N27	5	3	3	10	5.25
8	N28	6	0	9	5	5.00
9	N41	10	4	8	3	6.25
10	N52	2	2	8	3	3.75
11	N57	4	3	5	8	5.00
12	N59	6	5	12	1	6.00
	sum	71	37	106	54	
	Ø bands in a population	5.90	3.08	8.83	4.50	

Table 2
Detected polymorphic bands in five rhinoceros populations for 12 AFLP primer combinations, average of polymorphic loci over all populations for one primer combination and average number of bands in a population.

Results

The method AFLP was established for the rhinoceros. Genomic DNA was digested with the restriction-enzymes EcoRI and TaqI and a set of 64 primer combinations was tested. Of these primer combinations 12 were selected for further investigations. They produced an average of 60-80 bands per PCR reaction and animal in a range of 50 to 510/800 base-pairs. Only the major polymorphic bands were scored which could be well determined.

For 125 southern white rhino 71 polymorphic loci were analyzed (see table 2). The primer-combinations N02 and N41 showed most polymorphic bands. For the northern white rhino only 37 polymorphisms for the whole population could be detected with N25 showing the highest number of 8 polymorphic bands. This small number of 37 polymorphisms could be due to the small number of sampled animals. Northern white rhinoceros showed a similar fingerprint banding pattern as the southern white rhinoceros, but it could be clearly identified by single extra or absent bands as different species. For the black rhinoceros a total of 106 bands could be scored with N03 and N59 showing the biggest number of polymorphic bands. Even though the number of 6 sampled animals was small 54 polymorphic bands could be analyzed in the great Indian one horned rhino with N02 and N27 showing most polymorphic bands (see table 2). The different species showed for every primer combination their own banding patterns and could be clearly identified.

Population	number of individuals *N*	recessive Frequency *q*Ø	Heterozygosity *h*Ø	combined exclusion probability *P*
Css	69	0.60	0.36	0.96
Csw	56	0.60	0.36	0.96
Csc	5	0.65	0.40	0.90
Db	20	0.68	0.31	0.99
Ru	6	0.65	0.38	0.95

Table 3
Average frequencies of recessive allel (*q*), average heterozygosity (*h*) and combined exclusion probability (P) for all 5 rhinoceros populations.

Heterozygosity was determined after Nei (12) as: $h = 1 - \Sigma x_i^2$ where x_i is the frequency of allel *i*. Values between 0 and 0.5 are reached where 0 would be the least genetic diversity with a monomorph locus and 0,5 would be an equilibrium of allele distribution. With an average of 0.36 for the different rhinoceros species they seem to show still a high level of genetic variability in their populations. To reach highly probable exclusion rates for paternity testing the combined exclusion rate after Jamieson & Taylor (9) was used to calculate: $P = 1 - (1-P_1)(1-P_2)(1-P_3)....(1-P_k)$, where P_k is the exclusion probability of locus *k*. For the southern white rhinoceros, a combined exclusion probability of 96 %, and for the northern white rhinoceros of 90 %, was reached for parentage testing. For the great indian one horned rhino an exclusion probability of 95 % was reached. The highest level was reached for the black rhinoceros with an exclusion probability of 99 % (see table 3). A value of at least 95 %, better 99 %, would be wanted for commercial parentage testing in farm animals.

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

Table 4:
Number of loci for 13 calves which exclude paternity of each of 5 possible white rhinoceros bulls on a game farm in South Africa

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 with (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, recessive markers have a lower information content. Recessive markers of the best quality with an allele frequency of $q = 0.803$ would show a maximum exclusion probability at 0.081. Co-dominant markers where two alleles are observed would show for their best quality at an allele frequency $p = q = 0.50$, an exclusion probability of 0.187. So more recessive than co-dominant markers are needed to test parentage. Within 27 offspring with known decendency and both parents present from European Zoological Parks, only one animal showed a band which would have excluded parentage. As mutations occur, at least 2 bands are required for a reliable exclusion rate. So reliability of the AFLP markers for parentage testing could be confirmed. For the wild population of a game farm in South Africa for 13 calves with a known mother, the most probable sire of 5 possible bulls should be demonstrated. Table 4 shows that in animals 212, 228, 241, 252, 257 and 259 four of five sires could be excluded with two bands or more. As spontaneous mutations occur, a reliable exclusion is not possible for sires which are excluded only by 1 band. Nevertheless there is only one sire which could not be excluded for calves 209, 214, 222, 236 and 249. Only 2 of 13 animals show 2 possible sires. So more AFLP primer combinations will have to be analyzed for the white rhinoceros to increase accuracy.

Genetic distance and relationship

AFLP based genetic distances between individuals within each of five population were calculated as - ln(Gs) (13). Using the unweighed pair group method average (UPGMA) a dendrogram was constructed to visualize relationship.

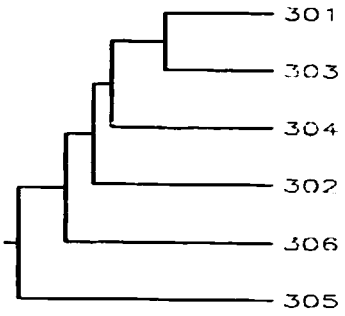

Figure 1
Example of UPGM phylogenetic relationship of 6 individuals of the indian rhinoceros

Figure 1 shows the closest relationship between bull 301 and animal 303 which is his offspring. Closest to them is 304, a bull which is the sire of 301 and grandsire to animal 303. Animal 302, which is the mother to animal 303, is a little bit further away. The biggest genetic distance shows an unrelated animal 305, which comes directly from Nepal.

Discussion and conclusions

To our knowledge this is the first report of the use of AFLP markers to determine genetic relationship in the rhinoceros. This study involved southern white rhinoceros from different European zoological parks and a wild population of a game farm in South Africa as well as northern white rhinoceros. It could not be differentiated between the zoo and wild population of southern white rhinoceros. Both showed the same amount of polymorphic bands and the same heterozygosity.
But it could be differentiated between the two subspecies southern and northern white rhinoceros. Both showed very similar banding patterns but they could be clearly identified by single extra or absent bands. For all rhinoceros species, polymorphic bands could be detected. The black rhinoceros showed the biggest amount of polymorphic bands and a mean heterozygosity of 0.31 even though only 20 animals where analyzed. This level of heterozygosity in the black rhinoceros which is lower than for other rhinoceros populations, might indicate a reduction in numbers of former large populations. But there is no evidence of depauperation and it shows that there is still a high degree of variability in this population. The estimate of high genetic variation among black rhinoceroses was also described by Swart et al. (18, 19) based on electrophoretic analysis of serum and red blood cell protein-encoding loci. The fact that for the southern white rhinoceros less polymorphic bands were scored even though 125 animals were analyzed could be due to using for this population only the best polymorphic loci which were detected in more than 3 animals, while faint or unclear bands were disgarded. So the effective amount of polymorphic loci is higher than the one scored for the analysis and heterozygosity might be according to it a little bit lower. But even a lower value than 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 would still suggest high levels of genetic variation. While a study of Merenlender et al. (10) based on allozymic loci found extremely small amounts of intraspecific variations, Stratil et al. (17) reports also of surprisingly high levels of variations in serum proteins in the same animals of the species northern white rhinoceros. For the great indian one-horned rhinoceros findings of Dinerstein et al. (4) based on protein electrophoresis 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 (14).

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, since a

large number of genetic markers from the uncharacterized genome can be generated. For more accuracy and higher exclusion rates, further primer-combinations will have to be tested.

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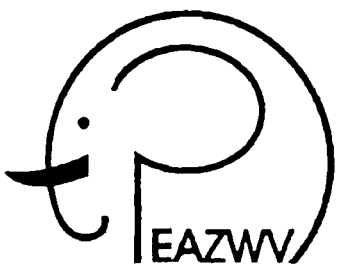

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