INVESTIGATION OF RAPDS AND MICROSATELLITES FOR USE IN SOUTH AFRICAN CRANES

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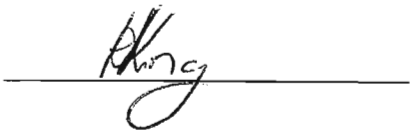
DECLARATION

I hereby declare that, unless specifically indicated,
this dissertation is the result of my own investigation.



Heather Anne King
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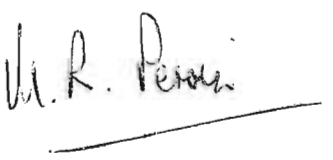

PREFACE

The results presented in this thesis follow from a study that was carried out at the School of Botany and Zoology, University of Natal, Pietermaritzburg under the supervision of Prof. Mike Perrin.

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To Stof, thank you for your constant support through good times and bad. Thank you for always believing in me and seeing the best in me and thank you for being the best friend I could ever wish to have.

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To my parents Marilyn and Tony, thank you for all the sacrifices you have made to get me here. Thank you for the support and love. Mom, your courage, strength and constant selflessness are an inspiration. I love you guys stax!

In memory:

Marilyn Mary King
8 June 1941 – 24 February 2004

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To my wonderful parents

"they are being offered thousands of rands for chicks" -Sunday Times

Syndicates now target Blue Cranes
- Natal Witness

"poachers rob their nests and sell their chicks on the black market for thousands of rands" -Sunday Times

"I had someone call me just the other day to say that he would pay me R5 000 for one chick."
-Sunday Times

First came poison, and now poachers
The blue crane, SA's national bird, faces a new threat in the Overberg
-Sunday Times

"The endangered blue crane is facing a new threat - illegal trading in young chicks - according to conservationists"
-Sunday Times

Blue crane chicks under threat
-Sunday Times

Farmers and farmworkers being offered money to catch and sell chicks.
-Sunday Times

"If I had to sell the birds I'd be a millionaire," said farmer Michael Fourie.
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"Penning said he believed that the stolen endangered birds were destined for the international collectors' market"
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South Africa's National Bird
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ABSTRACT

The three South African crane species, namely, the Wattled Crane (*Bugeranus carunculatus*), the Blue Crane (*Anthropoides paradisea*) and the Grey Crowned Crane (*Balearica regulorum regulorum*) are all threatened. South African legislation protects the cranes, however eggs and/or fledglings are sometimes illegally collected from the wild. These are then sold, often by registered breeders, who falsely claim them as the offspring of their captive breeding pair. DNA fingerprinting is one method to detect this crime.

Fifteen RAPD primers were screened for polymorphism in the three species. Seven primers produced polymorphic profiles in the Blue Crane and eight each in the Grey Crowned Crane and Wattled Crane, with an average of 14.57, 12.38 and 5.88 scorable loci per primer, respectively. The Band Sharing Coefficient for unrelated individuals was found to be 0.665, 0.745 and 0.736 for the Blue, Grey Crowned and Wattled Crane respectively.

Five microsatellite primers, originally developed for use in Whooping Cranes (*Grus american*), had previously been shown to be polymorphic in the Wattled Crane. This was also the case in this study with an average of 3.6 alleles per primer. Although all primers cross amplified, only a single primer each showed polymorphism in the Blue Crane (showing 6 alleles) and the Grey Crowned Crane (showing 5 alleles).

The RAPDs were found to be irreproducible, show high numbers of novel bands and had parent: offspring BSC values that were not significantly higher than those of unrelated individuals. Statistics showed that, in the Blue Crane, the probability that misassigned parents would be detected was low whilst there was an almost certainty that true parents would be incorrectly excluded.

The five microsatellite primers examined gave exclusionary powers of 0.869 and 0.641 where one or two parents were unknown in the Wattled Crane. The exclusionary powers for the Blue Crane and Grey Crowned Crane calculated at only one locus were much lower.

It was concluded that RAPDs were totally inappropriate for parentage analyses, however, microsatellites are a suitable technique and recommendations are made that other

microsatellites, developed for other species of crane, should be examined for their potential in this respect.

Chapter 1

INTRODUCTION

The law protects South African cranes and tampering with nests, eggs or chicks is illegal (Allan 1994). A permit is required to keep cranes in captivity, but fledglings are sometimes collected illegally from the wild for sale into captivity, possibly by registered breeders who then falsely claim their pair has produced eggs (Hudson 2000; Morrison 2002). The removal of chicks from the wild is a major threat to the cranes and it is for this reason that control measures need to be enforced to curb these illegal acts, which are draining an already strained resource (Allan 1994; South African Crane Working Group 2000).

DNA fingerprinting can be of great benefit in the fight against the illegal trade in wildlife. The term DNA fingerprinting is used in molecular biology to describe the unique banding pattern that can be produced from an individual's DNA, a pattern resembling a bar code (Bruford *et al.* 1998; Marin & Pinna 1999). DNA fingerprints are unique in all individual organisms, not only humans. By using the banding pattern produced by a DNA fingerprint, parentage can be determined because each fragment in a fingerprint must either have a maternal or paternal origin (Parker *et al.* 1998; Pena & Chakraborty 1994).

This thesis describes the examination of two DNA markers, microsatellites and Randomly Amplified Polymorphic DNA (RAPDs) that might be used to detect illegal birds and therefore reduce this threat. These two techniques are assessed as to their suitability for parentage testing with the long-term view of forensic applications.

1.1 CRANES OF SOUTH AFRICA

There are 15 species of crane worldwide of which three occur in South Africa (Johnsgard 1983; Walkinshaw 1973). Eleven of these species have been proposed for inclusion on the IUCN Red List of threatened species (Beilfuss *et al.* 2000). The three South African crane species, namely, the Blue Crane (*Anthropoides paradisea*), the Wattled Crane (*Bugeranus carunculatus*) and the Grey Crowned Crane (*Balearica regulorum regulorum*) (Figure 1. 1) are listed as threatened by the International Union for the Conservation of Nature and Natural Resources (IUCN), with the Grey Crowned Crane and Blue Crane being Vulnerable and the Wattled Crane Critically Endangered (Barnes 2000).

Figure 1. 1 Crane species found in South Africa: (a) Blue Crane, (b) Wattled Crane and (c) Grey Crowned Crane. (Photographs by: The South African Crane Working Group (a & c) and Heather King (b)).

The Blue Crane (Figure 1. 1) is South Africa's national bird. Its beauty and grace have captured the hearts of nature lovers for centuries. According to the National Crane and Habitat Action Plan (South African Crane Working Group 2000), the Blue Cranes global range is the most restricted of any crane species. It is endemic to southern Africa and, apart from small populations found at Etosha (northern Namibia) and western Swaziland, it is confined to South Africa (Figure 1. 2) (Allan 1997a). Unlike most cranes it does not require wetlands for its survival but instead usually nests on dry ground in grasslands and crop fields (Birdlife International 2000). Breeding occurs between August and April with clutch size usually being two, although clutches of three eggs have been recorded (Allan 1997a; Birdlife International 2000; Johnsgard 1983).

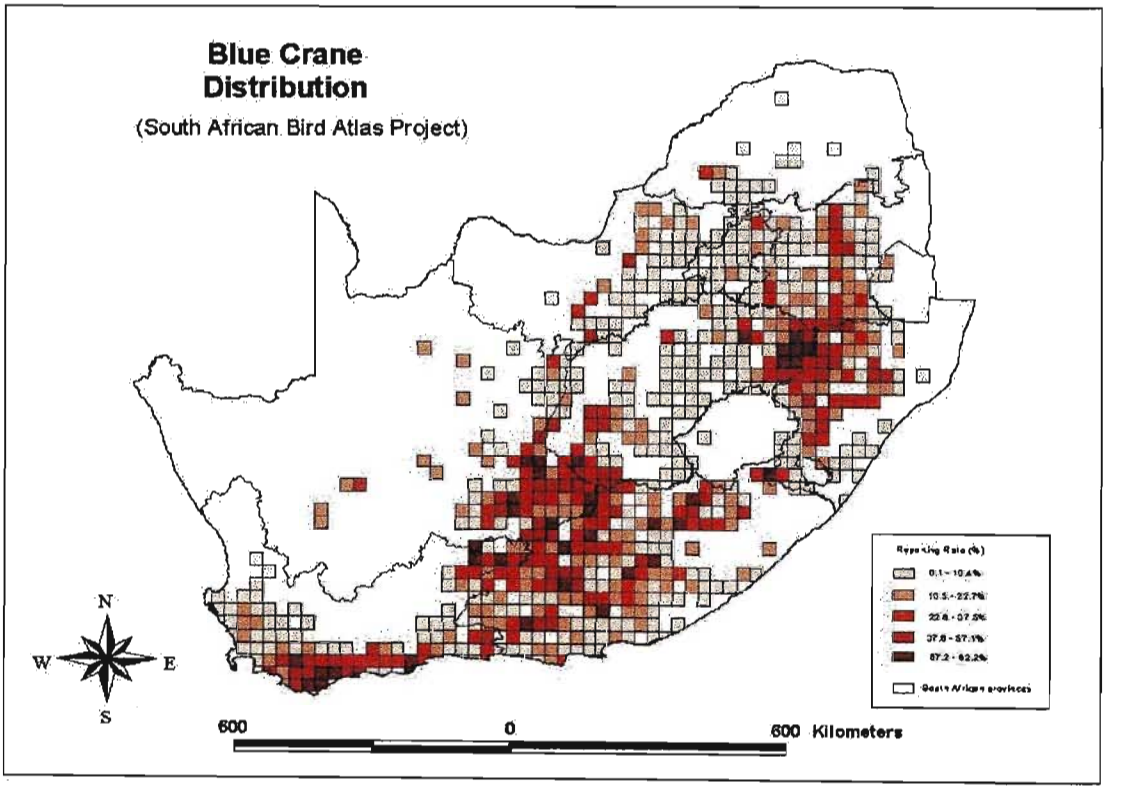

Figure 1. 2 Distribution of the Blue Crane as recorded in the South African Bird Atlas (Allan 1997a).

The Wattled Crane (Figure 1. 1) is South Africa's largest and most rare crane (Allan 1994; Allan 1997c). Although in the past the Wattled Crane was widespread throughout South Africa, its numbers have declined dramatically and only a very small population still remains in confined to a restricted area in the eastern parts of the country (Figure 1. 3) (South African Crane Working Group 2000). In 1982 there were just 102 breeding pairs left in South Africa (Allan 1997c) but these numbers have dropped to 81 in 2003 (K McCann, 2003, pers. comm.). This species requires expanses of pristine, high altitude wetland for breeding (Allan 1994). In the wild breeding occurs throughout the year with a peak in late autumn and winter (Urban 1993). The nest consists of a large exposed mound built in the wetland with either one or two eggs being laid (Allan 1996; Allan 1997c). In cases where two eggs are laid only one chick is usually raised and the second egg is deserted as soon as the first is hatched (Allan 1997c).

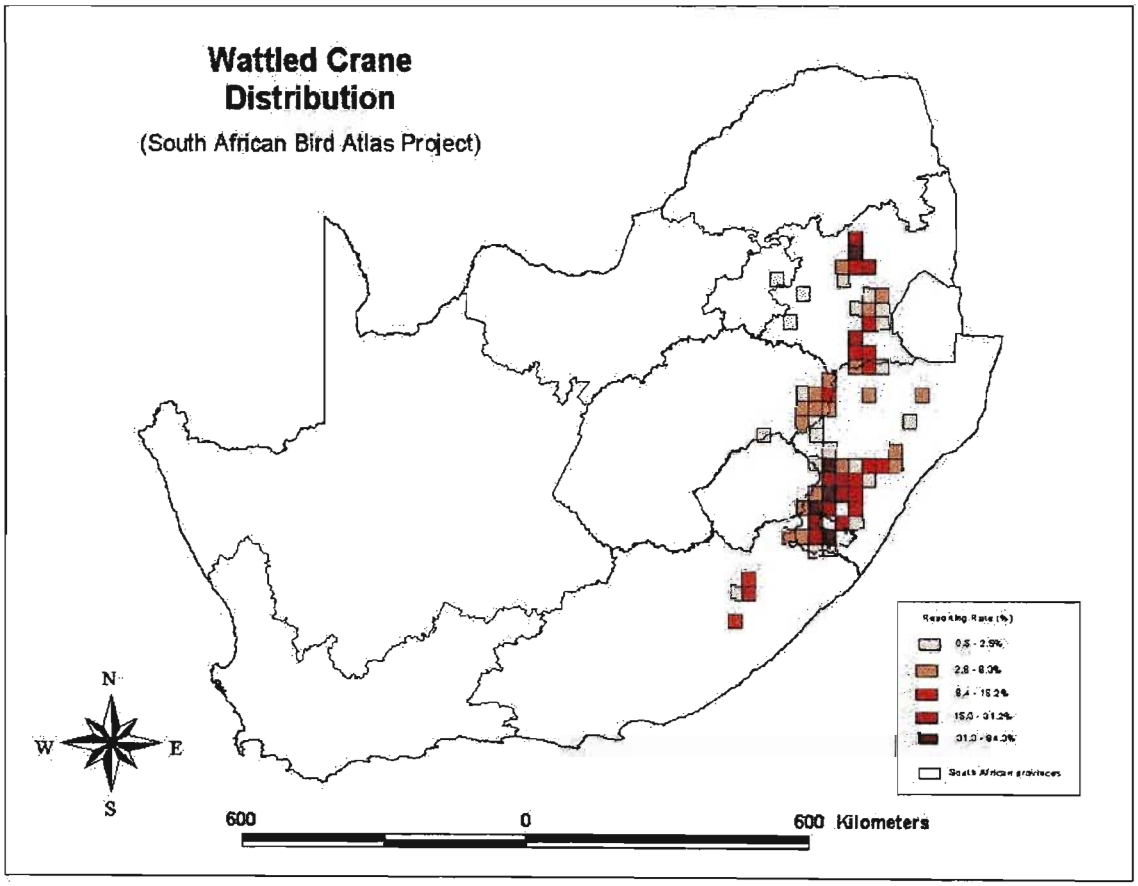

Figure 1. 3 Distribution of the Wattled Crane as recorded in South African Bird Atlas (Allan 1997c).

The Grey Crowned Crane (Figure 1. 1) is the most plesiomorphic extant species of crane (Allan 1997b) and is the most common in South Africa. Its distribution has not changed much over the last century and is fairly widespread over South Africa (Figure 1. 4)(South African Crane Working Group 2000). As with the Blue Crane, the Grey Crowned Crane is much more versatile than the Wattled Crane, when nesting (Allan 1994). It is usually associated with wetlands and nests from mid-October to May, making a simple nest of marsh vegetation, concealed in wetland habitat (Allan 1994; Walkinshaw 1973). Remarkably, Grey Crowned Cranes have also been recorded nesting in trees (Allan 1996; Long 1998; Steyn & Ellman-Brown 1974). Clutch sizes may be as large as four eggs although usually only one or two chicks are raised (Allan 1994; Allan 1996).

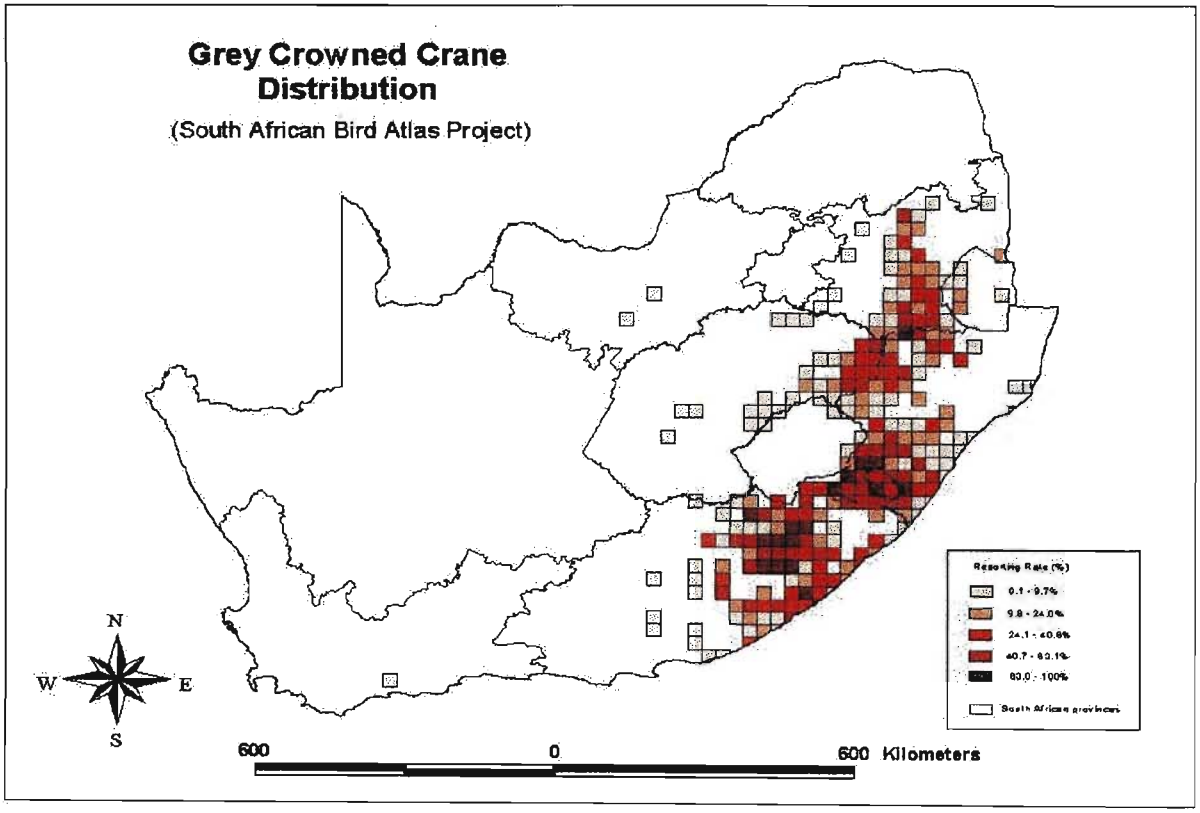

Figure 1. 4 Distribution of the Grey Crowned Crane as recorded in the South African Bird Atlas (Allan 1997b).

1.1.1 Threats facing the South African cranes

Population numbers of the South African cranes have decreased significantly over the last twenty years. According to the National Crane and Habitat Action Plan (South African Crane Working Group 2000), areas of Mpumalanga and KwaZulu-Natal have shown an 80% decline in population numbers with the most recent estimate at 21 000 individuals. Wattled Crane numbers have decreased by 36.1% with just 80 known active breeding pairs (South African Crane Working Group 2000). Although the most abundant of the crane species in South Africa, Grey Crowned Crane numbers have declined by 15% and are estimated between 85 000-95 000 (South African Crane Working Group 2000). From observations of Grey Crowned Crane nests over the 1999/2000 and 2000/2001 breeding seasons fledging success was 18.8% and 34.6%, respectively (Smallie 2002).

A key threat to the South African cranes is the loss of natural habitat (South African Crane Working Group 2000). The flooding of wetlands to create dams and the draining and development of wetlands for farmland has reduced the numbers of Grey Crowned Cranes and put the Wattled Crane on the brink of extinction (Allan 1994). Commercial afforestation has destroyed large expanses of the natural habitat of the Blue Crane putting them under threat. Afforestation with "thirsty" alien tree species may affect runoff and lead to the drying up of wetlands, again affecting the Grey Crowned Crane and Wattled Crane (Allan 1994).

Another major threat, especially to the Blue Crane, is the deliberate poisoning of these birds by farmers (Allan 1994; Tyson 1987). Cranes are attracted to croplands to forage where they may feed on and trample newly germinated crops (Allan 1994). Farmers retaliate by scattering poisoned grain around their fields to intentionally, and illegally, kill the cranes that come to forage. This has also had a major affect on many non-target bird species.

Collisions with power lines are another threat to the South African cranes (South African Crane Working Group 2000). The birds fail to see the lines when flying and collide with them causing electrocution and death (Allan 1994). Devices are now being attached to power lines in an attempt to make them more visible and in severe cases power lines are re-routed (McCann & Van Rooyen 2002).

Cranes nest on the ground, making them vulnerable to egg and chick removal for illegal practice (Allan 1994). It is this threat that this study is aimed at reducing. Egg collectors may remove eggs, while young chicks are captured for food or to be kept as pets (Hudson 2000; Morrison 2002). Yearly removal of chicks from the nest is suspected in one nest site in particular in South Africa due to human paths into the wetland and the presence of litter and cigarette butts in the area (Smallie 2002). The trade in wild animals has become a very profitable business with breeders selling legitimate Blue Cranes and Grey Crowned Cranes for approximately R8 000 per bird in 2003 (K McCann, 2003, pers. comm.).

As already mentioned cranes do not produce very large clutches, when they do breed at all, and so the loss of any offspring has a severe impact on the total population. In captive breeding an aviculturalist may suffer a great loss in income if a pair does not produce chicks and so some unscrupulous individuals may acquire chicks illegally from the wild, to be passed off as the offspring of their own pair. This is illegal under various sections of legislation including the National Environmental Management Act 107 of 1998, the Animal Protection Act 71 of 1962 and most importantly the Constitution of the Republic of South Africa Act 108 of 1996 (Appendix 1).

Unfortunately, up until now, there has been no way of proving if a chick is legitimate or if it has been illegally removed from the wild unless there have been independent witnesses. Thus, even though officials may suspect breeders of foul play, there is generally insufficient evidence to prosecute them.

1.1.2 Captive breeding

It must be recognised that captive breeding has its role in the conservation of the cranes (Mirande 1987). Well-managed, genetically viable captive breeding populations serve as an "insurance policy" against extinction when conservation efforts in the field fail. Captive breeding also helps conservation efforts in that they serve to educate the public, help in the training of field staff and provide information on the biology, disease and breeding behaviour of these birds (Mirande 1987).

Considerable knowledge and experience is required to maintain breeding birds in captivity. They are extremely sensitive to disturbance and in captivity worldwide fertility rates of Wattled Cranes were 28.7% and 19.4% in 1991 and 1992 respectively, which represents the typical annual fertility rate for captive Wattled Cranes (Beall 1996). In Africa in 1992, 18 of the 19 Wattled Cranes held in captivity were wild caught, the remaining bird having been hatched in captivity (Beall 1996).

Genetic technology can aid captive breeders by providing information relating to the sex of birds and the amount of genetic similarity between birds (Duan & Fuerst 2001; Jones *et al.* 2001; Yongtong *et al.* 1991). Reduced genetic variation, inbreeding, can have negative effects to the viability of the captive population (Snowbank & Krajewski 1995; Swengel 1987). Genetic technology can also be used to monitor that birds being sold by breeders are in fact captive bred and not wild caught. According to the National Guidelines for Trade and Keeping Cranes in Captivity (South African Crane Working Group 2003) the onus is on the breeder to prove that the cranes are captive bred.

1.2 CRANE GENETICS

Most of the genetic work to date on cranes has focused on phylogenetics and sequence divergence (Ingold *et al.* 1989; Krajewski 1989, 1994; Krajewski & Fetzner 1994; Krajewski & King 1996; Krajewski & Wood 1995, Wood & Krajewski 1996). In all cases the Balearica genus can be seen as the most anciently divergent group. As microsatellite primers generally only cross-amplify in closely related species (Ellegren 1992), this genetic distance may reduce the probability of the microsatellite primers, developed for Whooping Cranes (*Grus americana*), cross-amplifying in the Grey Crowned Crane.

The majority of molecular genetic research performed on cranes has been on the Whooping Crane. Studies have included allozyme research (Dessauer *et al.* 1992), MHC analysis (Jarvi *et al.* 1999), mitochondrial DNA sequencing (Glenn 1999; Snowbank & Krajewski 1995) and DNA fingerprinting using multilocus minisatellites (Longmire *et al.* 1992) and microsatellites (Glenn *et al.* 1997; Jones & Nicholich 2001; Jones *et al.* 2002).

Although not as numerous, similar studies have also been performed on the Sandhill Crane (*Grus canadensis*) (Dessauer *et al.* 1992; Jarvi *et al.* 1995, 1999), the Sarus Crane (*Grus antigone*) (Chen 1989; Dessauer *et al.* 1992; Krajewski & Wood 1995; Wood & Krajewski 1996) and the Siberian Crane (*Grus leucogeranus*) (Dessauer *et al.* 1992; Tokarskaya *et al.* 1994; Tokarskaya 1995).

Microsatellites have been developed for the Red-crowed Crane (*Grus japonensis*) (Hasegawa *et al.* 2000) and the Whooping Crane (Glenn *et al.* 1997). These have subsequently been used in the Wattled Crane, Sarus Crane and Sandhill Crane (Jones 2003) and will be discussed further in section 1.8.4.

1.3 DNA EVIDENCE

DNA evidence has become increasingly popular in prosecution. In the case of *People vs. Wesley*, 140 Misc.2d 306, 533 N.Y.S.2d 643 (Co. Ct. 1988) the state trial judge was quoted as saying DNA typing "can constitute the single greatest advance in the 'search for truth', and the goal of convicting the guilty and acquitting the innocent, since the advent of cross-examination". However, for DNA evidence to be admissible in court certain criteria have to be met. The evidence must first satisfy the test for admissibility, which ensures that only scientific evidence that is reliable is admitted. To determine the admissibility, the technique must be one in which the theory and technique have been used and tested by the scientific and forensic community sufficiently enough to have gained general acceptance (Melson 1990).

Another major factor is the qualifications of the expert witness giving testimony. The witness must be knowledgeable about the area of research as well as the opinions held by the scientific community (Melson 1990). Although the witness should be an expert in the field they should also be impartial. If the expert is a leading figure in the field, courts may question their impartiality to the technique (Melson 1990).

In most cases where DNA evidence has been deemed inadmissible in court, it has been due to the examining laboratory failing to follow accepted procedures (Melson 1990). This has become a particular area of scrutiny in the courts and in the case of Schwartz, 447 N.W.2d at

426, the Minnesota Supreme Court noted that the DNA test results were only as reliable and accurate as the procedures that the testing laboratory had used.

Finally, a major area of concern as far as admissibility is concerned is the chain of custody. According to Melson (1990) there are two objectives to the chain of custody. It connects the evidence to the place, object or defendant related to the case. Secondly, it rules out any tampering, alteration or substitution of the physical evidence between the time the evidence was collected and the time when it was analysed scientifically. While being analysed, there is always the possibility of human error (Thompson 1995; Lempert 1995). Often samples from the same case are processed together. Any inadvertent switching or cross-contamination could have serious effects on the outcomes of the tests. The probability of this occurring is reduced by having another individual witness every step of the procedure (Thompson 1995).

The quality of the genetic marker and the technique used depends on its ability to be consistent. Products must be consistently and objectively scored and must accurately reflect genetic variation (Thompson 1995).

1.4 REVIEW OF PCR

The polymerase chain reaction (PCR) is an *in vitro* technique developed in 1985 by Kary Mullis of the Cetus Corporation in California (Saiki *et al.* 1985). It is a powerful tool used to increase the amount of a specific sequence of DNA exponentially. Its development was a milestone in the field of molecular biology.

As seen in Figure 1. 5, the general principle is a simple one. The double stranded molecule of DNA must first be denatured and separated into two single stranded molecules. Two single stranded primers, situated on either side of the target DNA, are then annealed on opposite strands. DNA polymerase then extends the two primers and produces a new DNA molecule complementary to the original target strand. In the next cycle of PCR, these newly synthesized strands also become a template from which new strands can be synthesized. Thus each cycle of the PCR doubles the quantity of the target DNA, and as the process continues the quantity of DNA is selectively increased exponentially. In theory only a single DNA molecule

is needed for PCR. However, this makes the risk of contamination very high, and increases chance of any replication error having a significant effect on the resulting profile.

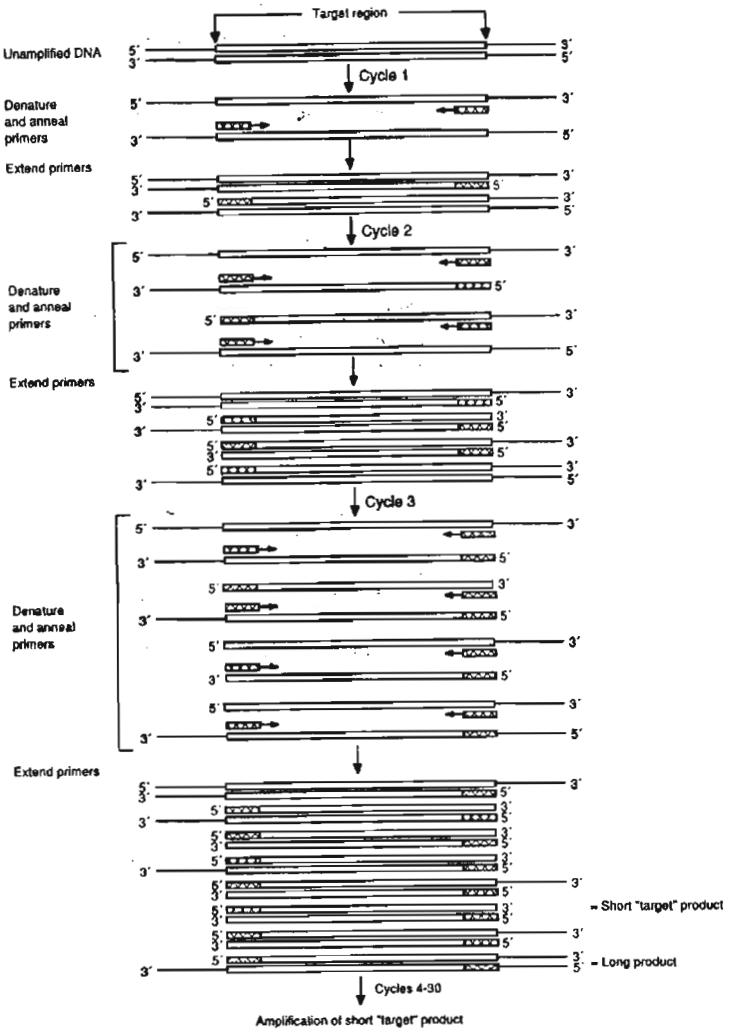

Figure 1. 5 The polymerase chain reaction showing how with each cycle of the reaction the product increases exponentially (after Newton & Graham 1994).

1.4.1 The PCR protocol

The PCR reaction consists of several cycles each containing a denaturation, annealing and elongation step. Each of these steps occurs at a different, specific temperature.

Prior to the cycles there is an initial denaturation step to completely denature all the genomic DNA. Denaturation is performed at 95-100°C. Each cycle then begins with another denaturation step to separate newly synthesized strands. Denaturation is followed by annealing to attach the primers to the DNA. The annealing temperature varies according to the base composition of the primers being used. The denaturation temperature can be determined through a simple formula: Tm = 4(G+C) + 2(A+T) °C (Newton & Graham 1994). From this the annealing temperature, Ta, should be optimised and is generally found to be a few degrees below the calculated Tm. Once the primers are annealed the new DNA strand can be synthesised. *Taq* polymerase requires an optimum temperature of 72°C to extend or synthesise the new DNA strand. A graphic example of this temperature profile can be seen in Figure 1. 6. The extension time required to synthesise the new strand will depend upon the length of the target sequence. A 2kb sequence requires approximately 1 minute for reliable amplification (Newton & Graham 1994). Generally 25-35 cycles produce sufficient quantities of the template sequence for observation on an agarose gel. The PCR reaction is then completed with a longer final elongation step to ensure all template strands are of full length.

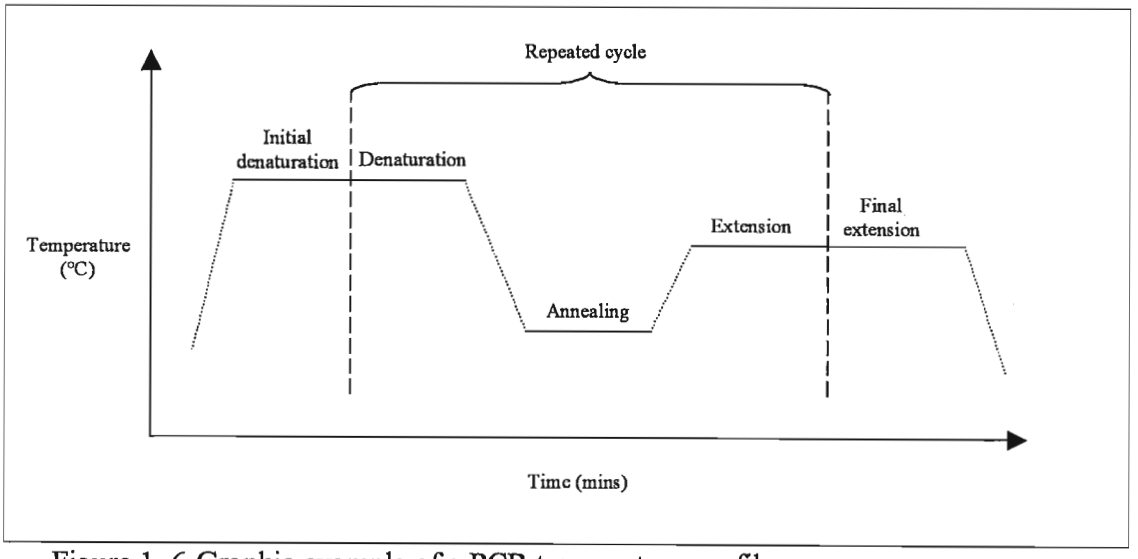

Figure 1. 6 Graphic example of a PCR temperature profile.

1.4.2 PCR reagents

In all PCR reactions it is recommended that a master mix be made up containing all the reaction components except the target DNA. This is done to reduce pipetting errors. Master

mixes are made up on ice in a laminar flow bench using filter tips to ensure no cross contamination occurs. *Taq* polymerase is added to the master mix last. The target DNA is added last to the thin walled PCR tube containing aliquots of the master mix.

1.4.2.1 PCR primers

In general, primers are designed to amplify unique segments of DNA. This requires knowledge of the sequence surrounding the target DNA. The sequence of the target DNA to be amplified need not necessarily be known. The nucleotide sequence of one of the primers will be complementary to the flanking region on one side of the target sequence and the other primer will be complementary to the flanking region on the other side. Thus, when the DNA is denatured the two primers can hybridise to their complementary sequences on either side of the target DNA. As seen in Figure 1. 7, the two strands of a DNA molecule run in opposite directions, the primers are produced so that they orientate themselves with their 3'-hydroxyl

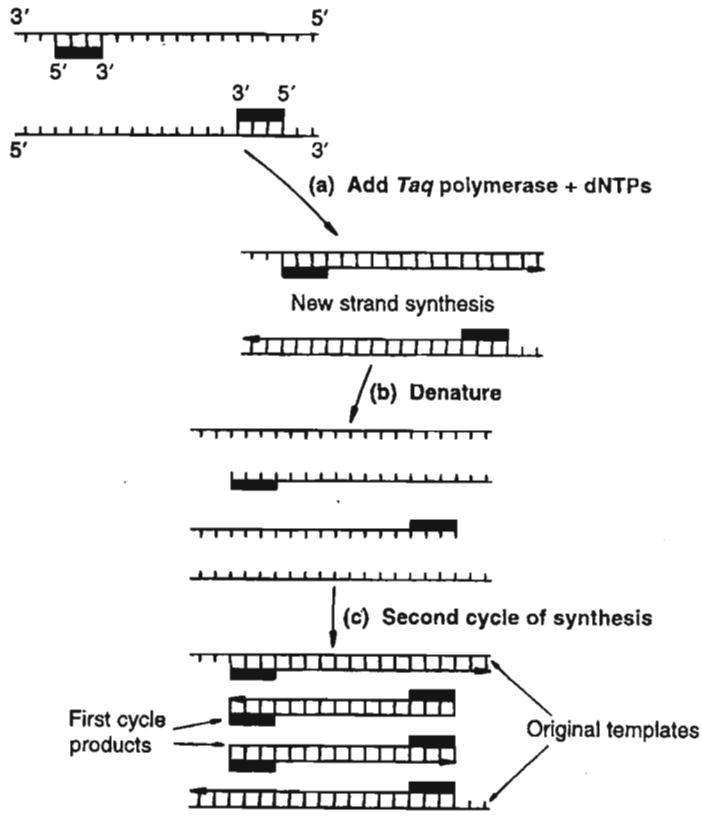

Figure 1. 7 Primers hybridise to the DNA so synthesis can occur in a 5' to 3' direction (adapted from Brown 1995).

group facing the sequence to be synthesized. DNA is synthesized by polymerase in a 3' to 5' direction (Brown 1995). The new strands that are synthesized include the primer sequence at each end permitting new primers to anneal for the next round of amplification.

1.4.2.2 DNA Taq Polymerase

PCR has only reached its full potential since the discovery of a thermostable DNA polymerase. Previously, the Klenow fragment of DNA polymerase I, isolated from *Escherichia coli,* was used (Sambrook *et al.* 1989). However, this became damaged at the high temperatures required for denaturation, and thus increasingly inactive, with each step in the PCR cycle. A new aliquot of enzyme was therefore necessary for the reaction during each new cycle, making the technique both expensive and tedious. The discovery of thermostable DNA polymerase, isolated from bacteria found living in hot springs solved this problem. As the enzyme was not denatured by elevated temperatures it withstood the high temperatures needed for the denaturation step required in each PCR cycle over the 30-40 cycles required. The first thermostable DNA polymerase was isolated from *Thermus aquaticus* and named *Taq* polymerase (Sambrook *et al.* 1989). This enzyme has a 5'-3' exonuclease activity but lacks 3'-5'exonuclease (Newton & Graham 1994). This is still the most commonly used polymerase although there are many on the market.

1.4.2.3 Other reagents

Deoxynucleoside triphosphates (dNTPs) are the precursors required for the synthesis of DNA. The dNTP mix contains deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP). The optimal dNTP concentration required for the reaction depends on several factors including the $MgCl_2$ concentration, the length of the fragment to be amplified and the number of cycles to be performed.

According to Newton and Graham (1994) Mg^{2+} ions stimulate the activity of the polymerase, the temperature and specificity of the primer annealing and are needed to form a soluble complex with the dNTPs, which is essential to their incorporation. Thus, the concentration of $MgCl_2$ used in the reaction affects both the yield and the specificity of the PCR product. The appropriate $MgCl_2$ concentration for a given reaction can be determined by

means of a $MgCl_2$ titration series (usually 0.5mM – 5mM) to indicate which concentration produces bright bands without a non-specific product. The presence of EDTA in a reaction (often found in the template DNA buffer and potentially carried over from blood collection and storage tubes) hinders the PCR reaction as it chelates Mg^{2+} ions out of the solution.

The most commonly used PCR buffer consists of 100mM Tris-HCl (pH8.3), 500mM KCl and 0.1% (w/v) gelatin. Commercial buffers usually also contain 15mM $MgCl_2$. This buffer is at a 10X concentration and needs to be diluted to a 1X concentration for use in the reaction.

Once optimised, the quantity of DNA added to the PCR reaction should remain constant. Thus, the concentration and the purity of each DNA template being used needs to first be established.

1.4.3 The PCR instrument

As already discussed the temperature and time intervals at each step of the PCR reaction are crucial for good results. Prior to the development of automated PCR machines it was necessary for one to manually transfer the reaction between heating or cooling blocks or water baths at different temperatures. This was a tedious and inaccurate process. The development of thermocyclers has been a huge advance to the PCR technique. Thermocyclers can now, automatically and within a single block, alter temperature conditions over extremely short spaces of time. This allows PCR products of a high quality and specificity to be obtained.

1.4.4 Hotstart PCR

To avoid non-specific bands being produced some researchers add the *Taq* polymerase component of the reaction mix only after the initial denaturation of the template DNA. This can either be done manually, by adding the enzyme only after the initial denaturation step. Alternatively, by separating the other reaction components from the enzyme with a layer of wax or Vaseline. As the temperature rises during the denaturation step the wax or Vaseline melts allowing the enzyme to mix with the other components and the reaction to continue as normal.

1.4.5 Factors affecting PCR specificity

Several factors affect the specificity of a PCR reaction. The major factors being $MgCl_2$ concentration and primer annealing temperature. Too little $MgCl_2$ in a reaction will result in low yields of product while too much $MgCl_2$ will result in the increase of non-specific products. If the primer annealing temperature is set too high the primers may not bind. However, if the annealing temperature is too low mismatches may occur between bases resulting in the primer binding in the incorrect position. This results in incorrect or non-specific products being produced.

1.5 REVIEW OF ELECTROPHORESIS

1.5.1 What is electrophoresis?

Electrophoresis is an effective way of separating out DNA fragments on the basis of size. It is also used in a range of applications e.g. DNA sequencing, quantifying DNA, checking sample quality and separating out whole chromosomes. The technique works on the principle that DNA has an overall negative charge. When a current is passed through a gel medium containing a DNA sample, the DNA will migrate through the gel towards the positive terminal. However the gel matrix retards fragments according to their size. Small fragments pass easily through the gel matrix whereas larger fragments are retarded more, thus separating the DNA by size. The mobility of the fragments has been found to be fairly independent of sequence or base composition (Sealy & Southern 1990)

1.5.2 Agarose gel electrophoresis

Agarose gels are used to accurately analyse double stranded DNA fragments in the size range of 70-50 000bp. Above this size other techniques such as pulsed field gel electrophoresis need to be used. For analysis of DNA fragments below this size polyacrylamide gels are generally used (Sealy & Southern 1990).

The concentration of the gel matrix also affects migration rate. By increasing the concentration, or percentage, of the agarose gel the pore size of the matrix decreases allowing greater separation of smaller fragments. Conversely by lowering the gel concentration the pore size of the matrix will be increased. This allows small fragments to travel easily through the gel while larger fragments will be retarded more and thus allow greater separation. By varying the concentration of the agarose gel a wide size range of DNA molecules can be resolved. The most efficient range for size separation at various agarose gel concentrations is seen in Table 1. 1.

Table 1. 1 Range of separation in gels containing different amounts of agarose (Sambrook *et al.* 1989).

Agarose concentration in gel (%[w/v])	Range of separation of linear DNA molecules (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

The horizontal slab gel apparatus was invented by Walter Schaffner (Sambrook *et al.* 1989) and was an improvement on the older vertical apparatus as, not only was the gel supported from beneath allowing lower gel concentrations to be used, but also gels of a variety of sizes could be made with the added advantage of being simpler to load, pour and handle (Sambrook *et al.* 1989). The apparatus is a submarine technique and consists of a tank, with lid, fitted at either end with platinum wire electrodes. These electrodes are connected to the power supply and provide the current across the gel. In the tank is a platform on which rests a tray, which will hold the gel. The apparatus also comes with a caster. The gel tray may be placed in the caster when a gel is poured. The caster also has a sample well comb, which is used to create the wells in which the samples will be placed. A power supply is necessary to deliver a current through the gel. Power packs, which deliver up to200mA DC at 200V, are sufficient for most applications (Sealy & Southern 1990).

Electrophoresis buffers provide ions, which are necessary for electrical conductance. Various different buffers may be used, however, for fragment analysis it is preferable to use a 0.5X TBE buffer rather than the usual 1X TAE. The buffering capacity of TAE is much less than that of TBE. For fragment analysis gels are generally run over long periods of time to ensure adequate separation of fragments, and so a buffer with a higher buffering capacity is necessary. The TBE can be prepared as a 5X stock and stored. If stored for too long a white precipitate may form indicating it must be discarded and replaced with a new stock. Buffer from the same batch and of the same concentration as that used to make the gel must be used to fill the tank when running a gel.

The most common and convenient way of visualising DNA in an agarose gel is by means of a fluorescent dye. Ethidium bromide is such a dye, which has the ability to intercalate with DNA molecules. When placed under UV light ethidium bromide, DNA complexes fluoresce in the red-orange region of the visible spectrum greater than unbound dye. This allows small quantities of DNA to be visualised.

Ethidium bromide has an overall positive charge, unlike DNA, and so when it is placed under an electric current it migrates to the negative pole (cathode). In cases where gels are run for long periods of time it may become necessary to add ethidium bromide to the tank buffer as well as to the gel. An alternative method is to stain the gel after electrophoresis by soaking it in a 0.5ug/ml EtBr solution.

Stock solutions of ethidium bromide (10mg/ml) should be stored at room temperature in dark containers. Ethidium bromide should be added to the gel and buffer to a final concentration of 0.5ug/ml.

1.5.2.1 Agarose gel preparation

The tank is levelled and filled with the appropriate buffer before the gel is inserted. The gel tray is placed in the caster ensuring the tray is well sealed at both ends. It is also necessary for the tray to be exactly level to ensure an even thickness when the gel is poured. The well comb should be placed exactly 90° to the direction of the run and suspended with the teeth of the comb positioned about 1mm above the base.

An agarose gel should be at least 3mm in thickness as these are easy to cast and handle. The agarose powder must first be melted into solution, in the chosen buffer, in a clean flask, by heating over an open flame or in a microwave. Special care must be taken when melting in a microwave to prevent superheating. This may lead to sudden boiling when the solution is removed from the microwave (Sambrook *et al.* 1989).

After the gel has cooled to approximately 55°C, it can be poured. In the case of larger volume gels it is necessary to place the container on a gentle rocking plate or in a waterbath while cooling. This ensures even cooling of the solution. If ethidium bromide is to be included in the gel it should be added to the cooled agarose and mixed in well before pouring. Absolutely no bubbles should be present in the poured gel.

The gel is allowed to set at room temperature. Once set the well comb should be carefully removed before removing the gel tray from the caster. The gel should not be left exposed to the air for long after setting as this can cause the gel to dry out and shrink. The set gel can now be submerged in the buffer in the tank.

Tracking dye must first be added to the sample before it can be loaded on the gel. Several different tracking dyes are available. Most contain bromophenol blue and/or xylene cyanol. The loading buffer intercalates with the DNA and is heavier than the tank buffer ensuring the DNA sample sinks into the well.

A micropipette is used to load the samples, mixed with dye, into the wells. The pipette tip is carefully directed into the top of the well. The sample is slowly and carefully transferred into the well and allowed to sink to the bottom. Bubbles can upset this settling process and so samples are centrifuged before loading.

It is essential to also load a size standard in the gel. Several size standards varying in size range are commercially available. These size standards are prepared from restriction digests of various plasmids or phages and the sizes of the fragments generated are precisely known. From this "ruler", fragment sizes of the DNA being examined are calculated.

1.5.2.2 Electrophoresis conditions

Once the samples and molecular weight markers are loaded the lid of the tank is closed. It is important to ensure the electrical leads are attached in such a way that DNA will migrate towards the positive terminal (anode).

Electrophoresis can usually be performed at room temperature. Overheating of the gel during electrophoresis can result in the bands becoming distorted. Circulating the buffer may help to stabilise temperature difference and cool the gel.

Larger fragments are best run at lower voltages over longer periods of time while smaller fragments can be run fairly quickly for a short period of time to maintain band sharpness. The longer a gel is allowed to run the greater the amount of separation that occurs between fragments. However smaller fragments tend to diffuse at very low voltages. It is necessary to establish what conditions are optimal to maintain band sharpness while still achieving adequate amounts of separation of the larger fragments. A voltage of 1-5V/cm is usually applied.

1.5.3 Capillary gel electrophoresis

In order to achieve the high resolution between fragments that may only be a single base difference in size, as is the case with microsatellite fragment analysis, capillary gel electrophoresis is required. The development of capillary gel electrophoresis in the early 1990s has allowed an enormous increase in the resolution and sensitivity of the standard electrophoresis technique (Altria 2000). It is a much simpler and faster version of the earlier technique where the electrophoresis is carried out automatically in capillaries containing polymers in solution acting as a molecular sieve.

1.6 MOLECULAR MARKERS FOR DNA FINGERPRINTING

Molecular markers cover a variety of different techniques that can be used to analyse genetic variation, including determining individual identification and exclusion and assignment

of parentage (Parker *et al.* 1998). Certain markers involve investigating a number of single loci, such as is the case with microsatellites, while others investigate multiple loci simultaneously, as is the case with the Randomly Amplified Polymorphic DNA (RAPD) and minisatellite techniques (Bruford *et al.* 1998). To be of use in determining genetic variation the marker, whether single-locus or multilocus, must be polymorphic. Certain markers investigate polymorphism on a phenotypic level investigating the proteins expressed by the DNA, rather than the DNA itself e.g. allozymes (Parker *et al.* 1998). However, examples of markers which investigate the DNA itself include Restriction Fragment Length Polymorphism (RFLP), RAPDs, Amplified Fragment Length Polymorphism (AFLP), minisatellites and microsatellites (Bruford *et al.* 1998; Parker *et al.* 1998; Vos *et al.* 1995). Within these DNA markers, a marker may be dominant or codominant (Table 1.2). With dominant markers, such as RAPDs, it is not possible to distinguish between different alleles at a locus preventing identification of individuals as homozygous or heterozygous (Micheli & Bova 1997). Codominant markers, such as microsatellites, provide this differentiation (Schlötterer 1998).

The term 'DNA Fingerprinting' was first coined by Alec Jeffreys, a geneticist at the University of Leicester, England in 1985. In his initial paper "Hypervariable 'minisatellite' regions in human DNA", the term 'DNA fingerprinting' was used to describe the technique that examined minisatellite variation (Jeffreys *et al.* 1985). However, this term has since become more universal, encompassing the majority of individual identification techniques and has been used in this manner throughout this thesis (Bruford *et al.* 1998). The term 'minisatellite analysis' is now generally used when referring to Jeffrey's initial technique. All the fingerprinting techniques use the polymorphisms seen in an individuals DNA to make comparisons between individuals.

A DNA fingerprinting technique is one that has the ability to reveal individual-specific patterns (Bruford *et al.* 1998). The specificity of DNA fingerprints lends itself to individual identification (Parker *et al.* 1998). Most fingerprinting techniques measure diversity at the genotypic level. Variation at this fine scale level is a major advantage in using DNA markers over phenotypic markers. The information can either be used to study relationships between

given individuals, or they may be used on a higher level to study populations (Bruford *et al.* 1998).

DNA fingerprinting has been used to screen genetic variation in plants and animals and provide answers to a wide range of questions including an individual's identification, reproductive success, rates of genetic divergence in a population, as well as assignment and exclusion of paternity (Bruford *et al.* 1998; Parker *et al.* 1998; Marin & Pinna 1999). A summary of the appropriateness of the most common molecular techniques, for studies involving parentage exclusion and parentage assignment, is presented (Table 1.2). Although there are numerous techniques to address these questions, none are ideal. An ideal technique would be easy to develop and use, distinguish loci with co-dominant alleles and have many loci that show a high degree of variation that can be scored consistently and objectively. Ideally, it would also be useful to score multiple loci on the same gel without the risk of confusing them.

Table 1. 2 Evaluation of appropriate DNA molecular techniques' ability to exclude and assign parentage (following Parker *et al.* 1998; Questiau *et al.* 1999).

Technique	Ease	Parentage	
		Exclusion	Assignment
RFLPs**	Extraction, digestion, electrophoresis, library screening, gel blot hybridisation; analysis straight forward	Fair if sufficient polymorphism available	Many polymorphic loci required
Multilocus minisatellites**	Extraction, digestion, electrophoresis, gel blot hybridisation; analysis problematic	**Excellent**	Good if potential number of parents is small
Microsatellites**	Primer development may be long (library screen, sequencing) thereafter extraction, PCR, electrophoresis; analysis straightforward	**Excellent**; number of primers necessary depends on polymorphism and population size	Good for large samples if sufficient numbers of highly polymorphic loci (primers) available
RAPDs*	Extraction, PCR; analysis problematic	Possible but may be problematic owing to artifactual bands	Good for relative contributions to large broods; more general application developing
AFLPs*	Extraction, digestion, ligation of linker, selective amplification, electrophoresis; analysis fair	Good	Fair if large numbers of primers

* Dominant marker; ** Co-dominant marker

The researcher must choose the marker that can provide the best answers to the questions being asked using the least time and expense. The choice of marker will depend on the amount of genetic polymorphism required to answer the question, the statistical analysis available for the technique and the constraints of time and cost (Parker *et al.* 1998).

1.6.1 Restriction Fragment Length Polymorphisms

RFLPs are a commonly used technique, and were the first type of fingerprinting to be used (Parker *et al.* 1998). The technique involves the restriction of the genomic DNA with an enzyme, followed by gel electrophoresis and Southern Blot hybridisation of the resultant fragments and probing for a gene or sequence of interest. Variation arises from processes such as point mutations, small deletions or insertions, which create or destroy restriction sites (Brdička & Nürnberg 1993).

The Southern Blotting process hampers research using RFLPs, as this is a time consuming and costly technique. Due to the fact that PCR is not used, another disadvantage of this technique is that relatively large quantities of high quality DNA are required. Parker *et al.* (1998) suggest a good technician should be able to produce approximately 1000 RFLP genotypes per year. Due to the need for restriction digests and the Southern Blotting techniques used, RFLP analysis running costs per sample in 1998 were estimated at approximately $7 per sample (Parker *et al.* 1998).

1.6.2 Randomly Amplified Polymorphic DNAs

RAPDs are created by amplifying fragments of DNA with the use of a primer of near-random sequence. The technique consists of a PCR followed by gel electrophoresis of the resultant fragments. For a fragment to be amplified, the primer must be able to anneal on opposing DNA strands within fairly close proximity to each other. Different genomes differ slightly in their base sequence composition and so annealing sites may or may not be present. This is, theoretically, a very simple technique, however, problems lie in the analysis of gels and repeatability of the technique. This technique is examined in greater detail and is discussed further in section 1.7.

1.6.3 Amplified Fragment Length Polymorphisms

AFLPs were originally described by Vos *et al.* (1995) to provide a universal DNA fingerprinting method. The technique involves the digestion of total cellular DNA with two restriction enzymes. Adaptor cassettes are ligated onto the resultant restriction fragments before selective amplification by PCR using two primers occurs. Resultant amplified fragments are then separated and analysed by electrophoresis.

The advantage of using AFLPs is the speed at which they can be processed (Mueller & Wolfenbarger 1999). Background interference is also less likely because complementary primers are used in the PCR. The markers are, however, termed dominant markers, as they do not distinguish between homozygous and heterozygous individuals (Mueller & Wolfenbarger 1999).

1.6.4 Minisatellites

Previously, before the advent of microsatellites, multilocus DNA minisatellites were the molecular marker of choice for relatedness and forensic studies (Epplen *et al.* 1992). Minisatellite DNA sequences are generally considered to be those containing regions where more than 10 bp of sequence are repeated in tandem (Brdička & Nürnberg 1993). They are found scattered throughout the genome. Minisatellite analysis requires restriction of the genomic DNA using one or more restriction endonucleases, which recognizes a cleavage site. Restriction fragments are separated by size using electrophoresis before being Southern Blotted and probed. Probing is done by hybridising a specific repeat sequence to DNA fragments that have been transferred onto the membrane. There are a variety of probes available. 'Jeffreys probes', including 33.6 and 33.15, were those originally developed by Jeffreys in his initial pioneering work (Jeffreys *et al.* 1985). These, as well as the M13 probes, have become very popular and are the most commonly used probes (Bruford *et al.* 1998). These probes were developed for use in humans and have been used extensively for this purpose, particularly in fields relating to forensics and paternity testing (Chakraborty & Kidd 1991; Chakraborty *et al.* 1992; Jeffreys *et al.* 1991; Lewontin & Hartl 1991). They have, however, also been used for research on a wide range of other organisms besides humans. A notable amount of work using

these probes, among others, generally to determine relatedness, has been undertaken on birds. Species include House Sparrows (*Passer domesticus*) (Burke & Bruford 1987; Wetton *et al.* 1987), Great Reed Warblers (*Acrocephalus arundinaceus*) (Bensch *et al.* 1994), Short-tailed Shearwaters (*Puffinus tenuirostris*) (Austin *et al.* 1993), Pukeko (*Porphyrio porphyrio*) (Jamieson *et al.* 1994), Zebra Finches (*Taeniopygia guttata*) (Birkhead *et al.* 1990), Waved Albatrosses (*Phoebastria irrorata*) (Huyvaert *et al.* 2000), Peregrine Falcon (*Falco peregrinus*) (Wetton & Parkin 1997), Merlin Falcon (*F. columbarius*) (Wetton & Parkin 1997), Kestrel (*F. tinnunculus*) (Wetton & Parkin 1997) as well as several species of parrot and cockatoo, including species of *Amazona, Ara, Aratinga, Psittacula* and *Cacatua* genera (Brock & White 1991; Madsen *et al.* 1992; Miyaki *et al.* 1992; Miyaki *et al.* 1997; Nader *et al.* 1999). Minisatellites have also been used in studies on cranes. The M13 probe was used to establish paternity in Whooping Cranes by Longmire *et al.* (1992). M13 has also been used in studies on captive populations of Siberian Crane (*G. leucogeranus*) (Torkarskaya *et al.* 1994, 1995).

Variability arises from the number of times the sequence is repeated. Variation originates at DNA replication when there is unequal crossing over or strand slippage related to the repeated nature of the minisatellite sequence (Jeffreys *et al.* 1988). Minisatellites can be performed as either multilocus or single locus, although the term is generally used to refer to multilocus analysis. Single-locus minisatellites require the development of locus specific probes, which is both costly and time consuming (Parker *et al.* 1998). Once again the disadvantage of the minisatellite technique lies in the need for Southern Blot hybridisation. Probing is also often done with radioactively labelled probes, which is hazardous and requires extreme care. A good technician can process approximately 1000 multilocus fingerprints per year but due to the restriction digests and Southern Blot analysis running costs per sample are estimated at approximately $7 as of 1998 (Parker *et al.*1998).

1.6.5 Microsatellites

Like minisatellites, microsatellites, which are sometimes referred to as Simple Sequence Repeats (SSRs) or Short Tandem Repeats (STRs), are also DNA sequences repeated in tandem throughout the genome, however, they are composed of repeated sequences of only 1-6bp, shorter than minisatellite repeats (Parker *et al.* 1998). They also originate through unequal

cross over or strand slippage (Schlötterer 1998). However the analysis of microsatellites is different from minisatellites. Analysis involves amplifying the repeated unit by PCR and determining the actual number of repeats by electrophoresis. The problem with this technique lies in the development of the primers required to amplify the microsatellite, as this is difficult, time consuming and costly, and in most cases 10-20 polymorphic primer sets are required, as not all primers may be polymorphic (Queller *et al.* 1993). The actual number of primers required will depend on the level of polymorphism observed.

According to Parker *et al.* (1998) once the technique is well established, which can take anything from a few months to a few years, samples can be processed at a very fast rate of several thousand per year. Once development and optimisation has been established it is estimated that running costs per sample should be less than $1 per sample per locus as of 1998, when using manual analysis. Costs using automated fragment analyses would be higher. More in depth information regarding microsatellites are provided in section 1.8.

1.6.6 Techniques used in this study

This study uses two of the techniques described above, RAPDs and microsatellites. As already discussed, RAPDs are a relatively cheap, quick and simple technique. It is a multilocus technique with dominant markers. In comparison, microsatellites are a single locus technique and are not dominant. Although microsatellites are overall a much more costly technique, the major costs relating to development has been avoided as the microsatellite primers used in this study had already been developed by Glenn *et al.* (1997). Laboratory facilities and equipment were available for both of these techniques making them suitable for this study. Due to funding constraints, very few microsatellite primers could be examined. This was unfortunate as these are the marker of choice for parentage and forensic work and as such offered the best opportunity to develop a system of forensic analysis for these species in relation to the illegal trade if DNA evidence is to be used in court proceedings. Although not ideal, RAPDs had the potential to provide a cheap and rapid analysis method, which may have been useful in some other research areas, and so were included in this study. A more appropriate multilocus technique would have been minisatellites, which have been used historically in forensic analysis, however, the facilities for using this method were not available. In addition, minisatellites are

now considered outdated following the advent of microsatellites and do not provide the reliability in analysis of microsatellites.

1.7 RANDOMLY AMPLIFIED POLYMORPHIC DNA

With the majority of amplification techniques some prior knowledge of the DNA sequence is required for primers to be developed. This often has a prohibitive effect, as acquiring such information can be time-consuming and costly. For this reason Williams *et al.* (1990) developed a technique which allows amplification of genomic DNA in the absence of such sequence information.

The technique uses primers of near arbitrary sequence to amplify random genomic fragments. Unlike a standard PCR, only one primer is used in the reaction instead of two and this single oligonucleotide acts as both a forward and reverse primer. Two other random amplification techniques are available at present, arbitrarily primed PCR (AP-PCR) and DNA amplification fingerprinting (DAF). They differ in the length of primer used and complexity of the resultant fingerprint (Micheli & Bova 1997). RAPDs generate the least complex patterns of the three techniques. It is also the least expensive, and the easiest and fastest technique of the three (Micheli *et al.* 1997). Together these techniques may collectively be termed multiple arbitrary amplicon profiling (MAAP) (Micheli & Bova 1997). The level of information provided by a single RAPD marker is very low as each marker provides only a limited level of polymorphism. It is only when several markers are used that this technique becomes useful. As greater polymorphism is seen, the probability of any two individuals sharing the same profile decreases. RAPDs have been shown to be useful in numerous organisms including bacteria, plants, invertebrates and vertebrates including primates and marsupials as well as several domestic animals (Bickel *et al.* 1993; Bishop *et al.* 2000; Fowler *et al.* 1998; Gwakisa *et al.* 1994; Koeleman *et al.* 1998; Latta & Mitton 1997; Neveu *et al.* 1996, 1998; Riedy *et al.* 1992).

In avian research much of the RAPD work done to date has been on various breeds of chicken (Singh & Sharma 2002; Smith *et al.* 1996; Tsuji *et al.* 1997; Wei *et al.* 1997). Studies have included population genetic relatedness and variability as well as species discrimination and identification. Similar studies have also been done on the Ostrich (*Struthio camelus*) (Bello & Sánchez 1999) and various Quail species (*Coturnix* spp.) (Sharma *et al.* 2000). RAPDs have even been used by Calvo *et al.* (2001) to identify the species used in poultry pâté. The RAPD technique has also been used for research on endangered birds, such as the Iberian Imperial Eagle (*Aquila adalberti*) (Padilla *et al.* 2000), Red-cockaded Woodpeckers (*Picoides borealis*) (Haig *et al.* 1994) and Light-footed Clapper Rail (*Rallus longirostris levipes*) (Nusser *et al.* 1996) due to the fact that only very small DNA samples are required for PCR, as is preferable with threatened species. However it is noted that all these studies were at a population level. Due to the heterogametic nature of avian sex chromosomes Lessells and Mateman (1998) have also used RAPDs to determine the sex of various species of birds. RAPDs have been used previously in cranes to determine the sex of an individual (Duan & Fuerst 2001).

1.7.1 RAPD primers

The primers used in the RAPD technique are 10-mer oligonucleotides with a G+C content of 40% or greater that lack palindromic sequences (Williams *et al.* 1990). For a fragment to be amplified two primers, which are identical in sequence, must be able to anneal in the correct orientation at sites on opposing DNA strands at a distance of less than 2kb from each other (Edwards 2001). The primers anneal at a number of randomly distributed sites throughout the genome. Any form of mutation, including substitutions, deletions, insertions and inversions, occurring in the DNA sequence will affect the annealing ability of the primers and result in a different set of amplified fragments. Even a single nucleotide change may affect the primer annealing site and introduce sufficient mismatch for amplification to occur (Micheli & Bova 1997). Occasionally the DNA fragment lying between two priming sites may contain a repeat unit. Variation in the number of the repeat units will affect the length of the DNA fragment lying between two priming sites. Such variation may also introduce variation in the resultant profile.

1.7.2 Advantages of RAPDs

The great advantage of this technique lies in its simplicity. The PCR is done with a single primer in a single cycling program. The resultant amplified products are run on an agarose gel and stained with ethidium bromide. This technique also has the advantage that the primers used are not species-specific. Thus a universal set of primers can be used. No previous information on the nucleotide sequence is required, which greatly reduces time and cost. Due to the fact that this is a PCR based technique, another major advantage of this technique over those such as RFLPs is that only nanogram amounts of genomic DNA are required on which to perform a reaction.

1.7.3 Disadvantages of RAPDs

Due to the randomness and sensitivity of the technique results have been known to be affected by DNA template quality, reaction conditions and temperature profiles. For this reason the reliability and repeatability of this technique has come into question. DNA quality seems to have the greatest effect on reproducibility and for this reason it is essential to ensure that only good quality high molecular weight DNA is used as a template. Degraded template may result in unreliable and irreproducible fingerprints and should not be used (Arribas *et al.* 1997, Weeden *et al.* 1992). To illustrate the irreproducible nature of RAPDs Jones *et al.* (2001) developed a RAPD exchange package, which was distributed to eight other European labs. The package contained two genomic DNA samples from two different clones of Poplar (*Populus x euramericana*), two decamer primers, *Taq* polymerase, 10X PCR buffer, agarose, a detailed protocol and a photograph of the RAPD profile obtained. As can be seen in the diagrammatic representation of the results (Figure 1. 8) only one of the eight labs was able to reproduce the profile exactly.

Another disadvantage of this technique lies in its dominant nature. Polymorphism is inherited in a Mendelian fashion but using this technique it is not possible to distinguish between heterozygous or homozygous individuals at a particular locus (Hadrys *et al.* 1992). RAPDs also have the disadvantage that alleles from different loci may be of similar size. The

alleles can then not be assigned to different loci and this makes estimating certain parameters difficult (Queller *et al.* 1993).

Another problem area with RAPDs lies in their analysis. RAPD profiles often have bands of varying intensity. It is often difficult to objectively score these bands, as human decision must define how faint a band can be before it is ignored (Queller *et al.* 1993).

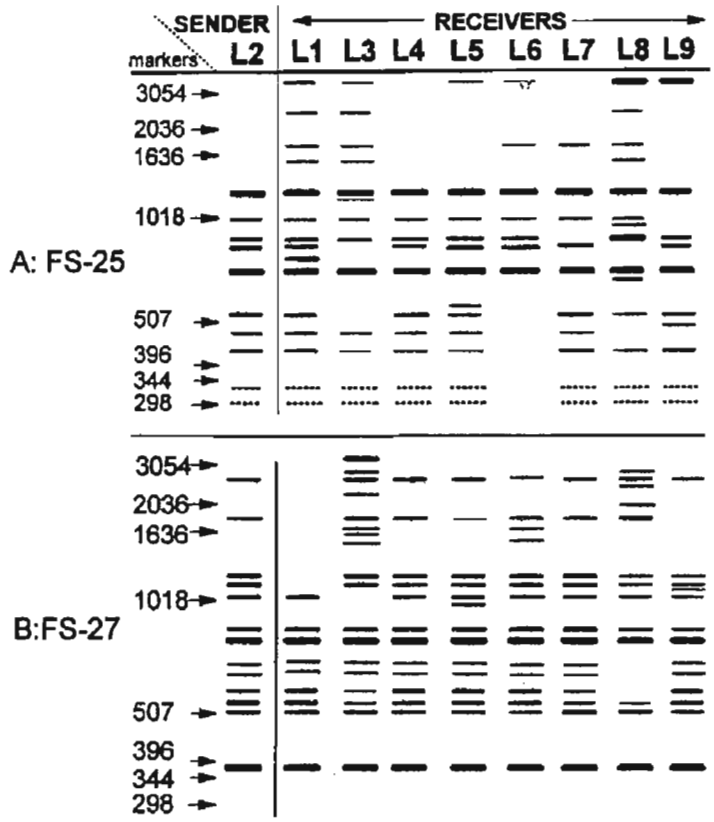

Figure 1. 8 Diagrammatic representation of the RAPD profiles obtained by the Milan group (L2) along with the other eight laboratories (after Jones *et al.* 2001).

1.8 MICROSATELLITES

Microsatellites are short tandemly repeated sequences of DNA scattered throughout the eukaryotic genome. The repeated units are 1-6bp in length and may be repeated 5 to about 100 times at each locus (Tautz 1993). Microsatellites can easily be amplified using PCR if the DNA sequences flanking the microsatellite have been identified and primers have been developed.

The technique consists of a simple PCR reaction using the two primers that have been identified to be complimentary to the sequences on either side of the microsatellite locus. Previously, analysis was done by running the resultant fragments on a polyacrylamide gel. However, with the development of the automated fragment analyser this process has been made much simpler and the results very precise due to the incorporation of an internal size standard (Edwards *et al.* 1991). When two fragment sizes are observed, this indicates the individual is heterozygous with each band representing an allele. However, when just one band is seen, the individual is assumed to be homozygous as both alleles are of the same size.

Microsatellite mutation rates range from 10^{-3} to 10^{-6}, which may be the gain or loss of, normally a single, repeat unit (Schlötterer 1998). Microsatellites with dinucleotide repeats have much higher mutation rates than those with trinuclotide repeats and those with larger numbers of repeats mutate faster than those with a smaller number (Schlötterer & Tautz 1992). The two main mechanisms thought to cause mutations in the number of repeats within a microsatellite locus are unequal cross over during meiosis and slippage (Levinson & Gutman 1987). Slippage is a mutation process that may occur during DNA repair or replication and it is thought to be the predominant cause of variation of microsatellite repeat number (Tautz 1989; Wolff *et al.* 1991). Although the frequency with which slippage mutations occur is not known, Tautz (1989) has shown that the frequency is not sufficient enough to occur in successive generations. It is, however, high enough to maintain a high degree of polymorphism in a population, which provides a useful tool for analysis of relatedness, such as parentage.

Microsatellites are an extensively used molecular marker and studies have included microsatellite locus identification, determination of population genetic variation and relatedness, and genetic mapping as well as species discrimination and identification. Research using microsatellites has been performed on plants including rice (*Oryza sativa* L.) and maize (*Zea mays* L.) (Ikeda *et al.* 2001; Senior *et al.* 1996), invertebrates including *Drosophila*, *Anopheles* mosquito and forest ants (*Myrmica punctiventris*) (Herbers & Mouser 1998; Irvin *et al.* 1998, Wang *et al.* 2001), mammals including various dog breeds, pilot whales (*Globicephala melas*), Alpine ibex (*Capra ibex* (*ibex*)), chimpanzees (*Pan troglodytes verus*) and cheetah (*Acinonyx jabatus*) (Amos *et al.* 1993; Gagneux *et al.* 1997; Harley *et al.* 2000;

Maudet *et al.* 2002; Pihkanen *et al.* 1996) and of course a wide range of birds including various swallows (Hirundinidae family), flycatchers, Darwin's Finches (*Geospiza fortis*), Peregrine Falcon and many more (Conrad *et al.* 2001; Ellegren 1992; Keller *et al.* 2001; Nesje *et al.* 2000).

The majority of previous research involving the use of microsatellites in paternity testing has been on birds that are socially monogamous to investigate extra-pair paternity for which they are a useful and reliable tool (Conrad *et al.* 2001; Ellegren *et al.* 1992; Keller *et al.* 2001; Primmer *et al.* 1995). Primmer *et al.* (1995) used six polymorphic microsatellite primers to resolve genetic relationships in the Barn Swallow (*Hirundo rustica*). An exclusion probability of 0.9996 was achieved and identified that 13 (30%) of the offspring in five broods were from extra-pair fertilization. Nine of the 13 extra-pair offspring were likely to be the offspring of a male of another family in the colony.

1.8.1 Development of microsatellite primers

The major drawback of microsatellites lies in the development of the primers. Although microsatellites are common in eukaryotic genomes they still have to be found. This is a time consuming and costly process. The regions upon which primers are designed are highly conserved in individual species. If the organism's genome has already been sequenced the microsatellites can easily be identified and primers developed, but this is rarely the case. If microsatellite primers have already been developed for a closely related species to the study species, the primers can be used in an attempt to amplify across species. However, if this is not the case one may need to construct a genomic library. A detailed description of the two alternative protocols to achieve microsatellite primer isolation is described by Schlötterer (1998). His first protocol uses the standard isolation procedure, which is recommended for the isolation of a limited number (<30) of dinucleotide loci. His second protocol, however, uses an enriched library for microsatellite locus identification. This protocol only clones pre-selected DNA fragments, containing a microsatellite motif, into the sequencing vector. Thus protocol two is much more efficient even though it may be more laborious

1.8.2 Advantages of microsatellites

Microsatellites are unmatched for their speed and cost effectiveness. However, numerous studies, including those on Atlantic salmon (*Salmo salar* L.), social wasps (*Polistes annularis*) and the *Anopheles* mosquito (Hughes & Queller 1993; Lehmann *et al.* 1996; Sánchez *et al.* 1996), have shown that even when very little allozyme variation is found, microsatellites show great diversity and provide the researcher with much more informative information.

A molecular marker's quality for DNA fingerprinting is dependant on its consistency, accuracy and how objectively it may be scored (Queller *et al.* 1993). Microsatellites match up to all of these aspects. Unlike RAPDs, which require very clean, high quality DNA template, microsatellites can make use of DNA that is not of optimal quality. This allows us to make use even of poorly preserved specimens as illustrated by Ellegren (1991) who performed microsatellite analysis using DNA extracted from feathers of museum birds which were more than 100 years old. Microsatellites only require minute quantities of DNA so feathers are a promising DNA source as it allows the use of a non-invasive sampling technique, which is very beneficial, particularly when working with endangered species.

With microsatellites, primers that amplify various loci sufficiently different in size may be pooled (Queller *et al.* 1993). When using automated machines, different fluorescent labels are used to distinguish between different loci, thus allowing several loci to be investigated at once (Edwards *et al.* 1991).

1.8.3 Disadvantages of microsatellites

As already discussed, the major drawback of microsatellites lies in their development, which is very costly and time-consuming. Once the primers have been developed there is still the possibility that the microsatellites obtained may not be polymorphic and this then first needs to be tested. This disadvantage is exagerated by the fact that microsatellite primers are very species-specific and so primers developed for one species are less likely to amplify or be polymorphic, as evolutionary distance from the species they were developed in increases (Primmer & Ellegren 1998).

When analysing microsatellite data from an automated fragment analyser stutter bands are often seen, particularly in the dinucleotide repeats. Stutter bands are a common occurrence in microsatellites and appear as a minor peak one repeat shorter than the true allele peak. They are produced during PCR amplification and are thought to result from slipped strand mispairing (Hauge & Litt 1993). When *Taq* DNA polymerase is used stutter bands are usually 10% of the height of the allele peak (Walsh *et al.* 1996). Resulting microsatellite profiles need to be manually checked to ensure stutter bands do not interfere with the true allele peak readings and to eliminate the potential for false positives.

1.8.4 Crane microsatellites

Thirty-seven microsatellite loci have been identified for Whooping Cranes by Glenn *et al.* (1997). Since then some of these loci have been used to resolve uncertain paternities occurring after multiple artificial inseminations in captive Whooping Cranes (Jones & Nicolich 2001) as well as to make improvements to the Whooping Crane studbook by developing comprehensive genetic pedigrees for the captive population (Jones *et al.* 2002). Both of these studies have greatly benefited the captive management of these endangered birds. These Whooping Crane microsatellite primers have also been used across species to investigate the genetic variation and structure among Wattled Crane, Sarus Crane (*Grus antigone*) and Sandhill Crane (*Grus canadensis*) (Jones 2003). In the Wattled Crane, Jones (2003) found that the South African population was an isolated group showing no gene flow with the Botswana and Zimbabwe population. The Sarus Cranes showed a limited gene flow across all populations in Asia. The Sandhill Cranes were seen to have a minimum gene flow across subspecies with the Canadian subspecies (*G. c. rowani*) being a transitional form of the two subspecies.

Microsatellites have also been developed for Red-crowned Cranes (*G. japonensis*) by Hasegawa *et al.* (2000). These seven microsatellites were found to cross-amplify in eight other crane species, including Blue Cranes and Wattled Cranes, however, they were not tested for polymorphism.

1.9 FORENSIC PARENTAGE TESTING

Parentage testing is often used in wildlife forensics (Ruth 1994; Shorrock 1998). Trade in wildlife has become a highly profitable business with both domestic and international trade in a wide variety of species (Bodasing & Mulliken 1996). TRAFFIC International (2003) has estimated annual global turnover at billions of dollars. The only requirement for international trade in wildlife is a certificate, from the country of export, stating that the animals are captive bred (Bodasing & Mulliken 1996). The high economic payoffs often lead breeders into the illegal and unethical trade in wild caught birds and animals (Shorrock 1998).

The ability to test parentage, therefore, is very beneficial. DNA evidence in human parentage testing is so convincing that in the USA, on average, less than 0.1% of cases continue to court (Kirby 1990). Genetic evidence has played a major role in identifying illegal conduct with regard to wildlife. According to Kirby (1990), Jeffrey's minisatellites were used to uncover the illegal sale of a number of macaws being sold as offspring of a group of breeding birds. Fingerprinting revealed that the offspring were in fact of wild origin and the guilty were convicted and sentenced. In 1991, the Royal Society for the Protection of Birds (RSPB) had a breakthrough in wildlife trade when a Liverpool man was convicted for the illegal sale of four Goshawks (*Accipiter gentiles*) with a market value of £3 000 (Shorrock 1998). Genetic profiling has since led to the conviction of at least 11 individuals to date. In 1987 a shipment of 20 "captive bred" Hyacinth Macaws (*Anodorhynchus hyacinthinus*) and 20 Palm Cockatoos (*Probosciger aterrimus*) were exported to the United Kingdom (Bodasing & Mulliken 1996). Due to the fact that from 1976 to 1987 only five Hyacinth Macaws and eight Palm Cockatoos were imported, and captive breeding data suggest these species have low reproductive rates, it is highly unlikely that the birds in question were captive bred (Bodasing & Mulliken 1996). The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) was drafted in 1973 by 80 countries due to growing concerns that international trade in wildlife may drive some species to extinction if not controlled (Mulliken 1995). South Africa became a Party of CITES when it entered into effect in 1975 (Mulliken 1995).

1.9.1 RAPDs and paternity

RAPDs are used for paternity testing due to their simplicity, speed and cost effectiveness (Fowler *et al.* 1998; Neveu *et al.* 1996). However, opinions vary as to their suitability. Riedy *et al.* (1992) found an excess of non-parental bands when determining paternity in chacma baboons (*Papio cynocephalus ursinus*). They concluded that these bands may have been due to mutation or PCR artefact and felt that these markers were not suitable for paternity studies due to likelihood of false exclusions. However, RAPDs have been used to determine paternity in the adder (*Vipera berus*), marine invertebrates, damselflies (*Calopteryx splendens xanthostoma*) and dragonflies (*Anax parthenope*) (Bishop *et al.* 2000; Hadrys *et al.* 1993; Hooper & Siva-Jothy 1996; Levitan & Grosberg 1993; Tegelström & Höggren 1994). This method was successful for these species as they produce large numbers of offspring and can be analysed using a 'synthetic offspring' approach. It is still noted that reproducibility of results is highly dependant on quality and concentration of the DNA, the polymerase used and the assay conditions. Although it is often mentioned that DNA fingerprinting or microsatellites may have provided better resolution for paternity testing, Neveu *et al.* (1996) feel that the limitations of RAPDs do not interfere sufficiently enough to prevent their use.

1.9.2 Microsatellites and paternity

Microsatellites are widely used in paternity investigations. These co-dominant markers are better for paternity studies as both alleles can be differentiated. The majority of paternity work using microsatellites has been to investigate extra-pair paternity in birds (Conrad *et al.* 2001; Ellegren 1992; Keller *et al.* 2001; Petren 1998; Primmer *et al.* 1995; Richardson *et al.* 2001). Microsatellites were used for the first time by Alderson *et al.* (1999) to investigate brood parasitism in the Brown-headed Cowbird (*Molothrus ater*). Although cheetah, considered an extremely inbred mammal, show almost no allozyme variability due to a population bottleneck, there was sufficient microsatellite variation for Harley *et al.* (2000) to be able to investigate paternity in a cheetah litter using microsatellite markers.

Jones and Nicolich (2001) used 12 of the 37 microsatellite loci previously identified for Whooping Cranes, developed by Glenn *et al.* (1997), to resolve uncertain paternities occurring

after multiple artificial inseminations in captive Whooping Cranes. Prior to this study, paternity assumptions were made according to the timing between insemination and albumin deposition, which occur 2 days before laying. Any potential sires were excluded if their microsatellite profile did not match that of the chick at any of the 12 loci. In cases where a maternal sample was not available, a partial maternal profile was generated from those of her offspring. Paternity was assigned when all but one of the potential sires had been excluded. Twenty of the 23 paternity cases in question were resolved with the remaining three being inconclusive. Results indicated that the previous method of paternity assignment, using insemination timing, was not a reliable indicator. It was also noted that cranes were capable of sperm storage.

1.10 REVIEW OF STATISTICAL ANALYSIS

1.10.1 Statistics for RAPDs

RAPD profiles produced on electrophoresis gels are scored on a binary system where fragments are scored as either present (1) or absent (0). According to Bruford *et al.* (1998) bands showing consistent segregation, or co-segregation, may be linked or be alleles of the same locus. When this is observed only one of the bands/allele bands showing consistent segregation or co-segregation should be retained for further analysis. Certain bands may also show sex linkage and these should also be removed before analysis.

The Band Sharing Coefficient (BSC) is the main index of similarity used in DNA fingerprinting (Bruford *et al.* 1998). The resultant value is a measure of the probability that a band in a given individual will be in any other random individual. This value is calculated by the formula $x = 2Nab/(Na + Nb)$ where Nab is the number of bands shared between two individuals a and b, Na is the total number of bands in a, and Nb is the total number of bands in b. If bands are independent markers, then the probability that all n bands occurring in a given individuals profile will be present in another random individual is x^n (Bruford *et al.* 1998). This is an important value to be able to calculate as once an individual is deemed to be the true parent of another individual, the probability that this is indeed the only individual that could be the parent must be determined.

Bruford *et al.* (1998) have shown that if it is assumed that all bands occur at equal frequency in a population, and shared bands are always identical alleles occurring at the same locus, then the allele frequency can be determined using the BSC by the equation $x = 2q-q^2$, where q is the allele frequency. Once allele frequency is known a parent:offspring BSC can be estimated by the equation $x = (1+q-q^2)/(2-q)$.

A novel band is one that is seen in the offspring that could not have been inherited from either parent or one that is not present in an offspring even though both parents possess it. When there are one or two novel bands in a profile there is the possibility that these bands may be the result of mutation. Minisatellites have a mutation rate in the order of 10^{-3} per fragment per gamete (Bruford *et al.* 1998; Burke & Bruford 1987) but there is no evidence that mutation rates have been calculated for RAPDs.

In some cases it occurs that misassigned parentage is not detected. Assuming the correct parents are not related to the putative parents, the probability of such an event occurring can be calculated by $I=(1-(1-x)^2)^n$. 1-I then would calculate the exclusion probability of false parentage (Bruford *et al.* 1998).

1.10.2 Statistics for microsatellites

1.10.2.1 Hardy-Weinberg Equilibrium and Linkage Disequilibrium

The 'Hardy-Weinberg Law' (H-W) states that over time and across generations gene frequencies will remain unchanged under certain assumptions. The breeding population must be large, exhibit random mating with no selection, no migration or mutation can occur, genotype frequencies must be equal initially and Mendelian segregation must occur (Lange 2002). The majority of statistical analyses make assumptions that the population will be in H-W equilibrium and therefore it is appropriate to examine for this, prior to using these statistics. However, it would be unusual to find any population with all of these characteristics, and this should be recognised when using these markers.

Certain loci may not show independent segregation as assumed by Mendel but may rather be linked during the formation of gametes at meiosis. This will have an effect on statistical estimates and so the amount of linkage needs to be determined. This is done by

estimating the amount of linkage disequilibrium. Although not significant, Jones *et al.* (2002) found some linkage disequilibrium in their study of Whooping Cranes. They suggest the disequilibrium is probably due to the population bottleneck experienced by the Whooping Cranes.

1.10.2.2 Polymorphism and Heterozygosity

Microsatellites have been known to be extremely polymorphic. Amos *et al.* (1993) found one locus to contain 54 different alleles in long-finned pilot whales. In birds, Dawson *et al.* (1997) found 46 alleles at one locus in the Yellow Warbler (*Dendroica petchia*), however, in cranes, there is a noticeable lack of variation (about 4 alleles per loci)(Jones 2003).

The amount of heterozygosity in a population is a simple way of measuring genetic variation. The amount of observed heterozygosity varies in the various species of cranes around the world. Hasegawa *et al.* (2000) found Red-crowned Cranes to have an observed heterozygosity of 0.586 while Tokarskaya *et al.* (1995) observed a heterozygosity of 0.85-0.72 in Siberian Cranes.

The five microsatellite loci used in this study were suggested by Jones (2003) as they have been found to amplify well and to be polymorphic in the Wattled Crane. Although these loci have previously been studied in the Wattled Crane they were re-examined on the eight samples available for this study. These primers were used to screen for polymorphism in Blue Cranes and Grey Crowned Cranes.

1.10.2.3 Wright's Fixation Index (F_{IS})

Sewall Wright developed the concept of a fixation index to measure the amount of inbreeding in subdivided populations (Excoffier 2001). F is a correlation between homologous genes at various levels relative to other levels. F_{IS} then may be termed the local inbreeding coefficient as it compares the correlation between individuals ($_I$) relative to the genes within a subpopulation ($_S$). Inbreeding would be indicated by a positive F_{IS} as there is an increase in homozygosity while negative F_{IS} values indicates a lack of inbreeding.

1.10.2.4 Parentage analysis

Parentage analysis involves excluding all other individuals as potential parents except for the true parents. Due to the Mendelian nature of microsatellites, any allele seen in an offspring must be either maternally or paternity derived. For this reason all combinations of putative parents should be screened for non-matching bands across all loci. Due to the high mutation rate of microsatellites (Bruford *et al.* 1998) there is the possibility that an odd band will not be identical to the profile seen in the potential parents.

In most cases of parentage testing the mother is known and a paternity test must be done. In the case of paternity testing the probability that an individual is the true father must be determined relative to the probability that he is not the father. This is represented by the Paternity Index (PI). Methods to determine this have been developed (Elston 1986; Ostrowski 2003). If X is the probability that the alleged father (AF) passed on a paternal gene and Y is the probability that a random male (RM) passed on the paternal gene then the PI is calculated as X/Y. If the alleged father is heterozygous X=0.5 and X=1 if he is homozygous. The probability that RM passed on the allele will depend on the frequency of the allele in the population. The PI is calculated for a single locus so the Combined Paternity Index (CPI) is for all available loci (Ostrowski 2003). This is calculated by multiplying the PI's of all loci together. Bayes' theorem is required to test the theory that the selected individual is indeed the father. This is the probability of paternity and is calculated by CPI/CPI+(1-PP)x100 where PP is the prior probability. Assuming the value is neutral and unbiased, 0.5 is usually used by laboratories for PP (Ostrowski 2003).

1.11 AIM OF THIS STUDY

This thesis investigates the suitability of RAPDs and microsatellites for their potential in forensic parentage investigations of South African cranes. In order to determine this, this dissertation :

RAPDs

- determines which of the 15 available RAPD primers generate polymorphic profiles;
- determines the amount of variation in unrelated captive birds within the population of Blue, Grey Crowned and Wattled Cranes;
- examines the appropriateness of RAPDs for parentage testing;
- determines if RAPDs are suitable for forensic applications of parentage testing;
- determines the amount of genetic variation/similarity in captive populations of cranes;

Microsatellites

- examines 5 microsatellite primers previously found to be polymorphic in Wattled Cranes to determine if they are conserved in the Blue Crane and Grey Crowned Crane;
- detemines the amount of variation in unrelated captive birds within the population of Blue, Grey Crowned and Wattled Cranes;
- examines the potential of microsatellites for parentage testing;
- determines if microsatellites are suitable for forensic applications of parentage testing.

Chapter 2

MATERIALS AND METHODS

2.1 DNA SAMPLES

2.1.1 Sample aquisition

A number of crane aviculturalists and zoological parks in South Africa were approached to donate blood samples from their captive cranes for DNA analysis including parentage analysis. Details of the organisations approached, their location within South Africa and the number of samples obtained from the various species are presented (Table 2. 1).

Table 2. 1 Numbers of blood samples of the three South African crane species collected from various sources.

Organisation	Location	Number of crane blood samples		
		Wattled	Blue	Grey Crowned
Amazona Endangered Parrot Breeding Facility	Assagai, KZN	4	5	8
Umgeni River Bird Park	Durban, KZN	2	9	3
Mitchell Park Zoo	Durban, KZN	2	2	4
Tygerberg Zoo	Cape Town, Western Cape	0	6	3
Monte Casino Bird Park	Johannesburg, Gauteng	0	3	0
Rehobath Birds	KZN	0	1	0
	Total	8	26	18

Birds were caught and a qualified vet exposed the brachial vein and wiped the area with a sterile swab. Samples were collected using a 1ml syringe with a 25Gx5/8" needle. Approximately 0.5ml of blood was drawn from each bird and transferred to an EDTA vacutainer tube, which was inverted several times to prevent the blood clotting. Samples were individually labelled with details as to the species, the unique transponder and/or ring number of the bird, its location and the collector's name (Appendix 3). In the laboratory, as samples were received, they were uniquely labelled in sequential numerical order. Blood samples were refrigerated at 4°C for a short period of time until a DNA extraction was performed and thereafter stored at –20°C.

2.1.2 DNA extraction

2.1.2.1 Overnight lysis

Extractions were optimised by examining various volumes of blood, ranging from 2.5µl – 20µl. It was established that best DNA extractions resulted from 15µl of blood. The blood was added to a 1.5ml microfuge tube containing 500µl 1XTNE lysis buffer, 50µl 1M Tris (pH8) (Roche), 1 unit Proteinase K (Roche), 7.5µl 25% SDS (Roche) and 7.5µl 10% Triton X-100 (BDH) and incubated overnight in a water bath at 37°C for cell lysis to occur.

2.1.2.2 DNA salt extraction

A salt extraction protocol, adapted from Bruford *et al.* (1998) was used to extract DNA (T. Taylor, 2003, pers. comm.). The microfuge tube containing the lysed cell mixture was removed from the water bath the following morning. 300µl of 5M NaCl (Saarchem) was added to the sample. Microfuge tubes were shaken hard for 15 seconds. Although this slightly increases the risk of the DNA being sheared it is an essential step for the dissociation of the DNA from any proteins (samples were later checked to ensure no significant damage had occurred – see below). Samples were centrifuged (Sigma 113 centrifuge) at 5000rpm for 15 minutes to pellet cell debris. The supernatant containing the DNA was carefully transferred to a new 1.5ml microfuge tube, ensuring none of the pellet was disturbed and avoiding any foam if present. At this point it was essential to make use of wide orifice tips for the transfer of the supernatant to prevent fragmentation of the DNA. This cycle, shaking,

centrifuge and transfer to a clean tube, was repeated several (usually 5) times until the supernatant was completely clear of any cell debris.

2.1.2.3 Ethanol precipitation

Two volumes of ice cold 100% ethanol were added to the clear supernatant and the tube inverted several times to precipitate the DNA. Samples were stored at –20°C for 30 minutes to increase the DNA yield. Following which the sample was centrifuged at 13 000rpm for 15 minutes to pellet the precipitated DNA. The supernatant was carefully removed and the pellet was washed with 1 volume of 70% ethanol. It was found that placing the sample on a shaker for 10 minutes at this point improved salt removal providing a cleaner end sample. The DNA was again pelleted by centrifuging at 13 000rpm for 10 minutes. The wash step was repeated several (usually 3) times to remove any remaining salt.

2.1.2.4 Resuspension

The final pellet was left to air dry, removing any remaining ethanol before adding 50µl 10mM Tris (pH8). The DNA was resuspended in a 37°C water bath overnight.

2.1.3 DNA quantification

Extracted DNA was run on a 0.6% 1XTAE agarose gel (Hispanagar) (described below) containing 0.5µg/ml ethidium bromide (Sigma) to ensure the DNA was of a good quality and high molecular weight. DNA concentrations were determined using a Pharmacia Biotech Ultrospec 2000 with a 100X dilution factor. Readings were taken at wavelengths of 260nm and 280nm and readings were used to determine concentration (using the equation: Concentration (µg/ml) = A260 x dilution factor x 50) and purity (using the equation: Purity = A_{260}/A_{280}). Only extractions giving purities of ±1.8 were used for further research.

2.2 RAPD PCR

2.2.1 RAPD primers

Fifteen RAPD primers were already available in the laboratory. These sequences had been selected from a previous study on Iberian Imperial Eagles (Padilla *et al.* 2000). Primers

that showed the highest number of scoreable bands were chosen. Sequences and names of the primers can be found in Table 2. 2. Primers were supplied by Integrated DNA Technologies (Whitehead Scientific). Dry primers were resuspended in 50µl TE. Working stocks were made to 100µM.

Table 2. 2 RAPD primer sequences used.

Primer Name	Primer Sequence	Sequence reference no.
OPA 003	CAG CCT CGG C	2601
OPA 004	GGG ACG CT C	2602
OPA 006	CCG CGC CGG T	2603
OPA 007	GTC CGA GGC C	2595
OPA 009	TCG GCG AGC C	2604
OPA 010	GCC ATC GGG C	2605
OPA 011	AAA CGG GCG G	2596
OPA 014	GAC CTG GCC G	2597
OPA 015	GTG CCT GCC G	2606
OPA 019	CTG GAG CGG C	2598
OPA 022	TGG GCA CGG C	2607
OPA 023	CGG CTC GGG T	2608
OPA 031	CAC GGT CGG C	2599
OPA 032	GCA CGC ACC G	2600
OPA 065	AAT CGG GTC G	2609

2.2.2 RAPD PCR components

A Roche Diagnostics PCR Core Kit was used and various optimisation reactions were performed. Annealing temperatures were varied between 34°C and 40°C, $MgCl_2$ concentration was varied from 1mM to 5mM and primer concentrations were also varied. Final reaction conditions are presented in Table 2. 3. DNA working stocks were made to a concentration of 100ng/µl. Master mixes were made and aliquoted out into PCR tubes already containing the DNA. Reaction volumes were made up to 25µl.

Table 2. 3 Optimised PCR reaction components.

PCR component	Required concentration
10X PCR buffer	1X
dNTPs	200µM
$MgCl_2$	1mM
Primer	2µM
Taq	1U
DNA	100ng

2.2.3 RAPD PCR protocol

Initial reactions were examined which established that a 'hotstart' protocol was not necessary. The optimised PCR protocol was as follows: 94°C for 3mins, followed by 40 cycles of 94°C for 1min, 40°C for 1 min and 72°C for 2mins and finally 72°C for 10 minutes. PCR was performed on an Applied Biosystems GeneAmp PCR system 9700.

2.3 MICROSATELLITE PCR

2.3.1 Microsatellite primers

Microsatellite primers were developed by Travis Glenn (University of Georgia, USA) for Whooping Cranes (Glenn *et al.* 1997). Primers were synthesized at the DNA Synthesis Laboratory at the University of Cape Town (UCT), South Africa. Forward primers were fluorescently labelled with either FAM or HEX enabling automated sequencing. A list of these primers is provided in Table 2. 4.

Table 2. 4 Microsatellite primers for use on cranes (T.C. Glenn unpublished data; Glenn 1997).

Primer Name	Primer Sequence (5'-3')	Fluorescent label	UCT ref. number
Gamμ3a	CAC ATT GCC AGA CTG TTG TAT	FAM	02-0825
Gamμ3b	ATC CCT GAA GCT AAC AAT AAA CC	-	02-0826
Gamμ6a	CAC CTT TTA TTG CGT ATG TAT TTT	HEX	02-0827
Gamμ6b	GGA TTA TGT TTT GGT TT GTT TTT	-	02-0828
Gamμ7a	TAA AGG AGT GGC TGC TGC TGT G	FAM	02-0829
Gamμ7b	CTG AGG CTC TGC TGT GGG AAA C	-	02-0830
Gamμ12a	GAG TGG GAG GGG ATA GGA TGG ATT	HEX	02-0831
Gamμ12b	AGC CTG ACA GCA AGA CCA AAG TAA	-	02-0832
Gamμ101c	CAG TAT AAA AAAC AAA CAG GTG AGA	FAM	02-0833
Gamμ101d	TGA AAA AAG TAC AGG AGA ACA TAG	-	02-0834

2.3.2 Microsatellite PCR components

The reactions were optimised individually rather than as multiplex reactions. In larger studies multiplexing provides a more time and cost effective method however this requires optimisation and with the limited number of samples in this study it was not necessary.

Five separate PCRs were performed for each of the primer sets. Optimised primer concentrations can be seen in Table 2. 5. Concentrations of the other PCR components were as follows: 2ng/µl of template DNA, 1X PCR buffer, 1.7mM MgCl2, 0.7mM dNTPs and 0.125U Taq polymerase. Reaction volumes were made up to 6µl.

Table 2. 5 Required concentrations of microsatellite primer in specific PCRs.

Primer sets	Required concentration (forward & reverse)
Gamµ3	0.67µM
Gamµ6	0.92µM
Gamµ7	0.08µM
Gamµ12	0.1µM
Gamµ101	1.34µM

2.3.3 Microsatellite PCR protocol

All PCR reactions began with a 2 minute denaturation at 94°C and ended with a 7 minute final extension at 72°C. Most reactions contained 34 cycles, however, in the Wattled Cranes with primer Gamµ101 only 27 cycles were needed. All PCR cycles began with a denaturation step at 95°C for 15 seconds and ended with an elongation step at 72°C for 30 seconds. Optimised annealing temperature differed for each primer set and varied between species. These conditions can be seen in Table 2. 6. The annealing step lasted for 30 seconds. As with the RAPDs, reactions were compared with and without a hotstart and it was determined that hotstart was not necessary. PCRs were performed on an Applied Biosystems GeneAmp PCR system 9700.

Table 2. 6 Annealing temperatures used for microsatellites.

Crane species	Primer set	Primer annealing temperature
Wattled Crane	Gamμ3	60°C
	Gamμ6	56°C
	Gamμ7	60°C
	Gamμ12	55°C
	Gamμ101	57°C
Blue Crane	Gamμ3	62°C
	Gamμ6	56°C
	Gamμ7	56°C
	Gamμ12	57°C
	Gamμ101	52°C
Grey Crowned Crane	Gamμ3	60°C
	Gamμ6	56°C
	Gamμ7	56°C
	Gamμ12	55°C
	Gamμ101	57°C

2.4 RAPD ANALYSIS

2.4.1 RAPD gels

Samples were loaded with loading buffer type III (Sambrook *et al.* 1989) and were run on a 1% agarose slab gel (200ml, 20cm) in 0.5X TBE with 0.5μg/ml ethidium bromide in both the tank and gel. Molecular weight marker X (Roche) was also loaded to estimate the size of resultant bands. Gels were run at 100volts for 4½ hours on a Hoefer Scientific Instrument PS 250/2.5amp Electrophoresis DC power supply.

2.4.2 Polymorphism testing

Polymorphism was first established by amplifying each of the primers on four unrelated individuals of each of the three species. Once polymorphic primers were identified a number of unrelated individuals were examined to establish the statistical level of polymorphism in the population. Due to the problems with reproducibility of RAPDs it is essential that individuals being compared are run on the same gel.

2.4.3 Reproducibility testing

Six DNA extractions were performed from a single blood sample to establish how reproducible the polymorphic profiles were from a single bird. Ideally, these extractions should have been done from six different blood samples taken from the same bird. However, it was not possible to obtain numerous blood samples from a single bird.

2.4.4 Paternity testing

Each known family group individual was amplified with available polymorphic primers. Again it is essential when comparing RAPD profiles that comparisons are made within a single gel and not across gels.

2.4.5 Statistical analysis

RAPD profiles produced were scored on a binary system where fragments are scored as either present (1) or absent (0). When bands showing consistent segregation, or co-segregation, was observed, only one of these bands/allele was retained for further analysis. Profiles were screened for sex linkage.

The Band Sharing Coefficient (BSC) was calculated by the formula $x = 2Nab/(Na + Nb)$ where Nab is the number of bands shared between two individuals a and b, Na is the total number of bands in a and Nb is the total number of bands in b (Bruford *et al.* 1998). BSCs were calculated for all combinations of unrelated individuals. The average BSC of unrelated individuals was calculated from these values. The BSC was also calculated for each parent: offspring combination within the Blue Cranes and Crowned Cranes. Novel bands seen in the offspring but not in either parent was counted as were those seen in both parents but not in the offspring.

The probability that individuals would share the same profile was calculated by the formula x^n, where n is the number of bands scored. The probability that misassigned parentage is not detected was determined by the equation $I=(1-(1-x)^2)^n$ (Bruford *et al.* 1998).

POPGENE Version 1.32 (Yeh *et al.* 1997) was used to conduct UPMGA (unweighted Pair Group Method with Arithmetric Averages) using Nei's coefficient of genetic similarity to

cluster the genotypes and produce a dendogram for individuals of each species to determine the amount of relatedness amongst individuals housed at the various breeding establishments.

2.5 MICROSATELLITE ANALYSIS

2.5.1 Microsatellite gels

A small aliquot of the PCR reaction (4µl) was run on a 1% agarose gel in 0.5X TBE with 0.5µg/ml ethidium bromide for 30 minutes at 100Volts. Samples were loaded with 1µl loading buffer and molecular weight marker X was used. By running this gel, only samples of high quality were identified and sent for automated fragment analysis, thus reducing costs.

2.5.2 Microsatellite fragment analysis

Microsatellite reactions were run on an ABi 3100 Prism Genetic Analyser. Volumes of between 1-2ul were loaded on the machine. A Rox internal size standard (Abi) was included with each sample. GeneScan provided a graphical image of the resultant size fragments. Stutter bands are a common occurrence with microsatellites, particularly with the dinucleotide repeats. For this reason manual acrylamide methods often give poor or misleading results (K Jones, 2002, pers. comm.). GeneScan images were manually screened to identify stutter bands to eliminate the potential for false positive scores.

2.5.3 Microsatellite polymorphism testing

Polymorphism was initially established by investigation of each of the primer sets on four unrelated individuals from each of the three species. Polymorphic primers were then used to examine all the individuals available to the study to establish the statistical level of polymorphism in the sample. Each individual allele within each locus was identified alphabetically in ascending order, enabling a genotype to be represented by two letters, e.g. AE would indicate that an individual was heterozygous containing alleles A and E at that locus.

2.5.4 Microsatellite paternity testing

Known paternity cases amongst the available samples already processed for polymorphism testing were examined. Parental genotypes were compared against offspring.

2.5.5 Statistical analysis

POPGENE Version 1.32 (Yeh *et al.* 1997) was used to test for H-W equilibrium and linkage disequilibrium. Linkage disequilibrium was estimated by POPGENE using Burow's composite measure between pairs of loci. The same package was used to determine the mean number of allele per locus, the observed heterozygosity (Ho), expected heterozygosity (He) and the F_{IS}.

All combinations of putative parents were screened for non-matching bands across all loci. CERVUS 2.0 (Marshall *et al.* 1998) was used to calculate the total exclusionary power of the available polymorphic loci where one or both parents were available for analysis.

Chapter 3

APPLICATION OF RAPDS TO THE SOUTH AFRICAN CRANES: RESULTS AND DISCUSSION

3.1 Results

3.1.1 Segregation analysis

Fifteen RAPD primers were screened for polymorphism. Although normally a much larger number of primers would be screened, due to funding restrictions only a limited number were available for this study. Where the sexes of individuals were known, profiles were manually scanned for the presence of sex-linked loci, however, none were observed.

Seven primers were found to show polymorphism (47%) in Blue Cranes providing 135 loci of which 102 were scorable (76%) with an average of 14.57 (±3.99) loci per primer (Table 3. 1). Eight primers produced polymorphic profiles (53%) in the Crowned Cranes providing 119 loci of which 99 were scorable (83%) with an average of 12.38 (±3.34) loci per primer (Table 3. 2). Eight primers showed polymorphism (53%) in the Wattled Cranes providing 98 loci of which 47 were scorable (48%) with an average of 5.88 (±1.89) loci per primer (Table 3. 3).

Primers were tested for reproducibility by performing the same PCR reaction on DNA from six extractions from a single blood sample of one individual. Identical profiles were obtained which initially suggested that these primers were reproducible. However, when parentage studies were performed, where the same individual's profile was generated and run more than once on the same gel, in some instances, these were not identical (discussed further in section 3.1.2.2).

Table 3. 1 Results of RAPD primer screening of Blue Cranes.

Primer tested	Polymorphic	No. of loci	No. of scorable loci	Percentage of polymorphic loci
OPA014	No	-	-	-
OPA019	No	-	-	-
OPA031	No	-	-	-
OPA032	No	-	-	-
OPA003	Yes	22	18	82%
OPA004	No	-	-	-
OPA006	Yes	27	17	63%
OPA009	No	-	-	-
OPA010	Yes	10	6	60%
OPA015	Yes	20	16	80%
OPA022	Yes	19	15	79%
OPA023	Yes	20	14	70%
OPA065	Yes	17	16	94%
Total		135	102	76%

Table 3. 2 Results of RAPD primer screening of Crowned Cranes.

Primer tested	Polymorphic	No. of loci	No. of scorable loci	Percentage of polymorphic loci
OPA014	Yes	21	17	85%
OPA019	Yes	10	8	80%
OPA031	Yes	16	13	81%
OPA032	No	-	-	-
OPA003	Yes	16	15	94%
OPA004	No	-	-	-
OPA006	Yes	16	15	94%
OPA009	No	-	-	-
OPA010	Yes	10	9	90%
OPA015	No	-	-	-
OPA022	No	-	-	-
OPA023	Yes	16	13	81%
OPA065	Yes	14	9	64%
Total		119	99	83%

Table 3. 3 Results of RAPD primer screening of Wattled Cranes.

Primer tested	Polymorphic	No. of loci	No. of scorable loci	Percentage of polymorphic loci
OPA014	Yes	14	6	43%
OPA019	Yes	8	3	38%
OPA031	Yes	14	7	50%
OPA032	No			
OPA003	Yes	11	6	55%
OPA004	Yes	5	3	60%
OPA006	Yes	16	7	44%
OPA009	No			
OPA010	Yes	10	7	70%
OPA015	No			
OPA022	No			
OPA023	Yes	20	8	40%
OPA065	No			
Total		98	47	48%

3.1.2 Band Sharing Coefficients

Fifteen unrelated Blue Cranes and Crowned Cranes were selected for establishing the average BSC of each species. Eight Wattled Crane samples were obtained from unrelated birds. RAPD profiles were scored (Appendix 4) following Bruford *et al.* (1998); where band/alleles showed consistent co-segregation, only one was retained for further analysis (Appendix 5).

3.1.2.1 BSC of non-related individuals

The BSC was manually calculated for each pair of individuals and then combined to obtain an average for unrelated individuals in each species, for the combined set of primers. All individuals of unknown pedigree were assumed to be unrelated, which is likely, given their origin. Average BSC for unrelated individuals was found to be: Blue Cranes 0.665 (±0.103) (Table 3. 4), Crowned Cranes 0.745 (±0.060) (Table 3. 5) and Wattled Cranes 0.736 (±0.056) (Table 3. 6). From these values the average allele frequency (q) was estimated to be 0.421 for Blue Cranes, 0.495 for Grey Crowned Cranes and 0.486 for Wattled Cranes (see Chapter 2).

Table 3. 4 Band sharing coefficients of unrelated Blue Cranes.

Individual	B2	B3	B7	B11	B12	B13	B14	B17	B18	B19	B21
B2	X										
B3	0.716	X									
B7	0.602	0.630	X								
B11	0.592	0.598	0.764	X							
B12	0.644	0.652	0.800	0.817	X						
B13	0.510	0.574	0.709	0.864	0.758	X					
B14	0.627	0.574	0.740	0.833	0.806	0.838	X				
B17	0.568	0.532	0.683	0.768	0.786	0.744	0.775	X			
B18	0.510	0.455	0.630	0.682	0.645	0.662	0.721	0.682	X		
B19	0.540	0.465	0.624	0.708	0.689	0.701	0.776	0.630	0.672	X	
B21	0.524	0.458	0.587	0.649	0.642	0.610	0.729	0.595	0.644	0.828	X
	B2	B3	B7	B11	B12	B13	B14	B17	B18	B19	B21

Individual

Table 3. 5 Band Sharing coefficients of unrelated Grey Crowned Cranes.

Individual	C2	C5	C8	C11	C12	C13	C14
C2	X						
C5	0.783	X					
C8	0.819	0.866	X				
C11	0.732	0.764	0.769	X			
C12	0.810	0.760	0.813	0.774	X		
C13	0.654	0.692	0.649	0.800	0.667	X	
C14	0.724	0.793	0.748	0.790	0.718	0.718	X
C15	0.804	0.696	0.739	0.661	0.708	0.626	0.778
	C2	C5	C8	C11	C12	C13	C14

Individual

Table 3. 6 Band Sharing coefficients of unrelated Wattled Cranes.

Individual	W2	W3	W6	W7	W8
W2	X				
W3	0.800	X			
W6	0.632	0.733	X		
W7	0.758	0.783	0.754	X	
W8	0.725	0.722	0.656	0.795	X
	W2	W3	W6	W7	W8

Individual

3.1.2.2 Parentage analysis

Only six known parentage cases were available for the Blue Cranes and three for the Grey Crowned Cranes (Table 3. 7). Profiles were scored in the same way as non-related

individuals (Appendix 6). For each primer, if co-segregating bands were seen, only one was retained for analysis (Appendix 7).

Table 3. 7 Known parentage cases for Blue Cranes (a) and Grey Crowned Cranes (b).

	Mother	Father	Offspring
a)	B2	B3	B4
	B18	B19	B20
	B21	B22	B23
	B24	B25	B5
			B6
			B26
b)	C17	C16	C18
	C5	C8	C6
			C7

Due to the nature of RAPDs it is not possible to compare the bands from individuals across different gels for practical reasons. Therefore, profiles generated in parentage gels did not include those generated for non-related individuals. The irreproducible nature is clearly seen when comparing Figure 3. 1a and b. For example, individual C8 was run in lane 7 on gel a and in lane 7 on gel b. Where samples of more than one offspring were obtained from the same parents, two PCRs were performed for each parent so they could be run on either side of the offspring in the RAPD agarose gel. However, an identical profile was found in only five out of the 16 cases (31%) where the same parent DNA template was used in separate PCRs (Appendix 6). For this reason only one of the two profiles produced for each parent was used in further analysis (Appendix 7).

The BSC was calculated for the combined set of primers for both maternal and paternal comparison with the offspring. A one tailed *z*-test was used to determine if the average BSC of unrelated individuals of that species were significantly higher from the parent: offspring dyads (Table 3. 8 and Table 3. 9). The average BSC of unrelated individuals should theoretically be lower than the BSC of related individuals. However, results were inconsistent with some parent: offspring BSCs being lower than BSC of unrelated individuals.

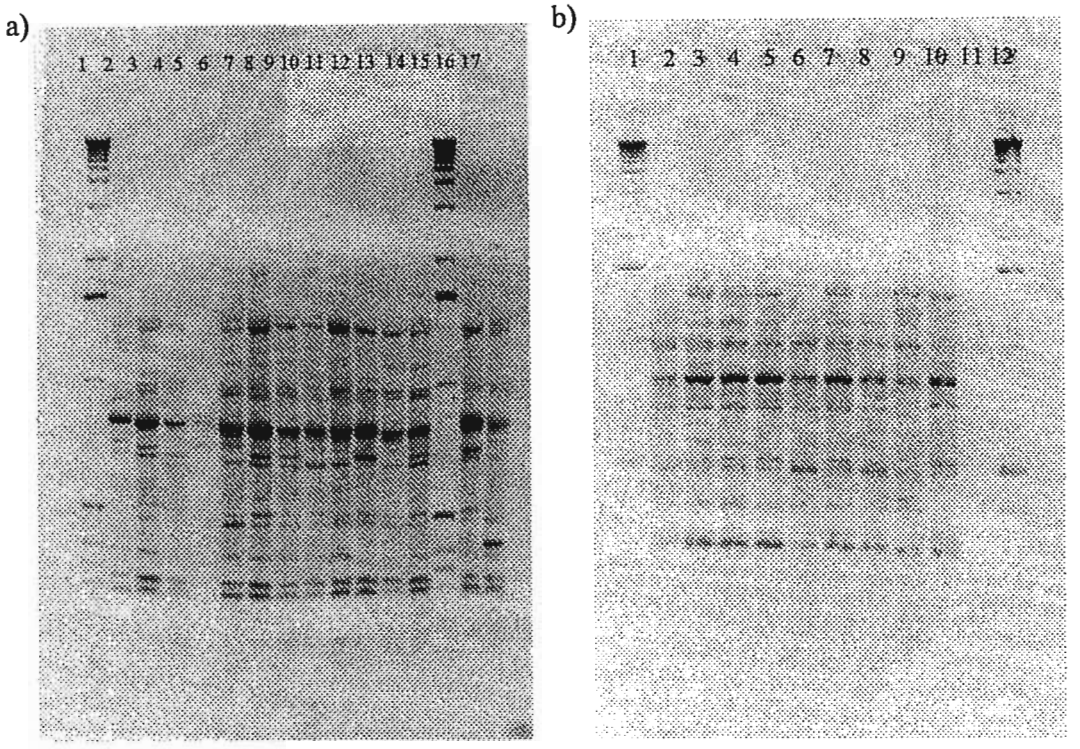

Figure 3. 1 a) RAPD profile produced from various unrelated Grey Crowned Cranes using primer OPA014. Lane 1: Lane 1: Molecular Weight Marker X (Roche), 2: C1, 3: C2, 4: C3, 5: C4, 6: C5, 7: C8, 8: C9, 9: C10, 10: C11, 11: C12, 12: C13, 13: C14, 14: Molecular Weight Marker X 15: C15, 16: C16, 17: C17.

b) RAPD profile produced from known Grey Crowned Crane parentage cases using primer OPA014. Lane 1: Molecular Weight Marker X (Roche), 2: C5, 3: C8, 4: C6, 5: C7, 6: C5, 7: C8, 8: C16, 9: C18, 10: C17, 11: negative control, 12: Molecular Weight Marker X (Roche).

Table 3. 8 Comparison of Blue Crane Band Sharing Coefficients (BSC) of parent: offspring combinations compared with the BSC of unrelated individuals (BSC=0.665).

Offspring	Comparison of offspring with mother			Comparison of offspring with father		
	Mother	BSC	Statistical significance	Father	BSC	Statistical significance
B4	B2	0.962	2.883*	B3	0.760	1.311*
B5	B24	0.868	1.971*	B25	0.724	0.573
B6	B24	0.807	1.379	B25	0.742	0.748
B20	B18	0.704	0.379	B19	0.870	1.990*
B23	B21	0.618	-	B22	0.480	-
B26	B24	0.786	1.175*	B25	0.820	1.505
Average:		0.791	1.223		0.733	1.000

*: $P>0.05$; **: $P>0.01$

Table 3. 9 Comparison of Grey Crowned Crane Band Sharing Coefficients (BSC) of parent: offspring combinations with the BSC of unrelated individuals (BSC=0.745)

Offspring	Comparison of offspring with mother			Comparison of offspring with father		
	Mother	BSC	Statistical significance	Father	BSC	Statistical significance
C6	C5	0.667	-	C8	0.837	1.533
C7	C5	0.769	0.400	C8	0.870	2.083*
C18	C17	0.778	0.550	C16	0.700	-
Average:		0.738	-		0.802	0.950

*: $P>0.05$

Mendelian inheritance expects each band present at a locus to be maternally or paternally inherited in an offspring. A number of novel bands were seen in the offspring that could not have been inherited from either parent. In addition, there were some cases where although both parents possessed a band, it was not observed in the offspring (Table 3. 10). Blue Cranes had an average of 3.5 novel bands and Grey Crowned had an average of 1.7 novel bands. A comparison of BSC of parent: offspring dyads with the number of novel bands seen are presented graphically in Figure 3. 2.

Normally, a greater number of novel bands, in conjunction with information from comparisons of BSCs, would be the method used to identify cases of non-parentage. However, there is a high degree of certainty that all parent: offspring groupings used in this study were legitimate. It must also be recognized, however, that there is always room for human error in the process, from the blood collection stage to the scoring of the profile on the gel. However, given the number of mismatches (Table 3. 10) novel bands are unlikely to be purely the result of personal and technical error.

It has been shown that by knowing the allele frequency, one can calculate the expected BSC that a parent: offspring would have (see Chapter 2). Thus the parent: offspring BSC is expected to be 0.788 in Blue Cranes and 0.831 in Grey Crowned Cranes.

In the everyday use of DNA analysis, parentage cases are not known but rather an attempt is being made to determine it. As an example, assume an investigation was underway to determine if individuals B2 and B3 were the parents of offspring B4. No novel bands were seen when comparing profiles (Table 3. 10). In addition the putative mother:

Table 3. 10 Numbers of unexpected bands, or lack thereof, seen in RAPD parentage profiles of Blue Cranes (a) and Grey Crowned Cranes (b).

	Offspring	Parents	No. of bands present in both parents but not in offspring	No. of bands present in offspring but in neither parent	Total no. of novel bands
a)	B4	B2 + B3	0	0	0
	B5	B24 + B25	1	2	3
	B6	B24 + B25	0	5	5
	B20	B18 + B19	2	2	4
	B23	B21 + B22	2	4	6
	B26	B24 + B25	0	3	3
				Mean :	3.5
b)	C6	C5 + C8	3	0	3
	C7	C5 + C8	1	0	1
	C18	C16 + C17	0	1	1
				Mean:	1.7

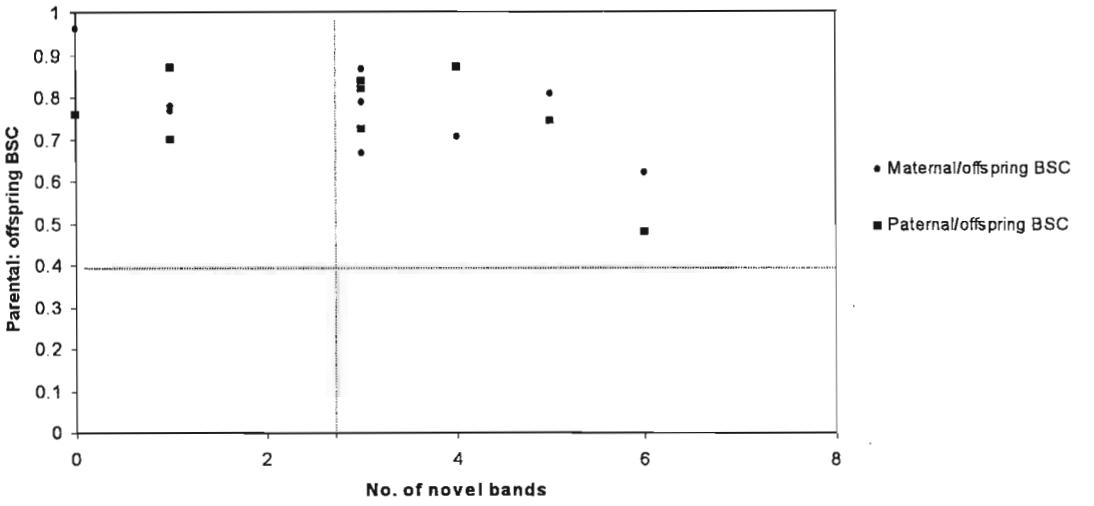

Figure 3. 2 Relationship between the number of novel bands seen in parent: offspring comparisons and their BSC. The dotted lines indicate approximate criteria for excluding parentage, so any individuals lying below or to the right of the dotted lines would be excluded as a parent (Bruford *et al.* 1998).

offspring and putative father: offspring BSC was significantly higher than the average BSC of non-relateds (Table 3. 8). This would indicate a strong likelihood that these two individuals are the true parents of B4. Given the information obtained from the selection of

RAPDs examined here, the probability that any second random individual of Blue Crane would share their profile with either parent would be 0.665^n or $2.340x10^{-11}$ when n=60 (60 bands were assessed in the paternity gels)(see Chapter 2). However, the probability that a misassigned pair of parents would not be detected (I) is $(1-(1-0.665)^2)^{60}$ or $7.910x10^{-4}$ (see Chapter 2).

If, the parents of B23 (B21 and B22) were not known to be the true parents one would exclude them as parents. Six novel bands were identified and the parent: offspring BSCs were in fact lower than the average BSC occurring in non-related individuals. Similarly, if the parents of B6 (B24 and B25) were not known to be the true parents they again would be excluded as parents as 5 novel bands were seen and the BSC of both parent: offspring combinations were not significantly greater than the average BSC of unrelateds. The probability that these individuals were falsely excluded would be $1-((1-(1-0.665)^2)^{60})$ or 0.999 (see Chapter 2).

3.1.3 Dendograms of unrelated individuals

Fifteen presumed unrelated individuals of Blue Crane (Figure 3. 3) and Grey Crowned Crane (Figure 3. 4) and eight Wattled Crane samples were examined (Figure 3. 5). This was done to examine the levels of genetic similarity among birds housed at the different establishments to reduce the potential for inbreeding depression should any of these birds be paired for breeding. The BSC of birds that shared the same branch of the dendogram and also originated from the same establishment (Appendix 3) were compared against the average BSC of unrelated individuals (Table 3. 11) to determine if they were significantly genetically more similar than unrelateds.

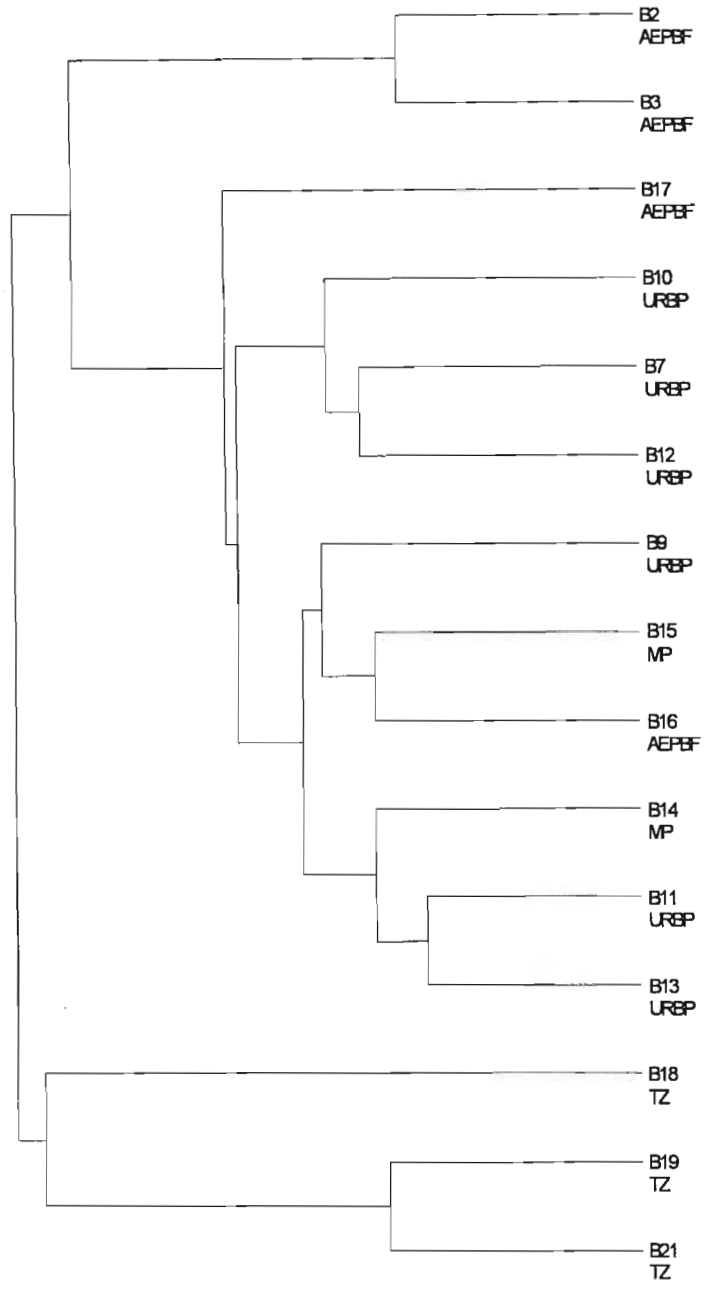

Figure 3. 3 Dendogram showing genetic similarity between 15 Blue Cranes.

AEPBF: Amazona Endangered Parrot Breeding Facility, Assagai, KZN
URBP: Umgeni River Bird Park, Durban, KZN
MP: Mitchell Park Zoo, Durban, KZN
TZ: Tygerberg Zoo, Cape Town, Western Cape

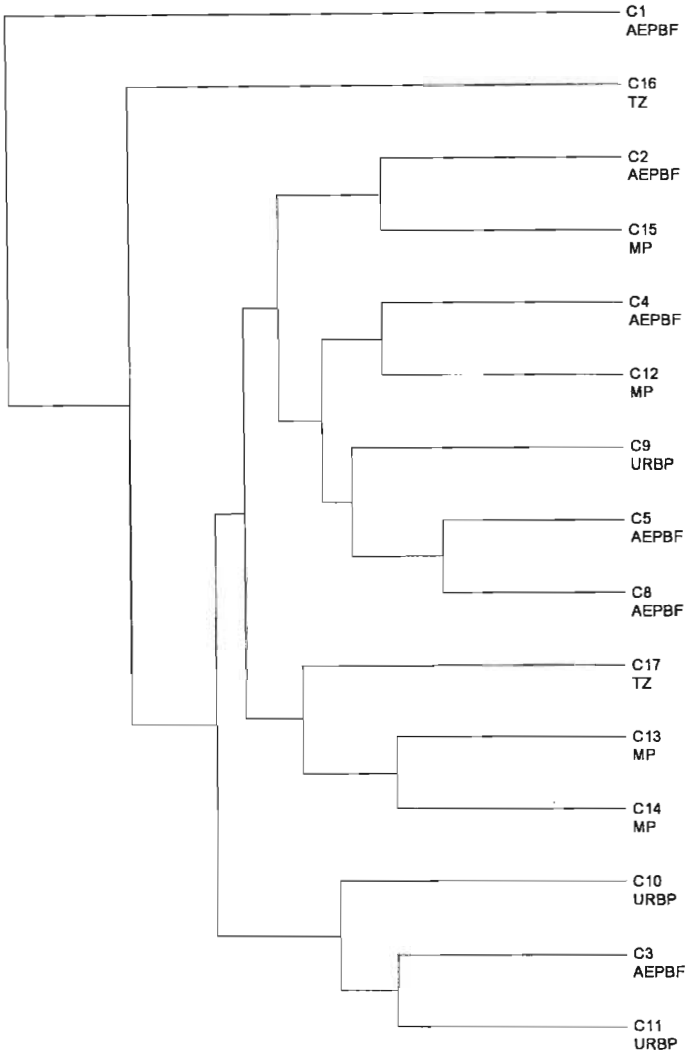

Figure 3. 4 Dendogram showing genetic similarity between 15 Grey Crowned Cranes.
AEPBF: Amazona Endangered Parrot Breeding Facility, Assagai, KZN
URBP: Umgeni River Bird Park, Durban, KZN
MP: Mitchell Park Zoo, Durban, KZN
TZ: Tygerberg Zoo, Cape Town, Western Cape

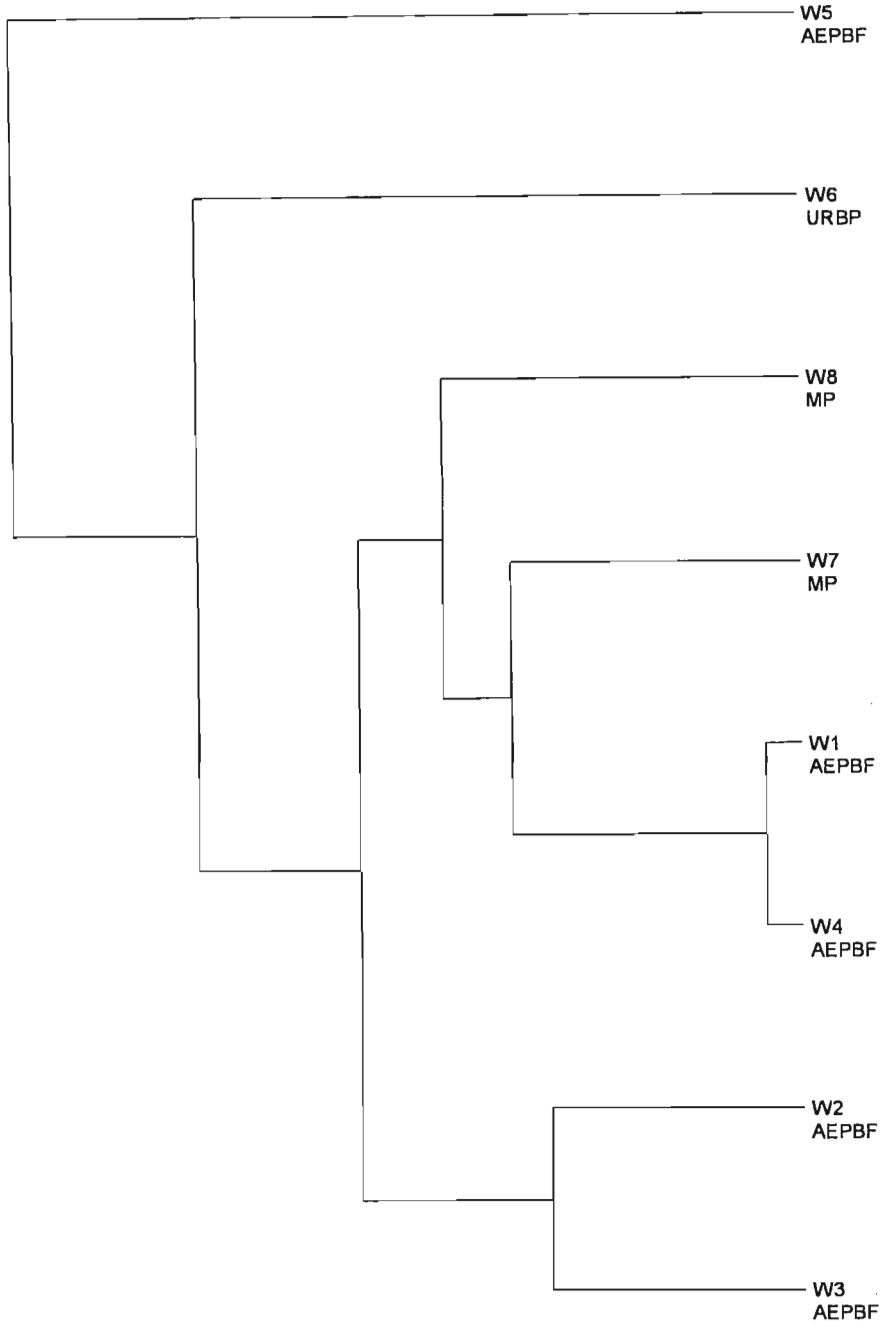

Figure 3. 5 Dendogram showing genetic similarity between 8 Wattled Cranes.

AEPBF: Amazona Endangered Parrot Breeding Facility, Assagai, KZN
URBP: Umgeni River Bird Park, Durban, KZN
MP: Mitchell Park Zoo, Durban, KZN
TZ: Tygerberg Zoo, Cape Town, Western Cape

Table 3. 11 Band Sharing Coefficients (BSC) among individual Blue Cranes (a), Grey Crowned Cranes (b) and Wattled Cranes (c) residing at the same establishments (Average BSC of unrelated Blue Cranes=0.665, Grey Crowned Cranes=0.745 and Wattled Cranes=0.736).

	Individual 1	Individual 2	Location	BSC	Statistical significance
a)	B2	B3	AEPBF	0.716	0.495
	B7	B12	URBP	0.800	1.311
	B11	B13	URBP	0.864	1.932*
	B19	B21	TZ	0.828	1.583
b)	C5	C8	AEPBF	0.866	2.017*
	C13	C14	MP	0.718	-
c)	W2	W3	AEPBF	0.800	1.143

*: $P>0.05$; **: $P>0.01$
AEPBF: Amazona Endangered Parrot Breeding Facility, Assagai, KZN
URBP: Umgeni River Bird Park, Durban, KZN
MP: Mitchell Park Zoo, Durban, KZN
TZ: Tygerberg Zoo, Cape Town, Western Cape

W1 and W4 (Figure 3. 5) were excluded from the analysis as only primer OPA010 produced a scorable profile for W1 resulting in just seven scorable loci (Appendix 7). Individuals B11 and B13 (Figure 3. 3), housed at URBP, and individuals C5 and C8 (Figure 3. 4), housed at AEPBF, both showed significantly higher BSC than unrelated individuals at the 95% level. Unfortunately, nothing is known about the pedigree of the Blue Cranes B11 and B13, so they may, in fact, be related or this result may have occurred by chance. However, C5 and C8 are both founders, obtained independently from the wild, C5 has been in captivity for at least 15 years whereas C8 was a recent victim of a power line collision. It is thus highly unlikely that they are related. There are, however, situations where unrelated individuals do, by chance, have similar profiles.

3.2 Discussion

This chapter investigated the use of RAPDs to examine relatedness and parentage in three species of cranes. Very few studies have been done using RAPDs for parentage studies and for this reason there is not a great deal of literature available for the comparison and

interpretation of the results. Where published results are available, they are not comparable with this study. Certain of these studies involved species with large numbers of offspring, which is not the case here, and the authors generated additional 'artificial offspring' to improve their powers of analysis. Alternatively, the results of the study indicated that RAPDs were an inappropriate tool for parentage investigations and therefore results are suspect (Riedy *et al.* 1992; Scott *et al.* 1992)

In this study, only 15 RAPD primers were screened, representing all that were available in the laboratory. Lewis and Snow (1992) suggest that more than 50 polymorphic RAPD loci should be scored for parentage exclusion cases. Although a greater number of primers would have provided a greater degree of discriminatory ability, the problems associated with RAPDs, clearly indicate that they are not an appropriate marker for parentage, especially for forensic investigations. These problems include the presence of novel bands, the inability to reproduce profiles for the same individual and difficulty in scoring profiles. Forensic parentage analysis was the long-term aim of this study and so further primers were not sought and RAPD markers were not investigated further.

The primers used in this study were selected from work previously done on Iberian Imperial Eagles (Padilla *et al.* 2000). Using 15 of the 60 primers used in that study, selected for their high levels of polymorphism, 47%, 53% and 53% were found to be polymorphic in the Blue Crane, Grey Crowned Crane and Wattled Crane respectively. The Iberian Imperial Eagle showed 59.7% polymorphism using 45 primers that included those used here (Padilla *et al.* 2000). Similar studies on the Red-Cockaded Woodpecker and Quail, using different primers, produced lower levels of polymorphism (38%, n=34 and 30%, n=20 respectively)(Haig *et al.* 1994; Sharma *et al.* 2000).

Although the profiles generated, based on the seven polymorphic primers for the Blue Cranes and eight each for the Grey Crowned Cranes and Wattled Cranes, provided numerous bands (135, 119 and 98 for Blue Cranes, Grey Crowned Cranes and Wattled Cranes respectively), of these, only 76% in the Blue Cranes and 83% in the Grey Crowned Cranes were scorable. The figure for Wattled Cranes was much lower with only 48% of bands eligible to be scored. The Iberian Imperial Eagle showed 60% polymorphism for the 614 bands obtained. In other studies, using other primers, 32% polymorphism was seen in the

Quail and 39% in the Marsh Wren (*Cistothorus palustris*) (Bowditch *et al.* 1993; Sharma *et al.* 2000). Just 1% of the 1338 bands seen in the Clapper Rail (*Rallus longirostris*) were polymorphic (Nusser *et al.* 1996). The value for the Clapper Rail is the lowest level of variability revealed by RAPDs in a vertebrate population (Nusser *et al.* 1996).

Blue Cranes, Grey Crowned Cranes and Wattled Cranes provide an average of 14.57, 12.38 and 5.88 scorable loci per primer respectively. These values were similar to the 13.6 seen in the Iberian Imperial Eagle although higher than the 3.16 seen in the Quail (Padilla *et al.* 2000; Sharma *et al.* 2000).

The Band Sharing Coefficient (BSC) is a measure of relatedness or genetic similarity between individuals. This was calculated as 0.665, 0.745 and 0.736 for presumed unrelated Blue Cranes, Grey Crowned Cranes and Wattled Cranes respectively.

The unreliable reproducibility of RAPD profiles is of great concern. Although the probability that any two unrelated Blue Crane individuals would share the same profile is extremely low (2.340×10^{-11}, n=60) this cannot be considered a reliable figure as single individuals have been shown to produce different profiles in separate amplifications. In only 31% of cases, where a PCR was performed twice on a single parent and run on the same gel, was the resultant profile the same. In addition, the high number of novel bands seen in parentage: offspring comparisons is cause for concern. The samples used in the parentage analysis are known to a high degree of certainty to be reliable. However, if these RAPD profiles had been presented as a test, using this general multilocus analysis, one would conclude that 6 of the 9 cases tested had been assigned parentage incorrectly. The fact that known parents are being excluded provides additional evidence that the comparability of parent: offspring profiles using RAPDs is unreliable. Scott *et al.* (1992) investigated the suitability of RAPDs for parentage analysis. They suggested that novel bands may result from contamination. Running a negative control on the RAPD analysis gel controls for contamination during the reaction. However, a contaminant derived from bacteria or parasites present during extraction could also potentially contaminate the sample. This is a possibility as the sample may have contained blood parasites. However, even if this were true, it would reinforce the fact that this technique is unsuitable for parentage testing as individuals could be excluded as parents, when they are indeed the true parents. The work by Riedy *et al.* (1992) on

baboons also concluded that RAPDs are unsuitable for parentage analysis because of the high frequency of novel bands, possibly due to PCR artifact and genomic mutation, as they can cause false exclusion.

The expected BSC of parent: offspring dyads were 0.788 and 0.831 for Blue Cranes and Grey Crowned Cranes respectively. As expected, these values were significantly greater (at the 95% level) than those of unrelated individuals, since related individuals should share more bands. Thus, the observed parent: offspring BSC values should also be significantly greater than the average BSC of unrelateds. However the results varied. Some values were significantly higher, as expected, but some were less than the unrelated value. These results imply that the related individuals shared fewer bands in common than two random, unrelated individuals. In the case of B23's comparison with parents B21 and B22, this result could have been cause by the high number of novel bands. However, only one novel band was seen in C18 that was not in either parent (C16 & C17) yet the paternal: offspring BSC was lower than that of unrelateds.

According to statistical analysis, the probability that missassigned parents are not detected in Blue Cranes is low (P=7.910x10^{-4}, n=60). However, more importantly, the probability that true parents are incorrectly excluded is very high (P=0.999, n=60). Thus, statistics show that there is a very low probability that incorrectly assigned parents will not be detected and an almost certainty that true parents could be excluded. This proves RAPDs are totally inappropriate for parentage testing in cranes.

Although RAPDs have been used in numerous population studies of birds looking at within and between population variability (Haig *et al.* 1994; Sharma *et al.* 2000; Singh & Sharma 2002), there is no literature where RAPDs have been successfully used for parentage testing in similar circumstances. There are papers where they are used in parentage cases of insects and other organisms that produce large numbers of offspring (Bishop *et al.* 2000; Hooper & Siva-Jothy 1996; Tegelström & Höggren 1994). However, due to the great number of offspring produced by these species, a 'synthetic offspring' approach can be applied, which is not the case with small numbers of offspring, such as birds.

The dendograms produced from the RAPD results indicated that there is minimal genetic similarity in birds housed at the same institutions. This implies that inbreeding

depression can be avoided at these establishments should these birds be paired in breeding. The information obtained in constructing the dendograms would allow breeders to plan mating in order to maintain genetic diversity.

Chapter 4

APPLICATION OF MICROSATELLITES TO THE SOUTH AFRICAN CRANES: RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Polymorphism testing

The five primers used in this study had previously been tested on Wattled Cranes and were shown to be polymorphic (Jones 2003). They had not, however, previously been tested on Blue Cranes or Grey Crowned Cranes. This study found that all primers cross-amplified, producing a single band on an agarose gel in Blue Cranes and Grey Crowned Cranes. However, only primer Gamμ6 of the Blue Cranes and Gamμ3 of the Grey Crowned Cranes produced polymorphic profiles (Table 4. 1; Appendix 8). The polymorphic primers were used to screen all available DNA samples. All eight Wattled Cranes samples were screened for the five primers. Twenty-six Blue Crane samples (of which 24 gave results) and 18 Grey Crowned Cranes samples were available. Only one primer (Gamμ101) in the Wattled Cranes was consistent with H-W expectations (Table 4. 1).

Table 4. 1 Table of descriptive statistics of Wattled Cranes from microsatellite data (n=8).

Species	Locus	No. of alleles	Allele size range	He	Ho	H-W Equilibrium
Wattled	Gamμ101	5	187-212	0.750	0.563	Yes
	Gamμ3	4	118-122	0.429	0.694	No
	Gamμ6	2	108-118	0.286	0.2449	No
	Gamμ7	4	170-192	0.375	0.695	No
	Gamμ12	3	132-140	0.857	0.5714	No
Mean		3.6		0.539	0.554	
Blue	Gamμ6	6	114-130	0.783	0.729	No
Crowned	Gamμ3	5	101-117	0.556	0.687	No

Linkage disequilibrium was not observed in the three species ($P<0.05$). Wright's fixation index (F_{IS}) was calculated as a measure of heterozygote deficiency or excess. Although within the Wattled Crane loci the F_{IS} values range from –0.500 to 0.461 the mean value is –0.031 indicative of a lack of inbreeding (Table 4. 2). The –0.074 seen for the Blue Cranes also indicates a lack of inbreeding (Table 4. 2). However, the mean F_{IS} for the Grey Crowned Cranes is 0.191, which indicates some inbreeding (Table 4. 2).

Table 4. 2 Wright's fixation index (FIS) of Blue Cranes (n=24), Wattled Cranes (n=8) and Grey Crowned Cranes (n=18).

	Wattled Cranes					Blue Cranes	Grey Crowned Cranes
	Gamμ101	Gamμ3	Gamμ6	Gamμ7	Gamμ12	Gamμ6	Gamμ3
F_{IS}	-0.333	0.383	-0.167	0.461	-0.500	-0.074	0.191

The number of alleles per locus, observed heterozygosity (Ho) and expected heterozygosity (He) were calculated (Table 4. 1). Locus Gamμ6 of the Wattled Cranes showed low levels of heterozygosity. One explanation for this might have been the presence of null alleles.

The mean number of alleles per locus was 3.6 (n=5) for the Wattled Crane. Allele frequency at each locus was established for each species with missing data excluded (Table 4. 3). Certain alleles were shown to be more common, and in the Wattled Crane, of the two

Table 4. 3 Allele frequencies seen in Blue Cranes (n=24), Wattled Cranes (n=8) and Grey Crowned Cranes (n=18).

Allele	Wattled Cranes					Blue Cranes	Grey Crowned Cranes
	Gamμ101	Gamμ3	Gamμ6	Gamμ7	Gamμ12	Gamμ6	Gamμ3
A	0.625	0.429	0.143	0.063	0.143	0.413	0.028
B	0.188	0.143	0.857	0.313	0.571	0.044	0.472
C	0.063	0.143		0.375	0.286	0.109	0.167
D	0.063	0.286		0.250		0.130	0.222
E	0.063					0.261	0.111
F						0.044	
Mean	0.200	0.250	0.500	0.250	0.333	0.167	0.200

alleles seen at Gamμ6, allele B had a frequency of 0.857. Locus Gamμ101 had 5 alleles but also had a common allele, A, with a frequency of 0.625. In the other two species, although occasionally certain alleles showed higher frequencies than these, they were all below 0.5. The mean allele frequency for Wattled Cranes was 0.273.

4.1.2 Parentage analysis

Twenty-six Blue Crane samples (of which 24 gave results) were available containing six known parentage cases, while 18 Grey Crowned Cranes samples were available with three known parentage cases (Appendix 8). Mendelian inheritance was seen in all parentage cases in both the Blue Crane and Grey Crowned Crane (Table 4. 5). The inherited allele is highlighted for the mother and father in each case.

Table 4. 4 Genotypes of known parentage cases confirm Mendelian inheritance.

Species	Mother		Offspring		Father	
	ID	Genotype	ID	Genotype	ID	Genotype
Blue Cranes	B2	**AB**	B4	BE	B3	**EE**
	B24	**AE**	B5	AA	B25	**AD**
	B24	**AE**	B6	AD	B25	**AD**
	B18	?	B20	AE	B19	**AC**
	B21	**AC**	B23	AD	B22	**DE**
	B24	**AE**	B26	AA	B25	**AD**
Grey Crowned Crane	C5	**BD**	C6	BD	C8	**BB**
	C5	**BD**	C7	BD	C8	**BB**
	C17	**BD**	C18	CD	C16	**AC**

When only a single locus is examined, the probability of two individuals sharing the same profile by chance is high. As would be expected, the total exclusionary power was very low in the Blue Crane for both one and two parents analysed at 0.327 and 0.505 and in the Grey Crowned Crane 0.273 and 0.448 (Table 4. 7). Five polymorphic primers in the Wattled Cranes provided a much higher exclusionary power at both levels (0.641 and 0.869).

The Paternity Index (PI) is calculated as X/Y where X is the probability that the alleged father passed on a paternal gene and Y is the probability that a random male passed on the paternal gene. This value is calculated per locus. As an example, individual B5 has a genotype

Table 4. 5 Total exclusionary power of available microsatellite loci

Crane Species	Number of loci	Total Exclusionary power	
		First parent	Second parent
Wattled	5	0.641	0.869
Blue	1	0.327	0.505
Crowned	1	0.273	0.448

AA. One of the A alleles originated from the mother, B24, whose genotype was AE. Thus the other A allele had a paternal origin. The father, B25, has a genotype AD, thus the probability that he contributed the other A allele is 0.5 as he is a heterozygous individual. The frequency of the A allele in the population is 0.413. Thus the PI for B25 at this locus is 1.210. To determine the probability of paternity the PI needs to be calculated across several loci. Because only one locus has been found to be polymorphic, this value cannot be calculated.

4.2 DISCUSSION

This chapter investigates the use of microsatellites in examining parentage in three species of cranes. The five microsatellite primers were originally isolated for Whooping Cranes and were selected due to their polymorphism in Wattled Cranes (Glenn *et al.* 1997; Jones 2003). All five microsatellite primers cross-amplified in the Blue Cranes and Grey Crowned Cranes to produce a single band on an agarose gel. Glenn *et al.* (1997) found a 90% cross-amplification success rate with eight of their primers, developed for Whooping Cranes, in studies in the family Gruidae. Hasegawa *et al.* (2000) found their 8 primers, developed for the Red-crowned Crane (*G. japonensis*), to cross-amplify in eight related crane species. In both studies cross-amplified products were not screened for polymorphism.

Although a primer may cross-amplify it is not necessarily polymorphic in the "new" species. Maudet *et al.* (2002) found that 90% of the primers tested, that had previously been designed for cattle (*Bos taurus*), sheep (*Ovis aries*) and goats (*Capra hircus*), cross amplified in Alpine ibex. However only 30% of these were polymorphic. Six of the ten primers tested in the root vole (*Microtus oeconomus*), five of which were previously developed for mice and the other five developed for various other species of vole, were found to be polymorphic (Papp *et*

al. 2000). In Blue Cranes, all primers produced a single band on an agarose gel in cross-amplification, but only one out of the five, Gamμ6, produced a polymorphic profile. This locus was relatively polymorphic and provided six alleles of a fairly large size range. Again in the Grey Crowned Crane although all primers cross-amplified, only one primer Gamμ3 was polymorphic. At this locus, five alleles were seen, also with a fairly large size range. So although only a single polymorphic locus was found out of the five available primers in both cases, each locus was relatively variable. This is unusual, as it has previously been seen that cranes generally are not very variable with approximately 4 alleles per locus (Hasegawa *et al.* 2000; Jones *et al.* 2002; Jones 2003). The Wattled Crane data followed this trend and the primers provided a relatively low average of 3.6 alleles per primer. However, work on the Peregrine Falcon showed a similar average of 4.25 alleles per locus (Nesje 2000). In this study the mean allele frequency of the Wattled Cranes was 0.273. This is quite high relative to the allele frequency (0.148-0.022) seen in the Yellow Warbler (Dawson *et al.* 1997). Studies done on artiodactyls have revealed much higher values of ≤ 14 alleles per locus (Engel *et al.* 1996).

Four of the five available loci in the Wattled Cranes and the single locus in both the Blue Crane and Grey Crowned Cranes did not conform to H-W expectations. Two of the 19 loci, tested on 24 individuals of Peregrine Falcon, also deviated from H-W equilibrium (Nesje *et al.* 2000). Conversely, studies on Alpine ibex, using 210 individuals saw no deviations from H-W proportions in their 19 loci (Maudet et al. 2002). Thus, the deviations from H-W (which assumes a large population size) seen in this study are likely a reflection of the very small sample sizes available to this study for the three species, as well as their nature of their origin. The samples used in this study were obtained from birds held in captivity. Full histories of the birds were not available but it is likely many originated from the wild and were obtained from separate subpopulations. Others would have been captive bred. All three species are endangered and their populations fragmented due to habitat degradation. Therefore, these factors influence the ability of the loci and samples to comply with the H-W expectations. These circumstances do not match the assumptions of the H-W equilibrium. Many statistical analyses assume H-W equilibrium in the sample analysed and therefore it needs to be recognised that this may have implications on the findings.

F_{IS} may be termed the local inbreeding coefficient. Inbreeding is indicated by a positive F_{IS} as there is an increase in homozygosity, while negative F_{IS} values indicate a lack of inbreeding (Excoffier 2001). The F_{IS} values in this study indicated a lack of inbreeding in the Wattled Crane. This was to be expected as all the Wattle Crane samples were from wild-caught birds and so were unlikely to be inbred. The Blue Crane F_{IS} value also suggests a lack of inbreeding. However, the Grey Crowned Crane value is positive suggesting some degree of inbreeding. Since an F_{is} value of 1.0 indicates an inbred population, the 0.191 F_{IS} value indicates levels of inbreeding that are very low. However, the F_{IS} cannot be a reliable measure in this case, particularly in the Wattled Cranes as, in this initial investigation of the markers, the sample size is far too low.

The observed heterozygosities were 0.729, 0.687 and 0.554 for the Blue, Grey Crowned and Wattled Crane respectively. This is comparable with levels seen in other vertebrates (He = 0.388-0.989) (Dawson *et al.* 1997). Low levels of heterozygosity may lead us to suspect null alleles (Richardson *et al.* 2001). However, in this case, where good levels of heterozygosity were seen, this is not likely the case.

Unfortunately no parentage cases were available for the Wattled Cranes and so the microsatellites could not be tested for this, in this species. For the polymorphic microsatellite locus in the Blue Cranes and Grey Crowned Cranes, the alleles appeared to be inherited in a Mendelian manner. Where data were available for known parentage cases, all offspring were seen to inherit one allele from the father and one from the mother. This verifies that these two markers conform to Mendelian inheritance and were suitable for relationship analyses in the respective species. It must be remembered, however, that only six parentage cases were available (with full data for only five cases) for the Blue Cranes and just three for the Grey Crowned Cranes. Although variable, one locus is not nearly adequate for paternity testing as the exclusionary powers are too low.

With only five primers tested, the probability of two individuals sharing the same profile by chance is relatively high in respect to the needs of forensic investigations. The calculated combined exclusionary probability for parentage testing in the Wattled Crane was 0.869 and 0.641 where one or both parents were unknown, respectively. This is quite high considering the small number of loci (n=5). Jones and Nicolich (2001) achieved a single parent

exclusionary probability of 0.948 from 12 primers in the Whooping Crane. A high exclusion probability of 0.9996, for one parent unknown, was found in the Yellow Warbler using just five loci (Dawson *et al.* 1997). While Primmer *et al.* (1995) achieved the same exclusion probability using six loci in the Barn Swallow. Nesje *et al.* (2000) reached a 0.99 and 0.94 exclusion probability where one or both parents were unknown, respectively, using 12 primers in the Peregrine Falcon.

The polymorphic loci found in this study are suitable for parentage testing. However, because of their limited number, they are inadequate, as the exclusionary power is too low. It is recommended that further investigations be required to discover more polymorphic loci in these species to increase the exclusionary power for parentage testing.

Chapter 5
DISCUSSION

The loss of genetic diversity is a current major global threat (Bothma & Galvovic 1992). Wildlife has become such a valuable, but threatened commodity, that it is necessary for the legal system to protect it. However, it is in the implementation of laws, already laid down, where the problem lies. Crane fledglings are sometimes collected illegally from the wild for sale into captivity, possibly by registered breeders who then falsely claim their captive pair has laid eggs and reared chicks (Allan 1994; Hudson 2000). South African legislation protects its wildlife, but without adequate measures to verify cases where illegal acts are suspected, these laws cannot be implemented, or the culprits prosecuted. DNA genotyping could be of great benefit in the fight to prevent the sale of wild caught birds. One way to accomplish this is to verify a suspect bird's parentage. For a genetic marker to be used in forensics it must be able to be consistently scored and must accurately reflect genetic variation. This thesis was aimed at determining the suitability of RAPDs and microsatellites for their potential in forensic parentage investigations so as to prevent the sale of wild caught Blue, Grey Crowned and Wattled Cranes.

RAPDs have previously been used in forensic applications for species identification (Congiu *et al.* 2000; Lee & Chang 1994) requiring completely different techniques than in this study. The forensic analysis was possible as only primers that were known to be monomorphic within a species were selected. Using these monomorphic primers, different species show a great deal of variation between their respective fingerprints, while within species very little, if any, variation is seen (Calvo *et al.* 2001; Lee & Chang 1994). This study has shown, however, that the RAPD technique is clearly inappropriate for forensic parentage testing.

A great concern with the use of RAPDs for forensics is their unreliable reproducibility. In only 31% of cases where a PCR was performed twice on a single individual and run on the same gel was the resultant profile the same. Irreproducibility of RAPDs has also been shown in other studies, for example, in the study conducted by Jones *et al.* (2001) several laboratories in Europe performed the same identical PCR using the same samples and reagents but produced different profiles. This has major implications in forensic work as it could render evidence inadmissible, as the defence may not be able to replicate the results. The quality of the genetic marker for forensic analysis depends on its ability to be consistent. Products must be consistently and objectively scored and must accurately reflect genetic variation.

The work by Riedy *et al.* (1992) on chacma baboons concluded that RAPDs are unsuitable for parentage analysis due to the high frequency of novel bands, which could lead to false exclusion. Results seen in this study agree with Riedy *et al.* (1992). In this study high numbers of novel bands were seen in parentage: offspring comparisons which would have led one to exclude known parents erroneously in 6 of the 9 known parentage cases.

In this study, RAPD primers were screened and polymorphic primers selected. Over 50 polymorphic loci were produced from the polymorphic primers (n=15) in the parentage gels, a value suggested by Lewis and Snow (1992) as necessary for parentage exclusion cases. The number of polymorphic bands in the Blue Crane and Grey Crowned Crane eligible to be scored (76% and 83% respectively) were higher than those seen in a number of other studies. In parent: offspring comparisons, BSC values also showed that results were unreliable with some values being significantly higher than those of unrelated individuals, as expected, while some were less than the unrelated value. RAPDs have been used in numerous population studies of birds (Haig *et al.* 1994; Sharma *et al.* 2000; Singh & Sharma 2002), however, there is no literature using RAPDs for avian parentage testing.

The most damning evidence against RAPDs in parentage testing is the fact that the probability of incorrect parent will not be detected is very low. Of even greater concern is the fact that the true parents are almost certain to be excluded.

The five microsatellite primers, selected from those originally developed for Whooping Cranes, had all previously been shown to be polymorphic in Wattled Cranes (Glenn *et al.*

1997; Jones 2003). These primers provided a relatively low average of 3.6 alleles per primer in the Wattled Crane. This was expected because the cranes have generally not been very variable with ~4 alleles per locus observed in previous studies (Hasegawa *et al.* 2000; Jones *et al.* 2002; Jones 2003).

Microsatellite primers are generally only polymorphic when cross amplifying them in closely related species (Ellegren 1992). In this study, all primers cross-amplified, however, only one out of the five, Gamμ6, produced a polymorphic profile in the Blue Crane although this locus was relatively polymorphic with 6 alleles. Similarly, in the Grey Crowned Crane only one primer, Gamμ3, was polymorphic. It was not unexpected that only one primer in the Grey Crowned Crane showed polymorphism as the Whooping Crane was estimated to diverge from this species 10-20 million years ago (Krajewski & King 1996). The Blue Crane and Wattled Cranes were estimated to diverge from the Whooping Crane 3-5 million years ago (Krajewski & King 1996). So the fact that, in the Blue Crane, only one primer of the five, developed for Whooping Cranes, was successful, was also not surprising. Hasegawa *et al.* (2000) developed eight microsatellite primers for the Red-crowned Crane and they were observed to cross amplify (although polymorphism was not tested in that study) in eight related crane species, including the Blue Crane and Wattled Crane. The Red-crowned Crane and Whooping Crane belong to the same genus and lie on the same clade, relative in evolutionary distance, to the Grey Crowned, Blue, and Wattled Cranes (Figure 5. 1). For this reason, the primers developed for the Red-crowned Crane might be expected to show similar levels of success, if examined in a future study, as those developed for the Whooping Crane.

Considering the small number of loci, the combined exclusionary probability for parentage testing in the Wattled Crane was found to be quite high in this study (0.869 and 0.641 where one or both parents were unknown respectively). However, higher levels of probability are required before these primers can be reliably used in parentage testing. This could be achieved through the use of additional microsatellite markers.

Five primers, each with heterozygosities of approximately 0.8, can be sufficient to result in a combined exclusion probability of 0.98-0.99 where one parent is unknown (Ellegren 1992). The observed heterozygosities seen for the Blue Crane (using primer Gamμ6) and the Grey Crowned Crane (using primer Gamμ3) are 0.783 and 0.556 respectively. This indicates

that these loci will be beneficial in future parentage work. Although this study is a good starting point, more polymorphic loci need to be found to increase the exclusionary power for parentage testing in the Blue Crane and Grey Crowned Crane.

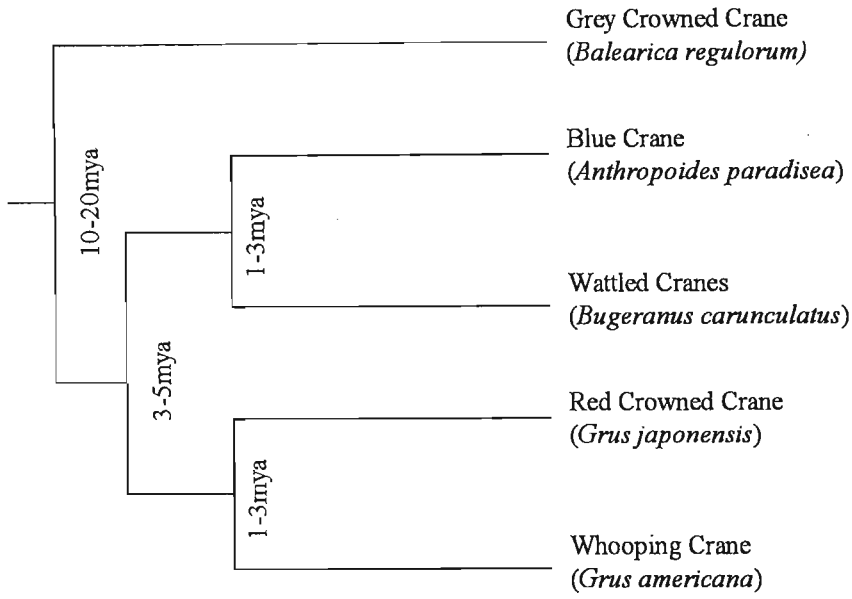

Figure 5. 1 A simplified phylogenetic tree of selected cranes adapted from Krajewski & King (1996) (not to scale).

Ellegren (1992) has reviewed the use of microsatellite loci for parentage analysis. Once developed, microsatellites are easily generated and unambiguously scored. They are robust and repeatable and it has been quoted as being a "system [that] is well suited as a tool for forensic investigations" (Lygo *et al.* 1994). Microsatellites, along with single locus minisatellites, are also the "preferred approach" for parentage assignment (Parker e*t al.* 1998).

Microsatellites are widely accepted in forensic analyses. Seven microsatellite markers were used to identify fraud in a fishing competition in Finland (Primmer *et al.* 2000). A contestant in the competition claimed he had caught a 5.5kg salmon in the lake. However, microsatellite analysis proved that the fish could not have originated from the lake in question but rather from one of the regions that supply the area's fish markets. Criminal charges were laid against the suspect.

Although in recent years South Africa has made improvements with respect to controlling illegal trade in wildlife, more attention needs to be placed on law enforcement. Genetic techniques provide a method to do this. Two techniques have been examined in this thesis as to their suitability in forensic parentage investigations. The reliability of RAPDs has been criticised by the scientific community and, as shown here, there are major problems with this technique. In addition, their dominant nature reduces their exclusionary power and, as seen not only here but also in other cases, unexplained novel bands hamper pedigree analysis. For DNA evidence to be admissible in court, the technique used must be one in which the theory and technique have been used and tested by the scientific community, sufficiently enough to have gained general acceptance for forensic work. It is there recommended that no further work go into examining this technique for forensic purposes.

The microsatellite technique, however, is very robust. It has been shown in other studies to be a suitable marker to accurately provide measures of probability in investigating issues of identity and parentage. Furthermore, it has been shown suitable and has been accepted by both the courts and the scientific community as an appropriate marker for investigating these issues including using the results as evidence for prosecutions, in illegal trade in wildlife.

It is recommended that further investigations be made as to the suitability of the outstanding microsatellite primers that were developed by Glenn *et al.* (1997) and Hasegawa *et al.* (2000) to determine which other loci are polymorphic in the South African cranes. A success rate of 20% was seen in this study, as, in both the Blue Crane and Grey Crowned Crane, only one of the five primers were polymorphic. Thus, a similar percentage is expected to be successful in further investigations with other primers. Thirty-two of the primers developed by Glenn *et al.* (1997) and all seven primers developed by Hasegawa *et al.* (2000) remain to be tested. Therefore, it is likely that approximately seven of the microsatellites currently developed for other crane species might be polymorphic in the South African cranes. Thus, it is likely that further microsatellites will need to be developed for these species before there are sufficient microsatellites available to provide a full forensic tool in order that prosecutions relating to the illegal trade in these species may be examined.

Once a sufficiently high exclusion probability, to satisfy the legal requirements of providing evidence 'beyond doubt', has been achieved, with additional polymorphic primers, the microsatellite technique will be useful in forensic parentage analysis. This method has strong potential to identify and prosecute breeders who are illegally selling wild caught crane chicks as there own captive bred birds. With the removal of this major threat to the South African cranes, natural populations can prosper. Hopefully, in time, this could lead to the South African cranes being removed from the endangered species lists. This project has made headway in achieving that goal.

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APPENDICES

APPENDIX 1
RELEVANT LEGISLATION FOR THE PROTECTION OF CRANES

1.1 Exert from: National Environmental Management Act 107 of 1998

NATIONAL ENVIRONMENTAL MANAGEMENT ACT
NO. 107 OF 1998

[ASSENTED TO 19 NOVEMBER, 1998]
[DATE OF COMMENCEMENT TO BE PROCLAIMED]
(English text signed by the President)

ACT

To provide for cooperative environmental governance by establishing principles for decisionmaking on matters affecting the environment, institutions that will promote cooperative governance and procedures for coordinating environmental functions exercised by organs of state; and to provide for matters connected therewith.

Preamble.—WHEREAS many inhabitants of South Africa live in an environment that is harmful to their health and wellbeing;

everyone has the right to an environment that is not harmful to his or her health or wellbeing;

the State must respect, protect, promote and fulfill the social, economic and environmental rights of everyone and strive to meet the basic needs of previously disadvantaged communities;

inequality in the distribution of wealth and resources, and the resultant poverty, are among the important causes as well as the results of environmentally harmful practices;

sustainable development requires the integration of social, economic and environmental factors in the planning, implementation and evaluation of decisions to ensure that development serves present and future generations;

everyone has the right to have the environment protected, for the benefit of present and future generations, through reasonable legislative and other measures that—
prevent pollution and ecological degradation;

promote conservation; and

secure ecologically sustainable development and use of natural resources while promoting justifiable economic and social development;

the environment is a functional area of concurrent national and provincial legislative competence, and all spheres of government and all organs of state must cooperate with, consult and support one another;

AND WHEREAS it is desirable—

that the law develops a framework for integrating good environmental management into all development activities;

that the law should promote certainty with regard to decisionmaking by organs of state on matters affecting the environment;

that the law should establish principles guiding the exercise of functions affecting the environment;

that the law should ensure that organs of state maintain the principles guiding the exercise of functions affecting the environment;

that the law should establish procedures and institutions to facilitate and promote cooperative government and intergovernmental relations;

that the law should establish procedures and institutions to facilitate and promote public participation in environmental governance;

that the law should be enforced by the State and that the law should facilitate the enforcement of environmental laws by civil society:

1.2 Exert from: Animal Protection Act 71 of 1962

ANIMALS PROTECTION ACT NO. 71 OF 1962

[ASSENTED TO 16 JUNE, 1962]
[DATE OF COMMENCEMENT: 1 DECEMBER, 1962]

(Afrikaans text signed by the State President)

as amended by
General Law Amendment Act, No. 102 of 1972
[with effect from 5 July, 1972 - see title GENERAL LAW AMENDMENT ACTS]
Animals Protection Amendment Act, No. 7 of 1972
Animals Protection Amendment Act, No. 54 of 1983
Animals Protection Amendment Act, No. 20 of 1985
Animals Protection Second Amendment Act, No. 84 of 1985

Protection of Animals Amendment Act, No. 7 of 1991
Animal Matters Amendment Act, No. 42 of 1993
Abolition of Corporal Punishment Act, No. 33 of 1997

[with effect from 5 September, 1997 - see title CRIMINAL LAW AND PROCEDURE]

GENERAL NOTE

In terms of Proclamation No. R.45 of 8 August, 1997, the administration of Act No. 71 of 1962 has been assigned to the Minister of Agriculture and Land Affairs.

ACT

To consolidate and amend the laws relating to the prevention of cruelty to animals.

1. **Definitions**

In this Act, unless the context otherwise indicates -

'animal' means any equine, bovine, sheep, goat, pig, fowl, ostrich, dog, cat or other domestic animal or bird, or any wild animal, wild bird or reptile which is in captivity or under the control of any person;

'Minister' means the Minister of Justice;

'owner', in relation to an animal, includes any person having the possession, charge, custody, or control of that animal;

'police officer' includes a member of any force established under any law for the carrying out of police powers, duties or functions;

'veterinarian' means a person registered as such under the Veterinary and Para-Veterinary Professions Act, 1982 (Act No. 19 of 1982).

[Definition of 'veterinarian' substituted by s. 12 of Act No. 7 of 1991.]

2. **Offences in respect of animals**

(1) Any person who -

a. overloads, overdrives, overrides, ill-treats, neglects, infuriates, tortures or maims or cruelly beats, kicks, goads or terrifies any animal; or

[Para. *(a)* substituted by s. 13 *(a)* of Act No. 7 of 1991.]

b. confines, chains, tethers or secures any animal unnecessarily or under such conditions or in such a manner or position as, to cause that animal unnecessary suffering or in any place which affords inadequate space, ventilation, light, protection or shelter from heat, cold or weather; or
c. unnecessarily starves or under-feeds or denies water or food to any animal; or

d. lays or exposes any poison or any poisoned fluid or edible matter or infectious agents except for the destruction of vermin or marauding domestic animals or without taking reasonable precautions to prevent injury or disease being caused to animals; or
e. being the owner of any animal, deliberately or negligently keeps such animal in a dirty or parasitic condition or allows it to become infested with external parasites or fails to render or procure veterinary or other medical treatment or attention which he is able to render or procure for any such animal in need of such treatment or attention, whether through disease, injury, delivery of young or any other cause, or fails to destroy or cause to be destroyed any such animal which is so seriously injured or diseased or in such a physical condition that to prolong its life would be cruel and would cause such animal unnecessary suffering; or
f. uses on or attaches to any animal any equipment, appliance or vehicle which causes or will cause injury to such animal or which is loaded, used or attached in such a manner as will cause such animal to be injured or to become diseased or to suffer unnecessarily; or
g. save for the purpose of training hounds maintained by a duly established and registered vermin club in the destruction of vermin, liberates any animal in such manner or place as to expose it to immediate attack or danger of attack by other animals or by wild animals, or baits or provokes any animal or incites any animal to attack another animal; or
h. liberates any bird in such manner as to expose it to immediate attack or danger of attack by animals, wild animals or wild birds; or
i. drives or uses any animal which is so diseased or so injured or in such a physical condition that it is unfit to be driven or to do any work; or
j. lays any trap or other device for the purpose of capturing or destroying any animal, wild animal or wild bird the destruction of which is not proved to be necessary for the protection of property or for the prevention of the spread of disease; or
k. having laid any such trap or other device fails either himself or through some competent person to inspect and clear such trap or device at least once each day; or
l. except under the authority of a permit issued by the magistrate of the district concerned, sells any trap or other device intended for the capture of any animal, including an), wild animal (not being a rodent) or wild bird, to any person who is not a *bonafide* farmer; or
m. conveys, carries, confines, secures, restrains or tethers any animal -
    i. under such conditions or in such a manner or position or for such a period of time or over such a distance as to cause that animal unnecessary suffering; or
    ii. in conditions affording inadequate shelter, light or ventilation or in which such animal is excessively exposed to heat, cold, weather, sun, rain, dust, exhaust gases or noxious fumes; or
    iii. without making adequate provision for suitable food, potable water and rest for such animal in circumstances where it is necessary; or

[Para. *(m)* substituted by s. 13 *(b)* of Act No. 7 of 1991.]

n. without reasonable cause administers to any animal any poisonous or injurious drug or substance; or
o. ...

[Para. *(o)* deleted by s. 2 of Act No. 42 of 1993.]

p. being the owner of any, animal, deliberately or without reasonable cause or excuse, abandons it, whether permanently or not, in circumstances likely to cause that animal unnecessary suffering; or
q. causes, procures or assists in the commission or omission of any of the aforesaid acts or, being the owner of any animal, permits the commission or omission of any such act; or

r. by wantonly or unreasonably or negligently doing or omitting to do any act or causing or procuring the commission or omission of any act, causes any unnecessary suffering to any animal; or

s. kills any animal in contravention of a prohibition in terms of a notice published in the *Gazette* under subsection (3) of this section,

[Para. (s) inserted by s. 21 *(b)* of Act No. 102 of 1972.]

shall, subject to the provisions of this Act and any other law, be guilty of an offence and liable on conviction to a fine not exceeding R4000 or in default of payment to imprisonment for a period not exceeding twelve months or to such imprisonment without the option of a fine, or, where any such act or omission is of a wilful and an aggravated nature, to a whipping not exceeding six strokes or to both such a fine and such a whipping or to both such imprisonment without the option of a fine and such a whipping.

[Sub-s. (1) amended by s. 3 of Act No. 54 of 1983, by s. 5 of Act No. 20 of 1985 and by s. 13 *(c)* of Act No. 7 of 1991.]

(2) For the purposes of sub-section (1) the owner of any animal shall be deemed to have permitted or procured the commission or omission of any act in relation to that animal if, by the exercise of reasonable care and supervision in respect of that animal, he could have prevented the commission or omission of such act.

(3) The Minister may by notice in the *Gazette* prohibit the killing of an animal specified in the notice with the intention of using the skin or meat or any other part of such animal for commercial purposes.

[Sub-s. (3) added by s. 21 *(c)* of Act No. 102 of 1972.]

1.3 Exert from: Constitution of the Republic of South Africa Act 108 of 1996

Constitution of the Republic of South Africa 1996

As adopted on 8 May 1996 and amended on 11 October 1996 by the Constitutional Assembly

Act 108 of 1996

ISBN 0-620-20214-9

Chapter 2

Bill of Rights

Environment

24. Everyone has the right

a. to an environment that is not harmful to their health or well-being; and
b. to have the environment protected, for the benefit of present and future generations, through reasonable legislative and other measures that
    i. prevent pollution and ecological degradation;
    ii. promote conservation; and
    iii. secure ecologically sustainable development and use of natural resources while promoting justifiable economic and social development.

APPENDIX 2
COMPOSITION OF VARIOUS SOLUTIONS

2.1 TNE lysis buffer

25ml 1M Tris base
10ml 5M NaCl
5ml 0.5M EDTA (pH7.5)
460ml dH_2O
Autoclave to sterilise
Store at room temperature

2.2 50X TAE (pH8)

484g Tris base
1.6l dH_2O
200ml 0.5M EDTA (pH8)
Adjust pH to 8 with glacial acetic acid (approx. 100ml)
Adjust volume to 2L with dH_2O
Autoclave to sterilise
Store at room temperature

2.3 Loading buffer type III

0.125g Bromophenol Blue
0.125g Xylene Cyanol
15ml glycerol
35ml sdH_2O
Store at 4-5°C
Use 1µl LB to every 5µl sample

2.4 5X TBE

54g Tris base
27.5g Boric Acid
20ml 0.5M EDTA (pH8)
Adjust volume to 1L with dH_2O

APPENDIX 3
SAMPLE INFORMATION

3.1 Blue Crane Sample Data

Sample ID No.	Bird Identification	Location	Collector	Date	Sex (if known)	Parents of Bird (if known)
B1	RAFDEC 2000	Reh. Birds	Dr. C. Kingsley	4/3/02	-	-
B2	Studbook #75 white ring right leg	AEPBF	Dr. C. Kingsley	24/04/02	Female	-
B3	Studbook #98 yellow ring left leg	AEPBF	Dr. C. Kingsley	24/04/02	Male	-
B4	Transponder: 435037460D	AEPBF	Dr. C. Kingsley	24/04/02	-	B2+B3
B5	Ring: 049	URBP	Dr. M. Penning	26/04/02	-	B24+B25
B6	Ring: 050	URBP	Dr. M. Penning	26/04/02	-	B24+B25
B7	Ring: 251	URBP	Dr. M. Penning	26/04/02	-	-
B8	Ring: 252	URBP	Dr. M. Penning	26/04/02	-	-
B9	Ring: 253	URBP	Dr. M. Penning	26/04/02	-	-
B10	Ring: 254	URBP	Dr. M. Penning	26/04/02	-	-
B11	Ring: 255	URBP	Dr. M. Penning	26/04/02	-	-
B12	Ring: 257	URBP	Dr. M. Penning	26/04/02	-	-
B13	Ring: 258	URBP	Dr. M. Penning	26/04/02	-	-
B14	Studbook #186	MP	Prof. M. Perrin	26/04/02	-	-
B15	Studbook #236	MP	Prof. M. Perrin	26/04/02	-	-
B16	Studbook #457 Transponder: 435631530A	AEPBF	Dr. C. Kingsley	24/04/02	-	-
B17	Transponder: 4350590C4C	AEPBF	Dr. C. Kingsley	24/03/02	Female	-
B18	Camp 15	TZ	Dr. S. Smith	13/05/02	Female	-
B19	Camp 15	TZ	Dr. S. Smith	13/05/02	Male	-
B20	Chick camp 15	TZ	Dr. S. Smith	13/05/02	-	B18+B19
B21	Camp 56	TZ	Dr. S. Smith	13/05/02	Female	-
B22	Camp 56	TZ	Dr. S. Smith	13/05/02	Male	-
B23	Chick camp 56	TZ	Dr. S. Smith	13/05/02	-	B21+B22
B24	Ring: 039	Monte	Unknown	30/05/02	Female	-
B25	Ring: 040	Monte	Unknown	30/05/02	Male	-
B26	"Athena"	Monte	Unknown	30/05/02	-	B24+B25

3.2 Crowned Crane Sample Data

Sample ID No.	Bird Identification	Location	Collector	Date	Sex (if known)	Parents of Bird (if known)
C1	Transponder: 4350657D4B	AEPBF	Dr. C. Kingsley	24/04/02	Female	-
C2	Transponder: 435D057D0F	AEPBF	Dr. C. Kingsley	24/04/02	Male	-
C3	Transponder: 43562A0345	AEPBF	Dr. C. Kingsley	24/04/02	Female	-
C4	Transponder: 43503B7960	AEPBF	Dr. C. Kingsley	24/04/02	Male	-
C5	Transponder: 4351044C13	AEPBF	Dr. C. Kingsley	24/04/02	Female	-
C6	Transponder: 43542F1459	AEPBF	Dr. C. Kingsley	24/04/02	Male	C5+C8
C7	Transponder: 435E165772	AEPBF	Dr. C. Kingsley	24/04/02	Male	C5+C8
C8	Transponder: 435108082B	AEPBF	Dr. C. Kingsley	24/04/02	Male	-
C9	Ring: 260	URBP	Dr. M. Penning	26/04/02	-	-
C10	Ring: 256	URBP	Dr. M. Penning	26/04/02	-	-
C11	Ring: 259	URBP	Dr. M. Penning	26/04/02	-	-
C12	Studbook #T00001	MP	Prof. M. Perrin	26/04/02	-	-
C13	Studbook #T00002	MP	Prof. M. Perrin	26/04/02	-	-
C14	Studbook #T00003	MP	Prof. M. Perrin	26/04/02	-	-
C15	Studbook #T00004	MP	Prof. M. Perrin	26/04/02	-	-
C16	Camp 17	TZ	Dr. S. Smith	13/05/02	Male	-
C17	Camp 17	TZ	Dr. S. Smith	13/05/02	Female	-
C18	Chick camp 17	TZ	Dr. S. Smith	13/05/02	-	C16+C17

3.3 Wattled Crane Sample Data

Sample ID No.	Bird Identification	Location	Collector	Date	Sex (if known)	Parents of Bird (if known)
W1	Studbook #81	AEPBF	Dr. C. Kingsley	24/04/02	-	-
W2	Studbook #83	AEPBF	Dr. C. Kingsley	24/04/02	-	-
W3	Studbook #95	AEPBF	Dr. C. Kingsley	24/04/02	-	-
W4	Studbook #96	AEPBF	Dr. C. Kingsley	24/04/02	-	-
W5	Studbook #82	URBP	Dr. M. Penning	26/04/02	-	-
W6	Studbook #89	URBP	Dr. M. Penning	26/04/02	-	-
W7	Studbook #98	MP	Prof. M. Perrin	26/04/02	-	-
W8	Studbook #113	MP	Prof. M. Perrin	26/04/02	-	-

AEPBF: Amazona Endangered Parrot Breeding Facility, Assagai, KZN
URBP: Umgeni River Bird Park, Durban, KZN
MP: Mitchell Park Zoo, Durban, KZN
TZ: Tygerberg Zoo, Cape Town, Western Cape
Monte: Monte Casino Bird Park, Johannesburg, Gauteng
Reh. Birds: Rehobath Birds, KZN

APPENDIX 4
BINARY REPRESENTATAION OF RAPD ELECTROPHORESIS GELS

RAPD gels were scored as 1 indicating band presence while 0 indicates no band observed. Spaces were left were data was unavailable.

4.1 Blue Crane RAPD data matrix

Primers: OPA003; OPA006; OPA010; OPA 015; OPA022; OPA023; OPA065

B2	10100010110100111101O1	1110100011100010010001110 11	1011110110	0001000000100000011111	1011001000110000011	1110010001000011110 1	0001110100000000O
B3	101000001101001111010 1	1100100011101000010001110 11	1101010110	0000000001010001011 1	1111101000010110011	1110010000000011110 0	0101110100000001 1
B7	1111101000101111110101	1100101011101100010011110 11	1011110110	1111000101011101111 1	1111111000010110011	1111011111110111111 1	0101110100000001 1
B9	101000110101101111010 1	1100100011111110010011110 11	1011110110	1011010001011101111 1	1111111111110111011	1111011101110111110 1	
B10	101010111001001101011 1	1100101011111110010001110 11	1101110110	1101010101011101111 1	1011111011110110011		01011101000000010
B11	011011111101101111011 1	1100101011111110010011110 11	1101110110	1111010001011101111 1	1011111110010110011	1111011010110111110 1	0011111101110001 0
B12	100111111111101111011 1	1100101011111100010011110 11	1011110110	0011010001011101111 1	0111111000110110011	1110011010110111110 1	0001110100001001 0
B13	011011111111001111011 1	1100101011111111010011110 11	1101110110	1110111001011101111 1	1011111101010111011	0110011110110111110 0	1001111111111001 1
B14	101111111101101111011 1	1110101011111110010011110 11	1011110110	1111111011011001111 1	1001101111110110011	1110011110110111110 1	0001111110011001 1
B15	011011111101001111011 1	1010110011111110010011110 11	1011111110	1111101111111101111 1	1011111010110111111	1110011101000111110 1	
B16	011111111101001111011 1	1100110011111110010111110 11	1011110110	0111011111011001111 1	1011111100110111111		1111111111111001 0
B17	011111111101111111011 1	1110110011111110010111110 11	1101010110	1101001011011101111 1	1001111000110111111	1110101010010111110 1	0001100110001001 0
B18	010111111101011111111 1	1101111111101010111001111 00	1111110111	1111111101111111111 1	0001101111111110111	1110011101010111110 1	0111100110000110 0
B19	101011110100101111111 1	1011111111111010111011111 01	1011110111	0011011001000001111 1	1000010011110110011	1110111110111111110 1	1111111110111011 1
B21	101011110100101111111 1	0010111011101010111001111 01	1011010111	0011011001001001111 1	1000000101110110011	0110011010111111110 1	0111101000000001 1

4.2 Crowned Crane RAPD data matrix

Primers: OPA014; OPA019; OPA031; OPA003; OPA006; OPA010; OPA023; OPA065

C1	0101000110011000001 11	0001011011	001000100010001 1			0010000000	0111101101111111	10101110011111
C2	0110111111001111111 11	1111111111	111110101110111 1	110100011	000010000010001 1	0011000000	0111101111111111	11111111111111
C3	0100101110000100111 11		011010101110011 1	101101011	000000000101011 0		0010100101011100	
C4		1111110111	011111101111101 1	111100011	110111111010111 1	1111101101	1111101111111111	11101111101111
C5	1111101101110111111 11	1111110111	011010101111111 1	111100011	010110011000011 1	0011000000	1111111101010111	1110111100111 1
C8	1111111111101111111 11	1111111111	011111111111111 1	111100011	110110011001000 1	0011000000	1111111111011100	11101111011111
C9	0111101101101111111 11	1100010111		101110011	000110000001011 1	0011000000	0111101101100110	10101111101111
C10	1111111011001001111 11	1111111111	011010100111101 1	111110111	010110000111011 1		0111101101101111	10101110111111
C11	0011111101100100111 11	1111111111	011010111111111 1	111111011	000010000111011 0	0111111111	1111110101011100	11101111101111
C12	1010111111001110110 11	1111111111	011111111111101 1	111010011	000110010010001 1	1111001100	1111101111110100	11101111101111
C13	0110111101100100110 11	1001101111	011000101111111 1	101110011	000000000101011 0	0111000011	1101110101100110	10100111101111
C14	0110101101111110111 11	1111111111	011000100101111 1	111110011	000110010101011 1	0111100000	1101110101101110	10101110101111
C15	0110011111001100111 11	1111111111	101000100010111 1	110110011	100110010010101 1	0011000000	1101110111101111	10101110011111
C16	0110011011000100011 11	1100011111			111011111100001 1	0011000000	1101111101 101111	10011111001010
C17		1101011111	011110101011111 1	110000011	000000000101001 0	0001000000	1100110111100110	10111111101011

4.3 Wattled Crane RAPD data matrix

Primers: OPA014; OPA019; OPA031; OPA003; OPA004; OPA006; OPA010; OPA023

W1							0000100000	
W2	110111101010111111111	111101111101111	00111000011	11101	01111011011111111	110111111	111101101010011111111	
W3	111111111111111111111	111100111001111	00111000111	11111	11110111111111111	110111111	111101111010011011111	
W4						0000110001		
W5			01011000001		01010011011010000000110000		000000000000011011101	
W6	000001111010111110111	111100000101001	10111011101	10000	01110111111111111	000011111	111101111111011011111	
W7	111111111111111110011	111100111111101	01111111111	11101	01111011111111111	010111111	111111111111011111111	
W8	000111110010111111011	111111111111111	11111111111	11101	11111011111111110	111111111	111101110111111111111	

RAPD gels were scored as 1 indicating band presence while 0 indicates no band observed. Spaces were left were data was unavailable.

5.1 Blue Crane RAPD data matrix

Primers: OPA003; OPA006; OPA010; OPA 015; OPA022; OPA023; OPA065

B2	10100010110100 1001	1111000000100 0011	101100	0001000001000001	101100000110000	1100100010000 1	0001101000000000
B3	10100000110100 1001	1101000010000 0011	110000	0000000001010000	111110000010100	1100100000000	0101101000000011
B7	11111010001011 1001	1101010011000 1011	101100	1111000101011101	111111000010100	1110111111011	0101101000000011
B9	10100011010110 1001	1101000111100 1011	101100	1011010001011101	111111111110110	11101110111001	
B10	10101011100100 0011	1101010111100 0011	110100	1101010101011101	101111011110100	0101101000000010	
B11	01101111110110 1011	1101010111100 1011	110100	1111010001011101	101111110010100	1110110101 1001	0011111011100010
B12	10011111111110 1011	1101010111000 1011	101100	0011010001011101	011111000110100	1100110101 1001	0001101000010010
B13	01101111111100 1011	1101010111110 1011	110100	1110111001011101	101111101010110	0100111011000	1001111111110011
B14	10111111110110 1011	1111010111100 1011	101100	1111111011011001	100110111110100	1100111011001	0001111100110011
B15	01101111110100 1011	1011100111100 1011	101110	1111101111111101	101111010110111	11001110100001	
B16	01111111110100 1011	1101100111101 1011	101100	0111011111011001	101111100110111	1111111111110010	
B17	01111111110111 1011	1111100111101 1011	110000	1101001011011101	100111000110111	1101010100 1001	0001001100010010
B18	01011111110101 1111	1101111010100 0100	111101	1111111101111111	000110111111101	1100111010 1001	0111001100001100
B19	10101111010010 1111	1011111110100 1101	101101	0011011001000001	100001011110100	1101111101 1101	1111111101110111
B21	10101111010010 1111	0011110010100 0101	101001	0011011001001001	100000101110100	0100110101 1101	0111010000000011

5.2 Crowned Crane RAPD data matrix

Primers: OPA014; OPA019; OPA031; OPA003; OPA006; OPA010; OPA023; OPA065

C1 01100110011000011 00010111 0010000001000 001000000 0111101011111 101010011
C2 01011111001111111 11111111 1111100111011 1101000 000010000100011 001100000 0111101111111 111111111
C3 01010110000001111 0110100111001 1011010 000000001010110 0010100001100
C4 11111101 01111101111110 1111000 110111110101111 111110101 1111101111111 111011101
C5 11110101110111111 11111101 01101001111111 1111000 010110110000111 001100000 1111111001011 111011001
C8 11111111101111111 11111111 01111111111111 1111000 110110110010001 001100000 1111111101100 111011011
C9 01110101101111111 11000101 1011100 000110000010111 001100000 0111101010010 101011101
C10 11111101100001111 11111111 01101000111110 1111101 010110001110111 0111101010111 101010111
C11 00111101100001111 11111111 01101011111111 1111110 000010001110110 011111111 1111110001100 111011101
C12 10011111001101101 11111111 01111111111110 1110100 000110100100011 111100100 1111101111000 111011101
C13 01011101100001101 10011011 01100001111111 1011100 000000001010110 011100011 1101110010010 101001101
C14 01010101111101111 11111111 01100000101111 1111100 000110101010111 011110000 1101110010110 101010101
C15 01001111001001111 11111111 10100000001011 1101100 100110100101011 001100000 1101110110111 101010011
C16 01001011000000111 11000111 111011111000011 001100000 1101111010111 100111000
C17 11010111 0111100101111 1100000 000000001010010 000100000 1100110110010 101111100

5.3 Wattled Crane RAPD data matrix

Primers: OPA014; OPA019; OPA031; OPA003; OPA004; OPA006; OPA010; OPA023

W1 0001000
W2 101101 111 1011101 001100 110 0111001 1101111 10010011
W3 111111 111 1001001 001101 111 1110111 1101111 10110010
W4 0001101
W5 010100 0100000 0001100 00000010
W6 000111 101 1000100 110101 101 0110111 0001111 10111010
W7 111111 100 1001110 101111 110 0111011 0101111 11111011
W8 001110 110 1111111 111111 110 1111010 1111111 10101111

APPENDIX 6
BINARY REPRESENTATAION OF RAPD PATERNITY ELECTROPHORESIS GELS

6.1 Blue Crane RAPD paternity data matrix

Primers: OPA003; OPA006; OPA010; OPA015

B2	010101111101101111110100100	000001000111000000000111011	1001001101 00011011010011
B4	010101111101101111110100100	000001000111000000000111011	1001001101 00001011110011
B3	011010100011000111110100100	000001000111000000000111011	1001001101 00001011111011
B24	101000111101101110101001 00	000001000111000000001111011	1001001101 00011010110001
b24	011111111011101110101011 00	000001000110011010001110011	1001001101 10111011010011
B25	010100111111101110101001 00	000001000111001100001111011	1001001101 00011010110001
b25	010100111111101110101011 00	000000100111001100000010011	1001111101 10111010110011
B5	111101111111101110101001 00	000001000111001010001111011	1001001101 00001010110001
B6	100101111111101110101001 00	100001000111001010001111011	1001001101 10111011111011
B26	101100111111101110111001 00	000000100111001100001011011	1001001101 10111011111011
B18	110110111111100111101111 10	000011011101001000110111100	1001001101 01011111111011
B20	100111111101001110101101 11	000010011011101001110111111	1111001111 01010011011101
B19	010111111101101111001101 11	000010011011101000110111111	1011101101 00010011011101
B21	100101111001101110101111 11	000000011010001000110111111	1001101101 00010010011101
B23	010100111101101111001101 10	001110011011001000110010000	1001101101 01001001011111
B22	010100100011010011001011 10	010000011010001000110100000	1001001101 01001001111111

6.2 Crowned Crane RAPD data matrix

Primers: OPA014; OPA031; OPA003; OPA006

C5	00111101010101 11010110111101001 01110 1101111110
c5	00111101010101 11000110111001011 01110 1101111110
C8	10111101110111 11111111111101101 11101 1101110111
c8	10111111110111 11011101111011101 11110 1101110111
C6	10111101110111 11000111011011001 11010 1101110111
C7	10111111110111 11010111111001101 11110 0101111110
C16	00111101011101 11000111111101101 11110 0101111111
C18	11111010011101 11010110111000001 11111 0111101111
C17	11011010110101 11110110001000001 11100 0111101111

APPENDIX 7
CORRECTED BINARY REPRESENTATAION OF RAPD PATERNITY ELECTROPHORESIS GELS

7.1 Blue Crane RAPD paternity data matrix

Primers: OPA003; OPA006; OPA010; OPA015

B2	010101111010111100000 000010011100000001 1101 10000 001101101001
B4	010101111010111100000 000010011100000001 1101 10000 000101111001
B3	011010100100011100000 000010011100000001 1101 10000 000101111101
B24	101000111010110100000 000010011100000011 1101 10000 001101011000
b25	010100111110110100100 000001011100110000 1001 10011 101101011001
B5	111101111110110100000 000010011100101011 1101 10000 000101011000
B6	100101111110110100000 100010011100101011 1101 10000 101101111101
B26	101100111110110110000 000001011100110010 1101 10000 101101111101
B18	110110111110011101110 000110110100100001 1110 10000 011111111101
B20	100111111000110101011 000100101110100101 1111 11100 011001101110
B19	010111111010111001011 000100101110100001 1111 10110 001001101110
B21	100101110010110101111 000000101000100001 1111 10010 001001001110
B23	010100111010111001010 001100101100100000 1000 10010 010100101111
B22	010100100101001000110 010000101000100001 0000 10000 010100111111

7.2 Crowned Crane RAPD paternity data matrix

Primers: OPA014; OPA031; OPA003; OPA006

C5	001110000 101010111010 01110 110110
c8	101111101 101101110111 11110 110101
C6	101110101 100011010110 11010 110101
C7	101111101 101011110011 11110 010110
C16	001110010 100011111011 11110 010111
C18	111101010 101010110000 11111 011011
C17	110101100 111010000000 11100 011011

APPENDIX 8
MICROSATELLITE GENOTYPES

Species	Individual	Locus				
		Gamμ101	Gamμ6	Gamμ3	Gamμ12	Gamμ7
Wattled Crane	W1	AE	BB	AC	AB	AD
	W2	AC	BB	CD	BC	CC
	W3	AA	AB	AA	AB	BB
	W4	AB	BB	AA	BB	CD
	W5	AD	..	..	..	BB
	W6	AA	BB	DD	BC	BC
	W7	AB	BB	BB	BC	CC
	W8	AB	AB	AD	BC	DD
Blue Crane	B2		AB			
	B3		EE			
	B4		BE			
	B5		AA			
	B6		AD			
	B7		CF			
	B8		DE			
	B9		CF			
	B10		CE			
	B11		AE			
	B12		AE			
	B13		DE			
	B14		AA			
	B16		AA			
	B17		AE			
	B19		AC			
	B20		AE			
	B21		AC			
	B22		DE			
	B23		AD			
	B24		AE			
	B25		AD			
	B26		AA			
Crowned Crane	C1			BB		
	C2			DE		
	C3			DE		
	C4			DE		
	C5			BD		
	C6			BD		
	C7			BD		
	C8			BB		
	C9			BE		
	C10			BB		
	C11			BB		

	C12			BB		
	C13			BB		
	C14			CC		
	C15			CC		
	C16			AC		
	C17			BD		
	C18			CD		