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USE OF MOLECULAR GENETICS IN RHINOCEROS CONSERVATION

E H Harley and C O'Ryan*

This paper will describe the different molecular genetic approaches which can provide information of value to conservation managers as well as some practical aspects relating to collection of material, costs, time to obtain results, etc.

Molecular genetics uses the differences in either proteins (allozymes) or DNA to provide information about differences between individuals, populations or species. The newest advances all involve DNA and these will constitute the primary focus of this discussion.

DNA in animals is found in two forms: nuclear DNA, which comprises some 99,9% of the total DNA in the cell, and mitochondrial DNA (mitDNA), which although small in quantity relative to the nuclear DNA, has some features which render it especially useful for certain applications. Analysis of DNA firstly requires selecting a region of DNA of appropriate size and structure for the comparison in question. This can be done in one of three ways:

1. Purifying that region of DNA. This can be done fairly easily for mitDNA provided sufficient material (e.g. 10 or more grams of heart muscle or fresh liver) is available, but this usually requires *post mortem* material or an aggressively invasive biopsy.

2. Using a DNA probe, usually radiolabelled, in a "Southern Blot" analysis, to hunt out and display its counterpart in the sample DNA preparation. This time-consuming method is tending to be replaced by newer methods (see below).

3. Using the Polymerisation Chain Reaction (PCR), a very powerful method which combines extreme specificity for the region of DNA of interest with extreme sensitivity in being able to amplify the target sequence over a million fold to make it available for subsequent analysis. The surprising ease, efficiency, and versatility of the PCR method are making it the method of choice for many applications.

Having selected a region of DNA, which will usually consist of a gene or non-coding region about which a lot is already known for other species, it may be analyzed by one of two main methods:

1. Gel electrophoresis to define the size of the DNA. Frequently this is done after cutting with a restriction enzyme to generate a number of fragments, the variation in number and size of which provide information on the amount of genetic differentiation between the samples.

2. Sequencing. This provides the ultimate genetic information about the region in question and is amenable to many methods of further statistical analysis.

MtDNA has a number of features which suit it to specific applications: it is of quite small size (about 16 000 base pairs in length), is circular, and exists in multiple copies per cell making it relatively easy to isolate. It is maternally inherited, shows no recombination, and evolves 5-10 times faster than most nuclear DNA sequences. The complete sequence of the mitDNA is known for an increasing number of organisms, including representatives of most mammalian orders (and including the white rhinoceros). These features make it useful for comparisons at both the between species and between subspecies level, so it has been widely used for construction of phylogenetic relationships and for identification of cryptic species. We have used a restriction enzyme-based

* Department of Chemical Pathology, University of Cape Town, Rondebosch, 7700, Rep. of South Africa

approach to define both between and within species variation for the African members of the Rhinocerotidae.

In the former case we measured[1] the genetic distance between black and white rhinoceros to estimate time of divergence of these two African species as about 3,5 million years before the present, a calculation based on the assumption of a relatively constant rate of evolution of mitDNA within most orders of the mammalia. Of more direct conservation relevance, studies using individuals from several black rhino subspecies[2,3] have shown that the genetic distances between the subspecies *minor, michaeli,* and *bicornis* are small, despite which fixed within-subspecies restriction site differences provide markers characteristic of the subspecies, and which could be used for the identification of e.g. captive individuals of uncertain origin. The measurement of genetic distance between the subspecies also enables recommendations to be made for both *in situ* and *ex situ* breeding programs. This has been addressed in detail elsewhere[3], but in summary suggests that wild populations should be managed as separate subspecific entities, and that this should also apply in captive breeding programs where this is possible, and especially if a breeding program has as its aim reintroduction to the wild, but that if no suitable mate exists for a captive individual, no problems from outbreeding depression would be anticipated from mating with another subspecies. This last point requires testing in a practical situation, however, especially since there are no published studies yet confirming the lack of chromosomal differences between the subspecies. For the two subspecies of white rhino, *Ceratotherium simum simum* and *C.s.cottoni*, the argument may be different since our preliminary results on individuals from Zaire and South Africa suggest a significantly greater degree of difference between the subspecies than that for black rhinoceros.

A useful practical application using DNA sequencing of PCR amplified mitDNA enabled us to provide forensic information (O'Ryan and Harley, in preparation) in a case where suspected poachers of white rhino had been found in possession of a blood-stained axe, which they claimed had only been used to slaughter a cow. DNA was amplified from the trace of blood and tissue from the axe and some 250 base pairs of sequence obtained. The sequence was compared with the corresponding sequences of rhino, human, and cow and showed a 100% match with the cow - in this case confirming the defendant's story. The implications for control of poaching, however, are obvious.

One of the most necessary requirements in conservation of dwindling numbers of any large mammalian species is a good method for quantitating both loss of genetic diversity and genetic subdivision (due to rapid genetic drift) in small, fragmented populations. Allozymes have, until recently, provided the only effective way of doing this, but suffer from the problem of insufficient variability to be ideal for the purpose. A powerful new method using nuclear DNA has recently been developed[4] which uses PCR to isolate individual highly variable loci termed microsatellites (named because they consist of very short repeats). These can provide typically between two and ten different alleles per locus and analysis of populations at a number of microsatellite loci (10 is ideal) enables excellent measures of heterozygosity and population differentiation to be obtained. The data is very clean and enables the rich diversity of population genetic theory to be applied in a way which was not possible with older methods such as DNA fingerprinting (or with newer, but theoretically more suspect, methods such as RAPDs). We have recently completed a microsatellite study on buffalo populations in Southern Africa (O'Ryan et al. in prep.) which show heterozygosities as high as 0,75 (on a scale of 0 to 1), and are in the process of performing a similar study on rhinoceros, to complement our previous mitDNA study[3].

Collection of samples for genetic studies can be tedious and time-consuming for those in the field. Methods are, however, being improved so that many studies now require less invasive procedures. Over the last few years we have established over 100 cell lines from black and white rhinoceros (using the ear nicks taken for marking purposes) which provide a unique genetic resource for present and future studies. Cell cultures are very expensive to establish, however, and would not be the standard route to follow for most studies on a new species. The basic molecular genetics required to characterise a species mitDNA or microsatellites is very time consuming, labour intensive, and expensive, but fortunately for the black rhinoceros and some bovid species much of this groundwork has now been done so that applied studies to address specific questions at the population or individual level will now be easier.

A typical study on the population genetics of a fragmented population, assuming microsatellite primers are available, and that samples had already been taken and stored, would cost several thousand Rands and take a skilled laboratory worker several weeks of full-time work. As a consequence most studies to date with conservation relevance have been spin-offs from academic studies in University laboratories funded by research grants. Contract work is likely to develop, however, as the value of the genetic information to conservation management becomes apparent. On the positive side, sampling of individuals for a microsatellite study will require only a few mℓ of blood or a very small biopsy for extraction of DNA, and even museum skin is amenable to analysis allowing retrospective studies of genetic diversity. Use of these techniques will also enable paternity (or maternity) to be established for an individual, and measures of population and individual heterozygosities in captive and wild populations can help in decision making with respect to translocations or mate choice, and in monitoring the extent of inbreeding and population subdivision.

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