THE SOURCES AND USES OF GENETIC MATERIAL IN THE STUDY OF *PETROGALE* (ROCK-WALLABIES) AND OTHER MAMMALS.

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THE application of molecular genetic techniques has the potential to greatly increase our knowledge of rock-wallaby (*Petrogale*) natural history, ecology, behaviour, evolution and conservation. Although several have recommended the use of molecular techniques on *Petrogale* (Kennedy 1992; Hall and Kinnear 1992; Maxwell, Burbidge and Morris 1996), relatively few studies have been published (e.g., Sharman, Close and Maynes 1990; Bee and Close 1992; Spencer, Odorico, Jones, Marsh, and Miller 1995; Pope, Sharp and Moritz 1996; Eldridge 1997; Eldridge and Close 1997). However many more studies are currently in progress. As the molecular genetic study of *Petrogale* is still in its infancy most of the examples used in this review concern investigations of other marsupials and eutherians. This however has the advantage of illustrating the universality of these techniques and will also make this review of interest to those working with other mammals.

Molecular genetic techniques are becoming increasingly useful to wildlife managers, ecologists and behaviouralists, as well as conservation and evolutionary biologists. However the great diversity of techniques and the associated proliferation of jargon and acronyms can be discouraging to those wishing to utilize these technologies for the first time. This paper therefore seeks to give a broad introduction to the most common molecular genetic techniques and summarise their uses. We also hope to describe the most appropriate sources of genetic material and their method of collection and storage. This paper is by no means exhaustive and we refer readers to recent publications which examine in detail the methodology, advantages, limitations and cost effectiveness of many of these techniques (Baverstock 1989; Hillis and Moritz 1990; Sherwin 1991; Moritz 1992; Bruford and Wayne 1993; Avise 1994; Smith and Wayne 1996).

As the establishment costs for many molecular genetic techniques are high it will often be easier for researchers who want samples analysed to collaborate with an established molecular genetics laboratory. The recipient laboratory should always be contacted well in advance so that the most appropriate methods of tissue collection, preservation and transportation can be recommended, along with advice on the required sample size and sampling strategy. It is often extremely useful for the recipient laboratory to also have one or two large tissue samples which can be used to trial and adapt the required technique/s to the species of interest. Large tissue samples are mostly acquired opportunistically and are usually derived from an animal that is unexpectedly found dead or has to be euthanased. Although these situations are unfortunate, they can provide an extremely valuable and large collection of tissues that would be otherwise unobtainable. This is particularly relevant for researchers who work with rare or little known species, especially in remote areas which are often poorly surveyed. Even if no laboratory is immediately interested in receiving the samples, opportunistic collections should still be made at every opportunity and the tissue (along with the skin and skull from the specimen) should be lodged with an appropriate museum. Several Australian museums have impressive storage facilities for tissues and more are currently being established (Moritz 1992). With many species continuing to decline, or likely to decline in the future, opportunistic collecting should be undertaken whenever possible because such samples may prove to be unobtainable in the future. It should also be noted that many regional wildlife parks, research facilities and zoos house species of native fauna that are poorly known (scientifically) and are rarely sampled.

GENETIC MARKERS

Genetic markers are identifiable characters that are passed from parents to offspring (i.e. heritable) and can be used to differentiate between individuals or populations of an organism. Initially genetic studies of animals used morphological features such as size, shape and colour. However from the 1960's biological macromolecules (e.g. proteins and DNA) have increasingly played an important role in the study of genetics and evolutionary biology (Moritz and Hillis 1990). The techniques developed to examine these molecules have generated a whole new area of biology, molecular biology (or molecular genetics). Molecular genetic techniques reveal an array of variable, heritable markers that can be used to examine genetic variation on a variety of levels and address diverse questions. Although variable markers are the "bread and butter" of molecular genetics, it is important to use markers with an amount of variation that is appropriate to the question being addressed. For example, if you wished to know whether species A was more closely related to species B or species C, then a highly variable marker may simply tell you that all three species are different from each other, while a marker with very low variability may tell you that all three species are essentially the same. In either case there is no satisfactory answer to the original question. What would be required in this situation is a moderately variable marker/s.

Molecular genetic techniques most commonly examine gene products (usually proteins) or genes themselves (i.e. DNA). Some techniques examine the variation present in one gene at a time (single-locus), while others simultaneously examine variation in many genes (multi-locus). Multilocus techniques (e.g. DNA fingerprinting) have the advantage of providing large amounts of information relatively quickly but are often difficult to interpret and analyse (see 3b). On the other hand single-locus techniques (e.g. microsatellites) may require more time to generate large data sets but these data are often easier to interpret and analyse using standard population genetic theory and parameters (see 3e).

MOLECULAR GENETIC TECHNIQUES

1. CYTOLOGY: THE EXAMINATION OF CHROMOSOMES

The DNA found within the nucleus of vertebrate cells consists of a series of very long molecules called chromosomes. Each chromosome carries a specific set of genes arranged in linear order. When a cell is about to divide the DNA, which is usually dispersed throughout the nucleus, becomes highly compacted and as a result each chromosome becomes visible as a small rod-like structure. The analysis of chromosomes is a relatively straightforward process in most vertebrates (Sessions 1990) and is mostly used in taxonomic, phylogenetic and evolutionary studies (Table 3) (e.g., Eldridge, Johnston and Close 1991; Eldridge and Close 1992; Eldridge, Johnston and Lowry 1992). It can also be useful in detecting cryptic species and hybrid zones (e.g., Briscoe, Calaby, Close, Maynes, Murtagh and Sharman 1982; Eldridge and Close 1992), as well as in sexing individual animals (e.g. new born pouch young). The usefulness of chromosome analysis is limited in those groups which show little or no chromosome variation, e.g. dasyurid marsupials (Rofe and Hayman 1985; Westerman and Woolley 1990).

The presence of cryptic species in *Petrogale* makes the field identification of many taxa very difficult (Eldridge and Close 1992). In many rock-wallaby species the traditional features used for field identification (e.g., skull/body morphology, coat colour and markings) are too highly variable to provide a reliable means of classification (Eldridge and Close 1992). Most *Petrogale* species, subspecies and races are however readily and most definitively identified by their karyotype; that is, the shape and number of their chromosomes (Sharman et al 1990; Eldridge and Close 1997). The discovery that the chromosomal complement tends to be constant within each *Petrogale* taxon but varies between taxa, has encouraged the development of techniques that should now enable any *Petrogale* population to be karyotyped, even those occurring in remote areas of Australia (Eldridge, Kinnear and Close 1994). Previous experience with *Petrogale* has shown that it is prudent to identify (via karyotyping) any previously unsampled population as the identity and chromosomal constitution of these populations can not always be assumed on the basis of geographic proximity to previously identified populations (e.g., Eldridge et al 1994; Close, Eldridge, Bell and Reside 1994). Also given the unparalleled genetic diversity found within *Petrogale* (Eldridge and Close 1997), it is possible that additional taxa await discovery and some of these may have highly restricted distributions.

To obtain a chromosome preparation living tissue is required in which the cells are dividing or can be induced to divide at a rapid rate. Although chromosomes can be obtained from a variety of tissues (including corneas and testes) the three most common sources are bone marrow, cultured lymphocytes and cultured fibroblasts (Table 1).

	Source of Tissue

	Recently dead animal		Long dead animal			Live (or recently dead) animal					
Uses	**Soft tissue**	**Bone marrow**	**Mummified remains**	**Bone**	**Museum skins**	**Ear/ Tail-tip**	**Blood**	**Hair**	**Semen**	**Urine**	**Faeces**
Chromosomes	+	+	-	-	-	+	+	-	-	-	-
Proteins:											
-Electrophoresis	+	-	-	-	-	+*	+	-	-	-	-
DNA:											
- RFLPs	+	-	-	-	-	+*	+	-	-	-	-
-Fingerprinting	+	-	-	-	-	+*	+	-	-	-	-
-Traditional Sequencing	+	-	-	-	-	+*	+	-	-	-	-
-PCR - RFLPs	+	-	+/-	+/-	+/-	+	+	+	+	+	+/-
-PCR - Sequencing	+	-	+/-	+/-	+/-	+	+	+	+	+	+/-
-PCR - Microsatellites	+	-	+/-	+/-	+/-	+	+	+	+	+	+/-
-PCR - RAPDs	+	-	?	?	?	+	+	+	+	+	?

Table 1. Sources of the most useful samples that can be obtained from *Petrogale* and other mammals for use in molecular genetic analysis. +, suitable samples; +/-, marginal samples; -, not suitable as samples. Recently dead animals are those from which samples can be collected within an hour (and preferably within minutes) of death. This includes collected specimens, euthanased animals and fresh road kills but also victims of predation, accidents etc. Long dead animals include collected specimens (usually a skin and skull) that have been deposited in museums and also mummified and skeletal remains often found in caves etc. * Ear and tail-tip biopsies can not often be used directly, but sufficient material for these techniques can be obtained from these samples if fibroblast cell lines are established (see Section 1c). N.B. The DNA and proteins in most soft tissues (particularly liver) will start to degrade once the animal has been dead for > one hour. This varies according to the size of the animal and the ambient conditions. However we have found that ear-biopsies from animals that have been dead for up to 10 hrs have produced viable fibroblast cell lines (see also Sherwin 1991).

a) Bone marrow

Obtaining chromosomes from bone marrow can be routinely performed by minimally equipped field researchers. It entails flushing the marrow from a suitable bone (in macropods usually the sternum or femur) with media containing a mitotic inhibitor (usually colchicine) and then treating the resultant cell suspension 1-2 hs later with hypotonic and fixative solutions. For a detailed protocol see Lee and Elder (1980) or Sessions (1990). The mitotic index, and therefore the number of chromosomes, can be increased by yeast pretreatment (Lee and Elder 1980). As this technique requires the sacrifice of the animal it is not always acceptable, especially when dealing with threatened species.

b) Cultured lymphocytes from peripheral blood

This procedure involves the short-term culture of lymphocytes (a type of white blood cell) obtained via a blood sample. For a general protocol see Sessions (1990) .

With macropods we have found that the best results are obtained using a modification of the method of Moorhead, Nowell, Mellman, Battips and Hungerford (1960). Using a 10 ml syringe that has been flushed with the anticoagulant heparin just prior to use, draw 5 - 10 ml of blood from the caudal vein. The blood should then be allowed to separate into upper (white blood cell) and lower (red blood cell) phases by standing the syringe upright (preferably at 37^{o}C) for 3 -5 hours. If necessary the settling of the blood can be aided by the addition of Dextraven 150 (1 : 4, Dextraven : blood). Once the blood is separated, 1ml of the white cell fraction (or up to 2ml if separation aided by Dextraven) should be added to 10ml of culture media (usually Hams F10 or Iscoves containing 10 - 15% foetal calf serum) and the tube incubate at 37^{o}C for 3 days (72 hrs). Prior to incubation, 0.2 ml of the mitotic stimulant PHA (phytohaemagglutanin) should be added to each 10 ml of culture media. Following 1-2 hours exposure to colchicine (1µg/ml final concentration) the cells can then be harvested according to the methods of Eldridge, Dollin, Johnston, Close and Murray (1988). In the field, lymphocyte cultures are usually incubated by keeping them close to the researcher's body (e.g., in an inner pocket); although they can be a nuisance and impede other field activities.

c) Cultured fibroblasts

The establishment of fibroblastic cell lines is the most expensive and labour intensive option. However, as the procedure results in a long-lived cell line that can be stored indefinitely and utilised as required it is worth the effort. Good fibroblast cell lines can also provide sufficient material for subsequent allozyme, and DNA studies (Table 1).

Fibroblasts can be established from a variety of soft tissues - kidney and diaphragm usually give the best results. We have had little success with heart and lung although other laboratories use these routinely for some species (Johnston and Eldridge unpublished data). The best results with live animals are obtained using ear biopsies from adults and tail tip biopsies from pouch young. Other laboratories routinely use skin biopsies or, for small mammals, toe clippings. Sterility is essential at all stages of these procedures as bacterial and/or fungal contamination will result in the failure of the culture. Therefore tissue biopsies are first placed in a collection media (which contains high levels of ant-bacterial and anti-fungal agents) for 3 - 4 hrs before being placed into a transport media for shipment to the laboratory. Fibroblasts from young animals tend to grow better than those from old animals. For a detailed description of methods see Cooper, Edwards, Sharman, VandeBerg and Marshall-Graves (1977) and Eldridge et al (1994).

For best results, tissue biopsies need to be transported to the laboratory as quickly as possible. However biopsies can remain viable for up to 2 weeks giving time for them to be transported from the field to the recipient laboratory (Eldridge et al 1994). During this time the biopsies must not be refrigerated or frozen but should be kept at ambient temperature (Table 2), avoiding obvious extremes. Tissue biopsies that have been frozen or preserved in alcohol can not be used in chromosome analyses but are useful for some types of DNA analysis (see Table 2).

2. PROTEIN ELECTROPHORESIS

Protein electrophoresis utilises variation in the electrophoretic mobility of specific blood and tissue enzymes (i.e., gene products) to indirectly estimate the level of variation in DNA sequences at individual gene loci. Changes in protein mobility result from an alteration of the net electrical charge on the protein molecule as a consequence of changes in the amino acid sequence encoded by the DNA. However not all changes in the DNA sequence result in amino acid substitutions and not all amino acid substitutions alter the net charge on the protein molecule.

Protein electrophoresis has been widely used (Murphy, Sites, Buth and Haufler 1990) to examine genetic variation within and between species (Table 3) (e.g., Close, Ingleby, van Oorschot, Gooley, Briscoe, and Sharman 1988; Southgate and Adams 1993). It has also proved useful in defining species boundaries and identifying cryptic species (e.g., Baverstock, Adams and Archer 1984; Adams,

	Preservation Method				
Uses	**Ambient temperature in culture media**	**Frozen and stored at -70^{o}C**	**80 - 100% ethanol**	**Dehydrated**	**Formalin**
Chromosomes	+	-	-	-	-
Proteins:					
Electrophoresis	-	+	-	-	-
DNA:					
- RFLPs	-	+	+	+/-	-
- Fingerprinting	-	+	+	+/-	-
- Traditional Sequencing	-	+	+	+/-	-
- PCR - RFLPs	-	+	+	+	+/-
- PCR - Sequencing	-	+	+	+	+/-
- PCR - Microsatellites	-	+	+	+	+/-
- PCR - RAPDs	-	+	+	+	?

Table 2. Preferred preservation methods for tissues to be used in genetic analysis. +, suitable preservation method; +/-, marginal preservation method; -, not suitable preservation method. For long-term storage of most frozen tissues -70^{o}C is essential. However tissues can be initially frozen at -20^{o}C and later transferred to -70^{o}C or could be kept at -20^{o}C if they are to be used immediately.

Reardon, Baverstock, and Watts 1988), as well as in determining the phylogenetic relationships amongst

divergent but closely related species (e.g., Close, Murray and Briscoe 1990; Colgan, Flannery, Trimble and Aplin 1993). The technique can be of limited use for distinguishing between very closely related species, which includes some rock-wallabies (Briscoe et al 1982; Eldridge and Close 1992).

For protein electrophoresis, tissue samples need to be promptly removed from freshly killed specimens and then frozen and stored (preferably) at - 70^{o}C (Table 2). See Richardson, Baverstock and Adams (1986) for detailed description of methods. Blood samples for protein electrophoresis are usually collected in the presence of an anticoagulating agent (e.g., heparin) and then rapidly processed. See VandeBerg and Johnston (1977), Cheng and VandeBerg (1987) and Sherwin (1991) for protocols. In many mammals the number of enzymes that can be analysed from blood cells is limited and soft tissues often give better results.

3. DNA ANALYSIS

The direct analysis of DNA can occur at several different levels of resolution, with the most appropriate technique being dependent on the samples available and the specific question/s being addressed (Table 3). Polymorphic DNA markers can be found in most species, even those which have previously shown low levels of variation (e.g. Hughes and Queller 1993; Paetkau and Strobeck 1994) with other techniques.

Fresh tissue or blood that has been rapidly frozen or fresh tissue that has been preserved in 80 - 100 % ethanol is the most useful for DNA analysis (Tables 1 and 2). This is because these samples provide large amounts of high molecular weight DNA that can readily be used in all available DNA based molecular techniques (Table 2). Occasionally large amounts of high molecular weight DNA can also be extracted from dehydrated tissue (Table 2), for example, the ears of road-killed rabbits (P. Sunnucks pers. comm.) and macropods (unpubl. data).

DNA is located at two major sites within the cells of all vertebrates. Most DNA is located in the nucleus and is referred to as the chromosomal or nuclear DNA (nDNA). Nuclear DNA contains thousands of functional genes, as well as a large amount of untranscribed DNA, which includes many repeated DNA sequences. The nDNA of sexual species is inherited from both parents and so mixing of genetic markers occurs with each generation.

In addition to the nDNA a small, circular molecule of double-stranded DNA is present in each mitochondrion, the cell organelle responsible for respiration. Mitochondrial DNA (mtDNA) contains 37 genes and is 15 - 20 kilobases (kb) in size in most animals (reviewed in Moritz, Dowling and Brown 1987; Avise 1994). Unlike nDNA, mtDNA is maternally (clonally) inherited (i.e. is passed from mothers to all offspring). Furthermore mtDNA is characterised by a relatively high rate of mutation and a lack of recombination (reviewed in Avise, Arnold, Ball, Bermingham, Lamb, Neigel, Reeb and Saunders 1987; Moritz et al 1987; Dowling, Moritz and Palmer 1990). These features make mtDNA extremely useful for examining population structure (e.g., Prinsloo and Robinson 1992; Hoelzer, Dittus, Ashley and Melnicks 1994; Pope et al 1996), migration (e.g., Baker, Slade, Bannister, Abernethy, Weinrich, Lein, Urban, Corkeron, Calmabokidis, Vasquez, and Palumbi 1994) and introgression (i.e., hybridisation and gene flow between species (e.g., Bee and Close 1992; Lehman, Eisenhawer, Hansen,

Question	Chromosomes	Protein Electrophoresis	mtDNA RFLPs	scnDNA RFLPs	Multi-locus Fingerprinting	Micro-satellites	RAPDs	DNA Sequencing
Identity of individuals	-	*	*	*	**	**	**	*
Parentage	-	*	*	**	**	**	**	**
Variation within species	*	**	**	**	*	**	**	**
Hybridisation	*	**	**	*	-	**	*	**
Species boundaries	*	**	**	*	-	**	*	**
Relationship between closely related species (0-50 mya)	*	**	**	*	-	-	-	**
Relationship between taxa (50 -100 mya)	*	*	-	-	-	-	-	**
Relationship between taxa (> 100 mya)	*	*	-	-	-	-	-	**

Table 3. Applications of molecular techniques. (After Hillis and Moritz 1990; Avise 1994). ** highly informative; * informative but not ideal or only informative in particular circumstances; - inappropriate. N.B. Not all categorizations are absolute. DNA sequence data is almost always informative but is not always the most time/cost effective method.

Mech, Peterson, Gogan and Wayne, 1991)), as well as phylogenetic relationships amongst closely related species (e.g., Cronin 1991; Cronin, Amstrup, Garner and Vyse 1991). MtDNA has been extensively studied in a wide range of organisms, with most researchers using either restriction fragment length polymorphism (RFLP) or sequence analysis (see 3a and 3c), although alternative methods are available (Lessa and Applebaum 1993).

DNA is obtained from tissue via a standard high molecular weight DNA extraction (for protocol see Hillis, Larson, Davis and Zimmer (1990)). The resultant DNA is referred to as genomic DNA, heterogeneous DNA or total cellular DNA as it contains both nDNA and mtDNA. If mtDNA sequence data or probes are available from the species of interest (or a closely related taxon) these can be used to examine mtDNA variation using the genomic DNA (see 3a, 3c). Otherwise purified mtDNA can be extracted (Dowling et al 1990) from fresh tissue (including blood or fibroblasts (unpubl. data)) and analysed directly (see 3a, 3c, 3d). The analysis of mtDNA using genomic DNA can however be complicated in some species by the existence of nuclear copies of segments of the mitochondrial genome (reviewed in Gellissen and Michaelis 1987). The transfer of copies of mitochondrial genes to the nucleus is known to occur in diverse taxa (including cats (Lopez, Yuhki, Masuda, Modi and O'Brien 1994), wombats (Taylor 1995) and bandicoots (Robinson 1992)) and can consist of multiple copies (Lopez et al 1994).

a) Restriction Fragment Length Polymorphisms (RFLPs)

Restriction fragment length polymorphism (RFLP) analysis examines only the variation in DNA molecules that occurs at small, specific sites, usually within a single gene (locus). A large variety of restriction enzymes are available that cut double-stranded DNA where a short specific sequence of bases is recognised in the DNA molecule. These "restriction sites" are mostly 4-6 bases in length and are unique for each enzyme. The number of these restriction sites present in a particular piece of DNA can vary as sites are gained or lost by mutation. As a result, when DNA is digested with specific restriction enymes the number and size of the resultant DNA fragments can also vary. This variation is referred to as RFLPs.

Most RFLP studies use mtDNA, single copy nuclear (scn) genes, or repetitive nuclear gene families (e.g., ribosomal genes). RFLP analysis is most useful (Table 3) in determining the levels of genetic variation within and between populations (e.g., Robinson 1995; Wettstein, Lager, Jin, States, Lamb and Chakraborty 1994), as well as the relationship between closely related species (e.g., Cronin et al 1991), but can also be used for gene mapping (e.g., McKenzie, Collet and Cooper 1993)). The complex RFLP patterns produced by hypervariable nuclear loci or some multi-gene families (e.g. Cooper, Holland, Rudman, Donald, Zehavi-Feferman, McKenzie, Sinclair, Spencer, Graves and Poole 1994) reveal high levels of intraspecific variation which can effectively function as a "DNA fingerprint" (see also 3b). Quality, high molecular weight DNA which has been extracted from rapidly frozen or alcohol preserved fresh tissue (Table 2) is essential for most RFLP studies as the technique requires the presence of long intact pieces of DNA.

b) DNA Fingerprinting (minisatellites or VNTRs)

DNA fingerprinting, a variation of RFLP analysis, is usually associated with the technique developed by Jeffreys in the 1980's (Jeffreys, Wilson and Thenin 1985a; 1985b). For a recent review see Burke, Hanotte and van Pijlen (1996). DNA fingerprinting examines variation at multiple hypervariable loci and results in a complex banding pattern that has the potential to distinguish between all individuals in a sexually reproducing population. Most probes used in multi-locus DNA fingerprinting hybridise to conserved repeated DNA sequences of between 16 - 64 base pairs (bp) long which are scattered throughout the genome in clusters of tandem repeats (reviewed in Avise 1994). These dispersed tandem repeats are referred to as minisatellite loci or variable number of tandem repeat (VNTR) loci. The number of repeat units in each cluster is highly variable and when digested by a restriction enzyme, that cuts the DNA between the clusters, produces a large number of different sized DNA fragments. When run out on a gel, these fragments produce a highly complex and individually specific banding pattern. Several fingerprinting probes (e.g. Jeffreys probes and M13) have been found to produce complex banding patterns in diverse taxa and have been widely used (reviewed in Avise 1994). However, the usefulness of these probes is not universal (e.g. Odorico, Spencer and Miller 1992)

DNA fingerprinting is most useful (Table 3) for confirming the identity of individuals and in assigning parentage (e.g., Signer, Schmidt and Jeffreys 1994) but can also be used to assess the levels of genetic variation within and between populations (e.g., Robinson, Murray and Sherwin 1993; Rassman, Arnold and Tautz 1994; Butler, Templeton and Read 1994). With DNA fingerprinting the similarity between individuals is usually assessed on the basis of band-sharing. This consists of an exhaustive series of pair-wise

comparisons from which the proportion of shared bands (or average percent difference) is calculated.

DNA fingerprinting requires high molecular weight DNA (Table 2) and can be technically demanding (see Bruford, Hanotte, Brookfield and Burke (1992) for methods). However, the major advantage of this technique is that it produces large amounts of data relatively quickly once the appropriate probe/restriction enzyme combination has been determined. Some DNA fingerprinting analyses do not always produce completely unambiguous results and there are difficulties comparing individuals between gels. The technique also has the disadvantage that specific alleles cannot often be determined and this limits the technique's usefulness in calculating accurate estimates of gene flow and other conventional population genetic parameters (Avise 1994).

Some of the disadvantages of multi-locus DNA fingerprinting are overcome by utilizing single locus minisatellites. That is, by examining particular minisatellites one at a time (reviewed in Avise 1994). However as the initial isolation of sufficient suitable minisatellites is difficult and time consuming this technique is unlikely to be widely used.

c) DNA Sequencing

Mammalian DNA consists of four nucleotides (or bases), adenine (A), thymine (T), cytosine (C) and guanine (G). Genetic information is encoded by DNA in the sequence of these four nucleotides along the molecule. Therefore in genetic studies the greatest resolution is obtained by directly determining the nucleotide sequence of the DNA molecule of interest. All other methods of assessing genetic variation and divergence are really indirect attempts to estimate changes in the DNA sequence. DNA sequencing has the advantage of being applicable to a wide range of genetic studies (Table 3). Sequence data are especially useful for studies of phylogeny and evolution with even distantly related taxa (> 100 million years) are able to be examined (e.g., Janke, Feldmaier-Fuchs, Thomas, von Haeseler and Pääbo 1994)

DNA sequencing requires the production of purified homologous DNA sequences from the region of interest. Prior to the development of PCR technology (see 3d) this was achieved by isolating and then cloning the required DNA region into a microbial vector (e.g. plasmid). This allowed the *in vivo* amplification of the cloned DNA segment by culturing the host bacterium. Subsequently the DNA sequence of the cloned segment could be obtained using the chain termination reaction (Sanger, Nicklen and Coulson 1977) or chemical cleavage method (Maxam and Gilbert 1977). For detailed discussion of techniques and protocols see Hillis et al (1990). Like RFLP analysis (see 3a), traditional DNA sequencing requires high molecular weight DNA which is most easily obtained from rapidly frozen or alcohol preserved fresh tissue (Table 2).

d) Polymerase Chain Reaction (PCR)

The recent development of the Polymerase Chain Reaction (PCR) has revolutionised molecular genetics (Mullis and Faloona 1987; Saiki, Gelfand, Stoffel, Scharf, Higuchi, Horn, Mullis and Erlich 1988). PCR involves the exponential *in vitro* amplification of a specific region of DNA by repeating cycles of denaturation and replication in the presence of a thermostable DNA polymerase. The region to be amplified is determined by using specific primers that flank the region of interest (acting as "start" and "stop" signals) and are designed to bind to homologous sequences on the target DNA molecule. This requires some *a priori* knowledge of the region under investigation. However, a series of conserved primers, which allow successful amplification of a variety of informative nuclear and mitochondrial DNA regions from diverse taxa, are now available (Kocher, Thomas, Meyer, Edwards, Pääbo, Villablanca and Wilson 1989; DeSalle, Williams and George 1993; Slade, Moritz, Heideman and Hale 1993; Avise 1994). Typically PCR products range from 100 base pairs (bp) to 2 000 bp (or 2 kb) in size, although much larger fragments have recently been obtained (Barnes 1994). For a detailed discussion of PCR and protocols see Innis, Gelfand, Sninsky and White (1990) and Mullis, Ferre and Gibbs (1994).

Using PCR, large amounts of DNA can be rapidly obtained even from small amounts of starting material (Table 1), including DNA extracted from single hair follicles (Higuchi, von Beroldingen, Sensabaugh and Erlich 1988; Taylor, Alpers and Sherwin 1997). The resultant PCR product can then be investigated by either RFLP or DNA sequence analysis, but also see Campbell, Harriss, Elphinstone and Baverstock (1995) and Girman (1996) . PCR based techniques are useful over a range of applications (Table 3) including individual identification (Taylor et al 1997), studies of genetic variation within and between populations (e.g., Suzuki, Kemp and Teale 1993), genetic divergence between species (e.g., Taylor, Sherwin and Wayne 1994; Gottelli, Sillero-Zubiri, Applebaum, Roy, Girman, Garcia-Moreno, Ostrander and Wayne 1994) and the investigations of phylogeny and evolution (e.g., Gatesy, Yelon, DeSalle and Vrba 1992). PCR based technologies will become increasingly useful to wildlife biologists as they allow largely non-invasive sampling (Taberlet and Bouvet 1992; Morin, Moore and Woodruff 1992; Morin and Woodruff 1996; Taylor et al 1997) and also the rapid screening

of large numbers of individuals.

A major advantage of the PCR technique is that it allows some useful data to be obtained using the partly degraded DNA found in dried skins (Thomas, Pääbo, Villablanco and Wilson 1990; Taylor et al 1994), bones (Höss and Pääbo 1993), formalin-fixed specimens (Impraim, Saiki, Erlich and Teplitz 1987), mummified remains (Pääbo 1989), and even feaces (Höss, Kohn, Pääbo, Knauer and Schroder 1992; Kohn, Knauer, Stoffella, Schroder and Pääbo, 1995). These developments are exciting and open up numerous possibilities including the utilisation of museum specimens to allow the molecular analysis of extinct species and populations (e.g., Higuichi, Bowman, Freiberger, Ryder and Wilson 1984; Pääbo 1989; Thomas, Schaffner, Wilson and Pääbo 1989; Wayne and Jenks 1991; Taylor et al 1994; Höss, Pääbo and Vereshchagin 1994). However, the fragmentary nature of degraded or ancient DNA places limitations on its usefulness as only relatively small regions (often < 200bp) are likely to be amplified. Furthermore these techniques remain problematic especially where chemical contaminants inhibit polymerase activity. As the successful amplification of degraded or ancient DNA can't be assured, the best chance of success remains with using DNA extracted from fresh tissue that has been frozen or preserved in alcohol immediately following collection. Researchers have also reported difficulties in obtaining PCR products from DNA extracted from blood, with heparin used as the anticoagulant (Beutler, Gelbart, Kuhl 1990). However, following treatment with Heparinase II successful amplification may be possible (Taylor et al 1994). Similar problems have not been reported using EDTA as an anticoagulant.

e) Microsatellites

Microsatellites consist of tandem repeats of simple DNA sequence motifs with each repeat unit usually less than five base pairs long (Tautz 1989). They are abundant and widely dispersed in mammalian genomes and are often highly polymorphic due to variation in the number of repeat units (Hamada, Petrino and Kakunaga 1982). Microsatellites with the general form $(CA)_n$ or $(GA)_n$ (i.e., dinucleotide repeats) are the most common in eutherian mammals, accounting for 0.5% of the human genome (Beckman and Weber 1992), and appear similarly common in marsupials (Odorico et al 1992; Taylor et al 1994; Spencer et al 1995). For a recent review see Bruford, Cheesman, Coote, Green, Haines, O'Ryan and Williams (1996).

Microsatellites can show lower levels of polymorphism than minisatellites but are more easily identified and interpreted. The large number of Mendelian inherited alleles present at many microsatellite loci means that allelic diversity and heterozygosity values are generally much higher than for equivalent protein or RFLP data (Hughes and Queller 1993; Paetkau and Strobeck 1994). As microsatellite loci are amplified using the PCR reaction (see 3c) data can be obtained from a range of starting material (Table 1). Alleles at dinucleotide repeat microsatellite loci are resolved on polyacrylamide DNA sequencing gels, while trinucleotide and tetranucleotide repeat loci are readily typed on non-denaturing polyacrylamide gels stained with ethidium bromide. Each allele can the be unequivocally assigned by reference to DNA size standards run on the same gel. Thus, unlike multilocus DNA fingerprints, results from different gels are directly comparable and specific alleles can be identified allowing the calculation of allelic frequencies and analysis of the data by standard population genetic procedures.

For these reasons microsatellite loci are rapidly becoming the marker of choice for molecular population genetic studies (Bruford and Wayne 1993), where they can be used to assess genetic variation (Paetkau and Strobeck 1994; Taylor et al 1994) and differentiation (Roy, Geffen, Smith, Ostrander and Wayne 1994), as well as determining population structure (Pope et al 1996), relatedness (Queller, Strassmann and Hughes 1993), parentage (Morin, Wallis, Moore and Woodruff 1994;) and detecting hybridization (Roy et al 1994; Gottelli et al 1994). As the genotype of each individual in a population can now be determined, microsatellite loci can also be used to investigate the interaction of kinship and social structure with genetic variance, effective population size and inbreeding (Bruford and Wayne 1993). In the future automated DNA sequencers will increasingly be used for microsatellite analysis, especially as individuals can be simultaneous typed at several loci.

f) Random amplified polymorphic DNA (RAPD) analysis

Another PCR based technique, random amplified polymorphic DNA (RAPD) analysis uses arbitrary short pieces of DNA (~ 10bp) as primers to produce anonymous multi-locus bands usually between 100bp and 2kb in size (Williams, Kubelik, Livak, Rafalski and Tingey 1990). RAPD has the advantage that no *a priori* knowledge of the study animal's genome is required and the technique is efficient and relatively inexpensive (Hadrys, Balick and Schierwater 1992). RAPD analysis has the same difficulties in analysis and interpretation as multi-locus DNA fingerprinting (see 3b) and there can also be some difficulties with repeatability and contamination (e.g. amplification of bands from symbiotic micro-organisms or parasites).

See Hadrys et al (1993) and Lynch and Milligan (1994) for detailed discussion of technique.

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