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Interspecific hybridisation in rhinoceroses: Confirmation of a Black × White rhinoceros hybrid by karyotype, fluorescence *in situ* hybridisation (FISH) and microsatellite analysis

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Abstract

Black and white rhinoceroses are among the most charismatic megaherbivores and have become flagship species for international conservation. They are often subject to intense management that includes being compressed unnaturally in space and density. We present chromosomal and microsatellite evidence to substantiate the first recorded instance of interspecific hybridisation between them. The data suggest that the genetic integrity of the African rhinoceros species probably depends on differences in behavioural and ecological preferences that offer semipermeable reproductive isolation. We caution against the retention of both species in captive and other population situations where disruption of species-specific behaviour patterns may result if there is an unnatural composition in terms of age and sex, and where access to conspecific mates is restricted or absent.

Introduction

Hybridisation between morphologically distinct mammalian species has resulted in a variety of hybrids encompassing both domesticated and non-domesticated animals (Gray 1971). Although interspecific crosses among free-living mammals are rarely reported, under conditions where access to conspecifics is denied or where species that exist naturally in allopatry are brought into artificial contact, hybridisation is not an unusual outcome. When the potential for interspecies reproduction is illustrated under artificial conditions, the implications for the management of natural populations can be significant, especially where the demographic profiles of the hybridising species have been substantially altered.

The black and white rhinoceroses are a case in point. Once widespread on the African continent, poaching, aided by inadequate protection in the vast areas where large populations of rhinoceroses once roamed, led to a precipitous decline in numbers throughout their former overlapping ranges. Consequently, natural populations are often small and fragmented being most pronounced in the critically endangered black rhinoceros.

In cases where both species co-occur in very low numbers, for example in some small private reserves and game ranches in southern Africa, compressed spacing and density, unnatural population composition and restricted, or no access to conspecifics may result. The instance of rhinoceros hybridisation documented herein occurred in an 800 ha enclosure of South Africa's National Zoological Gardens Game Breeding Centre, 200 km north of Pretoria, South Africa. A young white rhinoceros bull (aged at 4 years and 2 months) was held in the enclosure with two adult

white rhinoceros cows and an adult black rhinoceros bull. A female calf was born to one of the cows in late September 1988. Since male breeding success in the white rhinoceros is bound tightly to the acquisition of a territory and white rhinoceroses only become dominant territory holders from about 12 years of age (Owen-Smith 1988), although reproductively mature before then (Lindemann 1982), the presence of the black rhinoceros placed the provenance of the offspring in doubt.

Material and methods

We were unable to obtain tissue from the mother (referable to the *C. s. simum* subspecies) of the putative hybrid, or from either possible father precluding simply genotyping them to establish paternity. Consequently, we relied on data generated as part of a larger investigation into population structure in black and white rhinoceroses (Harley, unpublished) in determining provenance. The subspecies designation and origin of the 117 black rhinoceros included in our sample are: *bicornis* n = 51 (from Namibia and South Africa), *minor* n = 47 (from South Africa and Zimbabwe), and *michaeli* n = 19 (from South Africa). The collection of material from the specimens studied herein was done under permit to EH. The white rhinoceros sample included six specimens of the southern subspecies (*C. s. simum* collected in South Africa at the Hluhluwe Umfolozi National Park), and one specimen of the northern subspecies (*C. s. cottoni* from Garamba National Park, Democratic Republic of Congo). The putative F1 hybrid was biopsied after translocation to another breeding centre within the National Zoological Gardens system.

Fibroblast cultures were established from the putative hybrid and four of the *C. s. simum* and two of the *D. b. bicornis* specimens included in our sample. G- and C-banding of chromosomes followed Seabright (1971) and Sumner (1972), respectively. We used chromosome-specific painting probes made for Burchell's zebra (*E. burchelli*) by degenerate oligonucleotide polymerase chain reaction of flow-sorted chromosomes (Yang et al. 2003). Fluorescence *in situ* hybridisation follows Trifonov et al. (2003). Hybridisation signals were assigned to specific chromosomes, or chromosomal regions, using G-banding patterns obtained prior to *in situ* hybridisation.

DNA, extracted from skin biopsies or cells in tissue culture using conventional techniques, was diluted to a final concentration of 10–50 ng/μl for polymerase chain reaction (PCR) amplification. Variation at nine polymorphic microsatellite loci isolated from *D. b. bicornis* was investigated using the primers BR4, BR6, and BR17 (Cunningham et al. 1999) and DB1, DB14, DB44, DB49, DB52, and DB66 (Brown & Houlden 1999). The PCR amplification of BR4, BR6, and BR17 followed the conditions specified by Cunningham et al. (1999), and in the case of DB1, DB14, DB44, DB49, DB52 and DB66, those recommended by Brown and Holden (1999).

Genotypes were scored from autoradiographs and allele lengths (in base pairs) determined using a sequenced size ladder of M13 ssDNA. Allele frequencies at the nine loci were calculated from the pooled black and pooled white rhinoceros populations. Using these frequencies, and the alleles scored for the putative hybrid, assignment tests were performed following Paetkau et al. (1997).

Results

Cytogenetic analysis of the parental species

The G-banded and C-banded karyotypes of the white rhinoceros (2n = 82 and black rhinoceros (2n = 84) species have been described in detail elsewhere (Houck et al. 1994; Trifonov et al. 2003). The difference in diploid number between the rhinoceros species is due to a single fission event. This resulted in the second largest autosomal chromosome in white rhinoceros genome (*Csi* 2) being represented as two distinct elements in the black rhinoceros (*Dbi* 2 and *Dbi* 41; (Figure 1a, b).

Cytogenetic confirmation of the F1 hybrid

We have previously shown that a painting probe containing both the co-sorted *E. burchelli* (*Ebu*) X and *Ebu* chromosome 8 hybridises to three white rhinoceros chromosomes (*Csi* X, *Csi* 2 and *Csi* 37), and to four in black rhinoceros (*Dbi* X, *Dbi* 2, *Dbi* 37 and *Dbi* 41, Trivonof et al. 2003). The X was identified using a painting probe derived from the

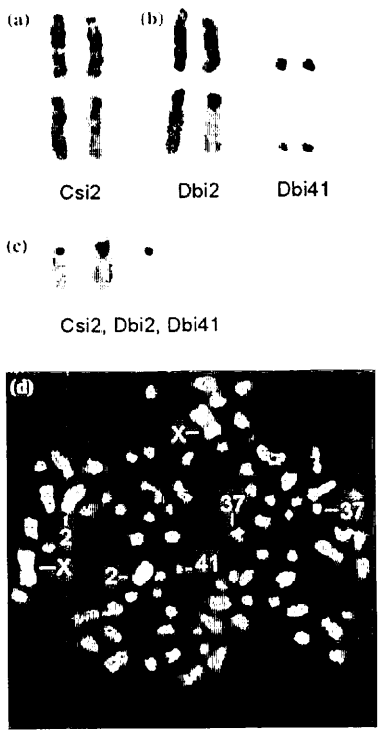

Figure 1. (a) White rhinoceros chromosome pair *Csi* 2 is represented as two separate pairs (b) in the black rhinoceros (*Dbi* 2 and *Dbi* 41). G-banded chromosomes in the upper row with the corresponding C-banded patterns beneath these. (c) The hybrid had a single copy of each of the diagnostic *Csi* 2, *Dbi* 2 and *Dbi* 41 chromosomes and showed species-specific differences in C-band patterns. (d) *In situ* hybridisation of a Burchell's zebra painting probe containing the co-sorted X chromosome and *Ebu* 8 to a metaphase spread of the putative hybrid. Signal is limited to a single copy of each of the diagnostic *Csi* 2, *Dbi* 2 and *Dbi* 41 chromosomes as well as to both X chromosomes and autosomal pair 37.

horse *E. caballus* (*Eca*) (Yang et al. 2003) allowing the isolation of the homologues of *Ebu* 8 in *C. simum* (*Csi* 2 + *Csi* 37) and *D. bicornis* (*Dbi* 2 + *Dbi* 37 + *Dbi* 41) respectively. In contrast to the two rhinoceros species, however, but consistent with its putative hybrid status, the calf's karyotype comprised a single copy of each of the diagnostic *Csi* 2, *Dbi* 2 and *Dbi* 41 chromosomes (Figure 1c, d). Additional support is provided by the C-band patterns of the two species. In general, the black rhinoceros has larger amounts of pericentromeric constitutive heterochromatin (Trifonof et al. 2003) than its white counterpart (Houck et al. 1994) as is clearly reflected in pair 2 (Figure 1c).

Microsatellite analysis

The hybrid was heterozygous at all nine test loci a feature not seen in any of the other rhinoceros specimens included in the study. This gave an observed heterozygosity of 1.0, compared with mean values of 0.52 for black and 0.32 for white rhinoceroses. Moreover, alleles private to both black rhinoceros and white rhinoceros were found at several loci (Table 1), the mean number of alleles per locus being 7.8 for black and 2.7 for white rhinoceroses. From a total of 18 alleles detected in the hybrid, six were private to *D. bicornis* and four to *C. simum*.

Assignment tests, in which the product of each individual's allele frequencies were compared with the overall frequency in either the black or the white rhinoceros population, showed that all black rhinoceros specimens (n = 117) were correctly assigned to the total *D. bicornis* population with a median likelihood value of 4.4×10^{-9}. All white rhinoceros (n = 7) were correctly assigned to the total *C. simum* population with a median likelihood value of 1.7×10^{-3}. The minimum likelihood ratio for assignation of a *D. bicornis* specimen to the *C. simum* population was 1.5×10^{7}; the corresponding value for a *C. simum* specimen being assigned to the *D. bicornis* population was 2.1×10^{16}. The hybrid showed a likelihood ratio of belonging to the *C. simum* sample population as opposed to the *D. bicornis* population of 1.1. Statistically, therefore, the hybrid was almost equally likely to belong to the one species as to the other. In summary, the results from all three genetic markers (karyotype, FISH and microsatellites) are fully consistent with the calf's F1 status as the inter-

Table 1. Allele sizes at nine microsatellite loci in the hybrid marked with B or W if private to Black or White rhinoceros, respectively

BR4		BR6		BR17		DB1		DB14		DB44		DB49		DB52		DB66	
129	109	152	142	133	117	132	130	282	274	182	174	163	155	222	212	201	199
B	W	–	–	B	W	B	W	B	W	–	–	–	B	–	B	–	–

specific progeny of a black × white rhinoceros cross.

Discussion

The most prominent phenotypic distinctions between the two African species are that *C. simum* has a square upper lip, a pronounced nuchal hump when the head is raised and several cranial characteristics that differ from *D. bicornis* with its pointed and prehensile upper lip, and no nuchal hump (Smithers 1983). The hybrid showed an admixture of phenotypic traits that are suggestive of its unique parentage (Figure 2).

What does this instance of hybridisation between *C. simum* and *D. bicornis* hold for the broader conservation of these species, and what is the degree of reproductive compatibility between them? Regrettably we have no idea whether hybrids of the black and white rhinoceroses show reproductive impairment. The animal was culled prior to reproductive age because it was thought by the Zoo's management to be a hybrid (at that point not proven), and therefore of no breeding or conservation value. The National Zoological Gardens also did not want to sell the animal given its suspected hybrid status, and all the more so since the hybrid was female and it is conceivable that females escape meiotic disruption whereas spermatogenesis is impaired in male hybrids (Haldane 1922).

This aside, the fact that hybridisation can occur between the critically endangered black rhinoceros and its numerically superior sister species, the white rhinoceros, suggests that assisted reproductive technologies could possibly be successfully applied to their conservation. Interspecific embryo transfer between species in which white rhinoceros cows are used as surrogate mothers for black rhinoceros embryos, or even possibly for the more endangered Sumatran and Javan rhinoceroses, may be a future reality. Clearly, although the Asian rhinoceroses could potentially benefit from embryo transfer, the genetic distance is greater (Tougard et al. 2001), and compatibility in terms of reproductive physiology is moot.

In the African context, however, particularly given the reality of small, heavily managed populations, the potential for some degree of infertility resulting from hybridisation, let alone the threat of introgression, is of concern. These findings have bearing on whether conservation efforts should be directed at protecting populations in their natural habitat (Leader-Williams 1993), or whether these should be devoted, at least in part, to captive breeding strategies and reintroduction (Foose 1993; Stanley Price 1993). Despite the recent successes with *in situ* conservation of the African species (Emslie and Brooks 1999), and given economic arguments that the protection of rhinoceroses is

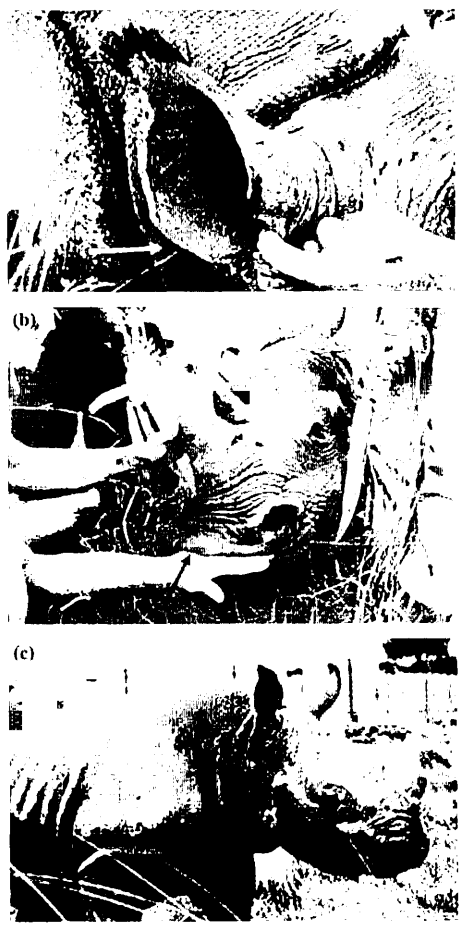

Figure 2. Phenotypic characteristics of the F1 rhinoceros hybrid. (a) Ear shape of the hybrid closely resembles that of a black rhinoceros with the posterior lobe rounded (arrowed) as is typically the condition for the black rhinoceros. (b) The hybrid had a fairly wide upper-lip more in keeping with that of the white rhinoceros, but it also exhibits a small upper-lip protrusion (arrowed) not unlike the prehensile upper-lip in black rhinoceros. (c) Head length in the hybrid was intermediate between the two African rhinoceros species.

expensive justifying the consolidation of both within the same protected area, there is clearly a cautionary tale. This is that under conditions of compressed densities and spacing that may characterise captive and semi-wild populations (Leader-Williams 1997) and those on small, privately owned reserves whose focus is ecotourism, prudent management should ensure that access to conspecific mates is unrestricted, and that the composition of populations is monitored. Where this is not possible the physical isolation of the African rhinoceros species should be considered.

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