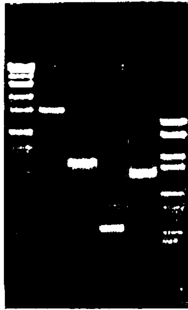

FAECAL DNA ANALYSIS WHAT DOES IT OFFER TO SAVE JAVAN RHINO?

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Molecular Approaches in Conservation

The Javan rhino, one of the rarest large mammals on earth, has received much attention for the last thirty years. Its only viable population in the Ujung Kulon National Park (UKNP) is poorly understood. Reliable data on the actual and effective population size, gender ratio, population structure, genetic variation, inbreeding depression etc. is not available. This information is, however, critical for designing sound management plans to conserve small population of an endangered species.

The main constraints to study the Javan rhino are its dense habitat and its tendency to avoid humans. Efforts have been made by using animal tracks such as (Schenkel and Schenkel 1969, Amman 1985 are two examples) and camera trapping (Griffith 1993). Both techniques have contributed to our understanding of Javan rhinos. However, their relatively high deviation gives rise a number of questions or scepticism, particularly when higher precision of data is needed for conservation management.

Meanwhile, an important development of molecular biology in the last three decades has brought the possibility (for the conservation biologist) to work on molecular-level characterisations of genes and their protein products. These molecular methods have opened for scrutiny the genetic diversity spectrum in any hierarchical levels ranging from micro-genetic diversity to macro-evolutionary. For instance, in the case of within-population diversity the frequently asked questions are : *What is the level of heterozygosity? Is the genetic variation decreased in the endangered species? Should the population be managed to increase the genetic variation and how? How serious a problem is inbreeding depression?* In the parentage and kinship case: *Who has bred with whom? How does this effect breeding and social structure? What is the effect of the mating system on effective population size and inbreeding?* And in the macro scale of

phylogeny: *What are the phylogenetic relationships of the endangered species and other and higher taxa?* (Avise 1996).

The immediate objective of conservation biology is to save endangered species from extinction, the intermediate objective is to make sure that the species is able to cope with the changing environment, while the ultimate goal is to allow the speciation process. These objectives cannot be separated from genetics. To adapt to environmental changes, a species trades to a great extent on its genetic diversity. For population geneticists, evolution is simply a change in allele frequencies within a population. The fittest individual passes on the most offspring, thus its genes or allele frequencies, to the next generation. Furthermore, reproductive success depends on complicated interactions between environmental variables and genome (Berry 1979). Only when these interactions are understood will significant advances take place in ecology and its applied arm, conservation management.

As biodiversity is the ultimate subject for conservation biologists, what we want to avoid is the loss of diversity. So when conservation biologists talk about preserving biodiversity they also imply protecting genetic variation. Below species level, diversity means genetic variation. DNA is the substance that makes up the organism which eventually composes the population. Variation among individuals may be due to genetic differences, environmental differences or interactions between them, and figuring out where the variation comes from is a significant part of what genetics is about. Identifying genetic variation to understand the differences between species and populations is the mainstay in conservation genetics.

The cheetah case is perhaps the best example of the potential for hidden hazards that threaten small populations from within. Known as the least genetically variable species among 37 members of the cat family, the cheetah has reproduction problems from its frequently found abnormal sperm in both free-ranging and captive populations. An extreme mortality of cheetah from outbreaks of a nearly benign domestic cat virus is also interpreted to be a consequence of its genetic homogeneity (O'Brien et al 1985). Immunological loci, particularly the MHC (Major Histocompability Complex) are highly variable in other feline and mammalian species. Another case is from Florida panther (*Puma concolor coryi*) which also has less genomic variation than other puma and where

sequences, for instance haemoglobin (Figure 1), show different band position, thus it tells us a variation.

Later molecular methods deal directly with the DNA, to name a few of them: RFLP (Restriction Fragment Length Polymorphism), Southern blotting, DNA fingerprinting. The procedure involves extracting the DNA substances from tissues, purifying the DNA, digesting them with restriction enzymes of which different length fragments will be produced, placing them on gel and running the electrophoresis. Then the gel is ready to be analysed. This paper will discuss a technique called PCR (Polymerase Chain Reaction) for its appropriate application to faecal DNA analysis.

Organismic phenotype	Genotype	Positions to which haemoglobin have migrated		Origin	Haemoglobin types present
Sickle-cell trait	$Hb^S\ Hb^A$	▭	▭	□	S and A
Sickle-cell anaemia	$Hb^S\ Hb^S$		▭	□	S
Normal	$Hb^A\ Hb^A$	▭		□	A

Figure 1 Electrophoresis of haemoglobin from an individual with homozygote sickle-cell anaemia, heterozygote (called *sickle-cell trait*), and a normal individual. The filled boxes show the positions to which the haemoglobin migrate on the gel (Griffiths et al 1996)

PCR was developed in 1983 and since then it brought a new horizon in molecular genetic analysis. This revolutionary technique permits genetic analysis to work on a small, degraded tissues such as fossil or hair of historic collections. A project to study the humpback whale has been successfully carried out of which DNA is extracted from sloughed skin cells of the animal while it swims. Divers simply swam at the back of the whale collecting the sloughed skin cells.

DNA has a double helix structure which is joined hydrogen bonds (G-C and A-T). This double-stranded structure can be separated into two single-stranded DNAs by heating. These two single-stranded DNAs will then serve as templates to create two new double-stranded DNAs. A primer (a very short section of a single-stranded DNA which is artificially made) then is added. It will find itself in the complementary position of the single-stranded, template DNA. This position will be the starting point for the primer to amplify the DNA which works as the template given appropriate DNA polymerase enzyme is available (see Figure 2). Once it has started, the amplified DNA will serve as

template DNAs for further amplification. Thus from one double-stranded DNA the reaction results in two, four, eight, eighteen, thirty six and so on. After twenty cycles, there are more than one million DNAs formed.

PCR is obviously useful when obtaining only a very small amount of substrate DNA, which is often the case for conservation biologists. Furthermore, no restriction digestion of the genome is needed because the primers will home in on the appropriate sequence; no lengthy cloning procedure is necessary since enough DNA is amplified so that clear bands on a gel is produced (Griffiths 1996). Consequently, the cost of the analysis is reduced. However, there are important points which should be kept in mind when applying this method, i.e. a specific primer that recognise the DNA of the given species might be needed. Universal primers may amplify "junk" DNA and nonhomologous loci (Palumbi and Baker 1996).

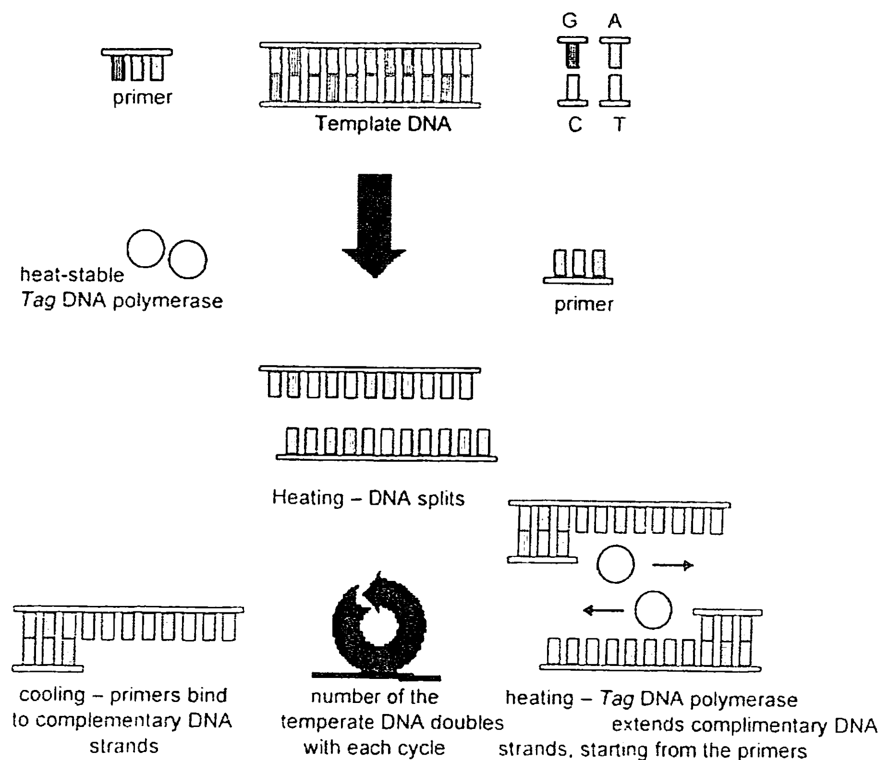
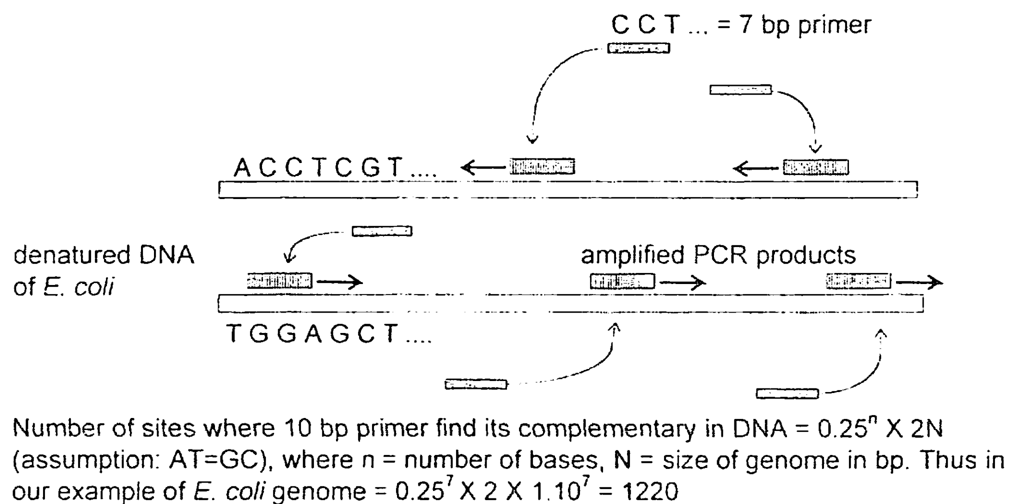


Figure 2 Schematic diagram of PCR reaction

Another recently developed, powerful PCR-based technique is RAPD (Random Amplification of Polymorphic DNA, pronounced "rapid") which requires very minimal knowledge about the species intended to study, is very cost-efficient and perhaps the easiest method. With this recently developed assay, DNA is amplified with any designed,

short primer, thus the different regions of genome are amplified in a random basis. In some places where the primer anneals, there will be two priming sites by chance close enough together and on opposite strands of the DNA that cycles of PCR will amplify the DNA between the primers. The result is a set of different-sized, amplified bands of DNA (Figure 3). However, this may involve a number of trials to find suitable primers that will show the polymorphic fragments.

RAPD, if applied correctly, can be used to survey genetic differentiation among populations, identify species on the basis of DNA pattern, scan polymorphic DNA sites, analyse paternity, and study phylogeny of closely-related species.



Number of sites where 10 bp primer find its complementary in DNA = 0.25^n X 2N (assumption: AT=GC), where n = number of bases, N = size of genome in bp. Thus in our example of *E. coli* genome = 0.25^7 X 2 X 1.10^7 = 1220

Figure 3 A rationale of RAPD assay

PCR Application to Conservation

One of the very powerful PCR application in many fields, including conservation, is DNA fingerprinting. It focuses on a specific section of gene, called VNTR (variable number tandem repeats) or minisatellite DNA which is repeats of short nucleotide sequences. VNTRs show variable number of different loci; in humans they spread 1 to 5 kb (kilo base pairs) sequences consisting of variable numbers of a repeating unit 15 to 100 nucleotides long. A PCR primer can be used to anneal at the edge of the satellites and amplifies them. They can then be separated by electrophoresis, and the set of fragments shown up are treated as a fingerprint to the individual (Figure 4). VNTRs are highly polymorphic in the number of repeats (Jeffreys et al 1985). Each individual of a

species has its specific number of repeats. As an illustration, minke whale (*Balaenoptera acutorostrata*) populations showed polymorphisms up to 98% (van Pijlen et al 1995). A procedure of using VNTRs in conservation is summarized in Figure 5.

Since then it has been used in a wide range of applications: forensic investigation (the opera-like O.J. Simpson's case for instance), population genetics and pedigree analysis: reproductive success and breeding strategies (e.g. Burke 1989, Packer et al 1991), paternity (e.g. Nybom and Schaal 1990, Rassmann et al 1996 - using microsatellites), and also population structure (e.g. van Pijlen et al 1995). Many findings from this research have contributed to conservation management. Wauters et al (1994) in their study of red squirrel (*Sciurus vulgaris*) for instance, found that the loss variability in isolated populations was due to reduced immigration (conservation management thus needs to encourage outbreeding). DNA fingerprinting can also identify a particular animal tissue comes within a population, but certainly the world-wide population DNA has to be obtained beforehand.

Matching identities of an individual from a population, which is the forensic case, is as simple as comparing the suspected DNA fingerprints with the evidence. The more bands shared, the higher probability they come from the same individual. We can also calculate the likelihood of the fingerprints match by chance. In U.S. legal forensic code, minimum shared bands are eight. If your DNA fingerprints match eight bands with the evidence DNA, you better find your identical twin because you are as unique as one in eighteen billion persons in the world. The chance of having bands match is $P = x^l$ where x is the mean unrelated bandsharing coefficient (0.069 for human) and l is number of shared bands. In paternity study, sharing bands of DNA fingerprints are used to determine the offspring's father (see Figure 6).

To illustrate the PCR application clearly, we will see a conservation case where PCR has been used. The forensic identification conducted by Baker and Palumbi (1996) will be our example. The International Whaling Commission produced a commercial whaling restriction in 1985/86. However, whaling continues mainly for scientific reasons and the animals killed can be legally sold, thus sustaining the commercial market. Some species are protected internationally and some can be hunted in some regions. Earthtrusts, an international NGO, called a trade monitoring to detect if whale meat sold

in the market was from legal "scientific" hunting and from regions where hunting is allowed.

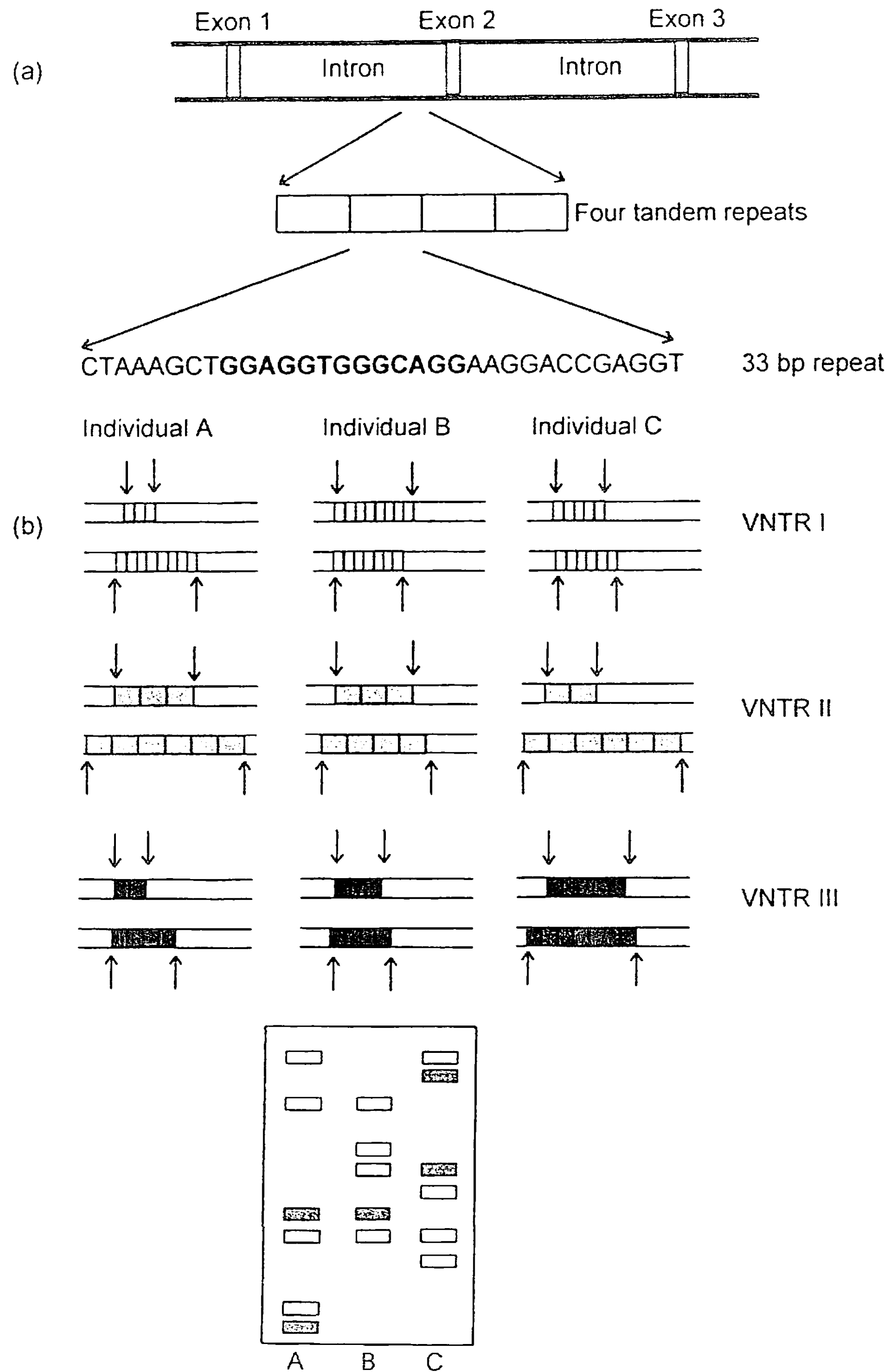
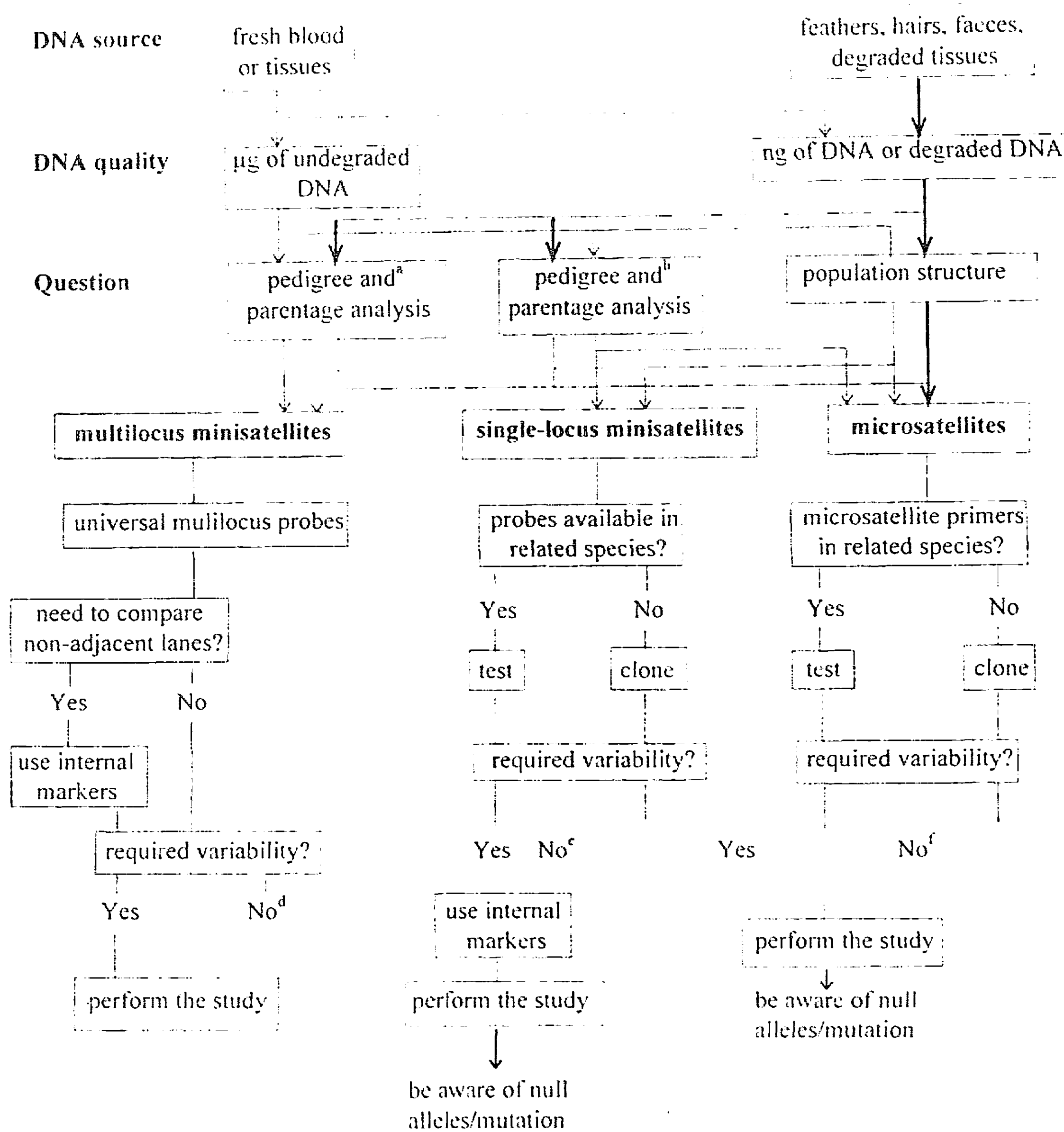


Figure 4 Obtaining a DNA fingerprint using a VNTR probe. (a) Preparation of the probe. The first intron of the gene has four repeats of the sequence shown, which contains a 13 bp core sequence (in bold). This core sequence is found at other VNTR loci, labelled VNTR I, II, III in this simple diagram. (b) The number of repeats at the three VNTR loci with the core sequence. The Southern blot has been probed with the 33 bp repeat in (a) and shows the DNA fingerprints of the three individuals (from JD Watson et al 1992)



a initial and small scale studies
b where many individuals or parents have to be tested
c for less genetically variable populations
d try additional multilocus probes, new restriction enzymes
e isolate new minisatellites loci or use microsatellite loci
f isolate new microsatellite loci or use minisatellite loci

Figure 5 Flow chart to emphasise the key factors to be considered when deciding which VNTR locus system to use in a population study (from Burke et al 1996)

To monitor the trade, PCR was the most suitable method to extract the substrate DNA from any dried meat to raw slice meat, skin or blubber sold in the market, and VNTRs were used as the marker of geographic origins. Sixteen samples of whale products from the Japanese market between February and April 1993 were identified.

The result was that eight samples were from minke whales, four from fin whales, one from either minke or humpback whales, one was either sperm whale or the harbour purpose, the rest was from the Delphinidae family (meaning dolphin meat was claimed by some Japanese retailers as whale meat). The minke whale products were likely from two oceanic populations (North Atlantic and Australia-Antarctic), fin whale products were from Iceland, Mediterranean, North Atlantic. After having checking ICW records of legal hunting, the researchers were confident to conclude that some whale products available on the Japanese retail market were from illegal sources.

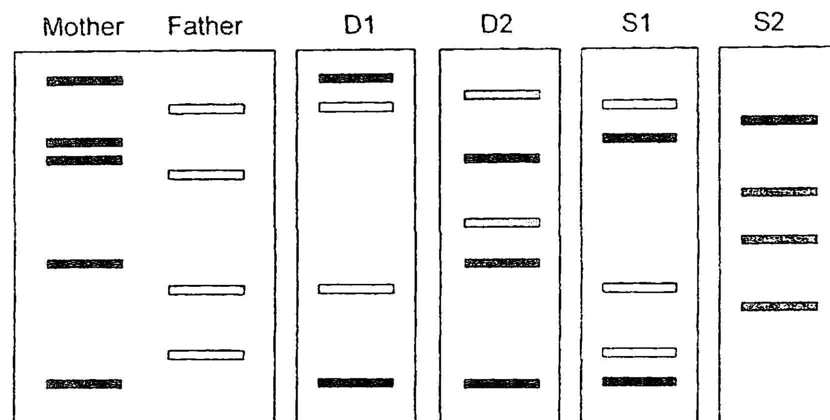


Figure 6 DNA fingerprints of parents with their two daughters and sons. A person's VNTRs come from genome donated by the father and the mother. D1 and S1 is the biological children of the parents, D2 is the father's step-daughter (a child of the mother and her former husband), S2 is their adopted son.

Faecal DNA Analysis of Javan Rhino

The application of PCR in Javan rhino conservation will give us the actual population size and gender ratio as well as better a understanding of home range, spatial distribution, paternity, relatedness between individuals, genetic variation and inbreeding depression. Using DNA fingerprinting techniques, we are able to differentiate accurately one individual from another while at the same time scan the sharing bands between the individuals.

The tissue collection will not employ immobilisation or anaesthesia. The DNA will be extracted faeces, thus PCR will be employed. What we aim to get from the faeces is epithelial cells sloughed from the internal wall of intestine. The project is expected to

start in July 1997 and end no shorter than three years. An initiation phase to verify the most cost-effective methods of faeces collection and PCR is needed. The PCR and DNA fingerprinting technique will be carried out by the Central Science Laboratory of the British Ministry of Agriculture, Food and Fisheries. The lab has had experience in extracting DNA from rat faeces. In the first phase, the lab will look for a method to amplify the faecal DNA.

Sample collection will employ the blanket cover technique in the peninsula. A number of teams each comprised of a biologist, a national park ranger and one or two locals needs to be initiated. The teams will track rhinos while collecting faeces and other related data such as footprints and urination. By combining appropriate field observation methods and DNA analysis, a comprehensive database will be produced. The database will also use GIS as a helping tool in home range and spatial distribution analysis. It should be noted that the occurrence of intensive tracking teams in the Ujung Kulon peninsula will also increase park protection.

Conclusion

Having understood the usefulnes as well as the shortcomings of molecular genetic approaches in conservation, we can be optimistic that much accurate population data on Javan rhinos will be gathered. However, a collaboration with a number of institutions, both governmental bodies and NGOs is needed in order to, first, secure funding (approximately USD 350,000 for a three-year project) by putting this study as a priority of Javan rhino conservation work; second, to establish field teams (approximately four teams) which will intensively work in the Ujung Kulon peninsular; and third, share data and information between different Javan rhino projects so that a more comprehensive understanding of the animals needed to design conservation management will be soon available.

Reference

Avise JC (1996) Introduction: The Scope of Conservation Genetics. In JC Avise and JL Hamrick (eds.) *Conservation Genetics: Case Histories From Nature*. New York: Chapman & Hall.

Ayala FJ (1976) *Molecular Evolution*. Sunderland, Mass: Sinauer.

Baker CS. RW Slade, JL Bannister, RG Abernethy, MT Weinricyh, J Lien, J Urban, P Corkerron, J Calambokidis, O Vasquez and SR Palumbi (1994) Hierarchical structure of mitochondrial DNA gene flow among humpback whales *Megaptera novaeangliae*, world-wide. *Molec. Ecol.* 3:313-327.

Bigg MA, PF Olesiuk, GM Ellis, JKB Ford and KC Balcomb (1990) Social organisation and genealogy of resident killer whales (*Orcinus orca*) in the coastal waters of British Colombia and Washington State. *Rep. Int. Whal. Comm.* Special Issue 12:383-406.

Bonnell ML and RK Selander (1974) Elephant Seal: Genetic Variation and Near Extinction. *Science* 184: 908-909.

Burke T, O Hanotte and I van Pijlen (1996) Minisatellite Analysis in Conservation Genetics. In TB Smith (ed.) *Molecular Genetic Approaches in Conservation*. Oxford: Oxford University Press.

Burke T (1989) DNA fingerprinting and other methods for the study of mating success. *Trends. Ecol. Evol.* 4:139-144.

Griffiths AJF, JH Miller, DT Suzuki, RC Lewontin and WM Gelbert (1996) *An Introduction to Genetic Analysis*. Sixth Ed. New York: WH Freeman and Co.

Hedrick PW and PS Miller (1992) Conservation Genetics Techniques and Fundamentals. *Ecol. Appl.* 2:30-46.

Hedrick PW (1996) Conservation Genetics and Molecular Techniques: A Perspective. In TB Smith (ed.) *Molecular Genetic Approaches in Conservation*. Oxford: Oxford University Press.

Jeffreys AJ, V Wilson and SL Thein (1985) Hypervariable 'Minisatellite' Regions in Human DNA. *Nature* 314:67-73.

Lewontin RC (1974) *The Genetic Basis of Evolutionary Change*. New York: Columbia University Press.

O'Brien SJ, DE Wildt, D Goldman, CR Merril and M Bush (1983) The Cheetah Is Depauperate in Genetic Variation. *Science* 221:459-462.

O'Brien SJ, JS Martenson, S Miththapala, D Janezewski, J Pecon-Slaterry, W Johnson, DA Gilbert, M Roelke, C Packer, M Bush, DE Wildt (1996) Conservation