UNIVERSITY OF KWAZULU-NATAL

INVESTIGATION OF THE UTILIZATION OF MICROSATELLITES FOR FINGERPRINTING IN THREE ENDANGERED SOUTHERN AFRICAN CRANE SPECIES

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INVESTIGATION OF THE UTILIZATION OF MICROSATELLITES FOR FINGERPRINTING IN THREE ENDANGERED SOUTHERN AFRICAN CRANE SPECIES

By

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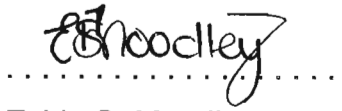
PREFACE

The experiment work described in this dissertation was conducted at the University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Annabel Fossey.

The results have not been submitted in any other form to another University and except where the work of other is acknowledged in the text, are the results of my own investigation.



Eshia S. Moodley
November 2006

I certify the above statement is correct.

Professor Annabel Fossey
Supervisor

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ABSTRACT

Cranes are large elegant birds that occur on all continents of the world except for South America and Antarctica. Of the fifteen species of crane worldwide, three predominantly occur in southern Africa; the Wattled crane (*Bugeranus carunculatus*), the Blue crane (*Anthropoides paradisea*) and the Crowned crane (*Balearica regulorum*). Crane numbers throughout the world are diminishing, mostly because of the destruction of their habitat and illegal bird trading. Efforts are underway to prevent species extinction, legally and through the compilation of a studbook that contains descriptions of physical attributes, ownership, location and possible kinships of birds in captivity. This investigation, first of its kind, was undertaken to assess whether twelve published and unpublished microsatellite primers developed for the related Whooping crane and Red-Crowned crane could be used to fingerprint the southern African crane species using cost effective polyacrylamide gel electrophoresis. The results obtained were then used to determine the extent of genetic variation within species and distance between species.

All primer sets amplified heterologous microsatellite loci in the three crane species, however, the unpublished primers produced poorly defined fingerprints even after extensive optimization. Of the twelve microsatellite loci investigated, the Blue crane and the Wattled crane revealed a high level of polymorphism. The Blue crane displayed 76% polymorphism and the Wattled crane 92%. In contrast, for the Crowned crane, that belongs to a different subfamily, Balearicinae, only 50% of the loci were polymorphic. The alleles displayed sizes similar to that of the species for which the primers were developed. Little variation in size, less than 10 bp, was noted for the different alleles of the polymorphic loci. The number of alleles, on the other hand, at each of the polymorphic loci was found to be low. The frequency of the most prevalent allele at most of the loci was generally reasonably high. These results therefore suggest that these primer sets are not suitable for individual identification and differentiation using polyacrylamide gel electrophoresis.

The observed heterozygosity of the three crane species was low; 12% in Blue crane; 7% in Crowned crane; and 13% in Wattled crane. Nei's identity further confirmed the high similarity between individuals; 66-100% for Blue crane; 55-100% for Crowned crane and 41-95% for Wattled crane. This low genetic variation is attributed to possible relatedness between birds supplied by aviculturists whom have a limited number of birds in captivity. A Hardy-Weinberg test for equilibrium revealed that most of the microsatellite loci displayed a deficiency of heterozygotes, while a few loci displayed an excess of heterozygotes. In general, the Hardy Weinberg test of equilibrium supported the notion that the individuals within each of the species might have been related.

Differentiation between the three crane species ranged from 3-5%, with Blue and Wattled crane displaying a higher degree of genetic similarity when compared to the Crowned crane, known to be the oldest extant crane species.

The limited allelic variation within the microsatellite loci tested, as well as the extensive genetic similarity between individuals suggests that a wide-ranging search for additional microsatellite loci that are more polymorphic and contain a larger number of alleles should be undertaken for the southern African crane species.

CHAPTER ONE
LITERATURE REVIEW

1.1 INTRODUCTION

Birds play a central role in the life of all humans, either for their beauty or for their irreplaceable role in ecosystems. However, in recent times dwindling numbers of birds in the wild and the extinction of species ensue great concern. Of the approximately 9 900 known species of birds worldwide, Birdlife International's *Threatened Birds of the World* (2000) lists 1 186 species as either endangered, threatened, or vulnerable.

Extinction is a continual threat faced by many species of birds. The decline in numbers has been ascribed to a range of human or 'anthropogenic' activities. The major contributing factors to the rapid decline in bird populations are thought to be habitat destruction, hunting, poisoning and illegal trade, where birds are stolen from the wild and sold into captivity. It has been predicted that persisting anthropogenic influences could put up to 12% of bird species at risk of becoming extinct within then next 100 years. (Birdlife International, 2000)

During the past 600 years approximately 100 species of birds have become extinct, but presently more than 1 000 species are considered to be nearing extinction (Wildlife Conservation International, 1992). Some of the bird species include the *Gallirallus owstoni, Zenaida graysoni, Vanellus macropterus, Campephilus imperialis, Anthropoides paradisea*, *Balearica regulorum*, *Bugeranus carunculatus*, *Paroreomyza maculata* and *Vermivora bachmanii*. In addition, more than 40 species of parrot are considered as endangered (Wildlife Conservation International, 1992).

Birdlife International (2000) estimated that 182 bird species would become critical with only a 50% chance of survival over the next three generations. It is also expected that a further 320 species will become threatened; and 680 bird species will be vulnerable if not protected.

1.2 ENDANGERED AVIAN SPECIES

1.2.1 Introduction

Endangered species are those bird species that display low numbers that require protection in order to survive and whose existence is threatened with immediate extinction. Critically endangered species are considered to have only a 50% chance of survival (Wildlife Conservation International, 1992). On the other hand, threatened species are likely to become endangered, whereas vulnerable species are at risk of becoming threatened (CITES, 2006). CITES, the Convention on International Trade in Endangered Species of Wild Fauna and Flora, aim to ensure that international trade of wild animals and plants does not threaten their survival. CITES has recently updated (12 January 2005) the appendices of most endangered and vulnerable avian species (Table 1.1).

Table 1.1 Number of endangered and vulnerable avian species, subspecies and populations, according to CITES (2006).

Appendices	Species	Subspecies	Populations
Appendix 1	146	19	2
Appendix 2	1401	8	1

Appendix 1=endangered species, subspecies and populations respectively; Appendix 2=vulnerable species, subspecies and population respectively.

Many bird species are endangered on a global scale. A bird population or species are considered to be endangered or threatened if (Wildlife Conservation International, 1992):

- The population or species consists of small numbers of individuals,
- The population or species is fluctuating in the number of individuals, and
- The population (s) is fragmented.

The number of endangered or threatened bird populations or species could decrease with an increase of global awareness of the threats facing the bird species.

1.2.2 Factors that impact bird populations

There are many reasons why a species may face extinction. Threats to bird populations include natural causes and a range of human or 'anthropogenic' activities (Allan, 1996). Natural causes such as the spread of disease amongst closely related individuals may contribute to the decline in bird populations. Other factors that could further contribute to decline in bird populations are changes in climate and worldwide sea level fluctuations. These global factors influence bird population migration and in that way may lead to a decline in numbers. Anthropogenic factors, such as wetland degradation, agricultural practises, land colonisation and the development of man-made structures, wild bird illegal trade, intentional poisoning and hunting, are human activities that predominantly contribute to the decline of bird populations (Allan, 1996).

An important natural cause of bird population decline can be attributed to the spread of avian influenza. Avian influenza is a rapid systemic illness that results in death of susceptible birds (World Health Organization, 2006). This highly contagious disease is of particular concern, because domestic birds come in contact with migratory birds, thereby depleting their numbers and also increasing the geographical range of the disease. In Italy, the avian influenza epidemic of 1999-2000 caused by the H7N1 strain resulted in the death of 13 million birds due to its ability to rapidly mutate into a highly pathogenic form within nine months (World Health Organization, 2006).

Bird habitats are often altered and degraded by anthropogenic activities that include wetland degradation and agricultural practices. Wetlands are destroyed by human activities such as the planting of forests, urban development, intensive agricultural practises and the production of plant monocultures (Allan, 1996). Other human activities include the alteration of the natural composition of wetlands. The flow of wetland water is often modified to increase water flow into the wetland, thereby causing a damming up of water, or water may be diverted out of the wetland for irrigation of agricultural crops. These practises alter the species composition and structure of the natural vegetation, thereby impacting on the survival of birds that depend on these wetlands (Allan, 1996). Agricultural practises such as tiling of the soil also destroys the species composition and

structure of the natural vegetation and therefore impact on the survival of the bird population dependent on the vegetation for food (Allan, 1996).

Habitat loss through land reclamation, or alternatively, colonisation further contributes to the decline in the number of birds of different species. Once a land is reclaimed, the number of people, vehicles, dogs and constructions increase; thereby reducing the availability of land for birds to roost, incubate their eggs, or just survive. Urban development such as, overhead powerlines and other man-made structures are a hazard for large birds such as eagles, vultures, storks and cranes. These birds are attracted to the powerlines because of their intention to utilize them as perches. When birds land or takeoff from the powerlines their wings touch the powerlines and they are electrocuted (McCann, 2000).

Illegal bird trade is an additional anthropogenic practice that affects species survival. Illegal bird trade is considered to be the largest immediate threat faced by many bird species (Wildlife Conservation International, 1992). The bulk of birds captured by traders and rural workers are sold as pets. Two industry surveys have indicated that 6 to 10%, which represents 14 to 30 million of American households, own a pet bird. In the last decade at least 8.5 million live birds were captured from the wild and smuggled into the United States (Wildlife Conservation International, 1992).

Hunting is an additional threat faced by many different bird species. Eagles and vultures are perceived as a major threat to live stock, while crane and geese appear to be a major threat to crop species (Allan, 1996). Farmers, therefore, actively seek out and kill these birds.

Poisoning, deliberate or accidental, has moreover contributed to the decline of bird populations in the recent years (McCann and Wilkins, 1998). Because farmers view vultures, large eagles and cranes as threats to livestock and grain they scatter grain soaked in commercial insecticides on agricultural fields and feedlots with the intention of killing all birds that congregate on their agricultural fields. The crane species in South

Africa have been particularly affected by this indiscriminate acts of poisoning (Allan, 1996).

Scientists, aviculturists and government authorities have therefore recognized the need to address the threats to many dwindling avian populations. The advent of molecular technology has in recent times become more and more important in the monitoring and protection of these avian species.

1.3 CRANE SPECIES

1.3.1 Introduction

The crane has inhabited earth for more than 60 million years and is found on all the world's continents, except South America and Antarctica. Of the 15 species worldwide, no less than 11 of these species are endangered. Six species of crane are native to the African continent. Three of these species of crane occur in South Africa (Allan, 1994).

Cranes are large, beautiful, graceful, majestic birds. Their long legs and necks are the outstanding characteristics of these bird species. The different species are distinguished by the colour of their head and facial features (Allan, 1994). These tall birds are known to be strong flyers and are able to embark on long distance flights. Many migrate over thousands of kilometres in bad weather and several hundred kilometres in favourable weather and plentiful roosting stopover points (Allan, 1994).

The population dynamics of the crane species contribute to their dwindling numbers. Cranes have low reproductive capability, which make it difficult for them to replace losses in population numbers caused by natural and human-caused disasters, habitat degradation, predation, hunting, poisoning and disease (Allan, 1996). Cranes begin nesting when they are between three to seven years in age. During the nesting period, cranes usually lay two eggs every year, of which, usually only one chick survives. The low reproduction rates, in addition to the indiscriminate human activities, result in the

depletion of their numbers, thereby signifying the urgency of crane conservation (Allan, 1996).

Wetland degradation has shown to impact on the number of crane that utilize these areas. In China and Russia the numbers of Siberian crane have been significantly affected by the loss of adequate wetland areas and if not addressed this species will be further endangered (Kanai *et al.*, 2002). In South Africa, wetland alteration has shown to affect the Wattled and Crowned crane, with the Wattled crane being the more sensitive of the two species to changes in the wetland habitat (Morrison and Bothma, 1998).

1.3.2 Southern African crane species

The Blue crane (*Anthropoides paradisea*) (Lichtenstein, 1793), the Crowned crane (*Balearica regulorum*) (Linnaeus, 1758), and the Wattled crane (*Bugeranus carunculatus*) (Gmelin, 1789), are the three crane species endemic to southern Africa. The Blue crane and the Crowned crane are considered as being vulnerable while the Wattled crane as being endangered (Birdlife International, 2000). Figure 1.1 depicts the graceful phenotypes of these three species.

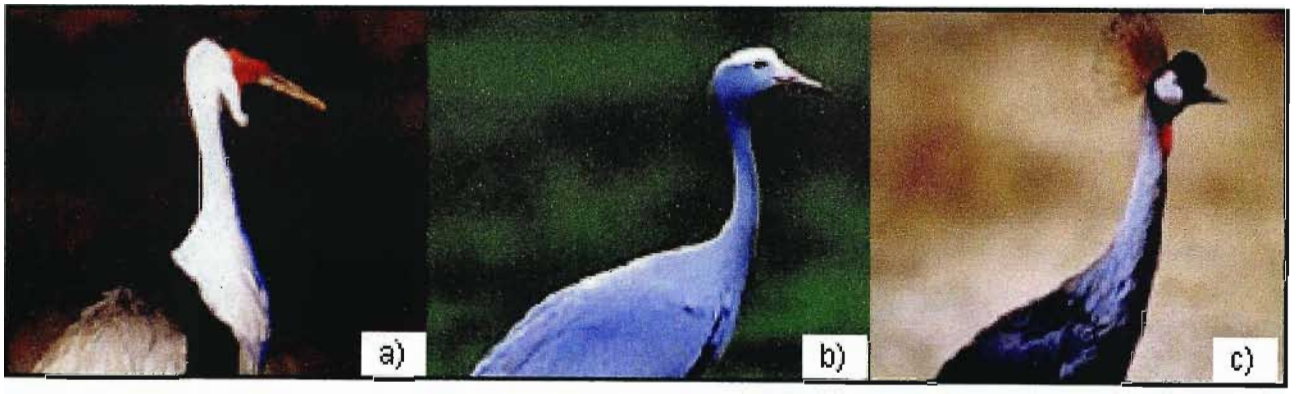


Figure 1.1 Representation of the three southern African crane showing their distinctive features: (a) Wattled crane, (b) Blue crane, and (c) Crowned crane (adapted from Maclean, 1985).

The widespread distributions of the three southern African crane species are represented in the map displayed in Figure 1.2.

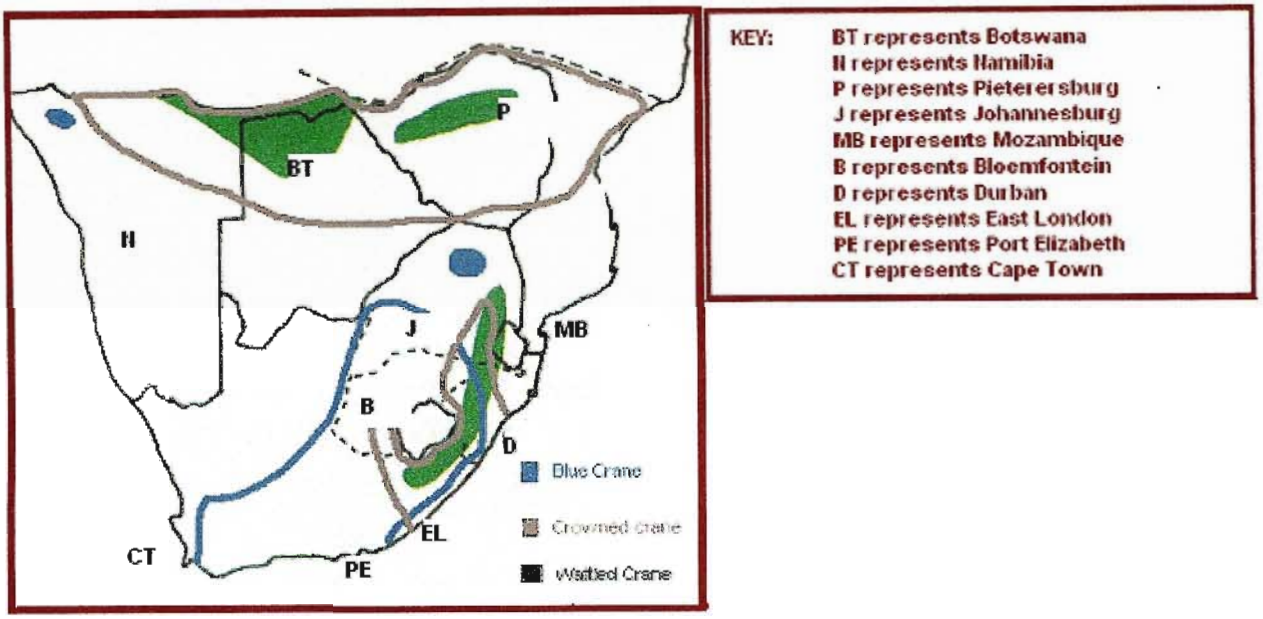


Figure 1.2 Southern African distribution of the Blue crane, Crowned crane and Wattled crane (adapted from Maclean, 1985).

A. Blue crane (*Anthropoides paradisea*)

The Blue crane, also known as the Stanley crane, is South Africa's national bird. The Blue crane species is distributed throughout southern Africa, however a few breeding pairs have been found in Swaziland, and a small isolated population in Namibia. Blue crane nest on dry ground and feed on frogs, reptiles, insects, fish and grain (Allan, 1994). Although most crane species are dependent upon wetlands, this is not the case with the Blue crane. Breeding occurs between the months of October and February. Clutches produced during the breeding period usually contain two eggs, occasionally one (Allan, 1994).

The Blue crane is known for feeding on cultivated agricultural fields and feedlots established by farmers for small livestock. The presence of these birds in such agricultural areas has contributed to their vulnerable status, because of poisoning by

farmers (Allan, 1994). In South Africa, Blue crane poisoning was reported as early as 1980, with the worst poisoning incident occurring in the eastern Cape, where 400 Blue cranes were killed after feeding on poisoned grain (Allan, 1994).

B. Crowned crane (*Balearica regulorum*)

The Crowned crane is thought to be the oldest extant crane species (Johnsgard, 1983). The Crowned crane species retains primitive bird characteristics such as the laying of unmarked eggs (Johnsgard, 1983). The Crowned crane has also retained primitive activities such as occasional breeding in trees, which no other crane species exhibits (Johnsgard, 1983). Predominantly, breeding occurs on the ground between the months of December and February producing egg clutch sizes that may be as large as four eggs. Two chicks are usually raised (Johnsgard, 1983).

The Crowned crane is found throughout southern Africa as well as Kenya and Uganda (Allan, 1994). The Crowned crane is associated with open country, in particular, grasslands near water. The grasslands represent suitable breeding grounds, but due to human activities such as forestry, the grasslands are undergoing significant transformation (Allan, 1994). In the past, the Crowned crane was found in wetland habitats, however over the past ten years, the South African population of Crowned crane has retreated from the wetland habitats due to the increased threat of poisoning.

C. Wattled crane (*Bugeranus carunculatus*)

The Wattled crane is South Africa's largest crane and is also found further north in Africa inhabiting, Zimbabwe, Mozambique, Botswana, Namibia, Angola, Zambia, Zaire, Ethiopia, Malawi and Tanzania (Allan, 1994). The Wattled crane is also dependent upon wetlands such as the Crowned crane. This species requires marshy area for feeding because their diet is comprised largely of aquatic vegetation (Allan, 1994).

Wattled crane require the wetland habitat for nesting. Some general field conservation studies carried out on the nesting habitats of cranes highlighted the use of possible crane

as an indicator of environmental quality in a habitat (Allan, 1995; Morrison and Bothma, 1998; Timoney, 1998). The destruction of wetlands indicates a decrease in environment quality and has largely contributed to the rapid decline in numbers of the species. In addition, the Wattled crane only produces one egg and thus only one chick is reared at each breeding attempt (West, 1977). The South African Crane Foundation (1992) reported that the Wattled crane eggs and chicks as being mostly susceptible to robbery. According to the recent statistics of South African Crane Foundation (2006), there are approximately 250 remaining Wattle crane individuals, of which, there are only 80 active breeding pairs. It has, therefore, become imperative that conservation and preservation of the Wattled crane, as well as the other southern African crane species is undertaken.

1.4 CAPTIVE BREEDING AND CONSERVATION OF SOUTH AFRICAN CRANE SPECIES

The keeping of crane in captivity is evidently an old practice (Johnsgard, 1983), but the reasons for their placement in captivity has changed with time. In the past these graceful birds were placed in captivity for their beauty and for recreational purposes. Due to the threats facing crane populations, cranes have been placed and reared in captivity for reproduction studies to facilitate in the conservation of these species. The early years of aviculture permitted cranes to incubate their own eggs and raise their own young. However, the success of this approach was dependent on favourable weather, freedom from predation and human disturbances. From the late 1800s, due to the difficulty in fulfilling these requirements, crane eggs have been taken from the wild and chicks hatched and reared in captivity (Johnsgard, 1983).

Captive breeding requires extensive knowledge and experience, and if managed well, plays a major role in species conservation (Johnsgard, 1983). In 1993, Nesbitt and Carpenter conducted an investigation on the survival strategy and the migration pattern of a population of Whooping crane that were reared in captivity and later reintroduced into the wild (Nesbitt and Carpenter, 1993). The study revealed that slow, calm releases were shown to be most successful. However, irrespective of its conservation role, captive breeding requires a large amount of time, patience, knowledge, and experience.

Captive breeding is also extremely sensitive to disturbances and should be regarded as an alternative "insurance policy" against extinction when all other conservation efforts fail. A management tool used in captive breeding programmes is that of artificial insemination (Gee and Mirande, 1996; Jones and Nicolich, 2001).

Artificial insemination is implemented mainly for three reasons (Gee and Mirande, 1996):

- Impaired reproduction due to physical impairment or behavioural difficulties,
- Manipulation of genetic composition without the disruption of existing pair bonds, and
- Minimisation of egg infertility by increasing fertility rates above that of natural mating.

As with captive breeding, artificial insemination is another conservation effort that requires extensive knowledge. A study performed around artificial insemination should retain extensive knowledge of the species in question. In addition to establish a successful conservation programme, biological and behavioural information concerning the species in question also needs to be known. To date, no information of successful artificial insemination on crane exists (Gee and Mirande, 1996).

Sexing of birds play an important role in successful captive breeding. Phenotypic discrimination of bird sexes is difficult in approximately half of the worlds species when the birds are adult, while nearly all chicks of the worlds 10 000 species are sexually indistinguishable (Griffiths *et al.*, 1998). Many bird species are morphologically monomorphic for external characters that differentiate their sexes. Furthermore it is particularly difficult to identify chromosomes. According to Saski and Takagi (1975) these diploid species have approximately 80 chromosomes. Although the sex chromosomes are heteromorphic, W and Z, with the females being the heterogametic sex (ZW) and the males the homogametic sex (ZZ), discrimination between the sexes is difficult through chromosome spreads, because of the number of chromosomes and their small size.

Avian molecular sexing technology has thus in recent years made vast strides in developing diagnostic tools applicable to many avian species. Griffiths *et al.* (1998) developed a molecular sex identification test, which has been found to be successful in non-ratite species. The test employs one set of primers that anneals to conserved exonic regions of the chromo-helicase-DNA-binding genes (CHD) on the sex chromosomes, that amplifies across an intron in both CHD-W and CHD-Z. Introns, noncoding regions, evolve more rapidly than coding regions and are therefore less conserved. The expectation is that the intron lengths amplified could have different sizes for the two genes CHD-W and CHD-Z, in different species. When PCR products are visualized through gel electrophoresis, a single band will be revealed in males, which have two Z sex chromosome, and two bands in females, which have both the Z and W chromosomes.

A female-specific DNA fragment linked to the W chromosome has been developed for the Whooping crane (*Grus americana*) (Duan and Fuerst, 2001). A set of PCR primers was developed which amplified a 227–230 bp female-specific fragment from all existing crane species and some non crane species. A larger duplicated version of this DNA segment (231-235 bp) was later found on the Z chromosome. Primer combinations for both these loci have provided an accurate sexing tool, which together with the sourcing of DNA from feathers, can now be used for the sexing of young crane chicks.

1.5 GENETIC FINGERPRINTING

Most crane species are experiencing a rapid decline in numbers making them vulnerable and in danger of extinction. Together with small egg clutches, any egg or chick lost, has a severe impact on an entire crane population (Allan, 1994). Furthermore, chicks are captured illegally from the wild and passed off as chicks produced by captive breeding pairs, thus hampering conservation efforts. The urgency for the development and establishment of well-controlled conservation practises has therefore been recognized. Molecular technologies have been identified as one of the possible major contributors to this conservation effort.

Molecular sexing and genotyping (genetic profiling) would greatly facilitate conservation efforts by contributing to breeding practises, individual identification and kinship determination.

Over the last decade a diverse array of molecular tools have become available to be utilized in individual identification (Parker *et al.*, 1998). These tools are either phenotypic or genotypic in nature. The most widely used phenotypic molecular tool is that of allozyme fingerprinting. These identification tools, which use differences in protein expression, are based on the variation of gene products due to differences in DNA sequences (Parker *et al.*, 1998). In genotypic analyses, a variety of fingerprinting procedures have been developed over the past years. These include single-locus fingerprinting such as restriction fragment length polymorphisms (RFLPs), single sequence repeats (SSRs) and multi-locus fingerprinting, such as random amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs). These genotypic fingerprinting tools analyze DNA variation directly at the DNA level, and are referred to as DNA markers.

1.5.1 Protein fingerprinting

Prior to the development of new DNA molecular techniques, protein assays were used to determine genetic variation between individuals. These protein assays were based on allozymes (Soltis and Soltis, 1989). These allozymes originate through amino acid alterations that cause changes in the conformation of enzyme molecules. Allozymes, thus indicate different allelomorphs at a particular locus. Therefore, different alleles are represented by allozymes that have different electrophoretic mobility. Electrophoresis verifies the changes in the amino acid within the nucleotide sequence of the respective coding gene (Karp *et al.*, 2001).

Allozymes possess the following advantages for fingerprinting (Karp *et al.*, 2001):

- The substrate specificity of the enzymes provides the basis for monitoring the genetic variation at a specific gene locus,
- Allozymes are commonly expressed as co-dominant markers enabling genotypes to be precisely distinguished as homozygous or heterozygous, and
- In addition, large samples of tissue can be processed in less time per sample when compared to DNA samples.

Although allozymes are useful in large-scale population structure studies, the levels of genetic accuracy and precision required for individual identification are far beyond the reach of routine allozyme analyses (Parker *et al.*, 1998; Smouse and Chevillon, 1998). Furthermore, allozyme studies target only a small portion of a genome, namely, the coding genes and not all allelic differences are picked up as different allozymes.

1.5.2 DNA fingerprinting

"DNA fingerprinting" is a term that was initially used to describe DNA profiling which utilized microsatellite sequence variation, but has become a collective term encompassing many of the profiling protocols. DNA fingerprinting is used to screen genetic variation in animals and plants and provide answers to a wide range of questions including an individual's identification, rates of genetic divergence in a population, reproductive success, and assignment and exclusion of parentage.

DNA fingerprinting is used to describe distinctive, unique banding patterns produced from an individual's DNA, which cannot be performed in phenotypic analysis. The type of profiling selected will depend on the question being asked, the statistical analysis available for identification, and the time and cost constraints (Parker *et al.*, 1998). The banding patterns differ depending on the profiling technique selected.

Molecular markers in DNA fingerprinting, are useful when they display genetic variation at a particular locus in a population; thereby containing more than one allele at the locus.

Such a locus is referred to as being polymorphic (Parker *et al.*, 1998). Each type of molecular marker displays particular types of genetic differences. These are either differences in nucleotide sequences, number of repeated DNA segments, presence or absence of restriction sites, or presence or absence of primer annealing sites (Parker *et al.*, 1998).

A. Single-locus markers

Single-locus markers represent genetic variation at a particular locus. Development of these markers is time consuming and costly and may require the identification of specific nucleotide sequence differences, polymorphic restriction sites and loci with variable number of repeated sequences. In addition these markers require the development of appropriate probes or primers to identify them. Variations in these markers are revealed through either PCR amplification or through DNA hybridisation with DNA probes. The most widely used single locus markers are Restriction fragment length polymorphisms (RFLPs) (Nei and Tajima, 1981) and Simple sequence repeats (SSRs) (Jeffreys *et al.*, 1985).

RFLPs

RFLPs were the first type of fingerprinting utilized in population genetics (Parker *et al.*, 1998). An RFLP occurs when variation in a particular restriction enzyme cleavage site is detected by either being present or absent. A restriction site in a particular DNA locus may be abolished through mutations of the enzyme target sequence, or a new restriction site may be created through mutations (Parker *et al.*, 1998). If a restriction site is present on a strand of DNA, the DNA will be cleaved by a restriction enzyme that targets the site. This would result in the DNA strand decreasing in size and thus showing up as two different bands on a gel. However, if the restriction site had been abolished through mutation, a larger sized band will be detected on a gel (Parker *et al.*, 1998). Although the number of alleles of RFLPs is limited, combinations of RFLPs and restriction enzymes provide for a greater number of genotypic possibilities. One of the important advantages

of RFLP markers is that they are co-dominant in nature, thus making it possible to distinguish between homozygous and heterozygous genotypes (Parker *et al.,* 1998).

SSRs

SSRs can be sub-divided into two major classes according to the number of nucleotides making up the repeat unit (Krawezak and Schmidtke, 1994). The SSR markers have characteristics that are useful because they are highly variable and are generally recognised as neutral, so that selection and environmental pressures do not influence their expression directly (Scotti *et al.*, 1999). Minisatellites, otherwise known as variable number tandem repeats (VNTRs), comprise of short tandem repeats of approximately 40 base pairs in length (Avise, 1994). Microsatellites, on the other hand, also known as simple sequence repeats (SSRs), are simpler in that they constitute of between one and four nucleotides per repeat unit. SSRs have a high polymorphic content brought about by the extensive variation in the number of repeats between individuals, thus producing a large number of different alleles that facilitate effective assessment of genetic relationships among individuals and populations (Parker *et al.,* 1998). The wide range of variation makes these markers popular fingerprinting tools especially in the identification of individuals (Scotti *et al.*, 1999). The different alleles of SSR markers are identified by their difference in size, either through PCR amplification or through enzyme digestion and probing.

B. Multi locus markers

Multi-locus fingerprints are primarily viewed as fingerprints that are generated from a number of loci and visualized in a single lane in a gel. They can either be generated by the amplification of several single loci, using the primers specific for each of the loci, or by amplification of many of the loci using one or a few arbitrary primers (Krawezak and Schmidtke, 1994). The use of arbitrary primers does not require any prior knowledge of the DNA composition and is much easier and less time-consuming than having to amplify several markers independently. Multi-locus fingerprints have a high information content and are useful for individual identification and parentage analyses. The most widely

used multi locus markers are Randomly Amplified Polymorphic DNAs (RAPDs) (Williams, 1990) and Amplified Polymorphic Length Polymorphisms (APLPs) (Vos *et al.,* 1995).

RAPDs

A single primer is used in RAPD analyses. The primer is arbitrary and consists of 10-11 nucleotides, which are able to anneal to multiple sites on the template DNA due to their limited number of nucleotides (Williams, 1990). When the primer anneals at two different places on the same DNA molecule and is at a suitable distance to allow for PCR amplification, the intervening sequence between the two annealing sites will be amplified. This results in a number of bands that can be visualized on agarose gels (Williams, 1990). Differences in genotypes are due to mutations in the annealing region of the primer or due to insertions or deletions of segments in the intervening sequence between the primer annealing sites (Parker *et al.,* 1998). RAPD alleles are, therefore, regarded as "presence" or "absence" alleles, where a fragment will be amplified if the primer anneals at two annealing sites, or will not be amplified if one or more primer annealing sites have been mutated. RAPD markers are thus dominant in nature, making it impossible to distinguish between homozygous and heterozygous individuals that contain a presence allele. Although limited in number of alleles detected and their dominant nature, they remain one of the more popular genetic tools mostly because they are relatively inexpensive, fast, require no prior knowledge of the DNA, and are able to assess a large number of loci at once (Smith and Wayne, 1996).

AFLPs

The AFLP genetic markers are based on repeated amplification of a subset of restriction fragments from a total digest of genomic DNA utilizing PCR (Desmarais *et al.*, 1998). The DNA is cut with two enzymes, a rare and a frequent cutter according to the length of their restriction site. Thereafter, each fragment is ligated to adapters that serve as a binding site for primers. Only fragments that contain both restriction sites, one at either end, will be amplified. This initial pre-selective amplification reduces the total number of restricted fragments present in the reaction. The PCR product is then used as a template for a

second round of amplification using primers with three additional selective nucleotides included at the 3' end (Desmarais *et al.*, 1998). This round of selective amplification, amplifies one out of 16 fragments (Desmarais *et al.*, 1998). The final product of these multiple amplification steps is a multi-locus fingerprint visualized on a gel (Desmarais *et al.*, 1998). These markers are, as was the case with RAPDs, also dominant in nature. Although AFLP fingerprinting is more robust than that of RAPDs, its major disadvantage is that it is significantly more expensive than RAPD analysis and is technically more challenging (Karp *et al.*, 2001).

1.6 GENETIC ANALYSIS OF DNA FINGERPRINTS

Molecular markers provide adequate tools for the analysis of genetic variation within populations and between populations, within species, as well as between species. Previously, traditional Mendelian methods of scoring the phenotypes were employed (Avise, 1994). However, these methods are insufficient for a detailed estimate of genetic variation, because the process is restricted to phenotypic characteristics that are limited in number (Avise, 1994). In addition, phenotypic differences do not pick up allelic differences. Molecular markers, on the other hand, provide the possibility for more detailed estimates of genetic variation due to the large number of different types of markers.

Genetic variation can be described by various statistics (Avise, 1994). The formulas of the different statistics are based upon either allele frequencies, or genotypic frequencies, or both. The formulas use observed values, expected values or both. Some of these statistics include observed heterozygosity, expected heterozygosity (Levene, 1949), also known as gene diversity and Nei's heterozygosity (Nei, 1972). Wright's (1978) inbreeding coefficient (F_{IS}) is also calculated to determine heterozygosity deficiency and thereby together with Hardy Weinberg Equilibrium tests provide a means to debate possible evolutionary forces. In addition, a number of genetic distance statistics have been formulated of which the most popular are Nei's (1972; 1978) and Roger's genetic distance measures (1972).

1.6.1 Measures of genetic variation

The frequency of polymorphic loci is a commonly used measure of quantifying genetic variation. It is the proportion of polymorphic loci located in a population, species, or taxon. The proportion of polymorphic loci is calculated by, firstly counting the number of polymorphic loci and then by dividing by the total number of loci examined (Hartl and Clark, 1997):

$$P = \frac{\text{Number of polymorphic loci}}{\text{Total number of loci scored}}$$

The polymorphic content, as a measure of quantifying genetic variation, is useful but has the disadvantage that it exhibits arbitrariness and imprecision. A locus displaying alleles with relatively high frequencies is equated similar to a locus containing an allele that is rare. Therefore, a locus of which the rare allele has a frequency less than 0.05, an arbitrary chosen value, is usually deemed monomorphic to compensate for this bias.

Average gene diversity (heterozygosity) utilizes the allele frequencies at many different loci. It measures the extent of genetic variation in a population, species, or taxon and is also known as the expected heterozygosity, under the assumption of Hardy Weinberg Equilibrium. A heterozygosity value is inferred using the allele frequencies with the assumptions of a larger, random mating population. Under unknown circumstances, the expected value is used rather than the observed as it depends only on allele frequency and can be used irrespective of the mating pattern of the population. Two popular estimates of expected heterozygosity used are those of Levene (1949) and Nei (1972).

Levene's (1949) heterozygosity is calculated using the formula below:

$$H = p(1-p)[1+1/2n-1)]$$

Where p is the frequency of allele i and n is the number of individuals in the sample.

Nei's (1972) is determined using the formula below:

$$H_j = 1 - \Sigma q_k^2$$

Where H_j is the heterozygosity estimated at locus j and

q_k is the frequency of the k^{th} allele at the locus.

Wright (1978) developed an approach to partition the genetic variation in a subdivided population that provides a description of differentiation based on the levels of heterozygosity. This approach consisted of three different F coefficients namely, F_{ST}, F_{IT}, and F_{IS}. The F_{ST} coefficient is a measure of genetic differentiation over subpopulations and its value is always positive. The F_{IT}, and F_{IS} coefficients are measures of the deviation from the Hardy-Weinberg proportions within subpopulations and in the total population, respectively, where positive values indicate a deficiency of heterozygotes and negative values indicate an excess of heterozygotes (Wright, 1978).

These three values are determined using the formulae below (Wright, 1978):

$$\overline{F}_{IS} = \frac{\overline{H}_S - \overline{H}_0}{\overline{H}_S}$$

$$\overline{F}_{IT} = \frac{\overline{H}_T - \overline{H}_0}{\overline{H}_T}$$

$$\overline{F}_{ST} = \frac{\overline{H}_T - \overline{H}_S}{\overline{H}_T}$$

Where F_{IS} is a measure of heterozygosity deficiency or excess,

F_{IT} is a measure if the accumulated inbreeding resulting from mating between remote relatives at all levels of the population hierarchy,

F_{ST} is a measure of the extent to which species is organized into subpopulations with restricted gene flow.

Wright's (1978) inbreeding coefficient (F_{IS}), as a measure of heterozygosity deficiency or excess, is also used to estimate the presence of a possible inbreeding effect in a particular subpopulation. F_{IS} can therefore be viewed as the inbreeding coefficient of a group of inbred organisms relative to the subpopulation or species to which they belong, where random mating is considered in each of the subpopulations.

The test for Hardy-Weinberg equilibrium evaluates the presence of possible evolutionary forces that impact on allelic frequencies. This test computes expected genotypic frequencies under random mating conditions, and compares it to the observed genotypic frequencies using chi-squared and likelihood ratio tests.

The Hardy-Weinberg Law (H-W) states that over time and across generations genotypic frequencies will remain unchanged under the assumptions of a large random mating population in which no effect of selection, migration, mutation or genetic drift exists.

1.6.2 Measures of genetic distance

Genetic distance is a measurement of genetic relatedness or unrelatedness of individuals between populations, species, or taxon. In 1972, Rogers and Nei independently derived ways of calculating genetic distance and similarity between taxa (Nei, 1972; Rogers, 1972). Both measures use allelic frequencies to estimate genetic distance, although their distance parameters have different properties.

Rogers (1972) devised an index of genetic distance as described below:

$$D = [0.5 \sum (x_i - y_i)^2]0.5$$

where x_i and y_i are the frequencies of the *i*th alleles in populations X and Y.

Nei's estimated the genetic identity (I) from allelic frequencies for alleles, from which the genetic distance (D) was calculated as the negative natural logarithm of (I). Nei's genetic identity (1972) is based on allelic frequencies and estimated genotypic frequencies across all loci.

$$I = J_{xy} / \sqrt{(J_x J_y)}$$

where J_{xy}, J_x and J_y represent the means across all loci of $\sum x_i y_i$, $\sum x_i^2$ and $\sum y_i^2$ respectively when x_i and y_i are the frequencies of the *i*th alleles in populations X and Y.

Nei's (1972) genetic distance (D) estimates genetic divergence between taxa. Genetic distance ranges from zero to infinity, where a value of zero indicates that the populations are identical and a high value that they are divergent or of a separate species (Cooke and Buckley, 1987).

$$D = -\ln (I)$$

Genetic identity values range between zero and one, where a value of zero indicates that the species being compared have no alleles in common and a value of one, that they are identical. Genetic distance ranges from zero to infinity, where a value of zero indicates that the populations are identical and a high value that they are divergent or of a separate species (Cooke and Buckley, 1987). Table 1.2 shows the expected values for genetic identity and distance that indicate the extent of relatedness of the individuals or species or populations being compared.

Table 1.2 Expected values for genetic identity and genetic distance (Cooke and Buckley, 1987).

STATUS	GENETIC IDENTITY	GENETIC DISTANCE
Closely related	> 0.9	< 0.1
Divergent or separate	< 0.8	> 0.2

1.7 APPLICATION OF DNA FINGERPRINTING IN THE CRANE

With the advent and development of sophisticated DNA fingerprinting techniques it has become possible to undertake a wide range of genetic analyses in crane. These include the genotypic analysis of individuals for forensic purposes as well as kinship determination. Other analyses at the population and species level include the assessment of genetic variation, determination of population substructure and evolutionary trends.

1.7.1 Individual fingerprinting

In forensic analysis and kinship determination it is of primary importance that a fingerprinting procedure is able to discriminate between individuals. A number of fingerprinting procedures are currently employed. The most popular is that of SSR's, in particular microsatellites. These markers are usually used in combination and are able to differentiate between individuals because of the large number of possible alleles at a particular locus. Other fingerprinting techniques used when simple SSR's are not available, include AFLP's and RFLP's.

Forensic analysis requires that fingerprints are sufficiently different so that differentiation between individuals is possible. In the illegal bird trade, birds are often confiscated and passed on as a progeny of captive parents. Fingerprinting is then of value to determine the uniqueness of such an individual's genotype or its relationship to known captive parents. It has been recognized that to conserve and preserve the endangered southern African crane species will require the extension of the current studbook to include molecular data of all birds in captivity. Very little genetic information has been gathered for the South African crane captive population, due to the lack of molecular investigations.

Individual identification is also of great value to breeders, especially when breeding pairs are formed. Breeders need to take special care as to avoid the potential parents within a breeding pairs being related. In that way, circumventing the possibility of inbreeding.

Microsatellite fingerprinting is currently not available for the three southern African species due to the lack of available primers. The only microsatellite loci for which primers are currently available for fingerprinting in crane are the 50 microsatellite primers developed for the Whooping crane by Glenn and Jones (personal communication) and the seven microsatellites isolated and characterized for the Red crowned crane by Hasegawa *et al.* (2000).

1.7.2 Genetic diversity and evolution of cranes

The endangered status of many of the crane species has stimulated evolutionary genetic investigations based mainly upon phylogenies and DNA relationships. In an attempt to resolve the evolutionary relationship amongst the different crane species Krajewski and Fetzner (1994) compared DNA sequences of the *Cytochrome-B* locus and suggested a rapid evolutionary diversification of crane lineages. This is supported by an investigation into centromeric repeat monomers by Madsen *et al.* (1992), who revealed that the centromeric repeats probably evolved from a common ancestral sequence that may date from the very early stages in the radiation of birds.

A number of investigations into genetic variation of crane species, captive and wild have been conducted. Within the endangered Siberian crane (*Grus leucogeranus),* Tokarskaya *et al.* (1995), using the M13 microsatellite probe, determined a high percentage of genetic differences and heterozygosity within a population, thus revealing a high percentage of genetic variability.

In the Whooping crane (*G. americana*), Glenn *et al.* (1999) assessed the genetic effect of a human-caused bottleneck by sequencing 314 base pairs (bp) of the mitochondrial DNA control region. This endangered bird species suffered a severe population bottleneck; only 14 adults survived in 1938. The DNA of cranes that lived before the bottleneck was obtained from museum specimens was compared to that of cranes that survived after the bottleneck. Six haplotypes were present among the prebottleneck individuals sequenced, and only one of these haplotypes persisted in the modern population. The most common modern haplotype occurred at a low frequency in the prebottleneck population, which

demonstrates the powerful effect of genetic drift in changing allele frequencies in small populations. By combining all available data, it was shown that no more than one-third of the prebottleneck haplotypes survived the human-caused population bottleneck. This data also demonstrated the significance of genetic effects such as loss of heterozygosity, loss of disease resistance and decrease in competitiveness. These data are supported by the findings of Longmire *et al.* (1992) who used the M13 microsatellite probe on a population of 42 individuals.

A number of investigations have been conducted to determine the subspecies status of the Sandhill crane *(Grus canadensis)* and the Sarus crane (*Grus antigore*). Glenn *et al.* (2002) sequenced a 437 bp segment from the mitochondrial DNA (mtDNA) control region of the Sandhill crane. The haplotypes that resulted indicated that the subspecies classified as *G. c. rowarm* and *G. c. tabida,* based on their morphology, did not differ genetically, but the subspecies *G. c. tabida* was genetically different from the other two subspecies. In a similar investigation in which Peterson *et al.* (2002) sequenced a 675 bp region of mtDNA of Sandhill crane, found that the data obtained supported the subspecies designations of *G. c. canadensis* and *G. c. tabida*. Genetic divergence ranged from 6.5 to 14.5% between *G. c. canadensis* and *G. c. tabida* and 0.5 to 6.6% within *G. c. canadensis* and 0.1 to 6.0% *G. c. tabida.*

1.8 MOTIVATION

Wildlife Conservation International (1992) and Birdlife International (2000) have recognized the need for cost effective molecular genotypic analyses (Selkoe and Toonen, 2006) of birds to facilitate the conservation effort and for inclusion of molecular data in the studbook of the three southern African crane species, Blue crane, Crowned crane and Wattled crane. Microsatellites used in fingerprinting, the preferred technology, have not been identified nor have primers for these three species been developed. However, five microsatellites were identified and primers developed by Glenn and Jones (personal communication) for the Whooping crane as well as seven loci and accompanying primers by Hasegawa *et al.* (2000) for the Red-Crowned crane. As it is known that the identification and development of microsatellite primers is very costly and

that microsatellite primers are often able to hybridise across species (Glenn and Jones, personal communication), an investigation was undertaken to test the applicability of these known primers to amplify heterologous microsatellite loci in the southern African crane species. It was envisaged that a technology that would be affordable to the crane industry would be assessed. Additionally to this primary investigation, the genetic variation within the three species was also investigated.

The investigation entailed the following components:

- The selection of suitable primers of published microsatellite loci and testing of their ability to amplify heterologous microsatellite loci in the three related southern African crane species,
- The generation of cost effective microsatellite fingerprints,
- The assessment of the ability of the selected microsatellites to distinguish between individuals, and
- The assessment of the genetic variation within each of the three crane species.

CHAPTER TWO
MATERIALS AND METHODS

2.1 INTRODUCTION

In South Africa of the three species of crane, the Crowned crane (*Balearica regulorum*) and Blue crane (*Anthropoides paradisea*) have been recognised as being vulnerable, while the Wattled crane (*Bugeranus carunculatus*) has been recently classified as an endangered species (Barnes, 2000). The depletion in the number of cranes in South Africa has been mostly due to illegal trafficking and the unlawful use of the cranes for medical purposes by the local community. Therefore, it has become imperative to devise means to conserve and control these species. DNA fingerprinting has been identified as a preferred means by which to profile and identify confiscated birds, as well as young chicks and adults in breeding colonies. Thereafter this information is lodged in a studbook (The South African Crane Foundation, 1992).

The SSR microsatellite fingerprinting has been deemed one of the most suitable DNA fingerprinting methods, as it is able to distinguish between individuals (Jones, 2003). In the case of cranes, a number of microsatellites have been identified and primers developed the use in the Red-Crowned crane and Whooping crane (Glenn and Jones, personal communication). DNA fingerprinting methodology can be used as a tool to identify individuals and it can be used for the monitoring of populations that are depleting in numbers. As microsatellites have not been identified and primers developed for all crane species, it was decided to assess whether the known microsatellites could be used for fingerprinting in the three South African crane species.

Twelve microsatellite loci were selected for this investigation, five developed by Glenn and Jones (personal communication) and seven by Hasegawa *et al.* (2000). These loci were selected based upon their polymorphic nature in the species that they were developed for. The five Glenn and Jones primer sets were developed for the Whooping crane (*Grus Americana*), as recommended by Jones, a co-worker of Glenn (personal

communication), and Ms. King a co-worker in the laboratory. The seven Hasegawa primer sets were developed for the Red-Crowned crane (*Grus japonensis)* (Hasegawa *et al.* 2000). Thus, the potential to undertake individual identification and the measurement of the inherent genetic variation within each of these species was investigated.

This investigation included three major areas of research:

Investigation 1

Determination of whether the known microsatellite primers of Red-Crowned crane and Whooping crane would cross-amplify heterologous microsatellite loci in the three South African crane species.

Investigation 2

To assess whether a more cost effective non-automated protocol would provide sufficient discrimination of genotypes instead of the costly automated protocol. A more cost effective protocol would be welcomed by the industry.

Investigation 3

Determination of the extent of genetic variation at the different microsatellite loci.

All recipes of solutions and buffers have been taken up in Appendix A.

2.2 MATERIALS

Cranes are not widely available and therefore, available birds of the three South African crane species were obtained from a limited number of crane breeders. DNA was isolated from venous blood using a sterile technique.

2.2.1 Selection of birds

Thirty-two cranes of the three South African crane species were obtained from zoological parks and aviculturalists around southern Africa. Although an attempt was made to include

only unrelated birds, it was later found that some of the birds in captivity were related making it impossible to include only birds that were unrelated. As the number of cranes available for this research was low, all birds, including those with uncertain pedigrees, were also included in the research. Bird supplier's information is summarised in Table 2.1.

Table 2.1 Sample information.

Sample number	Date of collection	Bird collector	Bird supplier	Province in South Africa	Supplier's identification
Blue crane					
B1	24/04/2002	Dr. C. Kingsley	W. Horsfield	KwaZulu-Natal	Ring No. 049
B2	26/04/2002	Dr. M. Penning	Umgeni Bird Park	KwaZulu-Natal	Ring No. 050
B3	26/04/2002	Dr. M. Penning	Umgeni Bird Park	KwaZulu-Natal	Ring No. 251
B4	26/04/2002	Dr. M. Penning	Umgeni Bird Park	KwaZulu-Natal	Ring No. 252
B5	26/04/2002	Dr. M. Penning	Umgeni Bird Park	KwaZulu-Natal	Ring No. 253
B6	26/04/2002	Dr. M. Penning	Umgeni Bird Park	KwaZulu-Natal	Ring No. 254
B7	26/04/2002	Dr. M. Penning	Umgeni Bird Park	KwaZulu-Natal	Ring No. 255
B8	26/04/2002	Dr. M. Penning	Umgeni Bird Park	KwaZulu-Natal	Ring No. 257
B9	26/04/2002	Dr. M. Penning	Umgeni Bird Park	KwaZulu-Natal	Ring No. 258
B10	13/05/2002	Dr. S. Smith	Tygerberg Zoo	Western Cape	Camp 56 (F)
B11	13/05/2002	Dr. S. Smith	Tygerberg Zoo	Western Cape	Camp 56 (F)
B12	13/05/2002	Dr. S. Smith	Tygerberg Zoo	Western Cape	Ring No. 039
B13	30/05/2002	Dr. S. Smith	Monte Casino	Gauteng	Ring No. 040
B14	30/05/2002	Dr. S. Smith	Monte Casino	Gauteng	Show Chick (2)
Crowned crane					
C1	24/04/2002	Dr. C. Kingsley	W. Horsfield	KwaZulu-Natal	T43506574B
C2	24/04/2002	Dr. C. Kingsley	W. Horsfield	KwaZulu-Natal	T434D057F
C3	24/04/2002	Dr. C. Kingsley	W. Horsfield	KwaZulu-Natal	T43562A035
C4	24/04/2002	Dr. C. Kingsley	W. Horsfield	KwaZulu-Natal	T43503B790
C5	26/04/2002	Prof. M. Perrin	Mitchelles Park	KwaZulu-Natal	T00002
C6	26/04/2002	Prof. M. Perrin	Mitchelles Park	KwaZulu-Natal	T00003
C7	26/04/2002	Prof. M. Perrin	Mitchelles Park	KwaZulu-Natal	T00004
C8	13/05/2004	Dr. S. Smith	Tygerberg Zoo	Western Cape	Camp No. 17 (A1)
C9	13/05/2004	Dr. S. Smith	Tygerberg Zoo	Western Cape	Camp No.17 (A2)
C10	13/05/2004	Dr. S. Smith	Tygerberg Zoo	Western Cape	Camp No.17 (C1)
Wattled crane					
W1	24/02/2002	Dr. C. Kingsley	W. Horsfield	KwaZulu-Natal	Stud No. 81
W2	24/04/2002	Dr. C. Kingsley	W. Horsfield	KwaZulu-Natal	Stud No. 83
W3	24/04/2002	Dr. C. Kingsley	W. Horsfield	KwaZulu-Natal	Stud No. 95
W4	24/04/2002	Dr. C. Kingsley	W. Horsfield	KwaZulu-Natal	Stud No. 96
W5	26/04/2002	Dr. M. Penning	Umgeni Bird Park	KwaZulu-Natal	Stud No. 82
W6	26/04/2002	Dr. M. Penning	Umgeni Bird Park	KwaZulu-Natal	Stud No. 89
W7	26/04/2002	Prof. M. Perrin	Mitchell's Park	KwaZulu-Natal	Stud No. 98
W8	26/04/2002	Prof. M. Perrin	Mitchell's Park	KwaZulu-Natal	Stud No. 113

2.2.2 DNA source

DNA was isolated from whole venous blood obtained from the birds using a sterile technique. The resident veterinarian collected blood from the brachial vein on the inside of the bird's wing as follows:

1. The wing was gently moved away from the body to expose the region containing the brachial vein.
2. An ethanol swab was used to clean the area under the wing.
3. The exposed vein was then pierced with a sterile surgical needle of a 1 ml syringe.
4. Once the syringe contained approximately 0.5 ml of venous blood, the blood was transferred into a vacutainer vial containing EDTA to prevent the coagulation of the blood.
5. The blood containing vial was then inverted a few times to ensure that blood coagulation did not occur.
6. Thereafter, the vial was closed tightly and appropriately labeled with the date of acquisition, the exclusive ring number or identification number of the respective bird, the species name, the collector's name and area location.
7. The vein was then swabbed with ethanol and pressure applied to the area to stop bleeding.
8. The vial containing the blood was then stored at $-20^{O}C$.

2.3 METHODS

2.3.1 DNA isolation

DNA was isolated from whole venous blood using the salting out protocol developed by Miller *et al.* (1988).

The protocol entailed four steps:

Step 1: Lysis of blood cells,

Step 2: Salting out of DNA,

Step 3: Ethanol precipitation of DNA, and

Step 4: Re-suspension of DNA in TE buffer.

The DNA isolation protocol entailed the following:

1. Various volumes of blood ranging from five to 25 µl of blood/EDTA samples were tested in order to find the most suitable volume of blood to use.
2. A vacutainer vial containing blood was removed from the refrigerator and left at room temperature for 30 minutes to thaw.
3. The blood was added to a 1.5 ml eppendorf tube containing 500 µl of TNE lysis buffer, 50 µl 1 M Tris-HCl (pH 8), 1 mg/ml proteinase K, 7.5 µl 25% sodium dodecyl sulfate (SDS) and 7.5 µl Triton X-100 in an eppendorf.
4. The eppendorf tube was then incubated overnight in a 37^{O}C water bath to allow lysis of the blood cells.
5. 300 µl of 5 M NaCl was added to the eppendorf containing the overnight mixture and thereafter the mixture was hand shaken for 15 seconds.
6. The eppendorf tube was then centrifuged in an Eppendorf Centrifuge S415 at 5 000 rpm for 15 minutes.
7. The supernatant containing the DNA was carefully removed using a wide bore tip and transferred to a fresh sterile eppendorf tube.
8. The eppendorf tube was then hand shaken for ten seconds and then centrifuged at 5 000 rpm for ten minutes.
9. The supernatant was removed once again and transferred to a fresh sterile eppendorf tube.
10. Steps 4-8 were repeated until the supernatant was clear of debris and free of whole blood lumps.
11. The DNA was then ethanol precipitated by adding two volumes of 100% ice-cold ethanol to the clear supernatant.
12 The eppendorf tube was then inverted several times to precipitate the DNA, thereafter the eppendorf tube was then placed in a –20 ^{O}C freezer for 45 minutes to an hour to facilitate DNA precipitation and yield.
13. Thereafter the eppendorf tube was inverted and centrifuged at 13 000 rpm for 15 minutes to precipitate the DNA into a pellet.
14. The supernatant was then removed and discarded.
15. The pellet was washed in 70% ethanol by placing the eppendorf tube on a shaker for ten minutes.
16. Lastly, the DNA containing solution was centrifuged at 13 000 rpm for a further ten minutes to pellet the DNA.
17. The washing steps 14 and 15 were repeated three times in order to remove all remaining salt.
18. The ethanol was gently poured off and the DNA pellet left to air dry for 30-60 minutes to remove any remaining ethanol.
19. The pellet was then re-suspended in approximately 50 µl of 10 mM Tris HCl (pH 8) depending on the size of the pellet.
20. This DNA containing solution was then left to fully re-suspend the DNA at 37^{O}C overnight.
21. Finally, the Tris-HCl containing DNA solution was stored in a –20 ^{O}C freezer until required.

In a number of instances the DNA yield was unsatisfactory due to the extended storage time of the whole blood; up to three months. Therefore, DNA isolation was performed up to six times in some instances until the desired concentration and purity was obtained.

2.3.2 DNA verification and quantification

Successful DNA isolation was verified using agarose gel electrophoresis. Isolated DNA (5 µl) mixed with loading dye (2 µl), was quantified by running 0.8% 1 X TAE agarose gel. The gel was prepared with 0.4 g agarose in 50 ml 1 X TAE and 1.25 µl ethidium bromide (20 mg/ml). A Roche molecular weight marker of size 10 (Roche Applied Science) was run alongside the isolated DNA to ensure that the desired product was isolated. The gel was left to run for 20 minutes at 100 volts and was thereafter viewed under ultraviolet light. The concentration of the DNA was estimated by comparing the thickness of the DNA band on the gel to that of a pencil thin band produced by 5 µl of DNA, which is equivalent to approximately 30 ng/µl.

DNA concentrations and purity were determined by spectrophotometric analyses using a Pharmacia Biotech GeneQuart ii RNA/DNA calculator. Readings were taken of a 100 times dilution of the resuspended DNA at wavelengths of 260 nm and 280 nm. The calculation of the concentration and purity of DNA was made by applying the following formulae (Sambrook *et al.*, 1989):

$$\text{Concentration } (\mu g/ml) = A_{260} \text{ X dilution factor X } 50$$

and

$$\text{Purity} = A_{260}/A_{280}$$

Concentrations and purities of all samples with a purity of 1.8 were included in this investigation, as this ratio indicated pure DNA.

2.3.3 Selection of microsatellite loci

Primer sets developed by Glenn and Jones (personal communication) for five microsatellite loci of the Whooping crane (*Grus Americana*) were used in this investigation. These loci were denoted G/J1-G/J5. All seven microsatellite loci published by Hasegawa *et al.* (2000) for the Red-Crowned crane (*Grus japonensis)* were also included in this investigation, denoted as H1-H7 (Table 2.2). The primer sets for all these loci were synthesized by the Molecular and Cellular Biology Sythethetic DNA Laboratory at the University of Cape Town using a Beckman Instruments Incorporated Oligo 1000M DNA synthesiser. Details of the primer sets are given in Table 2.2.

Table 2.2 Microsatellite primer sets selected.

Locus	Primer name	Primer sequence (5'-3')	Length (bp)	Orientation	Crane species	References
H1	8a	TCCGTCAAGCTTTTAGTCAT	20	F	Red-Crowned	Hasegawa *et al.* (2000)
	8b	TACAGTTAATGTGGGTGCAA	20	R	Red-Crowned	Hasegawa *et al.* (2000)
H2	11a	TGGGGTGCAGTTCAAATAAGCG	22	F	Red-Crowned	Hasegawa *et al.* (2000)
	11b	TCTGCATCCAAAAAGGACATGC	22	R	Red-Crowned	Hasegawa *et al.* (2000)
H3	13a	TCTGCATGCGTCCTGCCTCCAAGA	24	F	Red-Crowned	Hasegawa *et al.* (2000)
	13b	TGCCTTGCACAGGCAGGTGAAATG	24	R	Red-Crowned	Hasegawa *et al.* (2000)
H4	15a	TCTACCAGATATCATCAGAGCTTGC	25	F	Red-Crowned	Hasegawa *et al.* (2000)
	15b	TGCGAATGAACAGATGGCCCCAAGA	25	R	Red-Crowned	Hasegawa *et al.* (2000)
H5	34a	TGCTCAACATTCATCAGGATTTGGG	25	F	Red-Crowned	Hasegawa *et al.* (2000)
	34b	TCCCTCTGGTGTTGGCTGAAAATAC	25	R	Red-Crowned	Hasegawa *et al.* (2000)
H6	40a	TGGGAGAATCCTGCAAATTCTGCTA	25	F	Red-Crowned	Hasegawa *et al.* (2000)
	40b	TGAGGAATGAGCGATGCTTGTTTCA	25	R	Red-Crowned	Hasegawa *et al.* (2000)
H7	48a	TCCCGGCGACGTCCGAGTGCTGATG	25	F	Red-Crowned	Hasegawa *et al.* (2000)
	48b	TGCGAGGGACCCTCCACCGAGAAGC	25	R	Red-Crowned	Hasegawa *et al.* (2000)
G/J1	3a	CACATTGCCAGACTGTTGTAT	21	F	Whooping	Glenn and Jones(*)
	3b	ATCCCTGAAGCTAACAATAAACC	23	R	Whooping	Glenn and Jones(*)
G/J2	6a	CACCTTTTATTGCGTATGTATTTT	24	F	Whooping	Glenn and Jones(*)
	6b	GGATTATGTTTTGGTTTGTTTTT	23	R	Whooping	Glenn and Jones(*)
G/J3	7a	TAAAGGAGTGGCTGCTGCTGTG	22	F	Whooping	Glenn and Jones(*)
	7b	CTGAGGCTCTGCTGTGGGAAAC	22	R	Whooping	Glenn and Jones(*)
G/J4	9a	GAGTGGGAGGGGATAGGATGGATT	24	F	Whooping	Glenn and Jones(*)
	9b	AGCCTGACAGCAAGACCAAAGTAA	24	R	Whooping	Glenn and Jones(*)
G/J5	1c	CAGTATAAAAAACAAACAGGTGAGA	25	F	Whooping	Glenn and Jones(*)
	1d	TGAAAAAAGTACAGGAGAACATAG	24	R	Whooping	Glenn and Jones(*)

BP=base pairs; R=reverse; F=forward ; * represents personal communication; G/J=Glenn and Jones primer sets (Glenn and Jones, personal communication); H=Hasegawa primer sets (Hasegawa *et al.*, 2002).

2.3.4 Generation of microsatellite fingerprints

Microsatellite fingerprints were prepared for each individual of the three species. Prior to the generation of the microsatellite fingerprints, the optimal PCR conditions were firstly established.

A. Preparation of PCR reagents

The PCR Core Kit of Roche Diagnostics was utilized to amplify the respective microsatellite loci of the three species by preparing a master mixture of reagents contained in the PCR Core Kit. The kit contained deoxynucleoside triphosphates (dNTPs), magnesium chloride ($MgCl_2$), PCR buffer and DNA polymerase of *Thermus aquaticus* (Taq).

Two stock solutions were prepared, namely, a primer working stock solution and a DNA working stock solution.

Primer working stock solution

A working stock solution of each primer was prepared at a concentration of 100 μM. The volume of a particular primer working stock solution was determined by applying the following formulae (Sambrook *et al.*, 1989):

1. Primer Molecular Weight = Primer length (base pairs) X 330 daltons

2. Given: $C_1V_1 = C_2V_2$,
   Therefore final volume (V_2) = $\{C_1V_1\} / C_2$
   Where: C_1 = sample concentration in μM,
   V_1 = 10 μl (Volume of original primer solution),
   C_2 = 100 μM (Final primer concentration) and
   V_2 = Unknown (Volume of TE required to provide a 100 μM).
   While: (C_1) = $\{OD_{254\ nm}$ X 37 μg/ml X 1000$\}$ /Primer MW

Primer working stock solutions were thus prepared by (1) the determination of primer molecular weight, (2) the determination of C_1 using primer molecular weight formula (3) the determination of unknown in formula which represented the volume of TE buffer (V_2) required to be added to obtain the desired primer working stock solution, and lastly (4)

the subtraction of the original primer solution (V_1) from the calculated volume of TE buffer (V_2) as the V_2 volume was the final volume that would be added to obtain the desired primer concentration of the particular primer. The working stock solutions for the different primer sets of the H-loci were prepared according to Hasegawa *et al.* (2000) and those of the G/J –loci were recommended by Ms. King of the University of KwaZulu-Natal, who was working on a related project in the same laboratory and had previously devised the solutions (Table 2.3).

Table 2.3 Working stock solutions for the seven H-loci.

Primer number	Locus	Optical density	Concentration (C_1) µM	Volume (V_1) µl	Concentration (C_2) µM	Volume (V2) µl	Volume of TE buffer (µl)
8a	H1	574.6	3221.24	10	100	322.12	312.10
8b		586.9	3290.20	10	100	330.01	319.02
11a	H2	552.2	2814.24	10	100	281.42	271.00
11b		568.8	2898.84	10	100	289.88	280.00
13a	H3	576.0	2690.90	10	100	269.09	260.00
13b		622.3	2907.21	10	100	290.72	281.00
15a	H4	712.0	3193.21	10	100	319.32	309.00
15b		593.2	2660.41	10	100	266.04	256.00
34a	H5	761.4	3414.76	10	100	341.47	331.00
34b		661.5	2966.72	10	100	296.67	286.00
40a	H6	594.9	2668.03	10	100	266.80	256.00
40b		538.7	2415.98	10	100	241.59	231.00
48a	H7	550.9	2470.70	10	100	247.07	237.00
48b		582.2	2611.08	10	100	261.10	251.00

DNA working stock solution

Working stock solutions of template DNA containing a concentration of 100 ng/µl was prepared by applying the formula (Sambrook *et al.,* 1989):

Since $C_1V_1 = C_2V_2$

$(V_2) = \{C_1V_1\} / C_2$

Where: C_1 = sample concentration in ng/µl,

V_1 = 10 µl (Volume of original DNA solution),

C_2 = 100 ng/µl (Final DNA concentration) and

V_2 = Unknown (Volume of Tris-HCl required to provide 100 ng/µl).

The formula was used to determine the final volume of the Tris-HCl buffer required to provide the desired concentration of DNA working stock solution for each individual sample. In some instances the DNA concentration was too low to dilute and was therefore used undiluted. Table 2.4 provides a list of the various concentrations of the sample DNA.

Table 2.4 Working stock solutions of DNA (100 ng/µl).

Specimen	Concentration C_1 (ng/µl)	Volume V_1 (µl)	Concentration C_2 (ng/µl)	Volume V_2 (µl)	Volume (µl) of Tris HCl added
Blue crane					
B1	0.37	10	100	37.0	27.0
B2	0.47	10	100	46.5	36.5
B3	0.12	10	100	12.0	2.00
B4	0.21	10	100	20.5	10.5
B5	0.45	10	100	45.0	35.0
B6	0.40	10	100	40.0	30.0
B7	0.50	10	100	50.0	40.0
B8	0.10	10	100	10.0	0.00
B9	0.38	10	100	38.0	28.0
B10	0.20	10	100	19.5	9.50
B11	0.03	10	100	2.50	0.00
B12	0.20	10	100	20.0	10.0
B13	0.10	10	100	10.0	0.00
B14	0.03	10	100	3.00	0.00

Specimen	Concentration C_1 (ng/µl)	Volume V_1 (µl)	Concentration C_2 (ng/µl)	Volume V_2 (µl)	Volume (µl) of Tris HCl added
Crowned crane					
C1	0.15	10	100	15.0	5.00
C2	0.12	10	100	11.5	1.50
C3	0.24	10	100	24.0	14.0
C4	0.22	10	100	21.5	11.5
C5	0.08	10	100	7.50	0.00
C6	0.16	10	100	15.5	5.50
C7	0.15	10	100	14.5	4.50
C8	0.07	10	100	7.00	0.00
C9	0.12	10	100	12.0	2.00
C10	0.30	10	100	30.0	20.0
Wattled crane					
W1	0.650	10	100	65.0	55.0
W2	0.160	10	100	16.0	6.00
W3	0.170	10	100	17.0	7.00
W4	0.245	10	100	24.5	14.5
W5	0.190	10	100	19.0	9.00
W6	0.420	10	100	42.0	32.0
W7	0.165	10	100	16.5	6.50
W8	0.110	10	100	11.0	1.00

2.3.5 Optimization of microsatellite amplification conditions

Species cross-amplification of microsatellite loci often requires extensive optimization of the reagent concentrations and PCR conditions. Optimization was conducted by firstly, modifying reagent concentrations, followed by altering the annealing temperature of the primers, and lastly by changing the number of PCR cycles.

The reagent concentrations and PCR cycling conditions utilized for the amplification of the G/J-loci were employed as suggested by Heather King, whom had optimized the conditions earlier in the same laboratory (Table 2.5).

Table 2.5 Optimized reagent and PCR cycling conditions for the G/J-loci.

Reagents	Original Concentration	Required Concentration	Volume used (µl)
PCR reaction buffer	10 ×	10 ×	2.40
$MgCl_2$	25 mM	1.50 mM	1.62
DNTPs	10 mM	10.0 mM	1.60
Primer	100 µM	10 µM	1.68
Taq polymerase	5 U	1 U	0.10
Sterilized distilled water	–	–	12.6
DNA template	100 ng/µl	25 ng	5.00

PCR cycling conditions

93°C for two minutes, 35 cycles of: 30 seconds at 90°C, 30 seconds at an annealing temperature of 58°C, and 20 seconds at 72°C (Perkin Elmer GeneAmp PCR system 9700).

In the case of the H-loci, the published reagent concentrations and PCR cycling conditions were used to test the amplification of the H-loci on two individuals of each species (Table 2.6). The amplification products were then separated on a 0.8% agarose gel and assessed for suitability for fingerprinting analysis. The amplification products in most instances appeared to be unclear and unsuitable for subsequent analyses. After this initial amplification further optimization steps were undertaken.

Table 2.6 Composition of PCR reagents used in initial PCR, Hasegawa *et al.* (2000).

Reagents	Stock solution concentrations	Required Concentration	Volume used (µl)
PCR reaction buffer	10 ×	1 ×	2.00
$MgCl_2$	25 mM	1.50 mM	0.60
DNTPs	10 mM	0.20 mM	0.20
Primer	100 µM	0.25 µM	0.25
Taq polymerase	5 U	0.50 U	0.15
Sterilized distilled water	–	–	6.78
DNA template	100 ng/µl	30-300 ng (50ng)	1.25

PCR cycling conditions

93°C for two minutes, 30 cycles of: 30 seconds at 90°C, 20 seconds at an annealing temperature of 60°C, and 20 seconds at 72°C (Perkin Elmer GeneAmp PCR system 9700).

A. Optimization of reagent concentrations

For this investigation primers of the H-loci were required to anneal to the DNA of the other crane species, thus creating the need for extensive optimization of the reagent concentrations in the PCR reaction.

The optimization of the Hasegawa *et al.* (2000) reagents was undertaken by the modification of $MgCl_2$ concentrations ranged from 1.5 to 4.5 mM with 1 mM increments. In the case of the DNA template, concentrations ranged from 2 to 6 ng/μl with 1 ng/μl increments. Primer concentrations ranged from 0.1 μg to 0.7 μg of 0.25 μM of each primer, within 0.1 μg increments being tested. Firstly an initial PCR reagents composition was established for the Hasegawa *et al.* (2000) protocol. The initial amplification reactions were conducted on two individuals of each species and the amplification products separated by electrophoresis on a 0.8% agarose gel.

B. Optimization of PCR cycling conditions

The parameters set for the thermal cycling conditions also influence successful PCR amplification. It is therefore important to optimize annealing temperatures, as well as the number of cycles to obtain suitable and sufficient amplification products.

The thermal cycling procedure is performed by incubating the samples at three different temperatures that correspond to three different steps, namely, DNA denaturation, annealing of primers and elongation or extension of primers. The initial denaturation of the genomic DNA normally occurs at 95-100^{O}C. Thereafter, each cycle begins with a denaturation step at 92-95^{O}C. The particular primer annealing temperature is thus another vital component in the optimization of a PCR. Thus, a range of primer annealing temperatures was assessed for each primer pair, ranging from 50^{O}C to 60^{O}C, with an increment of 1^{O}C. For the elongation and extension of the primers a standard temperature of 72^{O} C was utilized (Newton and Graham, 1994).

The final number of cycles was optimized by modifying the annealing temperature of the different primer sets. Generally 25-35 cycles are enough to produce an adequate quantity of DNA. Reactions that utilize more than 40 cycles show an increase in unnecessary artifactual products and rarely increase the quantity of required product. Thus, in this investigation optimization was attempted using between 30 and 35 cycles.

2.3.6 Verification of amplification products

Agarose gels were prepared to verify the successful amplification of the different microsatellite loci. All microsatellite amplification products were run on a 2% agarose gel (1.0 g of agarose, 50 ml of 0.5 X TBE) at 80 volts for 30 minutes. The gel was stained with 0.0001 mg/ml of ethidium bromide, which enabled visualization and thus verification of the presence of the amplified product. Five microlitres of the amplification product together with one microlitre of loading buffer was loaded into each well of the gel. The Roche molecular weight marker was also run alongside these PCR products, to determine whether the desired PCR product had been amplified. The gel was visualized under ultra violet light.

2.3.7 Generation of microsatellite fingerprints

Microsatellite fingerprints were generated by separating the amplification products of each locus for each individual on a 20% polyacrylamide gel. The polyacrylamide gel was prepared by dissolving all the reagents, as listed in Table 2.7, while continually stirring with a glass rod to avoid the formation of bubbles. This mixture was then poured into a 20 mm X 20 mm gel cast. The gel was then allowed to set for 45–60 minutes. Twenty microlitres of the PCR products that remained after verification, was loaded into a gel well together with 4 μl of loading buffer. The gel was then run at 60 volts for two hours, or until the products had separated sufficiently. The gel was then stained by immersion in 2.5 microlitres of ethidium bromide in 100 ml of distilled water solution, covered and left on a shaker for 30 minutes. The gel was then immersed in distilled water to stop the staining process and thereafter visualized by ultra violet light illumination.

Table 2.7 Composition of reagents used to make polyacrylamide gels.

Reagents	Volume (ml)
30% Acrylamide	6.66
Distilled water	1.27
5 X TBE	2.00
10% Ammonium persulfate	0.07
*TEMED	3.50

*Added just before use.

2.3.8 Analysis of microsatellite fingerprints

The microsatellite fingerprints that were generated for the three South African crane species in this investigation were analysed in terms of the following:

- The description of each individual's genotype to ascertain whether each individual's fingerprint was unique,
- The determination of the extent of genetic variation within each species, and
- The comparison of genetic compositions and variation of the three species.

Four different software packages were employed to estimate allele sizes, analyse molecular genetic data and to construct dendrograms:

- UVIgelstartMW (version 11.01) for windows (1999-2003), a proprietary image analysis software package was used to determine the sizes of the microsatellite amplification products.

- The Windows word processor Notepad was used to record each individual's genotype. This document was used for all subsequent analyses in POPGENE (Version 1.30) by Yeh (1999).

- POPGENE (Version 1.30) by Yeh (1999) is a software package used to calculate genetic variation between and within populations and species using dominant and co-dominant markers. Most types of population genetic measures, such as allele and genotypic frequencies, diversity indices, neutrality tests and genetic distances could be calculated using POPGENE. POPGENE, a free software package, is obtainable from the website www.ualberta.ca/~fyeh/.

- The Treeview programme was employed to view the dendrograms generated by POPGENE.

The microsatellite fingerprints were analysed according to the following steps:

Step 1: The different alleles (sizes) at each locus were identified using UVIgelstartMW (version 11.01).

Step 2: Digital fingerprints (phenotypes) of each individual were constructed by converting microsatellite gel fingerprints into digital fingerprints using Notepad-Microsoft Windows internal processor.

Step 3: POPGENE was employed to calculate different measures of genetic variation between individuals within each species.

Step 4: POPGENE was then used to estimate the genetic distances between the three different species by calculating Nei's (1987) Unbiased Measures of Genetic Identity (I) and Genetic distance (D).

Step 5: Dendrograms displaying within species relationships and between species relationship were constructed based upon Nei's (1978) Genetic distance using unweighted pair group method using arithmetic average means (UPGMA) of POPGENE. The Treeview programme was then employed to view the dendrograms generated by POPGENE.

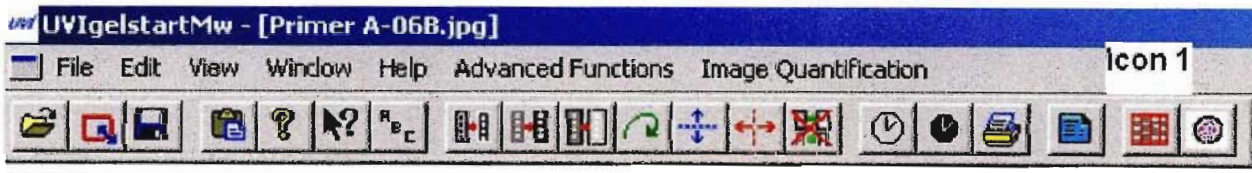
Step 6: The data were interpreted.

A. Determination of allele size

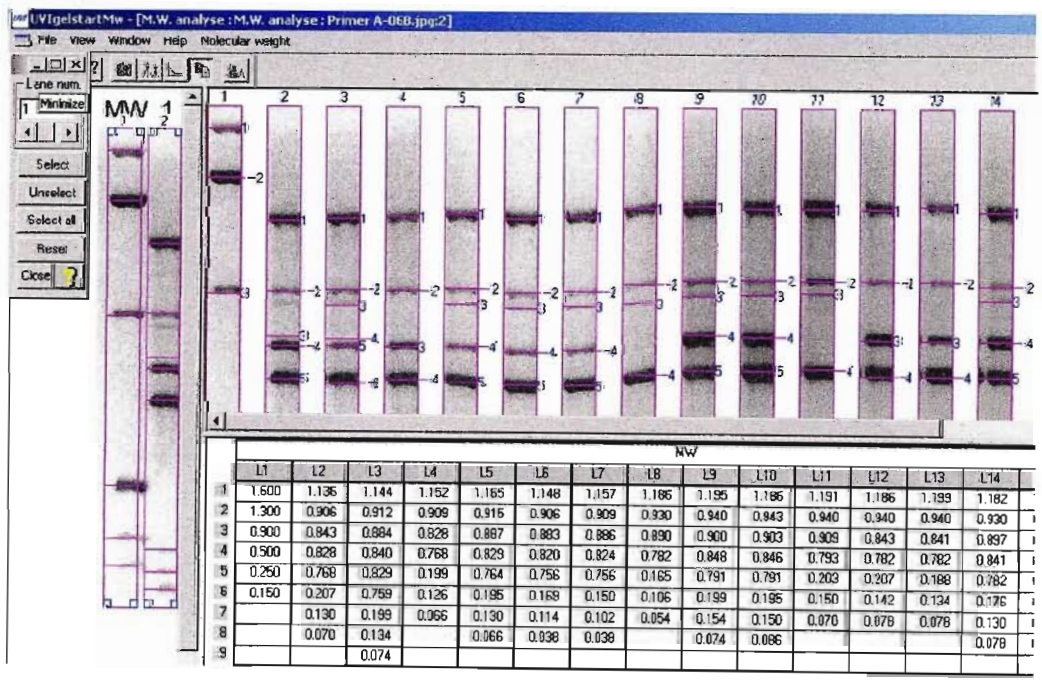
The different alleles of microsatellite loci differ in the number of repeats. Thus, different alleles of a particular locus produce amplification products of different sizes. The

software programme UVIgelstartMW version 11.01 for windows (1999-2003) was used to determine the fragment sizes of the twelve microsatellite loci. The programme permitted the calculation of electrophoretic distance of band fragments of each primer pair according to the molecular weight marker that was run alongside the amplification products on the particular gel. This was achieved by first loading the electronic gel image into the UVIgelstartMW programme and then displaying it on the video screen. The wells of the image were placed on the working window with the lanes running vertically down the screen. Icon one on Figure 2.1(a) was then used to construct vertical lines separating the lanes to the wells. A window was then drawn around the area of interest and thereafter each of the gel lanes was separated. The reference of the molecular weight sizes was then recorded and the fragment sizes of the bands detected as in Figure 2.1(b).

Figure 2.1 **(a)** Representation of a window depicting options available by UVIgelstartMW programme.



(b) Representation of an output window of fragment sizes after image analyses.



B. Construction of individual genotypes

The molecular fingerprints were converted into digital fingerprints by assigning alphabetic letters to the alleles of each microsatellite locus for each individual and recorded in the word processor of Windows, Notepad. These digital fingerprints were regarded as a representation of an individual's genotype (Figure 2.2). A single band on a gel represented a homozygous genotype and was identified by a single letter of the alphabetic, for example AA or BB. An individual that was heterozygous was represented by two different letters of the alphabet, for example AB. This Notepad file, when saved was a *.txt* file, which was compatible with the software package utilized in subsequent analyses.

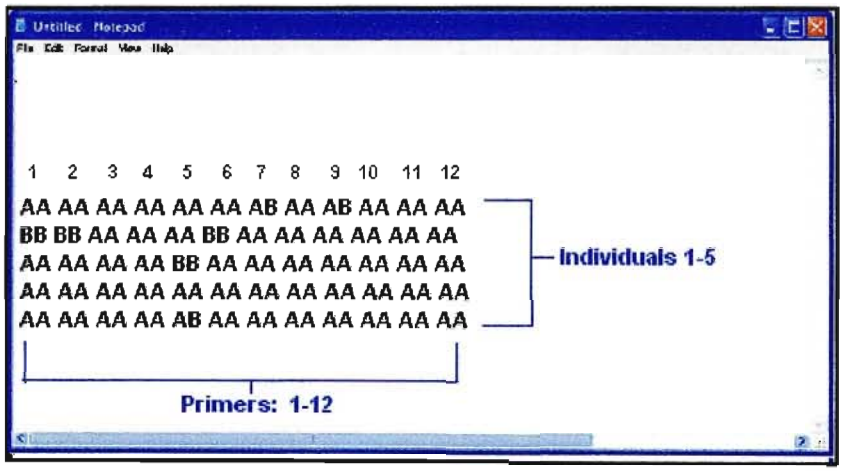


Figure 2.2 Representation of digital genotypes in Notepad of five Blue crane individuals.

C. Analysis of genetic variation and distance

A number of different measures of genetic variation and distance were determined using the software programme POPGENE (Version 1.30) by Yeh (1999). The text file *.txt* of the genotypic data recorded in Notepad was opened in POPGENE and the respective analyses were requested and performed.

Determination of allelic variation

A number of measures of allelic variation were determined. Firstly, polymorphic (P) microsatellite loci were identified; loci with two or more alleles in the population (crane species). Loci containing a single allele in the population (species) was deemed monomorphic (M) microsatellite loci. The proportion of polymorphic loci was then calculated by, counting the number of polymorphic loci and then by dividing by the total number of loci examined as below (Hartl and Clark, 1997):

$$P = \frac{\text{Number of polymorphic loci}}{\text{Total number of loci scored}}$$

The allele frequencies for each locus within each of the three species were also calculated using the formula below (Hartl and Clark, 1997):

$$\text{Allele frequency} = \frac{\text{Number of observed alleles}}{\text{Total number of alleles scored}}$$

Allelic variation was further determined using the Shannon's information index (Lewontin, 1972). The Shannon's information index (I), is a measure of allelic variation utilizing the observed number of alleles (n_a) and effective number of alleles (n_e). The effective number of alleles are the number of equally frequent alleles that would produce the same heterozygosity. Shannon's diversity index is scaled from a value of zero to one, where a value of zero equals the minimum diversity attainable and a value of one equals the maximum diversity attainable. Shannon's index can be calculated using the following formula (Lange, 2002):

$$I = -\sum {}^{*}P_i \log_2 {}^{*}P_i$$

Where ${}^{*}P_i$ is the frequency of the presence or absence of alleles.

Determination of genotypic variation

Genotypic variation was assessed in terms of the observed and expected amount of heterozygosity residing within each of the three species. The observed heterozygosity was calculated by counting the number of heterozygotes in the population (species) and then dividing by the total number of individuals. The expected heterozygosity, under Hardy Weinberg equilibrium conditions (HWE), was calculated by first determining the frequency of heterozygotes at each locus and then averaging these frequencies over all loci (Levene, 1949; Nei 1978). Nei's (1978) expected heterozygosity at any one locus was determined using the formula below:

$$H_j = 1 - \Sigma q_k^2$$

Where H_j represents heterozygosity estimated at locus j and q_k is the frequency of the k^{th} allele at the locus.

Levene's (1949) heterozygosity was calculated using the formula below:

$$H = p\,(1-p)\,[1+1/2n-1)]$$

Where p is the frequency of allele i and n is the number of individuals in the sample.

The test of Hardy-Weinberg equilibrium was performed for each locus of each species to determine if any possible evolutionary forces that impact on allelic frequencies were present.

The Wright's (1978) inbreeding coefficient (F_{IS}) indicated whether there is reduction in heterozygosity within a population (species) due to inbreeding and was calculated using the formula below:

$$F_{is}=H_s-H_i/H_s$$

H_s represents expected heterozygosity

H_i represents observed heterozygosity

Genetic distance and similarity

Nei's original (1972) and unbiased (1978) method of measurement of genetic identity (I) and genetic distance (D) were used to determined the genetic similarity between individuals within each of the species and between the three crane species. Nei's two measures were calculated according to the formulae (Nei, 1972) below:

$$I = J_{xy} / \sqrt{(J_x J_y)}$$

where J_{xy}, J_x and J_y represent the means across all loci of $\Sigma x_i y_i$, Σx_i^2 and Σy_i^2 respectively when x_i and y_i are the frequencies of the *i*th alleles in populations X and Y.

and

$$D = -\ln (I)$$

D. Construction of dendograms

Genetic identity and distance measures were used to construct dendrograms depicting the extent of the relatedness between individuals within crane species, as well as between crane species. The dendrogram was constructed in POPGENE using UPGMA, which is an adaption of the NEIGHBOR and PHYLIP version 3.5c by Joe Felsenstein (1993). The TREEVIEW programme was then employed to view the dendrograms generated by POPGENE.

CHAPTER THREE
RESULTS

3.1 INTRODUCTION

This investigation was undertaken to assess whether microsatellite primers developed for crane species related to the three southern African crane species could be used in individual identification and the determination of genetic variation within and between populations. The primer sets developed for these related species were assessed for their ability to cross anneal and amplify heterologous microsatellite loci in the three southern African crane species; the Blue crane (*Anthropoides paradisea*), the Crowned crane (*Balearica regulorum*), and the Wattled crane (*Bugeranus carunculatus*). The resulting fingerprints were assessed for their ability to uniquely differentiate between individuals of the same species. These data were then further analyzed to provide information about genetic variation within each of the three species and genetic distance between the three species.

The results of this investigation are presented as follows:

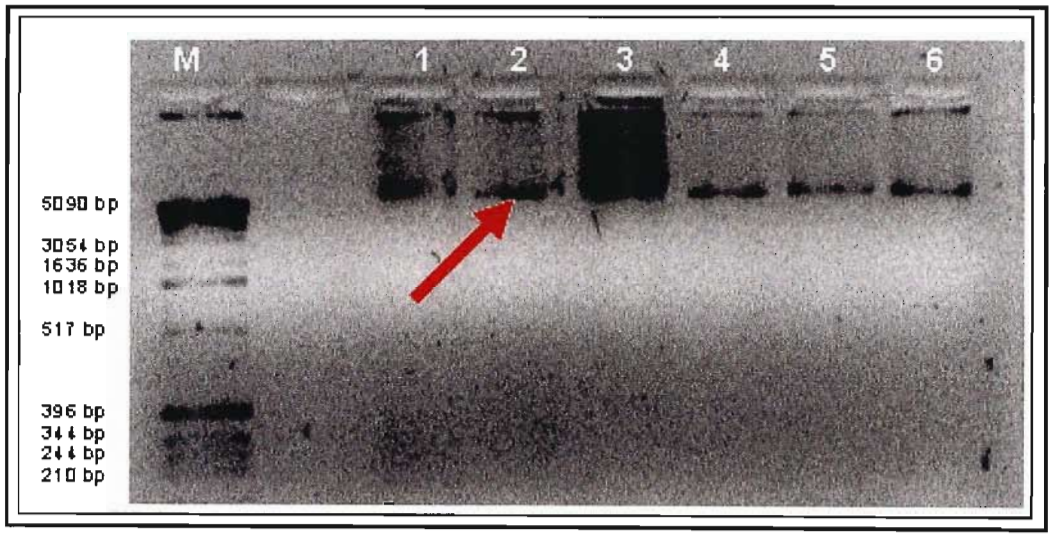
- DNA isolation,
- Optimization of microsatellite amplification,
- Assessment of microsatellite fingerprints:
    - determination of polymorphic and monomorphic loci,
    - determination of alleles,
- Analysis of genetic variation within species, and
- Determination of genetic distance between species.

Computer print-outs of all statistical analyses have been taken up in Appendix B.

3.2 DNA ISOLATION

DNA was successfully isolated from venous blood with the salting out method developed by Miller *et al.* (1988). High molecular weight DNA in an isolate was confirmed by running a 0.8% agarose gel containing the Roche molecular weight marker of size 10 (Roche Applied Science) for 20 minutes at 100 volts and viewing under ultraviolet light. High molecular weight DNA presented a single, well-defined band on the gel (Figure 3.1).

Figure 3.1 Gel electrophoresis verification of high molecular weight DNA in DNA isolates.



Lane M: Molecular weight marker 10.
Lanes 1- 6: DNA samples.

The concentration and quality of the isolated DNA was determined through spectrophotometric analysis. DNA from most samples was of acceptable concentration and quality (Table 3.1). The DNA concentration of the different samples ranged from 25 µg/µl to 650 µg/µl. In instances where the DNA concentration was less than 100 µg/µl, which could be attributed to extended storage, additional isolations were performed until the required concentrations were obtained. The purity of all samples fell within the range of acceptable purity of 1.7 to 1.9.

Table 3.1 Concentration and purity of DNA samples.

Individuals	Absorption A260	Absorption A280	Concentration (µg/µl)	Purity (260 nm/280 nm)
Blue crane				
B1	0.076	0.042	370	1.81
B2	0.091	0.052	465	1.75
B3	0.022	0.013	120	1.70
B4	0.041	0.023	205	1.78
B5	0.091	0.050	450	1.81
B6	0.081	0.045	400	1.8
B7	0.099	0.055	500	1.79
B8	0.019	0.011	100	1.77
B9	0.076	0.042	380	1.81
B10	0.039	0.022	195	1.77
B11	0.005	0.003	25	1.66
B12	0.037	0.022	200	1.69
B13	0.019	0.011	100	1.75
B14	0.005	0.003	30	1.81
Crowned crane				
C1	0.030	0.017	150	1.76
C2	0.023	0.013	115	1.70
C3	0.048	0.026	240	1.84
C4	0.043	0.024	215	1.79
C5	0.016	0.009	75	1.77
C6	0.031	0.017	155	1.82
C7	0.029	0.016	145	1.81
C8	0.014	0.008	70	1.75
C9	0.024	0.013	120	1.84
C10	0.057	0.013	300	1.73
Wattled crane				
W1	0.130	0.073	650	1.78
W2	0.032	0.018	160	1.77
W3	0.034	0.019	170	1.79
W4	0.049	0.028	245	1.75
W5	0.038	0.021	190	1.81
W6	0.084	0.048	420	1.78
W7	0.033	0.018	165	1.83
W8	0.022	0.012	110	1.83

3.3 OPTIMIZATION OF MICROSATELLITE LOCUS AMPLIFICATION

Microsatellite fingerprints were generated for the thirty-two crane individuals using primer pairs of the twelve selected microsatellite loci. As these microsatellite loci were developed for related species, their accompanying primers were not completely homologous with the DNA of the three southern African species. Therefore, to obtain successful microsatellite amplification, optimal reagent concentrations and PCR cycling conditions are required. The reagent concentrations and the PCR conditions that were used to amplify the G/J-loci were applied as suggested by Ms. King (personal communication) while optimization was required for the H-loci to facilitate cross hybrization of the primers of these loci. Optimization of the conditions for the H-loci was achieved by the modification of the published reagent concentration and PCR conditions (Hasegawa *et al.* 2000) (Table 3.2).

Table 3.2 Optimized PCR reagent concentrations for the H-loci.

Reagents	Proposed Hasegawa concentration	Optimized concentration
PCR reaction buffer	1X	1X
$MgCl_2$	1.50 mM	2.50 mM
DNTPs	0.20 mM	0.20 mM
Primer	0.25 µM	0.25 µM
Taq polymerase	0.50 U	1.00 U
Sterilized distilled water	–	–
DNA template	50 ng	25 ng

The optimized PCR cycling conditions involved the modification of the primer annealing temperature and the changing of the number of PCR cycles (Figure 3.2).

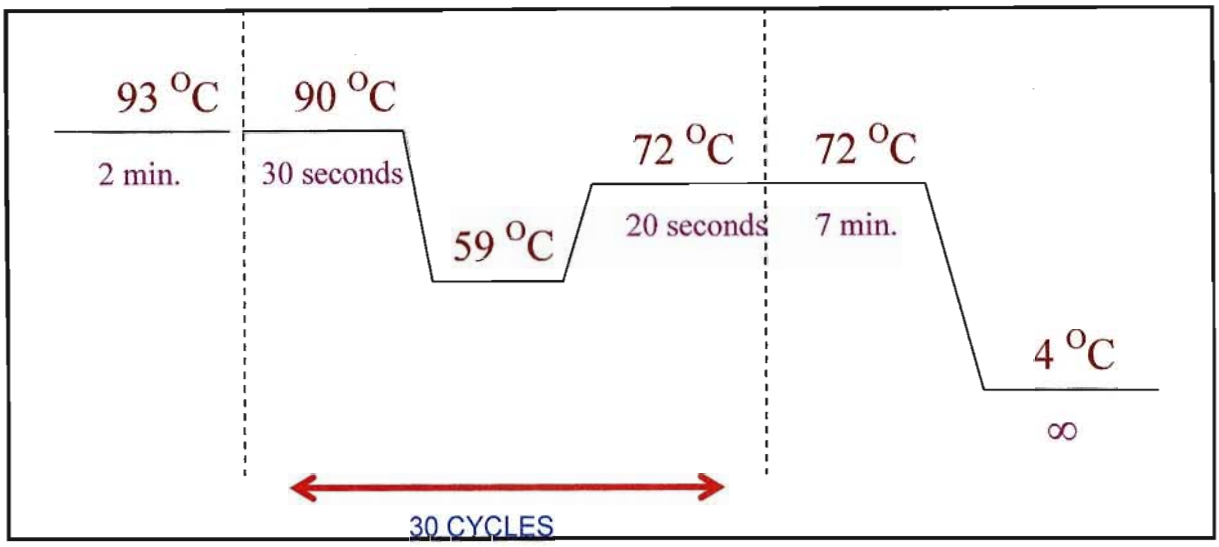


Figure 3.2 Representation of the cycling conditions utilized in the amplification of H- loci.

The optimized amplification conditions successfully amplified all 12 microsatellite loci in all three species (Figure 3.3). However, the amplification products generated from the G/J- loci were not always clear and distinct. This was also found by Ms. King (personal communication), whom had attempted extensive optimization, of these primers. The problem could be attributed to DNA homology differences between the related southern African crane species.

Figure 3.3 Agarose gel of successful amplification of one locus for nine individuals.

Lane M: Molecular weight marker 10.
Lanes 1- 9: Amplification product.
Lane 5 and 6: Two alleles at a polymorphic locus

3.4 ASSESSMENT OF MICROSATELLITE FINGERPRINTS

Fingerprints were assessed in terms of genetic variation within a locus; whether a locus was polymorphic or monomorphic. In the case of polymorphic loci the different alleles were identified in each of the three species.

3.4.1 Determination of polymorphic loci

Discrimination between individuals through molecular fingerprinting requires genetic variation at the loci involved in the fingerprinting procedure. In the microsatellite fingerprinting of the individuals of the three crane species, polymorphic loci were identified by establishing which of the loci generated bands of different sizes. These bands equate to different alleles at a particular locus, each differing in the number of repeats, thus producing amplification products of different sizes. In contrast, when only a single band could be identified in all individuals of a species, the locus was termed monomorphic. Of the twelve loci investigated, only five were polymorphic in all three species (Table 3.3). All other loci were monomorphic in one or two of the species. None of the loci were monomorphic in all three species. The percentage of polymorphic loci differed greatly amongst the three species, with the Crowned crane showing only 50% polymorphic loci, followed by the Blue crane with 84% and the Wattled crane with 92%.

Table 3.3 Polymorphic and monomorphic loci in the three crane species.

Microsatellite locus	Polymorphic and monomorphic loci in crane			Polymorphic in all species
	Blue	Crowned	Wattled	
H1	P	P	P	YES
H2	P	M	P	NO
H3	P	M	P	NO
H4	M	P	P	NO
H5	P	P	P	YES
H6	P	M	P	NO
H7	P	M	M	NO
G/J1	M	M	P	NO
G/J1	P	P	P	YES
G/J3	P	M	P	NO
G/J4	P	P	P	YES
G/J5	P	P	P	YES
Frequency monomorphic loci	0.16	0.50	0.08	
Frequency polymorphic loci	0.84	0.50	0.92	

H=Hasegawa microsatellite loci; G/J=Glenn and Jones microsatellite loci;
P=polymorphic locus; M=monomorphic locus.

3.4.2 Determination of alleles

After generating fingerprints of all microsatellite loci for all individuals, the different alleles at each locus were determined. The molecular fingerprints were captured as electronic fingerprints and opened in the UVIgelstartMW version 11.01 for windows (1999-2003) software package to determine the allele sizes. The sizes of the molecular weight marker's fragments were used as references from which the sizes of the fragments of the fingerprints could be determined. The different alleles of all loci in all species were within a narrow size range, approximately 5 bp. It was therefore difficult to

ascertain the number of alleles at a particular locus. With reference to the sizes of the fragments of lane five and six of Figure 3.3, only two different alleles for each polymorphic locus could be observed. These data thus revealed that using non-automated technology was not suited to assess fingerprints using these twelve microsatellite loci in these cranes. However, polymorphic loci were awarded two arbitrary alleles making it possible to estimate genetic variation within the species and to compare genetic distance between the species.

These data further revealed that the allele sizes of all the H-loci were distinctly larger than that of the expected sizes found for the Red-Crowned crane by Hasegawa *et al.* (2000) (Table 3.4). In the case of the G/J-loci, allele size comparisons were not possible as the range of the allele sizes for the Whooping crane for which the primers were developed had not been published at the time of writing this dissertation. It was interesting to note that the alleles of the different loci displayed similar size ranges in the three crane species.

Table 3.4 Allele sizes published and determined, for all twelve loci for three crane species.

Microsatellite locus	Published allele size	Determined allele size (±5 bp)
H1	108	210
H2	186	800
H3	158	396
H4	100	300
H5	149	340
H6	116	220
H7	132	235
G/J1	-	200
G/J2	-	120
G/J3	-	120
G/J4	-	180
G/J5	-	140

-not published

3.5 GENETIC ANALYSIS OF THE THREE CRANE SPECIES

3.5.1 Introduction

There are a number of different methods to quantify genetic variation within and between a species using microsatellite loci. In this investigation the quantification of genetic variation firstly required the description of the genotypes within each species, thereafter, genetic variation within species was calculated in terms of allelic variation, heterozygosity according to Nei (1973) and Levene's (1949); Shannon's information index (Lewontin, 1972) and Wright's (1978) inbreeding coefficient (F_{IS}). Finally Nei's (1978) genetic identity and genetic distance between species were estimated.

3.5.2 Genetic analysis of the Blue crane

A. Genotypes

The two arbitrary alleles at each of the polymorphic loci were denoted A and B and used to describe the genotypic composition of each locus for each individual. Loci were either recorded as AA or BB for the homozygous condition, or AB for the heterozygous condition (Table 3.5). The choice of these symbols was in compliance with the software used in the subsequent genetic analyses. All H-loci in this population of Blue crane were polymorphic, while two of the five G/J-loci were polymorphic and three monomorphic. The H4-locus, although polymorphic, was homozygotic in all individuals, either AA or BB. No heterozygous individuals for the H4-locus occurred in this population. The heterozygosity within individuals between loci ranged from 0 to 33%, while heterozygosity within locus between individuals ranged from 0 to 29%.

Table 3.5 Genotypes of all individuals of the Blue crane sample population.

Individual	Microsatellite locus												Frequency heterozygous loci within individuals
	H1	H2	H3	H4	H5	H6	H7	G/J1	G/J2	G/J3	G/J4	G/J5	
B1	AA	AA	AA	AA	AA	AA	AB	AA	AB	AA	AA	AA	0.16
B2	BB	BB	AA	AA	AA	BB	AA	AA	AA	AA	AA	AA	0.00
B3	AA	AA	AA	AA	BB	AA	AA	AA	AA	AA	AA	AA	0.00
B4	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	0.00
B5	AA	AA	AA	AA	AB	AA	AA	AA	AA	AA	AA	AA	0.08
B6	BB	AA	AA	AA	BB	AA	AA	AA	AB	AA	AA	AA	0.08
B7	AA	AA	AA	AA	AB	AA	AA	AA	AA	AA	AA	AA	0.08
B8	BB	BB	AA	AA	BB	AA	AA	AA	BB	AA	AA	AA	0.00
B9	AB	AB	AB	AA	AB	AA	AA	AA	AA	AA	AA	AA	0.33
B10	AB	AB	AB	AA	AB	AA	AA	AA	AA	AA	AA	AA	0.33
B11	AA	AA	AA	AA	AB	AA	AA	AA	AB	AA	AA	AA	0.16
B12	AB	AA	AA	AA	BB	AA	AA	AA	AB	AB	AA	AA	0.25
B13	AB	BB	BB	BB	AA	AB	AA	AA	BB	AA	AA	AA	0.16
B14	AA	BB	BB	BB	AA	AA	AA	AA	BB	AA	AA	AA	0.00
Frequency of heterozygous loci between individuals	0.29	0.14	0.14	0.00	0.21	0.07	0.07	0.00	0.29	0.07	0.00	0.00	

B. Allelic variation

The frequency of the alleles was determined for each of the loci in the Blue crane sample population (Table 3.6). It was found that the frequency of the most prevalent allele at each of the polymorphic loci ranged from 54% (H5-locus) to 96% (H7-locus and G/J3-locus).

Table 3.6 Allele frequencies of the twelve loci of the Blue crane.

Allele	Microsatellite locus											
	H1	H2	H3	H4	H5	H6	H7	*G/J1	G/J2	G/J3	*G/J4	*G/J5
A	0.64	0.64	0.78	0.86	0.54	0.90	0.96	1.00	0.64	0.96	1.00	1.00
B	0.36	0.36	0.22	0.14	0.46	0.10	0.04	0.00	0.36	0.04	0.00	0.00

*Monomorphic loci

Shannon's information index (I) (Lewontin, 1972), a measure of allelic variation, was estimated utilizing the observed number of alleles (n_a) and effective number of alleles (n_e). Shannon's information index for the polymorphic loci ranged from approximately 15 to 69% (Table 3.7).

Table 3.7 Shannon's information index for the Blue crane.

Locus	*n_a	*n_e	Shannon's information index (I)
H1	2.0000	1.8491	0.6518
H2	2.0000	1.8491	0.6518
H3	2.0000	1.5077	0.5196
H4	2.0000	1.3243	0.4101
H5	2.0000	1.9898	0.6906
H6	2.0000	1.2366	0.3405
H7	2.0000	1.0740	0.1541
G/J1	1.0000	1.0000	0.0000
G/J2	2.0000	1.8910	0.6518
G/J3	2.0000	1.0740	0.1541
G/J4	1.0000	1.0000	0.0000
G/J5	1.0000	1.0000	0.0000
Mean	1.7500	1.3961	0.3520
Standard Deviation	0.4523	0.3919	0.2806

*n_a=observed number of alleles;n_e=effective number of alleles.

Shannon's information index (Lewontin, 1972) was the lowest in the H7-locus and G/J3-locus (15.41%), which reflects the abundance of the prevalent allele (96%) and the sparseness of the rarer allele (4%). In the case of the H5-locus, the higher value of the Shannon's information index is explained by the relatively higher frequency of the two alleles at this locus.

C. Genotypic variation

Genotypic variation was assessed by computing a number of different statistics. The observed heterozygosity (H_o) and expected heterozygosity (H_e), (Levene, 1949; Nei, 1972) were determined for each locus of the Blue crane sample population (Table 3.8). The mean observed heterozygosity for this sample population was approximately 12% which is about 50% that of the expected heterozygosities. Four of the loci were completely homozygous, while the observed homozygosity in the remaining loci ranged from 64 to 100%. The observed heterozygosity, on the other hand, ranged from 7 to 36%. The unbiased estimates of Levene (1949) and Nei (1972) of heterozygosity were determined and found to be similar. However, to quantify the differences between these two estimates, their differences from the observed heterozygosity values were determined. Two of the Levene estimates of heterozygosity revealed the same value as the observed value of heterozygosity, while the deviation for the other loci ranged between 13 and 33%. In contrast, all estimates of heterozygosity according to Nei (1972) differed from the observed heterozygosity values, ranging from -0.02 to 32%.

Table 3.8 Observed heterozygosity and homozygosity, Levene's expected homozygosity and heterozygosity and Nei's heterozygosity for the Blue crane.

Locus	Observed homozygosity	Observed heterozygosity	Levene's expected heterozygosity	H_e-H_o(Levene)	Nei's expected heterozygosity	H_o-H_e(Nei)
H1	0.7143	0.2857	0.4762	0.1905	0.4592	0.1435
H2	0.8571	0.1429	0.4762	0.3333	0.4592	0.3163
H3	0.8571	0.1429	0.3492	0.2063	0.3367	0.1938
H4	1.0000	0.0000	0.2540	0.2540	0.2449	0.2449
H5	0.6429	0.3571	0.5159	0.1588	0.4974	0.1329
H6	0.9286	0.0714	0.1984	0.1270	0.1913	0.1199
H7	0.9286	0.0714	0.0714	0.0000	0.0689	-0.0025
G/J1	1.0000	0.0000	-	-	-	-
G/J2	0.7143	0.2857	0.4762	0.1905	0.4592	0.1735
G/J3	0.9286	0.0714	0.0714	0.0000	0.0689	-0.0025
G/J4	1.0000	0.0000	-	-	-	-
G/J5	1.0000	0.0000	-	-	-	-
Mean	0.8810	0.1190	0.2407		0.2321	
Standard Deviation	0.1268	0.1268	0.2104		0.2029	

Wright's (1978) inbreeding coefficient (F_{IS}), as a measure of heterozygosity deficiency, was calculated to estimate the presence of a possible small population effect (captive breeding effect). Inbreeding was indicated by a positive F_{IS} because there is an increase in homozygosity, while a negative F_{IS} is indicative of a lack of inbreeding. This calculation compares the observed heterozygote frequency (H_o) with the expected heterozygote frequency (H_e) under random mating. The results revealed that most of the loci presented as heterozygous deficient in the population, except for locus H4. On the other hand, locus G/J3 was the only locus that displayed heterozygosity excess (Table 3.9).

Table 3.9 Wright's inbreeding coefficient (F_{IS}) for the Blue crane.

Locus	F_{IS}
H1	0.3800
H2	0.6900
H3	0.5800
H4	1.0000
H5	0.2800
H6	0.6300
H7	0.0400
G/J2	0.3800
G/J3	-0.0400

A test for the Hardy-Weinberg equilibrium (HWE) was conducted for each locus to identify the presence of possible evolutionary forces (Table 3.10). Of the nine polymorphic loci tested, five loci were in HWE. The Chi-square test on the remaining four loci proved to be highly significant for three of the four loci, and significant for the fourth. This significant deviation from the Hardy-Weinberg equilibrium could be attributed to the small population size, which causes a random change in genotypic frequencies, particularly if the population is very small. It also could be attributed to inbreeding which causes an increase in homozygosity within the species.

Table 3.10 Chi-squared test estimates for Hardy-Weinberg equilibrium assessed for the twelve loci of Blue crane species.

Locus number	Chi-square value	Probability	Significance	HWE
H1	2.4471	0.11	NS	Yes
H2	7.4941	0.00	**	No
H3	5.7065	0.01	*	No
H4	18.0870	0.00	**	No
H5	1.4285	0.23	NS	Yes
H6	8.3200	0.00	**	No
H7	0.0000	1.00	NS	Yes
G/J2	2.4471	0.11	NS	Yes
G/J3	0.0000	1.00	NS	Yes

Significant at p=0.05; ** Highly significant at p=0.01; NS=Non-significant.

D. Distance measures

In an attempt to quantify genetic differences between the different Blue cranes Nei's (1978) genetic identity and genetic distance were calculated using the statistical software programme, POPGENE Version 1.32 (Yeh 1999). From the values presented as distance matrices, dendrograms were constructed depicting the relationships between individuals of the sample population.

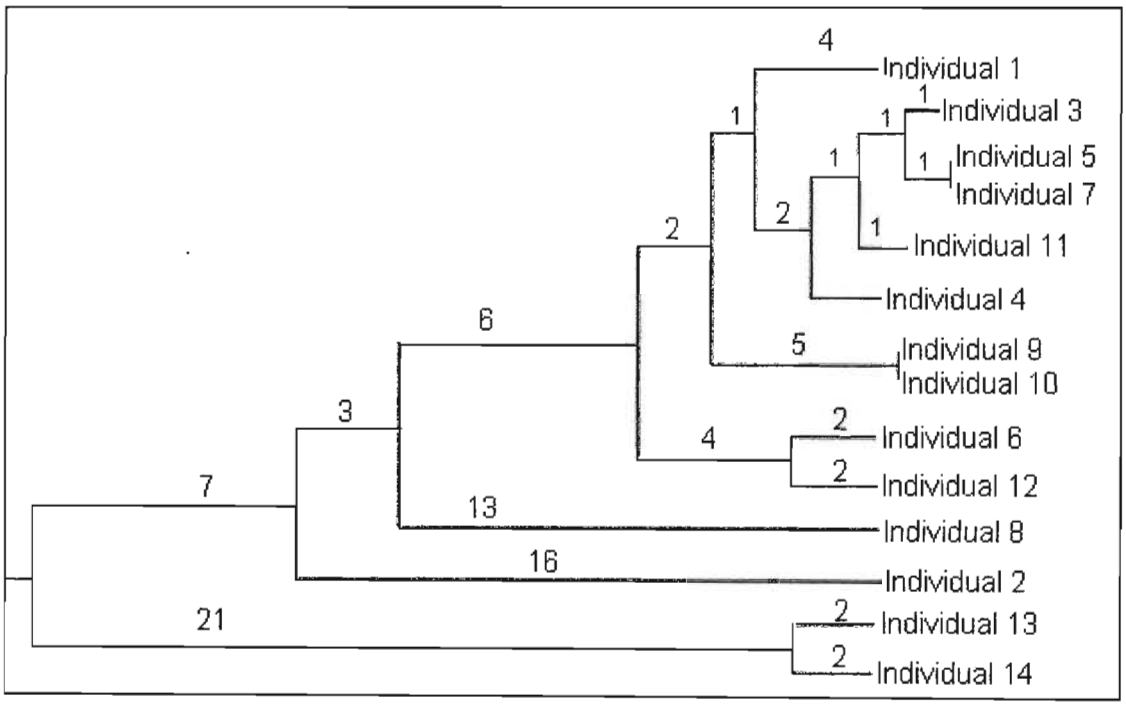
Similarity, identity (I), between Blue crane individuals ranged from approximately 66 to 100%, whereas genetic distance (D) ranged from 0 to 64% (Table 3.11).

Table 3.11 Nei's genetic identity (top diagonal) and genetic distance (bottom) diagonal) of the Blue crane sample population.

Individual	1	2	3	4	5	6	7	8	9	10	11	12	13	1
1	****	0.6963	0.8704	0.9574	0.9336	0.8002	0.9336	0.6963	0.8581	0.8581	0.9545	0.8374	0.6364	0.6
2	0.3620	****	0.6667	0.7500	0.7236	0.7236	0.7236	0.7500	0.8216	0.8216	0.6963	0.6682	0.6963	0.5
3	0.1388	0.4055	****	0.9167	0.9789	0.8938	0.9789	0.7500	0.9129	0.9129	0.9574	0.9354	0.5222	0.5
4	0.0435	0.2877	0.0870	****	0.9789	0.8087	0.9789	0.6667	0.9129	0.9129	0.9574	0.8463	0.6093	0.6
5	0.0687	0.3236	0.0213	0.0213	****	0.8696	1.0000	0.7236	0.9325	0.9325	0.9780	0.9100	0.5779	0.6
6	0.2229	0.3236	0.1123	0.2123	0.1398	****	0.8696	0.8938	0.8859	0.8859	0.8891	0.9555	0.5779	0.5
7	0.0687	0.3236	0.0213	0.0213	0.0000	0.1398	****	0.7236	0.9325	0.9325	0.9780	0.9100	0.5779	0.6
8	0.3620	0.2877	0.2877	0.4055	0.3236	0.1123	0.3236	****	0.8216	0.8216	0.7833	0.8463	0.6963	0.6
9	0.1530	0.1965	0.0912	0.0912	0.0699	0.1212	0.0699	0.1965	****	1.0000	0.9058	0.8783	0.7151	0.7
10	0.1530	0.1965	0.0912	0.0912	0.0699	0.1212	0.0699	0.1965	0.0000	****	0.9058	0.8783	0.7151	0.7
11	0.0465	0.3620	0.0435	0.0435	0.0222	0.1175	0.0222	0.2442	0.0989	0.0989	****	0.9305	0.6364	0.6
12	0.1774	0.4032	0.0668	0.1668	0.0943	0.0455	0.0943	0.1668	0.1298	0.1298	0.0721	****	0.5583	0.5
13	0.4520	0.3620	0.6496	0.4955	0.5483	0.5483	0.5483	0.3620	0.3353	0.3353	0.4520	0.5829	****	0.9
14	0.3620	0.5390	0.5390	0.4055	0.4487	0.5918	0.4487	0.4055	0.3143	0.3143	0.3620	0.5483	0.0436	**

A dendrogram based on Nei's genetic distance (1978) was constructed using the unbiased pair group method using arithmetic averages (UPGMA). The dendrogram (Figure 3.4) shows two distinct clades, a small clade consisting of two individuals obtained from Gauteng (13 and 14) and a larger clade consisting of the remainder of the individuals. Within the large clade a number of smaller clades were also distinguishable, but no particular lineages to the origins of the birds could be made.

Figure 3.4 Dendrogram of Blue crane sample population.



Individuals 1-9=KwaZulu-Natal; Individuals 10-12=Western Cape and individuals 13-14=Gauteng

3.5.3 Genetic analysis of the Crowned crane

The genetic analysis of the Crowned crane followed the same pattern as that of the Blue crane.

A. Genotypes

The genotypic composition of each individual of the Crowned crane sample was constructed using the AA, BB, and AB notations. One of the seven H-loci of this

population was monomorphic, while three of the five G/J-loci were monomorphic. The H2-locus and the H6-locus, although polymorphic, all individuals were homozygous. The heterozygosity within individuals between loci ranged from 7 to 14% while heterozygosity within locus between individuals ranged from 0 to 30% (Table 3.12).

Table 3.12 Genotypes of all individuals of the Crowned crane species.

Individual	Microsatellite locus												Frequency heterozygous loci within individuals
	H1	H2	H3	H4	H5	H6	H7	G/J1	G/J2	G/J3	G/J4	G/J5	
C1	AA	AA	AA	AA	AA	BB	AA	AA	AA	AA	AA	AA	0.00
C2	BB	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	0.00
C3	AB	AA	AA	AA	AA	AA	AA	BB	AA	AA	AA	AA	0.07
C4	AB	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	0.07
C5	AA	AA	AA	AA	AB	AA	AA	AA	AA	AA	AA	AA	0.07
C6	AA	BB	BB	BB	AA	AA	AA	AA	BB	AA	AA	AA	0.00
C7	AB	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	0.07
C8	AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AA	0.07
C9	AA	AA	AA	AB	AB	AA	AA	BB	AA	AA	AA	AA	0.14
C10	AA	AA	AB	AB	AA	AA	AA	AA	AA	AA	AA	AA	0.14
Frequency of heterozygous loci between individuals	0.30	0.00	0.10	0.20	0.20	0.00	0.00	0.00	0.10	0.00	0.00	0.00	

B. Allelic variation

The frequency of the alleles was determined for each of the loci in the Crowned crane sample population (Table 3.13). It was found that the frequency of the most prevalent allele at each of the polymorphic loci ranged from 75% (H1-locus) to 90% (H2-locus, H5-locus, and H6-locus).

Table 3.13 Allele frequencies of the twelve loci of the Crowned crane.

Allele	Microsatellite locus											
	H1	H2	H3	H4	H5	H6	*H7	G/J1	G/J2	*G/J3	*G/J4	*G/J5
A	0.75	0.90	0.85	0.80	0.90	0.90	1.00	0.80	0.85	1.00	1.00	1.00
B	0.25	0.10	0.15	0.20	0.10	0.10	0.00	0.20	0.15	0.00	0.00	0.00

*Monomorphic loci

Shannon's information index (I) (Lewontin, 1972), a measure of allelic variation, ranged from approximately 32 to 56% (Table 3.14).

Table 3.14 Shannon's information index for the Crowned crane.

Locus	*n_a	*n_e	Shannon's information index (I)
H1	2.0000	1.6000	0.5623
H2	2.0000	1.2195	0.3251
H3	2.0000	1.3423	0.4227
H4	2.0000	1.4706	0.5004
H5	2.0000	1.2195	0.3251
H6	2.0000	1.2195	0.3251
H7	1.0000	0.0000	0.0000
G/J1	2.0000	1.4706	0.5004
G/J2	2.0000	1.3423	0.4227
G/J3	1.0000	1.0000	0.0000
G/J4	1.0000	1.0000	0.0000
G/J5	1.0000	1.0000	0.0000
Mean	1.6667	1.2404	0.2820
Standard Deviation	0.4924	0.2107	0.2210

*n_a=observed number of alleles;n_e=effective number of alleles.

Shannon's information indexes (Lewontin, 1972), across all these loci, was indicative of relatively large differences in frequencies between the prevalent and rare alleles at each locus.

C. Genotypic variation

The observed heterozygosity (H_o) and expected heterozygosity (H_e), (Levene, 1949; Nei, 1972) were determined for each locus of the Crowned crane sample population (Table 3.15). The mean observed heterozygosity for this sample population was approximately 7% which is approximately 39% that of the expected heterozygosities. Seven of the loci were completely homozygous, while the observed homozygosity in the remaining loci ranged from 70 to 100%. The observed heterozygosity, on the other hand, ranged from 10 to 30%. The differences between the two heterozygosity estimates and observed heterozygosity values were similar; ranged between -1 to 32% for Levene's estimate and -2 to 34% for Nei's estimate.

Table 3.15 Observed heterozygosity and homozygosity, Levene's expected homozygosity and heterozygosity and Nei's heterozygosity for the Crowned crane.

Locus	Observed homozygosity	Observed Heterozygosity	Levene's expected heterozygosity	H_e-H_o(Levene)	Nei's expected heterozygosity	H_o-H_e(Nei)
H1	0.7000	0.3000	0.3947	0.0947	0.3750	0.0750
H2	1.0000	0.0000	0.1895	0.1895	0.1800	0.1800
H3	0.9000	0.1000	0.2684	0.1684	0.2550	0.1550
H4	0.8000	0.2000	0.3368	0.1368	0.3200	0.1200
H5	0.8000	0.2000	0.1895	-0.0105	0.1800	-0.0200
H6	1.0000	0.0000	0.1895	0.1895	0.1800	0.1800
H7	1.0000	0.0000	-	-	-	-
G/J1	1.0000	0.0000	0.3368	0.3368	0.3200	0.3200
G/J2	0.9000	0.1000	0.2684	0.1684	0.2500	0.1500
G/J3	1.0000	0.0000	-	-	-	-
G/J4	1.0000	0.0000	-	-	-	-
G/J5	1.0000	0.0000	-	-	-	-
Mean	0.9250	0.0750	0.1811		0.1721	
Standard Deviation	0.1055	0.1055	0.1480		0.1406	

Wright's (1978) inbreeding coefficient (F_{IS}) results revealed that most of the loci presented as heterozygous deficient in the population, except for H2, H6 and G/J1-

locus. However, locus H5 was the only locus that displayed heterozygosity excess (Table 3.16).

Table 3.16 Wright's inbreeding coefficient (F_{IS}) for the Crowned crane.

Locus	F_{IS}
H1	0.2000
H2	1.0000
H3	0.6078
H4	0.3750
H5	-0.1111
H6	1.0000
G/J1	1.0000
G/J2	0.6078

The tests for HWE (Table 3.17) revealed that five of the eight loci were not in HWE; three of these loci were highly significant and two significant. This significant deviation from the Hardy-Weinberg equilibrium could be attributed to the small population size and inbreeding which causes an increase in homozygosity within the species.

Table 3.17 Chi-squared test estimates for Hardy-Weinberg equilibrium assessed for the twelve loci of Crowned crane species.

Locus number	Degree of freedom	Chi-square value	Probability	Significance	HWE
H1	1	0.6943	0.40	NS	Yes
H2	1	19.0588	0.00	**	No
H3	1	5.6471	0.01	*	No
H4	1	2.1125	0.14	NS	Yes
H5	1	0.0588	0.80	NS	Yes
H6	1	19.0588	0.00	**	No
G/J1	1	12.8000	0.00	**	No
G/J2	1	5.6470	0.01	*	No

Significant at p=0.05; ** Highly significant at p=0.01; NS=Non-significant.

D. Distance measures

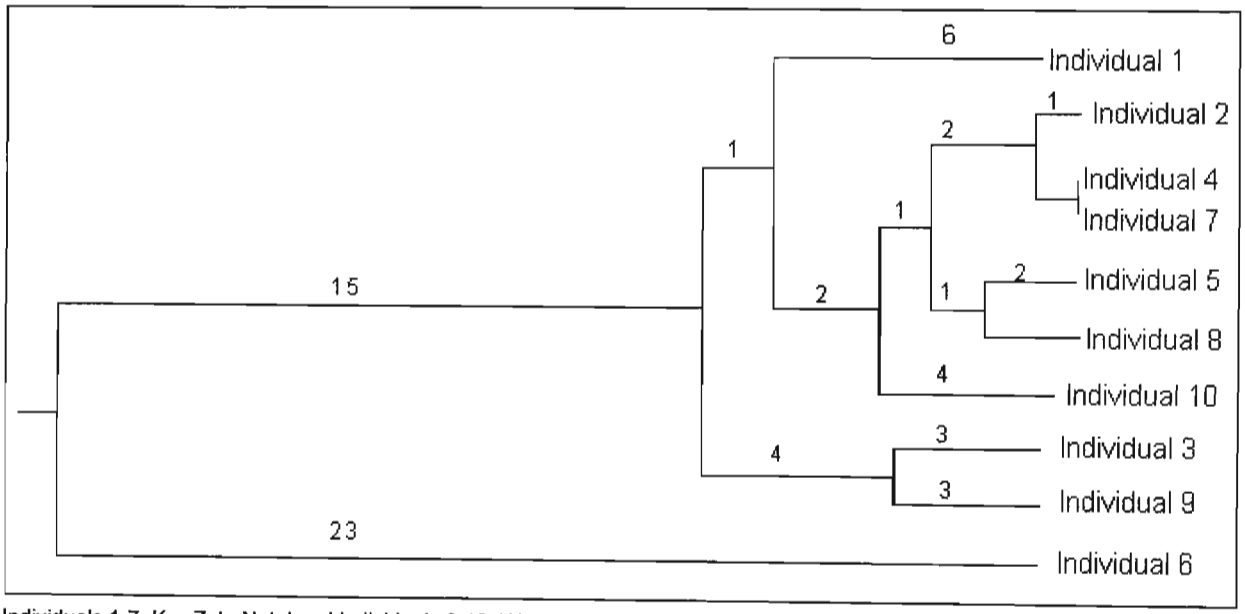
Similarity, identity (I), between crowned crane individuals ranged from approximately 55 to 100%, whereas genetic distance (D) ranged from 0 to 59% (Table 3.18).

Table 3.18 Nei's genetic identity (top diagonal) and genetic distance (bottom diagonal) of the Crowned crane sample population.

Individual	1	2	3	4	5	6	7	8	9	10
1	****	0.8333	0.8087	0.8938	0.8938	0.5833	0.8938	0.8938	0.7833	0.8704
2	0.1823	****	0.8938	0.9789	0.8938	0.5833	0.9789	0.8938	0.7833	0.8704
3	0.2123	0.1123	****	0.9130	0.8696	0.5533	0.9130	0.8696	0.9336	0.8447
4	0.1123	0.0213	0.0910	****	0.9565	0.6384	1.0000	0.9565	0.8447	0.9336
5	0.1123	0.1123	0.1398	0.0445	****	0.6384	0.9565	0.9565	0.8891	0.9336
6	0.5390	0.5390	0.5918	0.4487	0.4487	****	0.6384	0.7236	0.6093	0.7833
7	0.1123	0.0213	0.0910	0.0000	0.0445	0.4487	****	0.9565	0.8447	0.9336
8	0.1123	0.1123	0.1398	0.0445	0.0445	0.3236	0.0445	****	0.8447	0.9336
9	0.2442	0.2442	0.0687	0.1688	0.1175	0.4955	0.1688	0.1688	****	0.8636
10	0.1388	0.1388	0.1688	0.0687	0.0687	0.2442	0.0687	0.0687	0.1466	****

The dendrogram (Figure 3.5) shows two distinct clades, a small clade consisting of one individual (6) and a large clade consisting of nine individuals. Within the large clade a number of smaller clades were also distinguishable. No particular link between the position of an individual in the dendrogram and origin could be established.

Figure 3.5 Dendrogram of Crowned crane sample population.



Individuals 1-7=KwaZulu-Natal and individuals 8-10=Western Cape.

3.5.4 Genetic analysis of the Wattled crane

The genetic analysis of the Wattled crane followed the same pattern as that of the Blue crane and the Crowned crane.

A. Genotypes

All of the twelve loci were polymorphic except for two of the H-loci (H4 and H7-locus). Four loci (H4-locus, G/J2, 3, 4-loci) although polymorphic, all individuals were homozygous. The heterozygosity within individuals between loci ranged from 0 to 83% while the heterozygosity within locus between individuals ranged from 0 to 38% (Table 3.19).

Table 3.19 Genotypes of all individuals of the Wattled crane species.

Individual	Microsatellite locus												Frequency heterozygous loci within individuals
	H1	H2	H3	H4	H5	H6	H7	G/J1	G/J2	G/J3	G/J4	G/J5	
W1	BB	AA	AB	BB	AA	AA	AA	BB	AA	AB	AA	AA	0.17
W2	AA	AB	AA	BB	BB	AA	AA	BB	BB	AA	BB	AA	0.08
W3	AA	AA	AA	AA	AB	AA	AA	BB	AA	BB	AA	AA	0.08
W4	AB	BB	AA	AA	AB	AA	AA	AA	BB	AA	BB	AB	0.25
W5	AA	AA	AA	AA	AA	AA	AA	AB	AA	BB	AA	AA	0.83
W6	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	0.00
W7	AB	AA	AA	AA	AB	AA	AA	AA	AA	AA	AA	AB	0.25
W8	AA	AA	AA	AA	AA	AB	AA	BB	AA	BB	AA	AB	0.17
Frequency of heterozygous loci between individuals	0.25	0.13	0.13	0.00	0.38	0.13	0.00	0.00	0.00	0.13	0.00	0.38	

B Allelic variation

The frequency of the alleles was determined for each of the loci in the Wattled crane sample population. It was found that the frequency of the most prevalent allele at each of the polymorphic loci ranged from 56% (G/J1-locus and G/J-3-locus) to 94% (H3-locus and H6-locus) (Table 3.20).

Table 3.20 Allele frequencies of the twelve loci of the Wattled crane.

Allele	Microsatellite locus											
	H1	H2	H3	H4	H5	H6	*H7	G/J1	G/J2	G/J3	G/J4	G/J5
A	0.75	0.81	0.94	0.75	0.69	0.94	1.00	0.56	0.75	0.56	0.75	0.81
B	0.25	0.19	0.06	0.25	0.31	0.06	0.00	0.44	0.25	0.44	0.25	0.19

*Monomorphic loci

Shannon's information index (I) (Lewontin, 1972), a measure of allelic variation, ranged from approximately 23 to 68% (Table 3.21).

Table 3.21 Shannon's information index for the Wattled crane.

Locus	*n_a	*n_e	Shannon's information index (I)
H1	2.0000	1.6000	0.5623
H2	2.0000	1.4382	0.4826
H3	2.0000	1.1327	0.2338
H4	2.0000	1.6000	0.5623
H5	2.0000	1.7534	0.6211
H6	2.0000	1.1327	0.2338
H7	1.0000	1.0000	0.0000
G/J1	2.0000	1.9692	0.6853
G/J2	2.0000	1.6000	0.5623
G/J3	2.0000	1.9692	0.6853
G/J4	2.0000	1.6000	0.5623
G/J5	2.0000	1.4382	0.4826
Mean	1.9167	1.5195	0.4728
Standard Deviation	0.2887	0.3115	0.2094

*n_a=observed number of alleles;n_e=effective number of alleles.

Here too, the Shannon's information indexes (Lewontin, 1972), across all these loci, were relatively large for most loci, thereby indicating a sizable difference between the allele frequencies of the prevalent and rare alleles at each locus.

C. Genotypic variation

The observed heterozygosity (H_o) and expected heterozygosity (H_e), (Levene, 1949; Nei, 1972) were determined for each locus of the Wattled crane sample population (Table 3.22). The mean observed heterozygosity for this sample population was approximately 14% which is approximately 44% that of the expected heterozygosities. Four of the loci were completely homozygous, while the observed homozygosity in the remaining loci ranged from 63 to 100%. The observed heterozygosity, on the other hand, ranged from 13 to 38%. The differences between the two heterozygosity estimates and observed heterozygosity values were similar; ranged between -5 to 40% for Levene's estimate and -0.8 to 37% for Nei's estimate.

Table 3.22 Observed heterozygosity and homozygosity, Levene's expected homozygosity and heterozygosity and Nei's heterozygosity for the Wattled crane.

Locus	Observed homozygosity	Observed Heterozygosity	Levene's expected heterozygosity	H_e-H_o(Levene)	Nei's expected heterozygosity	H_o-H_e(Nei)
H1	0.7500	0.2500	0.4000	0.1500	0.3750	0.1250
H2	0.8750	0.1250	0.3250	0.2000	0.3047	0.1797
H3	0.8750	0.1250	0.1250	0.0000	0.1172	-0.0078
H4	1.0000	0.0000	-	-	-	-
H5	0.6250	0.3750	0.4583	0.0833	0.4297	0.0547
H6	0.8750	0.1250	0.1250	0.0000	0.1172	-0.0078
H7	1.0000	0.0000	-	-	-	-
G/J1	0.8750	0.1250	0.5250	0.4000	0.4922	0.3672
G/J2	1.0000	0.0000	-	-	-	-
G/J3	0.8750	0.1250	0.5250	0.2000	0.4922	0.3672
G/J4	1.0000	0.0000	-	-	-	-
G/J5	0.6250	0.3750	0.3250	-0.0500	0.3047	-0.0445
Mean	0.8646	0.1354	0.3340		0.3132	
Standard Deviation	0.1355	0.1355	0.1665		0.1531	

Wright's (1978) inbreeding coefficient (F_{IS}) results revealed that most of the loci presented as heterozygous deficient in the sample population, except for H4, G/J2 and G/J4-locus. However, locus H3, H6 and G/J5-locus displayed an excess of heterozygosity (Table 3.23).

Table 3.23 Wright's inbreeding coefficient (F_{IS}) for the Wattled crane.

Locus	F_{IS}
H1	0.3333
H2	0.5897
H3	-0.0667
H4	1.0000
H5	0.1273
H6	-0.0667
G/J1	0.7460
G/J2	1.0000
G/J3	0.7460
G/J4	1.0000
G/J5	-0.2308

The tests for HWE (Table 3.24) revealed that five of the eleven polymorphic loci were not in HWE; three of these loci were highly significant and two were significant. This significant deviation from the Hardy-Weinberg equilibrium could be attributed to the small population size and inbreeding, which causes an increase in homozygosity within the species.

Table 3.24 Chi-squared test estimates for Hardy-Weinberg equilibrium assessed for the twelve loci of Wattled crane species.

Locus number	Degree of freedom	Chi-square test (x^2 =)		Probability	HWE
		Chi-square	Significance		
H1	1	1.4318	NS	0.2314	Yes
H2	1	4.3076	*	0.0370	No
H3	1	0.0000	NS	1.0000	Yes
H4	1	10.1818	**	0.0014	No
H5	1	0.3181	NS	0.5727	Yes
H6	1	0.0000	NS	1.0000	Yes
G/J2	1	10.1818	**	0.0014	No
G/J3	1	5.3333	*	0.0209	No
G/J4	1	10.1818	**	0.0014	No
G/J5	1	0.2692	NS	0.6034	Yes

Significant at p=0.05; ** Highly significant at p=0.01; NS=Non-significant; -=monomorphic

D. Distance measures

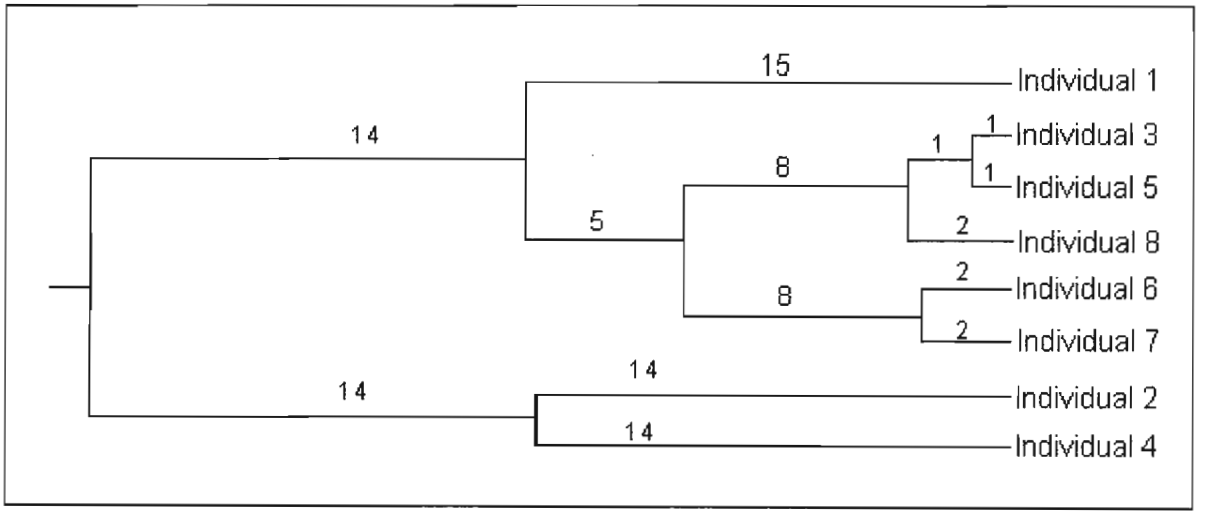
Similarity, identity (I), between Wattled crane individuals ranged from approximately 41 to 95%, whereas genetic distance (D) ranged from 4 to 87% (Table 3.25).

Table 3.25 Nei's genetic identity (top diagonal) and genetic distance (bottom diagonal) of the Wattled crane species.

Individual	1	2	3	4	5	6	7	8
1	****	0.5779	0.7557	0.4187	0.7557	0.6963	0.6979	0.7273
2	0.5483	****	0.6087	0.7280	0.5217	0.5533	0.5460	0.4890
3	0.2801	0.4964	****	0.5005	0.9565	0.8087	0.7735	0.9336
4	0.8706	0.3174	0.6921	****	0.5460	0.6682	0.7143	0.4652
5	0.2801	0.6506	0.0445	0.6051	****	0.8938	0.8190	0.9336
6	0.3620	0.5918	0.2123	0.4032	0.1123	****	0.9354	0.7833
7	0.3597	0.6051	0.2568	0.3365	0.1996	0.0668	****	0.7444
8	0.3185	0.7154	0.0687	0.7652	0.0687	0.2442	0.2952	****

The dendrogram (Figure 3.6) shows two distinct clades, a small clade consisting of two individuals (2 and 4) obtained from one supplier, and a large clade consisting of the remainder of the individuals. No particular link between the position of an individual in the dendrogram and origin could be established.

Figure 3.6 Dendrogram of Wattled crane sample.



Individuals 1-8=KwaZulu Natal with individuals 1-4 from one owner=W. Horsfield; Individuals 5-6=Dr. M. Penning and individuals 7-8=Prof. M.Perrin

3.6 COMPARATIVE GENETIC ANALYSIS OF THE THREE CRANE SPECIES

A comparative analysis of the different genetic variation statistics was undertaken to compare the inherent genetic variation that resided in the different species. These statistics included Nei's identity and distance (1978), Shannon's Information Index (Lewontin, 1972), Nei's heterozygosity (1972), and the number of polymorphic loci.

Similarity, identity (I), between the Blue, Wattled and Crowned crane species ranged from approximately 92 to 96%, showing a high degree of genetic similarity, with the Blue crane and Wattled crane displaying marginally a closer relationship than the Blue and the Crowned and the Wattled and the Crowned crane, which is supported by the genetic distance (D) that was reasonably low; ranging from approximately 3 to 5% (Table 3.26).

Table 3.26 Nei's genetic identity (top diagonal) and genetic distance (bottom diagonal) of the Blue, Wattled, and Crowned crane species.

Species	Blue	Wattled	Crowned
Blue	****	0.9690	0.9226
Wattled	0.0315	****	0.9497
Crowned	0.0806	0.0516	****

The number of polymorphic loci, mean observed heterozygosity, Shannon's allelic diversity and Nei's heterozygosity were compared for all three species (Table 3.27). These genetic variation statistics revealed that the Wattled crane ranked number one, harbouring the highest amount of inherent genetic variation, with the largest number of polymorphic loci, the highest amount of allelic variation and heterozygosity; followed by the Blue crane and the Crowned crane.

Shannon's information index, revealed an expected higher estimation of genetic variation than that of Nei (1972). This is attributed to the formulation of these estimates. Shannon's information index is based on the number of alleles, n_a, and the effective number of alleles, n_e, where n_e is the number of equally frequent alleles it will take to achieve a given level of expected heterozygosity. Nei, on the other hand employs only the allele frequencies to estimate heterozygosity

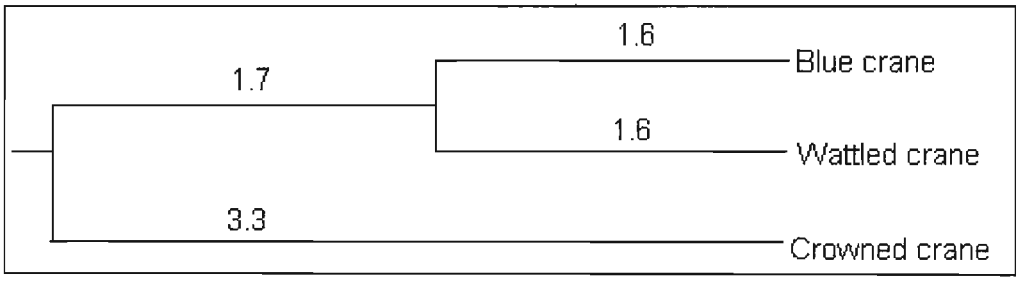
Table 3.27 Comparative analysis of genetic variation statistics between the thee crane species.

Species	Genetic variation statistic				
	Observed heterozygosity (%)	Number of polymorphic loci	Mean Shannon's index (I)	Mean Nei's heterozygosity (H_e)	*Mean SI–Mean H_e (%)
Blue	12	9	0.35	0.23	12
Crowned	7	6	0.28	0.17	11
Wattled	14	11	0.47	0.31	16

*Difference between mean Shannon's information index and Nei's heterozygosity (H_e).

A dendrogram was constructed using Nei's genetic distance (1978) to reveal the genetic relationships between the different crane species (Figure 3.7). The dendrogram shows that the Blue crane and the Wattled crane were closer related to one another than the Crowned crane. The dendrogram also supports the findings that the Crowned crane contained the least amount of inherent genetic variation placing this species further away from the other two crane species in the dendrogram.

Figure 3.7 Dendrogram of Blue, Wattled and Crowned crane.



CHAPTER FOUR
DISCUSSION AND CONCLUSION

4.1 INTRODUCTION

For centuries the beauty of cranes have enthralled people. They are of the most elegant of all birds, known for their trumpeting calls, carefree courtship and lifelong devotion shown by mated pairs (50-60 years lifespan). Crane species occur on all continents of the world except for South America and Antarctica (Allan, 1994). Of the fifteen species of crane worldwide, three predominantly occur in southern Africa; the Wattled crane (*Bugeranus carunculatus*), the Blue crane (*Anthropoides paradisea*) and the Crowned crane (*Balearica regulorum*) and are easily be recognized (Allan, 1994). The Wattled crane is the largest of the three southern African crane species and is identifiable by the presence of a wattle. The Blue crane is recognized by its typical silvery, bluish-grey colouring and is almost entirely restricted in distribution to South Africa (Allan, 1994). Blue crane is also a symbol of peace and royalty in Zulu culture, thus not surprisingly is also the national bird of South Africa. The Crowned crane is the oldest species of living cranes (Johnsgard, 1983) and can be recognized by its golden-crowned plumage.

In the last few decades, crane numbers throughout the world have displayed a drastic decline due to various factors, of which the most important has been the destruction of their habitat (Allan, 1996). Unfortunately, their beauty and elegance has contributed to the ever-growing illegal trade, thus also contributing to their diminishing numbers. All three southern African species are listed by the Red Data Bird list (CITES, 2005); the Wattled crane as being critically endangered and the Blue and Crowned crane as both being vulnerable.

South Africa is world-renown for its biodiversity; grasslands and wetlands support a rich diversity of species. Cranes promote ecotourism and job creation in South Africa, with birding trips in particular, growing at a rapid rate. Crane species also

act as indicators of the health of the environment, in particular grasslands and wetlands, where declining numbers are indicative of grassland and wetland degradation (Allan, 1995). In recognition of the threat on these crane species numerous efforts are underway to prevent species extinction. All three southern African species are protected by law; they may not be disturbed, persecuted or removed from the wild (The South African Crane Foundation, 1992). A number of NGOs, especially Birdlife, Endangered Wildlife Trust and The Southern African Crane Working Group, initiate, monitor and coordinate habitat conservation efforts by encouraging safe environmental practices, environmental education and sustainable utilization of these resources for the benefit of all. A studbook is also being compiled of birds in captivity.

Data recorded in this studbook includes descriptions of physical attributes, ownership, location and possible kinships. The need for the inclusion of genetic information, such as fingerprinting is becoming more and more important as a growing number of birds are confiscated each year, which are often passed off as "non-wild". Therefore, the development of genetic tools that can be used to identify kinships would be useful in the illegal bird trade as well as for avicultural practices (Selkoe and Toonen, 2006).

In this investigation microsatellite fingerprinting was selected as the molecular tool of choice because of the general abundance of microsatellite loci in many species, their large number of alleles per locus and known primers for a number of microsatellite loci published for the related crane species (Scotti *et al.*, 1999; Hasegawa *et al.*, 2000; Glenn and Jones, personal communication). It has been estimated that microsatellites mutate relatively faster than other loci, 10^{-3} to 10^{-5} mutations per gamete, thereby generating many alleles at a particular locus. (Edwards *et al.*, 1992; Bowcock *et al.*, 1994; Forbes *et al.*, 1995). Although birds have the smallest known genome sizes among vertebrates and a calculated microsatellite density that is much lower than that of the human genome (Primmer *et al.*, 1997), microsatellites are still regarded as a suitable marker for individual

fingerprinting and kinship determination. Furthermore, it was also deemed important to select a fingerprinting methodology that was reasonably rapid and cost effective for breeders and conservation monitoring groups, while still allowing for accurate discrimination between individuals (Selkoe and Toonen, 2006). The data generated in this investigation was then further analysed to estimate the inherent genetic variation within each of the species and genetic distance between the species.

4.2 SUITABILITY OF SELECTED MICROSATELLITE MARKERS FOR INDIVIDUAL FINGERPRINTING

Primer sets developed for a particular species to amplify microsatellite loci have in a number of instances been used to amplify heterologous microsatellite loci in related species. A negative relationship exists between microsatellite cross-species performance and evolutionary distance. The greater the evolutionary distance between the original species and the tested species, the less likely that cross-species amplification will be successful (Primmer *et al.*, 1997; Galbusera *et al.*, 2000).

In bird species, a wide range of microsatellite markers has been tested for cross-species amplification. Primmer *et al.* (1997) found that swallow and pied flycatcher microsatellite markers successfully amplified 162 heterologous loci in 48 bird species. Reed *et al.* (2000) found that 54% of 520 chicken markers successfully amplified heterologous turkey loci, producing amplification products of similar size. Baratti *et al.* (2001), on the other hand, found that only 16% of 154 chicken microsatellite markers successfully amplified heterologous loci in pheasants, while 34% heterologous turkey microsatellites were successfully amplified in pheasants. Kayang and co-workers (2002) isolated 100 quail microsatellite markers and successfully amplified 42 chicken and 20 guinea fowl heterologous loci. Huang *et al.* (2005) also tested 35 duck microsatellite markers on turkey, goose and peacock and found that only 2 amplified in chicken, 14 in goose and none in the peacock. In

an extensive investigation of cross-amplification ability in passerine species, Galbusera *et al.* (2000) found that the 40 passerine markers tested displayed cross-species amplification in a wide range of passerine species.

In cranes, Glenn (1997) found that eight Whooping crane primer sets were able to amplify 90% heterologous microsatellite loci in related crane species, while Hasegawa *et al.* (2000) used seven Red-Crowned crane microsatellite primer sets to cross-amplify heterologous loci in eight related crane species. The primer sets developed by Glenn and Jones (personal communication) showed cross-amplification in the Wattled crane, while the Hasegawa *et al.* (2000) primer sets amplified heterologous microsatellite loci of the Blue crane.

In this investigation seven published primer sets (Hasegawa *et al.*, 2000) and five unpublished primer sets (Glenn and Jones, personal communication) developed for related crane species were tested in the three southern African crane species. As was expected, all these primer sets were able to amplify heterologous microsatellite loci in these three related crane species, especially the Hasegawa *et al.* (2000) microsatellite primer sets. However, the amplification products of some of the Whooping crane primers, developed by Glenn and Jones (personal communication) were poorly defined in the Wattled crane, even after extensive optimization attempts, which can be explained by the fact that the Whooping crane and the Wattled crane belong to two different species groups of the subfamily Gruinae; Whooping crane belongs to the Americana and the Wattled crane to the Anthropoides species group (Krajewski and King, 1996). It was interesting to note that the Whooping crane primer sets were able to amplify heterologous loci in the Blue crane that also belongs the Anthropoides species group. These results may be attributed to the smaller sample size of Wattled crane (8 individuals) in comparison to the relatively larger sample size of Blue cranes (14 individuals).

The closer the phylogenetic distance between two species, the greater the probability that heterologous microsatellite loci will be polymorphic (Ellegren, 1992;

Primmer *et al.,* 1997; 2002; Galbusera *et al.*, 2000). In a number of cross-species investigations, it was found that the microsatellite polymorphic content ranged from 14 to 61% depending on the phylogenetic distance between the species being compared (Reed *et al.*, 2000; 2003; Kayang *et al.*, 2002, Huang *et al.*, 2005). Of the twelve microsatellite loci investigated, the Blue crane and the Wattled crane revealed a high level of polymorphism. An expected result, as these two species belong to the same subfamily, Gruinae (Krajewski and King; 1996), as the Red-Crowned and Whooping crane from whom the microsatellite primers were developed. The Blue crane displayed 76% polymorphism and the Wattled crane 92%. In contrast, the Crowned crane, that belongs to a different subfamily, Balearicinae (Krajewski and King; 1996), revealed only 50% polymorphic loci.

An analysis of the allele sizes also revealed that the alleles of the heterologous microsatellite loci were mostly of similar size to that of the species for which the primers were developed; Red-Crowned crane (Hasegawa *et al.*, 2000) and Whooping crane (Glenn and Jones; personal communication). However for the alleles that differed in size, a large difference was observed in comparison to published results. These changes could be attributed to natural selection and difference in mutational rates selected for in nature. In addition the alleles of the Red-Crowned species (published allele sizes) originate from a different crane species, in comparison to the present study, which originated from Asia and is located in a different continent in comparison to Africa. Due to the different populations of cranes differences in allele sizes are expected. Little variation in size, a little more than 5 bp, was noted for the different alleles of the polymorphic loci. The number of alleles, on the other hand, at each of the polymorphic loci was found to be low. Each polymorphic loci only exhibited two alleles because the variation of the amplified fragments sizes was so little on the gel that only two alleles could be observed. These results may have varied if automated sequencing could have been performed. Similar results were also observed, as has been found in a number of other investigations. The mean number of alleles of chicken heterologous loci in turkey was 1.4 (Reed *et al.*, 2000) and 2.3 (Reed *et al.*, 2003)

and the number of alleles of quail heterologous loci in chicken and guinea fowl was 3.7 (Kayang *et al.*, 2002).

In this investigation the frequency of the most prevalent allele at most of the loci was generally reasonably high, ranging from 54 to 96% in the Blue crane, from 75 to 90% in the Crowned crane and from 56 to 94% in the Wattled crane. These results therefore suggest that these primer sets (Hasegawa *et al.*, 2000; Glenn and Jones, personal communication) are not suitable for individual identification and differentiation using polyacrylamide gel electrophoresis. With such a low number of alleles at the different polymorphic loci, these primers would probably also not be suitable in an automated system.

4.3 GENETIC VARIATION AND DISTANCE MEASURES

Microsatellites have proven to be very useful for the purpose of unveiling genetic diversity (Scotti *et al.*, 1999). They are not only highly variable, but are generally recognised as neutral markers, where selection and environmental pressures do not influence their expression directly (Scotti *et al.*, 1999).

Molecular studies assessing intra-population and inter-population variation within bird species are few. The observed heterozygosity differ greatly between populations of the same species. The observed heterozygosity for mottled duck ranged from 13 to 85% (Williams *et al.*, 2006); for yellow warbler from 38 to 99% (Dawson *et al.*, 1997); for hawk from 69 to 95% (Bollmer *et al.*, 2005); for large-billed scrubwren from 14 to 91% (Bardeleben *et al.*, 2005); for burrowing owl from 34 to 54% (Korfanta *et al.*, 2005) and for peeking duck from 4 to 97% (Huang *et al.*, 2005). However, in parrot the observed heterozygosity within different populations was exceptionally low when compared to other species; ranging from 2 to 7% (Madsen *et al.*, 1992).

Species variation in cranes has only been studied in Siberian crane and Red-Crowned crane. Heterozygosity in captive populations of Siberian crane ranged from 72 to 85% (Tokarskaya *et al.*, 1995), however in contrast, Hasegawa *et al.* (2000) confirmed that heterozygosity in the Red-Crowned crane was low. In this investigation, the observed heterozygosity of the three southern African species was also found to be rather low; 12% in Blue crane; 7% in Crowned crane; and 13% in Wattled crane. Nei's identity (I) (1978) further confirmed this outcome, where similarity between individuals within each of the species ranged from 66 to 100% for Blue crane; 55 to 100% for Crowned crane and 41 to 95% for Wattled crane. The low genetic variation within each of the species can probably be attributed to possible relatedness between the individuals that were all supplied by various aviculturists whom have limited number of birds.

Hardy-Weinberg test for equilibrium of the various loci revealed that most of the microsatellite loci displayed a deficiency of heterozygotes, while a few loci did have an excess of heterozygotes. Low heterozygosity could be attributed to the small population size. The small population size further lowers heterozygosity by inbreeding, which was supported by the results obtained from Wright's (1978) inbreeding coefficient. The Hardy-Weinberg test of equilibrium, in general, was also supportive of the notion that the individuals within each of the species might have been related. Natural selection also acts to remove certain genotypes from a population, which would further contribute to the low heterozygosity observed.

Interspecific comparisons of heterologous microsatellite loci are rare. A study of interspecific differentiation between species of parrot (Madsen *et al.*, 1992) revealed little differentiation between two species of cockatoo, less than 3%, while differentiation between cockatoo and other parrot species was 45%. Differentiation between the three southern African crane species investigated showed differentiation ranging between 3 and 5%, with Blue and Wattled crane displaying a higher degree of genetic similarity when compared to the Crowned crane, an

expected result as it is believed that the Crowned crane is the oldest extant crane species (Johnsgard, 1983).

4.4 CONCLUSION

This comparative investigation of molecular fingerprinting and molecular genetic variation assessment of southern African crane species is the first of its kind. The research carried out in the study would undeniably provide helpful information to the use of microsatellites in conservation attempts. It has become apparent that the low number of polymorphic microsatellite loci (Primmer *et al.*, 1997) and the high genetic similarity between individuals within crane species could hamper effective individual fingerprinting and kinship analysis. A comparative analysis of allozyme, RAPD and microsatellite polymorphisms on chickens (Zhang *et al.*, 2002) revealed that microsatellite analysis showed the highest heterozygosity or gene diversity (0.7561) in comparison to 0,2209 in allozyme analysis and a 0.2632 in RAPD analysis. Therefore an extensive search for additional microsatellite loci that are more polymorphic and contain a larger number of alleles should be undertaken.

Although more expensive, other molecular fingerprinting techniques might be alternative routes of investigation, such as the use of AFLPs and the use of the multilocus minisatellite DNA fingerprinting (Jeffreys *et al.*, 1985). AFLP fingerprinting has not been popular in avian molecular studies, due to their expensive nature. However, the use of multilocus minisatellite DNA fingerprinting (Jeffreys *et al.*, 1985) has become more popular over the past few years because of their success in differentiating between closely related individuals (Eimes *et al.*, 2004) and might be a potential DNA fingerprinting prospect to be considered for the management and conservation of the Blue, Crowned an Wattled crane species.

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APPENDIX

A. Recipes of solutions and buffers

B. Statistical analyses output

A. Recipes of solutions and buffers

Acrylamide (30%)

29 g acrylamide

1.0 g *N,N'* – methylenebisacrylamide

100 ml water

Heat the solution to 37^{O}C to dissolve the chemicals.

Large Agarose gel (0.8%)

2.0 g per 250 ml TAE (1X)

6.0 µl Ethidium Bromide (10 mg/ml) for each 250 ml 1X TAE

Dissolve well, microwave for 4 min and run at 100 V for 60 min.

Ammonium Persulfate (APS- 10%)

1.0 g ammonium persulfate

10 ml distilled water

Ethanol (70%)

70 ml ethanol

30 ml distilled water

Ethidium Bromide (EtBr 10 mg/ ml)

0.01 g ethidium bromide

1 ml distilled water

EDTA (0.5 M)

37.23 g ethylene diamine tetra-acetic acid di-sodium salt

50 ml distilled water

Dissolve well, adjust the pH to precisely pH 8 with NaOH and make up to 300 ml with distilled water.

Glycerol (30%)

30 ml glycerol

100 ml distilled water

Loading Buffer (20%)

0.012 g bromophenol blue

0.0125 g xylene cyanol

1.5 ml glycerol (30%)

3.5 ml sterile distilled water

Store at 4-5^{O}C, use 1 µl of Loading Buffer to 5 µl sample.

Polyacrylamide solution (20%)

6.66 ml acrylamide (30%)

1.27 ml water

2.0 ml TBE (5X)

*0.07 ml ammonium persulfate (10%)

*Added just before use.

Dissolve with stirring bar, ensure there are no bubbles and pour gently into cast. Allow setting for 45 min–60 min. Use quickly so that gel does not dry out.

Proteinase K (10 mg/ml)

0.01 g proteinase K

1 ml sterile distilled water

Store at 4-5^{O}C.

SDS (25%)

20 g SDS 65

100 ml distilled water

Incubate at 65^{O}C to dissolve. Store at room temperature.

Sodium Chloride (5 M)

146,1 g NaCl

300 ml sterile distilled water

Dissolve well, make up to 500 ml with distilled water.

TAE buffer (50X)

48.46 g tris base

200 ml EDTA (0.5 M)

1.6 L distilled water

Adjust pH to 8.0 with glacial acid and then make up to 2000 ml with distilled water.

Autoclave.

TAE buffer (10X)

48.46 g tris base

4.1 g anhydrous sodium acetate

3.72 g EDTA

Adjust pH to 7.8 with glacial acid and then make up to 1000 ml with distilled water.

TAE buffer (1X)

20 ml TAE (10X)

980 ml distilled water

TBE buffer (5X)

54 g tris base

27.5 g boric acid

20 ml EDTA (0.5 M)

Make up to 1 L with distilled water.

TBE buffer (0.5X)

100 ml TBE (5X)

900 ml distilled water

TE buffer (1X)

12.11 g tris base

3.72 g EDTA

700 ml distilled water

Make up to 1 L with distilled water. Adjust pH to 8.0 with concentrated HCL.

TNE buffer (1X)

3.25 g tris base

2.92 g NaCl

50 ml EDTA (0.5 M)

Make up to 500 ml with distilled water.

Tris-HCl (10mM)

20 µl tris (1M)

180 µl distilled water

Tris-HCl (1M)

12.11 g tris base

800 ml sterile distilled water

Adjust pH to 8.0 with concentrated HCL.

Triton X-100 (10%)

1 ml triton X-100

9 ml distilled water

B. Statistical analysis output

1. GENOTYPIC DATA OF BLUE, WATTLED AND CROWNED CRANE

Multi-populations Descriptive Statistics

Overall @ Locus : 1

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	18	5.7143	0.3325	4.8889
(B, A)	9	3.5714	1.5398	-7.3934
(B, B)	5	143	1.9248	6.1091

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 3.797129
Degree of freedom : 1
Probability : 0.051341

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 3.604587
Degree of freedom : 1
Probability : 0.057620

Overall @ Locus : 2

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	23	18.6667	1.0060	9.6027
(B, A)	3	11.6667	6.4381	-8.1487
(B, B)	6	1.6667	11.2667	15.3712

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 18.710714
Degree of freedom : 1
Probability : 0.000015

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 16.825187
Degree of freedom : 1
Probability : 0.000041

Overall @ Locus : 3

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	25	22.7143	0.2300	4.7941
(B, A)	4	8.5714	2.4381	-6.0971
(B, B)	3	0.7143	7.3143	8.6105

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 9.982390
Degree of freedom : 1
Probability : 0.001580

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 7.307475
Degree of freedom : 1
Probability : 0.006867

Overall @ Locus : 4

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	25	21.0476	0.7422	8.6044
(B, A)	2	9.9048	6.3086	-6.3995
(B, B)	5	1.0476	14.9113	15.6292

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 21.962053
Degree of freedom : 1
Probability : 0.000003

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 17.834124
Degree of freedom : 1
Probability : 0.000024

Overall @ Locus : 5

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	17	15.0159	0.2622	4.2196
(B, A)	10	13.9683	1.1273	-6.6840
(B, B)	5	3.0159	1.3053	5.0555

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 2.694865
Degree of freedom : 1
Probability : 0.100672

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 2.591031
Degree of freedom : 1
Probability : 0.107470

Overall @ Locus : 6

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	28	26.2381	0.1183	3.6396
(B, A)	2	5.5238	2.2479	-4.0637
(B, B)	2	0.2381	13.0381	8.5129

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 15.404356
Degree of freedom : 1
Probability : 0.000087

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 8.088804
Degree of freedom : 1
Probability : 0.004454

Overall @ Locus : 7

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	31	31.0000	0.0000	0.0000
(B, A)	1	1.0000	0.0000	0.0000
(B, B)	0	0.0000	0.0000	0.0000

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 0.000000
Degree of freedom : 1
Probability : 1.000000

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 0.000000
Degree of freedom : 1
Probability : 1.000000

Overall @ Locus : 8

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	25	20.2381	1.1204	10.5655
(B, A)	1	10.5238	8.6188	-4.7073
(B, B)	6	1.2381	18.3150	18.9382

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 28.054299
Degree of freedom : 1
Probability : 0.000000

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 24.796399
Degree of freedom : 1
Probability : 0.000001

Overall @ Locus : 9

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	21	17.1587	0.8599	8.4846
(B, A)	5	12.6825	4.6538	-9.3079
(B, B)	6	2.1587	6.8352	12.2669

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 12.348887
Degree of freedom : 1
Probability : 0.000441

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 11.443633
Degree of freedom : 1
Probability : 0.000717

Overall @ Locus : 10

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	27	24.4444	0.2672	5.3694
(B, A)	2	7.1111	3.6736	-5.0740
(B, B)	3	0.4444	14.6944	11.4573

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 18.635227
Degree of freedom : 1
Probability : 0.000016

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 11.752640
Degree of freedom : 1
Probability : 0.000608

Overall @ Locus : 11

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	$2*O*Ln(O/E)$
(A, A)	30	28.0952	0.1291	3.9358
(B, A)	0	3.8095	3.8095	0.0000
(B, B)	2	0.0952	38.0952	12.1781

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 42.033898
Degree of freedom : 1
Probability : 0.000000

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 16.113927
Degree of freedom : 1
Probability : 0.000060

Overall @ Locus : 12

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	$2*O*Ln(O/E)$
(A, A)	29	29.0476	0.0001	-0.0952
(B, A)	3	2.9048	0.0031	0.1936
(B, B)	0	0.0476	0.0476	0.0000

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 0.050820
Degree of freedom : 1
Probability : 0.821643

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 0.098405
Degree of freedom : 1
Probability : 0.753752

Overall Allele Frequency :

Allele \ Locus	1	2	3	4	5	6	7	8
Allele A	0.7031	0.7656	0.8438	0.8125	0.6875	0.9062	0.9844	0.7969
Allele B	0.2969	0.2344	0.1562	0.1875	0.3125	0.0938	0.0156	0.2031

Allele \ Locus	9	10	11	12
Allele A	0.7344	0.8750	0.9375	0.9531
Allele B	0.2656	0.1250	0.0625	0.0469

Overall Summary Statistics:

Summary of Genic Variation Statistics for All Loci

Locus	Sample Size	na*	ne*	I*
1	64	2.0000	1.7167	0.6082
2	64	2.0000	1.5598	0.5445
3	64	2.0000	1.3581	0.4334
4	64	2.0000	1.4382	0.4826
5	64	2.0000	1.7534	0.6211
6	64	2.0000	1.2047	0.3111
7	64	2.0000	1.0317	0.0805
8	64	2.0000	1.4787	0.5047
9	64	2.0000	1.6397	0.5789
10	64	2.0000	1.2800	0.3768
11	64	2.0000	1.1327	0.2338
12	64	2.0000	1.0981	0.1892
Mean	64	2.0000	1.3910	0.4137
St. Dev		0.0000	0.2463	0.1769

[See Nei (1987) Molecular Evolutionary Genetics (p. 176-187)]
* na = Observed number of alleles
* ne = Effective number of alleles [Kimura and Crow (1964)]
* I = Shannon's Information index [Lewontin (1972)]

Summary of Heterozygosity Statistics for All Loci

Locus	Sample Size	Obs_Hom	Obs_Het	Exp_Hom*	Exp_Het*	Nei**
1	64	0.7188	0.2812	0.5759	0.4241	0.4175	0.4031
2	64	0.9062	0.0938	0.6354	0.3646	0.3589	0.3146
3	64	0.8750	0.1250	0.7321	0.2679	0.2637	0.2363
4	64	0.9375	0.0625	0.6905	0.3095	0.3047	0.3133
5	64	0.6875	0.3125	0.5635	0.4365	0.4297	0.3690
6	64	0.9375	0.0625	0.8274	0.1726	0.1699	0.1628
7	64	0.9688	0.0312	0.9688	0.0312	0.0308	0.0230
8	64	0.9688	0.0312	0.6711	0.3289	0.3237	0.2707
9	64	0.8438	0.1562	0.6037	0.3963	0.3901	0.3631
10	64	0.9375	0.0625	0.7778	0.2222	0.2188	0.1870
11	64	1.0000	0.0000	0.8810	0.1190	0.1172	0.1250
12	64	0.9062	0.0938	0.9092	0.0908	0.0894	0.1016
Mean	64	0.8906	0.1094	0.7364	0.2636	0.2595	0.2391
St. Dev		0.0975	0.0975	0.1364	0.1364	0.1343	0.1201

* Expected homozygosty and heterozygosity were computed using Levene (1949)
** Nei's (1973) expected heterozygosity

The number of polymorphic loci is : 12
The percentage of polymorphic loci is : 100.00 %

Wright's (1978) inbreeding coefficient (Fis) as a measure of heterozygote deficiency or excess

Allele \ Locus	1	2	3	4	5	6	7	8
Allele A	0.3263	0.7388	0.5259	0.7949	0.2727	0.6322	-0.0159	0.9035
Allele B	0.3263	0.7388	0.5259	0.7949	0.2727	0.6322	-0.0159	0.9035
Total	0.3263	0.7388	0.5259	0.7949	0.2727	0.6322	-0.0159	0.9035

Allele \ Locus	9	10	11	12
Allele A	0.5995	0.7143	1.0000	-0.0492
Allele B	0.5995	0.7143	1.0000	-0.0492
Total	0.5995	0.7143	1.0000	-0.0492

Summary of F-Statistics and Gene Flow for All Loci

Locus	Sample Size	Fis	Fit	Fst	Nm*
1	64	0.3089	0.3175	0.0125	19.7500
2	64	0.7162	0.7354	0.0675	3.4513
3	64	0.4811	0.4976	0.0317	7.6331
4	64	0.7872	0.7898	0.0121	20.4386
5	64	0.1581	0.2489	0.1079	2.0666
6	64	0.5979	0.5998	0.0047	53.0954
7	64	-0.0370	-0.0120	0.0241	10.1250
8	64	0.8461	0.8901	0.2859	0.6244
9	64	0.6459	0.6593	0.0379	6.3435
10	64	0.6499	0.7536	0.2962	0.5942
11	64	1.0000	1.0000	0.1818	1.1250
12	64	-0.2308	-0.0667	0.1333	1.6250
Mean	64	0.5332	0.5774	0.0947	2.3913

[See Nei (1987) Molecular Evolutionary Genetics (p. 159-164)]
* Nm = Gene flow estimated from Fst = 0.25(1 - Fst)/Fst.

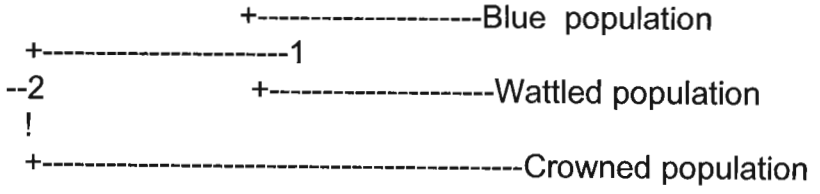
Nei's Original Measures of Genetic Identity and Genetic distance

pop ID	1	2	3
1	****	0.9690	0.9226
2	0.0315	****	0.9497
3	0.0806	0.0516	****

[See Nei (1972) Am. Nat. 106:283-292)]
Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Dendrogram Based Nei's (1972) Genetic distance: Method = UPGMA

--Modified from NEIGHBOR procedure of PHYLIP Version 3.5



*** File Name: dgram1.plt**

Between	And	Length
2	1	1.73112
1	Blue	1.57360
1	Wattled	1.57360
2	Crowned	3.30472

2. BLUE CRANE MICROSATELLITE INDIVIDUAL ANALYSIS

Multi-populations Descriptive Statistics

* Overall @ Locus : 1 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	7	5.6667	0.3137	2.9583
(B, A)	4	6.6667	1.0667	-4.0866
(B, B)	3	1.6667	1.0667	3.5267

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 2.447059
Degree of freedom : 1
Probability : 0.117745

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 2.398442
Degree of freedom : 1
Probability : 0.121456

* Overall @ Locus : 2 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	8	5.6667	0.9608	5.5174
(B, A)	2	6.6667	3.2667	-4.8159
(B, B)	4	1.6667	3.2667	7.0037

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 7.494118
Degree of freedom : 1
Probability : 0.006190

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 7.705306
Degree of freedom : 1
Probability : 0.005506

* Overall @ Locus : 3 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	10	8.5556	0.2439	3.1201
(B, A)	2	4.8889	1.7071	-3.5753
(B, B)	2	0.5556	3.7556	5.1237

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 5.706494
Degree of freedom : 1
Probability : 0.016902

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 4.668549
Degree of freedom : 1
Probability : 0.030720

* Overall @ Locus : 4 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	12	10.2222	0.3092	3.8482
(B, A)	0	3.5556	3.5556	0.0000
(B, B)	2	0.2222	14.2222	8.7889

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 18.086957
Degree of freedom : 1
Probability : 0.000021

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 12.637122
Degree of freedom : 1
Probability : 0.000378

* Overall @ Locus : 5 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	5	3.8889	0.3175	2.5131
(B, A)	5	7.2222	0.6838	-3.6772
(B, B)	4	2.8889	0.4274	2.6034

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 1.428571
Degree of freedom : 1
Probability : 0.231998

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 1.439276
Degree of freedom : 1
Probability : 0.230257

* Overall @ Locus : 6 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	12	11.1111	0.0711	1.8471
(B, A)	1	2.7778	1.1378	-2.0433
(B, B)	1	0.1111	7.1111	4.3944

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 8.320000
Degree of freedom : 1
Probability : 0.003921

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 4.198212
Degree of freedom : 1
Probability : 0.040467

* Overall @ Locus : 7 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	13	13.0000	0.0000	0.0000
(B, A)	1	1.0000	0.0000	0.0000
(B, B)	0	0.0000	0.0000	0.0000

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 0.000000
Degree of freedom : 1
Probability : 1.000000

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 0.000000
Degree of freedom : 1
Probability : 1.000000

* Overall @ Locus : 8 *

Monomorphic locus: No further analysis !!!

* Overall @ Locus : 9 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	7	5.6667	0.3137	2.9583
(B, A)	4	6.6667	1.0667	-4.0866
(B, B)	3	1.6667	1.0667	3.5267

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 2.447059
Degree of freedom : 1
Probability : 0.117745

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 2.398442
Degree of freedom : 1
Probability : 0.121456

* Overall @ Locus : 10 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	13	13.0000	0.0000	0.0000
(B, A)	1	1.0000	0.0000	0.0000
(B, B)	0	0.0000	0.0000	0.0000

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 0.000000
Degree of freedom : 1
Probability : 1.000000

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 0.000000
Degree of freedom : 1
Probability : 1.000000

* Overall @ Locus : 11 *

Monomorphic locus: No further analysis !!!

* Overall @ Locus : 12 *

Monomorphic locus: No further analysis !!!

Overall Allele Frequency :

Allele \ Locus	1	2	3	4	5	6	7	8
Allele A	0.6429	0.6429	0.7857	0.8571	0.5357	0.8929	0.9643	1.0000
Allele B	0.3571	0.3571	0.2143	0.1429	0.4643	0.1071	0.0357	

Allele \ Locus	9	10	11	12
Allele A	0.6429	0.9643	1.0000	1.0000
Allele B	0.3571	0.0357		

Overall Summary Statistics:

Summary of Genic Variation Statistics for All Loci

Locus	Sample Size	na*	ne*	I*
1	28	2.0000	1.8491	0.6518
2	28	2.0000	1.8491	0.6518
3	28	2.0000	1.5077	0.5196
4	28	2.0000	1.3243	0.4101
5	28	2.0000	1.9898	0.6906
6	28	2.0000	1.2366	0.3405
7	28	2.0000	1.0740	0.1541
8	28	1.0000	1.0000	0.0000
9	28	2.0000	1.8491	0.6518
10	28	2.0000	1.0740	0.1541
11	28	1.0000	1.0000	0.0000
12	28	1.0000	1.0000	0.0000

Mean	28	1.7500	1.3961	0.3520
St. Dev		0.4523	0.3919	0.2806

[See Nei (1987) Molecular Evolutionary Genetics (p. 176-187)]
* na = Observed number of alleles
* ne = Effective number of alleles [Kimura and Crow (1964)]
* I = Shannon's Information index [Lewontin (1972)]

Summary of Heterozygosity Statistics for All Loci

Locus	Sample Size	Obs_Hom	Obs_Het	Exp_Hom*	Exp_Het*	Nei**	Ave_Het
1	28	0.7143	0.2857	0.5238	0.4762	0.4592	0.1429
2	28	0.8571	0.1429	0.5238	0.4762	0.4592	0.0714
3	28	0.8571	0.1429	0.6508	0.3492	0.3367	0.0714
4	28	1.0000	0.0000	0.7460	0.2540	0.2449	0.0000
5	28	0.6429	0.3571	0.4841	0.5159	0.4974	0.1786
6	28	0.9286	0.0714	0.8016	0.1984	0.1913	0.0357
7	28	0.9286	0.0714	0.9286	0.0714	0.0689	0.0357
8	28	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
9	28	0.7143	0.2857	0.5238	0.4762	0.4592	0.1429
10	28	0.9286	0.0714	0.9286	0.0714	0.0689	0.0357
11	28	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
12	28	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
Mean	28	0.8810	0.1190	0.7593	0.2407	0.2321	0.0595
St. Dev		0.1268	0.1268	0.2104	0.2104	0.2029	0.0634

* Expected homozygosty and heterozygosity were computed using Levene (1949)
** Nei's (1973) expected heterozygosity

The number of polymorphic loci is : 9
The percentage of polymorphic loci is : 75.00 %

Wright's (1978) inbreeding coefficient (Fis) as a measure of heterozygote deficiency or excess

Allele \ Locus	1	2	3	4	5	6	7	8
Allele A	0.3778	0.6889	0.5758	1.0000	0.2821	0.6267	-0.0370	****
Allele B	0.3778	0.6889	0.5758	1.0000	0.2821	0.6267	-0.0370	****
Total	0.3778	0.6889	0.5758	1.0000	0.2821	0.6267	-0.0370	****

Allele \ Locus	9	10	11	12
Allele A	0.3778	-0.0370	****	****
Allele B	0.3778	-0.0370	****	****
Total	0.3778	-0.0370	****	****

Summary of F-Statistics and Gene Flow for All Loci

Locus	Sample Size	Fis	Fit	Fst	Nm*
1	28	-1.0000	0.3778	0.6889	0.1129
2	28	-1.0000	0.6889	0.8444	0.0461
3	28	-1.0000	0.5758	0.7879	0.0673
4	28	****	1.0000	1.0000	0.0000
5	28	-1.0000	0.2821	0.6410	0.1400
6	28	-1.0000	0.6267	0.8133	0.0574
7	28	-1.0000	-0.0370	0.4815	0.2692
8	28	****	****	0.0000	****
9	28	-1.0000	0.3778	0.6889	0.1129
10	28	-1.0000	-0.0370	0.4815	0.2692
11	28	****	****	0.0000	****
12	28	****	****	0.0000	****
Mean	28	-1.0000	0.4872	0.7436	0.0862

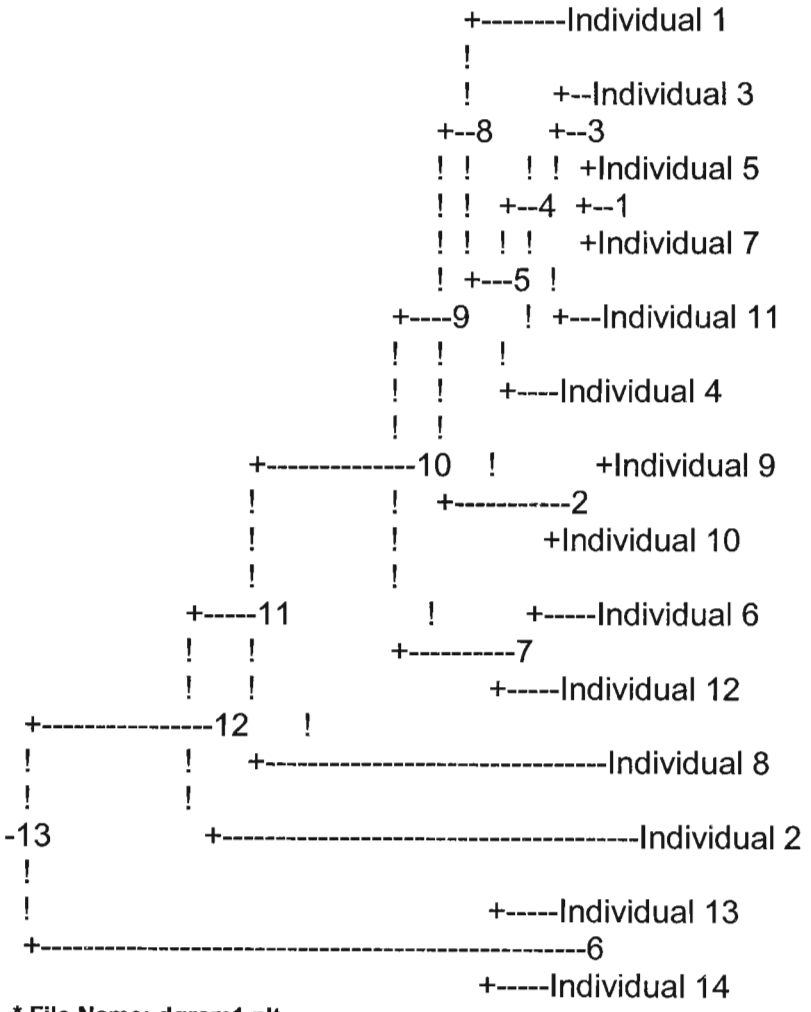
[See Nei (1987) Molecular Evolutionary Genetics (p. 159-164)]
* Nm = Gene flow estimated from Fst = 0.25(1 - Fst)/Fst.

Nei's Original Measures of Genetic Identity and Genetic distance

ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	****	0.6963	0.8704	0.9574	0.9336	0.8002	0.9336	0.6963	0.8581	0.8581	0.9545	0.8374	0.6364	0.6963
2	0.3620	****	0.6667	0.7500	0.7236	0.7236	0.7236	0.7500	0.8216	0.8216	0.6963	0.6682	0.6963	0.5833
3	0.1388	0.4055	****	0.9167	0.9789	0.8938	0.9789	0.7500	0.9129	0.9129	0.9574	0.9354	0.5222	0.5833
4	0.0435	0.2877	0.0870	****	0.9789	0.8087	0.9789	0.6667	0.9129	0.9129	0.9574	0.8463	0.6093	0.6667
5	0.0687	0.3236	0.0213	0.0213	****	0.8696	1.0000	0.7236	0.9325	0.9325	0.9780	0.9100	0.5779	0.6384
6	0.2229	0.3236	0.1123	0.2123	0.1398	****	0.8696	0.8938	0.8859	0.8859	0.8891	0.9555	0.5779	0.5533
7	0.0687	0.3236	0.0213	0.0213	0.0000	0.1398	****	0.7236	0.9325	0.9325	0.9780	0.9100	0.5779	0.6384
8	0.3620	0.2877	0.2877	0.4055	0.3236	0.1123	0.3236	****	0.8216	0.8216	0.7833	0.8463	0.6963	0.6667
9	0.1530	0.1965	0.0912	0.0912	0.0699	0.1212	0.0699	0.1965	****	1.0000	0.9058	0.8783	0.7151	0.7303
10	0.1530	0.1965	0.0912	0.0912	0.0699	0.1212	0.0699	0.1965	0.0000	****	0.9058	0.8783	0.7151	0.7303
11	0.0465	0.3620	0.0435	0.0435	0.0222	0.1175	0.0222	0.2442	0.0989	0.0989	****	0.9305	0.6364	0.6963
12	0.1774	0.4032	0.0668	0.1668	0.0943	0.0455	0.0943	0.1668	0.1298	0.1298	0.0721	****	0.5583	0.5791
13	0.4520	0.3620	0.6496	0.4955	0.5483	0.5483	0.5483	0.3620	0.3353	0.3353	0.4520	0.5829	****	0.9574
14	0.3620	0.5390	0.5390	0.4055	0.4487	0.5918	0.4487	0.4055	0.3143	0.3143	0.3620	0.5463	0.0435	****

[See Nei (1972) Am. Nat. 106:283-292)]
Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Dendrogram Based Nei's (1972) Genetic distance: Method = UPGMA
--Modified from NEIGHBOR procedure of PHYLIP Version 3.5



*** File Name: dgram1.plt**

Between	And	Length
13	12	7.02906
12	11	2.68776
11	10	6.47389
10	9	1.83512
9	8	1.12040
8	Indiv 1	3.66333
8	5	1.49988
5	4	0.69750
4	3	0.40197
3	Indiv 3	1.06399
3	1	1.06399
1	Indiv 5	0.00000
1	Indiv 7	0.00000
4	Indiv 11	1.46596
5	Indiv 4	2.16346
9	2	4.78373
2	Indiv 9	0.00000

Between	And	Length
2	Indiv 10	0.00000
10	7	4.34455
7	Indiv 6	2.27429
7	Indiv 12	2.27429
11	Indiv 8	13.09274
12	Indiv 2	15.78050
13	6	20.63428
6	Indiv 13	2.17528
6	Indiv 14	2.17528

3. CROWNED CRANE MICROSATELLITE INDIVIDUAL ANALYSIS

Multi-populations Descriptive Statistics

* Overall @ Locus : 1 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	6	5.5263	0.0406	0.9869
(B, A)	3	3.9474	0.2274	-1.6466
(B, B)	1	0.5263	0.4263	1.2837

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 0.694286
Degree of freedom : 1
Probability : 0.404710

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 0.623944
Degree of freedom : 1
Probability : 0.429585

* Overall @ Locus : 2 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	9	8.0526	0.1115	2.0021
(B, A)	0	1.8947	1.8947	0.0000
(B, B)	1	0.0526	17.0526	5.8889

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 19.058824
Degree of freedom : 1
Probability : 0.000013

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 7.890939
Degree of freedom : 1
Probability : 0.004968

* Overall @ Locus : 3 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	8	7.1579	0.0991	1.7796
(B, A)	1	2.6842	1.0568	-1.9748
(B, B)	1	0.1579	4.4912	3.6917

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 5.647059
Degree of freedom : 1
Probability : 0.017485

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 3.496490
Degree of freedom : 1
Probability : 0.061499

* Overall @ Locus : 4 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	7	6.3158	0.0741	1.4400
(B, A)	2	3.3684	0.5559	-2.0852
(B, B)	1	0.3158	1.4825	2.3054

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 2.112500
Degree of freedom : 1
Probability : 0.146100

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 1.660175
Degree of freedom : 1
Probability : 0.197580

* Overall @ Locus : 5 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	8	8.0526	0.0003	-0.1049
(B, A)	2	1.8947	0.0058	0.2163
(B, B)	0	0.0526	0.0526	0.0000

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 0.058824
Degree of freedom : 1
Probability : 0.808365

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 0.111350
Degree of freedom : 1
Probability : 0.738612

* Overall @ Locus : 6 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	9	8.0526	0.1115	2.0021
(B, A)	0	1.8947	1.8947	0.0000
(B, B)	1	0.0526	17.0526	5.8889

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 19.058824
Degree of freedom : 1
Probability : 0.000013

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 7.890939
Degree of freedom : 1
Probability : 0.004968

* Overall @ Locus : 7 *

Monomorphic locus: No further analysis !!!

* Overall @ Locus : 8 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	8	6.3158	0.4491	3.7822
(B, A)	0	3.3684	3.3684	0.0000
(B, B)	2	0.3158	8.9825	7.3833

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 12.800000
Degree of freedom : 1
Probability : 0.000347

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 11.165527
Degree of freedom : 1
Probability : 0.000833

* Overall @ Locus : 9 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	8	7.1579	0.0991	1.7796
(B, A)	1	2.6842	1.0568	-1.9748
(B, B)	1	0.1579	4.4912	3.6917

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 5.647059
Degree of freedom : 1
Probability : 0.017485

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 3.496490
Degree of freedom : 1
Probability : 0.061499

* Overall @ Locus : 10 *

Monomorphic locus: No further analysis !!!

* Overall @ Locus : 11 *

Monomorphic locus: No further analysis !!!

* Overall @ Locus : 12 *

Monomorphic locus: No further analysis !!!

Overall Allele Frequency :

Allele \ Locus	1	2	3	4	5	6	7	8
Allele A	0.7500	0.9000	0.8500	0.8000	0.9000	0.9000	1.0000	0.8000
Allele B	0.2500	0.1000	0.1500	0.2000	0.1000	0.1000		0.2000

Allele \ Locus	9	10	11	12
Allele A	0.8500	1.0000	1.0000	1.0000
Allele B	0.1500			

Overall Summary Statistics:

Summary of Genic Variation Statistics for All Loci

Locus	Sample Size	na*	ne*	I*
1	20	2.0000	1.6000	0.5623
2	20	2.0000	1.2195	0.3251
3	20	2.0000	1.3423	0.4227
4	20	2.0000	1.4706	0.5004
5	20	2.0000	1.2195	0.3251
6	20	2.0000	1.2195	0.3251
7	20	1.0000	1.0000	0.0000
8	20	2.0000	1.4706	0.5004
9	20	2.0000	1.3423	0.4227
10	20	1.0000	1.0000	0.0000
11	20	1.0000	1.0000	0.0000
12	20	1.0000	1.0000	0.0000
Mean	20	1.6667	1.2404	0.2820
St. Dev		0.4924	0.2107	0.2210

[See Nei (1987) Molecular Evolutionary Genetics (p. 176-187)]
* na = Observed number of alleles
* ne = Effective number of alleles [Kimura and Crow (1964)]
* I = Shannon's Information index [Lewontin (1972)]

Summary of Heterozygosity Statistics for All Loci

Locus	Sample Size	Obs_Hom	Obs_Het	Exp_Hom*	Exp_Het*	Nei**	Ave_Het
1	20	0.7000	0.3000	0.6053	0.3947	0.3750	0.1500
2	20	1.0000	0.0000	0.8105	0.1895	0.1800	0.0000
3	20	0.9000	0.1000	0.7316	0.2684	0.2550	0.0500
4	20	0.8000	0.2000	0.6632	0.3368	0.3200	0.1000
5	20	0.8000	0.2000	0.8105	0.1895	0.1800	0.1000
6	20	1.0000	0.0000	0.8105	0.1895	0.1800	0.0000
7	20	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
8	20	1.0000	0.0000	0.6632	0.3368	0.3200	0.0000
9	20	0.9000	0.1000	0.7316	0.2684	0.2550	0.0500
10	20	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
11	20	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
12	20	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
Mean	20	0.9250	0.0750	0.8189	0.1811	0.1721	0.0375
St. Dev		0.1055	0.1055	0.1480	0.1480	0.1406	0.0528

Expected homozygosty and heterozygosity were computed using Levene (1949)
** Nei's (1973) expected heterozygosity

The number of polymorphic loci is : 8
The percentage of polymorphic loci is : 66.67 %

Wright's (1978) inbreeding coefficient (Fis) as a measure of heterozygote deficiency or excess

Allele \ Locus	1	2	3	4	5	6	7	8
Allele A	0.2000	1.0000	0.6078	0.3750	-0.1111	1.0000	****	1.0000
Allele B	0.2000	1.0000	0.6078	0.3750	-0.1111	1.0000	****	1.0000
Total	0.2000	1.0000	0.6078	0.3750	-0.1111	1.0000	****	1.0000

Allele \ Locus	9	10	11	12
Allele A	0.6078	****	****	****
Allele B	0.6078	****	****	****
Total	0.6078	****	****	****

Summary of F-Statistics and Gene Flow for All Loci

Locus	Sample Size	Fis	Fit	Fst	Nm*
1	20	-1.0000	0.2000	0.6000	0.1667
2	20	****	1.0000	1.0000	0.0000
3	20	-1.0000	0.6078	0.8039	0.0610
4	20	-1.0000	0.3750	0.6875	0.1136
5	20	-1.0000	-0.1111	0.4444	0.3125
6	20	****	1.0000	1.0000	0.0000
7	20	****	****	0.0000	****
8	20	****	1.0000	1.0000	0.0000
9	20	-1.0000	0.6078	0.8039	0.0610
10	20	****	****	0.0000	****
11	20	****	****	0.0000	****
12	20	****	****	0.0000	****
Mean	20	-1.0000	0.5642	0.7821	0.0697

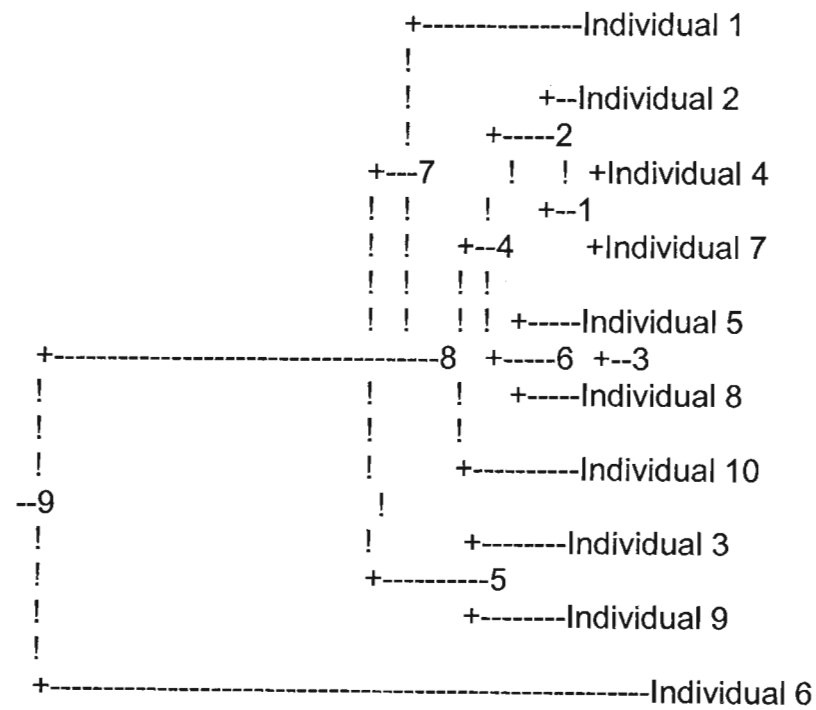
[See Nei (1987) Molecular Evolutionary Genetics (p. 159-164)]
* Nm = Gene flow estimated from Fst = 0.25(1 - Fst)/Fst.

Nei's Original Measures of Genetic Identity and Genetic distance

ID	1	2	3	4	5	6	7	8	9	10
1	****	0.8333	0.8087	0.8938	0.8938	0.5833	0.8938	0.8938	0.7833	0.8704
2	0.1823	****	0.8938	0.9789	0.8938	0.5833	0.9789	0.8938	0.7833	0.8704
3	0.2123	0.1123	****	0.9130	0.8696	0.5533	0.9130	0.8696	0.9336	0.8447
4	0.1123	0.0213	0.0910	****	0.9565	0.6384	1.0000	0.9565	0.8447	0.9336
5	0.1123	0.1123	0.1398	0.0445	****	0.6384	0.9565	0.9565	0.8891	0.9336
6	0.5390	0.5390	0.5918	0.4487	0.4487	****	0.6384	0.7236	0.6093	0.7833
7	0.1123	0.0213	0.0910	0.0000	0.0445	0.4487	****	0.9565	0.8447	0.9336
8	0.1123	0.1123	0.1398	0.0445	0.0445	0.3236	0.0445	****	0.8447	0.9336
9	0.2442	0.2442	0.0687	0.1688	0.1175	0.4955	0.1688	0.1688	****	0.8636
10	0.1388	0.1388	0.1688	0.0687	0.0687	0.2442	0.0687	0.0687	0.1466	****

[See Nei (1972) Am. Nat. 106:283-292)]
Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Dendrogram Based Nei's (1972) Genetic distance: Method = UPGMA
--Modified from NEIGHBOR procedure of PHYLIP Version 3.5



*** File Name: dgram1.plt**

Between	And	Length
9	8	14.75566
8	7	1.48879
7	Indiv 1	6.41786
7	6	2.27987
6	4	0.78541
4	2	2.28859
2	Indiv 2	1.06399
2	1	1.06399
1	Indiv 4	0.00000
1	Indiv 7	0.00000
4	3	1.13000
3	Indiv 5	2.22259
3	Indiv 8	2.22259
6	Indiv 10	4.13799
8	5	4.46936
5	Indiv 3	3.43730
5	Indiv 9	3.43730
9	Indiv 6	22.66232

4. Wattled Crane Microsatellite Individual analysis

Multi-populations Descriptive Statistics

* Overall @ Locus : 1 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	5	4.4000	0.0818	1.2783
(B, A)	2	3.2000	0.4500	-1.8800
(B, B)	1	0.4000	0.9000	1.8326

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 1.431818
Degree of freedom : 1
Probability : 0.231468

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 1.230901
Degree of freedom : 1
Probability : 0.267232

* Overall @ Locus : 2 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	6	5.2000	0.1231	1.7172
(B, A)	1	2.6000	0.9846	-1.9110
(B, B)	1	0.2000	3.2000	3.2189

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 4.307692
Degree of freedom : 1
Probability : 0.037940

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 3.025063
Degree of freedom : 1
Probability : 0.081987

* Overall @ Locus : 3 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	7	7.0000	0.0000	0.0000
(B, A)	1	1.0000	0.0000	0.0000
(B, B)	0	0.0000	0.0000	0.0000

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 0.000000
Degree of freedom : 1
Probability : 1.000000

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 0.000000
Degree of freedom : 1
Probability : 1.000000

* Overall @ Locus : 4 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	6	4.4000	0.5818	3.7219
(B, A)	0	3.2000	3.2000	0.0000
(B, B)	2	0.4000	6.4000	6.4378

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 10.181818
Degree of freedom : 1
Probability : 0.001418

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 10.159611
Degree of freedom : 1
Probability : 0.001436

* Overall @ Locus : 5 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	4	3.6667	0.0303	0.6961
(B, A)	3	3.6667	0.1212	-1.2040
(B, B)	1	0.6667	0.1667	0.8109

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 0.318182
Degree of freedom : 1
Probability : 0.572702

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 0.302997
Degree of freedom : 1
Probability : 0.582010

* Overall @ Locus : 6 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	7	7.0000	0.0000	0.0000
(B, A)	1	1.0000	0.0000	0.0000
(B, B)	0	0.0000	0.0000	0.0000

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 0.000000
Degree of freedom : 1
Probability : 1.000000

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 0.000000
Degree of freedom : 1
Probability : 1.000000

* Overall @ Locus : 7 *

Monomorphic locus: No further analysis !!!

* Overall @ Locus : 8 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	3	1.4000	1.8286	4.5728
(B, A)	1	4.2000	2.4381	-2.8702
(B, B)	4	2.4000	1.0667	4.0866

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 5.333333
Degree of freedom : 1
Probability : 0.020921

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 5.789276
Degree of freedom : 1

Probability : 0.016124

* Overall @ Locus : 9 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	6	4.4000	0.5818	3.7219
(B, A)	0	3.2000	3.2000	0.0000
(B, B)	2	0.4000	6.4000	6.4378

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 10.181818
Degree of freedom : 1
Probability : 0.001418

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 10.159611
Degree of freedom : 1
Probability : 0.001436

* Overall @ Locus : 10 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	4	2.4000	1.0667	4.0866
(B, A)	1	4.2000	2.4381	-2.8702
(B, B)	3	1.4000	1.8286	4.5728

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 5.333333
Degree of freedom : 1
Probability : 0.020921

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 5.789276
Degree of freedom : 1
Probability : 0.016124

* Overall @ Locus : 11 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	6	4.4000	0.5818	3.7219
(B, A)	0	3.2000	3.2000	0.0000
(B, B)	2	0.4000	6.4000	6.4378

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 10.181818
Degree of freedom : 1
Probability : 0.001418

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 10.159611
Degree of freedom : 1
Probability : 0.001436

* Overall @ Locus : 12 *

Genotypes	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln(O/E)
(A, A)	5	5.2000	0.0077	-0.3922
(B, A)	3	2.6000	0.0615	0.8586
(B, B)	0	0.2000	0.2000	0.0000

Chi-square test for Hardy-Weinberg equilibrium :

Chi-square : 0.269231
Degree of freedom : 1
Probability : 0.603848

Likelihood ratio test for Hardy-Weinberg equilibrium :

G-square : 0.466398
Degree of freedom : 1
Probability : 0.494649

Overall Allele Frequency :

Allele \ Locus	1	2	3	4	5	6	7	8
Allele A	0.7500	0.8125	0.9375	0.7500	0.6875	0.9375	1.0000	0.4375
Allele B	0.2500	0.1875	0.0625	0.2500	0.3125	0.0625		0.5625

Allele \ Locus	9	10	11	12
Allele A	0.7500	0.5625	0.7500	0.8125
Allele B	0.2500	0.4375	0.2500	0.1875

Overall Summary Statistics:

Summary of Genic Variation Statistics for All Loci

Locus	Sample Size	na*	ne*	I*
1	16	2.0000	1.6000	0.5623
2	16	2.0000	1.4382	0.4826
3	16	2.0000	1.1327	0.2338
4	16	2.0000	1.6000	0.5623
5	16	2.0000	1.7534	0.6211
6	16	2.0000	1.1327	0.2338
7	16	1.0000	1.0000	0.0000
8	16	2.0000	1.9692	0.6853
9	16	2.0000	1.6000	0.5623
10	16	2.0000	1.9692	0.6853
11	16	2.0000	1.6000	0.5623
12	16	2.0000	1.4382	0.4826
Mean	16	1.9167	1.5195	0.4728
St. Dev		0.2887	0.3115	0.2094

[See Nei (1987) Molecular Evolutionary Genetics (p. 176-187)]
* na = Observed number of alleles
* ne = Effective number of alleles [Kimura and Crow (1964)]
* I = Shannon's Information index [Lewontin (1972)]

Summary of Heterozygosity Statistics for All Loci

Locus	Sample Size	Obs_Hom	Obs_Het	Exp_Hom*	Exp_Het*	Nei**	Ave_Het
1	16	0.7500	0.2500	0.6000	0.4000	0.3750	0.1250
2	16	0.8750	0.1250	0.6750	0.3250	0.3047	0.0625
3	16	0.8750	0.1250	0.8750	0.1250	0.1172	0.0625
4	16	1.0000	0.0000	0.6000	0.4000	0.3750	0.0000
5	16	0.6250	0.3750	0.5417	0.4583	0.4297	0.1875
6	16	0.8750	0.1250	0.8750	0.1250	0.1172	0.0625
7	16	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
8	16	0.8750	0.1250	0.4750	0.5250	0.4922	0.0625
9	16	1.0000	0.0000	0.6000	0.4000	0.3750	0.0000
10	16	0.8750	0.1250	0.4750	0.5250	0.4922	0.0625
11	16	1.0000	0.0000	0.6000	0.4000	0.3750	0.0000
12	16	0.6250	0.3750	0.6750	0.3250	0.3047	0.1875
Mean	16	0.8646	0.1354	0.6660	0.3340	0.3132	0.0677
St. Dev		0.1355	0.1355	0.1665	0.1665	0.1561	0.0677

Expected homozygosty and heterozygosity were computed using Levene (1949)
** Nei's (1973) expected heterozygosity

The number of polymorphic loci is : 11
The percentage of polymorphic loci is : 91.67 %

Wright's (1978) inbreeding coefficient (Fis) as a measure of heterozygote deficiency or excess

Allele \ Locus	1	2	3	4	5	6	7	8
Allele A	0.3333	0.5897	-0.0667	1.0000	0.1273	-0.0667	****	0.7460
Allele B	0.3333	0.5897	-0.0667	1.0000	0.1273	-0.0667	****	0.7460
Total	0.3333	0.5897	-0.0667	1.0000	0.1273	-0.0667	****	0.7460

Allele \ Locus	9	10	11	12
Allele A	1.0000	0.7460	1.0000	-0.2308
Allele B	1.0000	0.7460	1.0000	-0.2308
Total	1.0000	0.7460	1.0000	-0.2308

Summary of F-Statistics and Gene Flow for All Loci

Locus	Sample Size	Fis	Fit	Fst	Nm*
1	16	-1.0000	0.3333	0.6667	0.1250
2	16	-1.0000	0.5897	0.7949	0.0645
3	16	-1.0000	-0.0667	0.4667	0.2857
4	16	****	1.0000	1.0000	0.0000
5	16	-1.0000	0.1273	0.5636	0.1935
6	16	-1.0000	-0.0667	0.4667	0.2857
7	16	****	****	0.0000	****
8	16	-1.0000	0.7460	0.8730	0.0364
9	16	****	1.0000	1.0000	0.0000
10	16	-1.000	0.7460	0.8730	0.0364
11	16	****	1.0000	1.0000	0.0000
12	16	-1.0000	-0.2308	0.3846	0.4000
Mean	16	-1.0000	0.5676	0.7838	0.0690

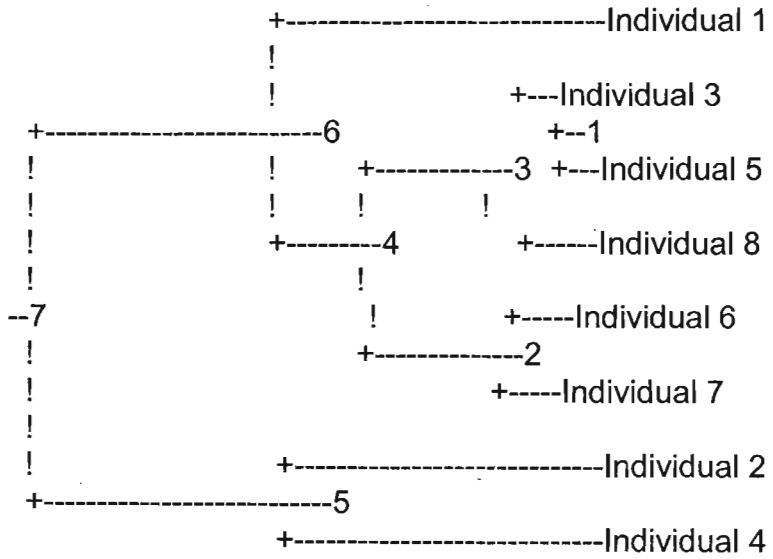
[See Nei (1987) Molecular Evolutionary Genetics (p. 159-164)]
* Nm = Gene flow estimated from Fst = 0.25(1 - Fst)/Fst.

Nei's Original Measures of Genetic Identity and Genetic distance

ID	1	2	3	4	5	6	7	8
1	****	0.5779	0.7557	0.4187	0.7557	0.6963	0.6979	0.7273
2	0.5483	****	0.6087	0.7280	0.5217	0.5533	0.5460	0.4890
3	0.2801	0.4964	****	0.5005	0.9565	0.8087	0.7735	0.9336
4	0.8706	0.3174	0.6921	****	0.5460	0.6682	0.7143	0.4652
5	0.2801	0.6506	0.0445	0.6051	****	0.8938	0.8190	0.9336
6	0.3620	0.5918	0.2123	0.4032	0.1123	****	0.9354	0.7833
7	0.3597	0.6051	0.2568	0.3365	0.1996	0.0668	****	0.7444
8	0.3185	0.7154	0.0687	0.7652	0.0687	0.2442	0.2952	****

[See Nei (1972) Am. Nat. 106:283-292)]
Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Dendrogram Based Nei's (1972) Genetic distance: Method = UPGMA
--Modified from NEIGHBOR procedure of PHYLIP Version 3.5



*** File Name: dgram1.plt**

Between	And	Length
7	6	14.33213
6	Indiv 1	16.00255
6	4	4.99932
4	3	7.56594
3	1	1.21471
1	Indiv 3	2.22259
1	Indiv 5	2.22259
3	Indiv 8	3.43730
4	2	7.66495
2	Indiv 6	3.33828
2	Indiv 7	3.33828
7	5	14.46370
5	Indiv 2	15.87098
5	Indiv 4	15.87098