Assessment of Genetic Diversity and DNA Fingerprinting of the Cape Parrot (*Poicephalus robustus*) Using Randomly Amplified Polymorphic DNA (RAPD)

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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 others is acknowledged in the text, are the results of my own investigation.

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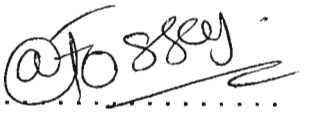
Gillian Blue

April 2004

I certify the above statement is correct.



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Professor Annabel Fossey

Supervisor

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LIST OF ABBREVIATIONS

AFLPs	amplified fragment length polymorphisms
AMOVA	analysis of molecular variance
bp	base pairs
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
dH_2O	distilled water
DNA	deoxyribose nucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylene diamine tetra-acetic acid di-sodium salt
GC	guanine and cytosine
ICBP	International Council for Bird Preservation
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
NJ	neighbour joining
OD	optical density
PBFDV	psittacine beak and feather disease virus
PCR	polymerase chain reaction
RAPDs	randomly amplified polymorphic DNA
RFLPs	restriction fragment length polymorphisms
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SNPs	single nucleotide polymorphisms
SPSS	statistical processing for the social sciences
SSRs	simple sequence repeats
TAE buffer	tris-sodium acetate-ethylene diamine tetra-acetic acid di-sodium salt
TBE buffer	tris-boric acid-ethylene diamine tetra-acetic acid di-sodium salt
TE buffer	tris-ethylene diamine tetra-acetic acid di-sodium salt
TNE buffer	tris-sodium chloride-ethylene diamine tetra-acetic acid di-sodium salt

UPGMA	unweighted pair-group method using arithmetic mean
UV	ultra violet
VNTRs	variable number tandem repeats

ABSTRACT

The Cape parrot (*Poicephalus robustus*) is South Africa's only endemic parrot. It has become increasingly rare in recent years, with fewer than 500 birds left in the wild, and is now regarded as endangered. Possible factors contributing to this rapid decline in numbers include habitat loss, food shortage, disease and illegal trafficking and trading in the species. Habitat loss and food shortage have been brought about by the rapid destruction of the yellowwood trees in the afromontane forests in South Africa and have played a role in reducing the population numbers. The Psittacine beak and feather disease virus (PBFDV) has also contributed to the loss of some individuals, however it is the illegal trafficking of this rare and valuable species that has become of great concern. As the Cape parrot is becoming increasingly rare and therefore highly sought after, its commercial value has multiplied to the extent that illegal black market trapping is on the rise.

The industry involved in breeding and conservation of endangered bird species, has a need for the proper establishment of studbooks, containing all available information on captive as well as tagged birds. Most of the information found in studbooks is based on morphological attributes of individual birds. Although this is useful, there is a need to add molecular information in order for complete identification of individuals, particularly in a species threatened by illegal trading and theft. A preliminary analysis of the amount of variation present in the population of interest is therefore required so that appropriate methods and techniques can be developed to identify individual birds. A RAPD analysis was conducted to assess the amount of variation in the Cape parrot and lay the foundations for the establishment of individual identification in the species.

Blood samples from 30 parrots, consisting of both related and unrelated individuals, were obtained from three separate locations: Amazona in Assagay, Rehoboth Farm in Dargle, as well as from the Eastern Cape. 15 random primers were selected and

used to conduct a randomly amplified polymorphic DNA (RAPD) analysis. RAPDs are extremely useful in situations where relatively inexpensive first approximations of the genetic variation are needed, such as in rare and endangered species. After successful optimisation of the technique in the species, the 15 primers were screened for all 30 individuals and the individual DNA fingerprints, analysed.

Clear, distinctive and reliable DNA fingerprints were obtained for all individuals however, it was interesting to note despite the analysis of 85 loci using the 15 primers almost identical DNA fingerprints were produced between the individual birds. A population analysis into the amount of variation present between and within the three populations, as well as for the representative population as a whole, was conducted. Using various statistical programmes such as POPGENE and ARLEQUIN, heterozygosities, genetic distance measures, diversity indices, Wright's fixation index and AMOVAs were estimated.

The amount of polymorphism detected in this investigation was 33 % and the heterozygosity, 0.37, which is a relatively high value for the uniformity displayed in the DNA profiles. The high GC content of the primers however, could be a possible explanation thereof. Relationship and kinship determination, sex determination as well as population assignment was possible despite not being able to identify each individual based on unique DNA fingerprints.

The AMOVA analysis indicated significant variation on both the between (5.59 %) and within (94.41 %) levels of analysis. Little variation or differentiation was observed between the three subpopulations, which was confirmed with an F_{ST} value of 0.056. The variation experienced within each subpopulation was analysed using Shannon's index of phenotypic diversity. The Amazona population displayed the most variation with a value of 0.286 and the Rehoboth population, the least with 0.195. This was expected, with the individuals from the latter population comprising one extended family. Nei's measures of genetic identity revealed that the individuals from Amazona

were more similar to the Eastern Cape population, which was again expected with regular exchanging of chicks between the two breeders.

RAPD technology was successful in laying the foundations for individual identification in the Cape parrot. It was also successful in producing reproducible DNA fingerprints in the species that were able to determine relatedness to some extent, determine the sex of individuals and identify individuals from a particular subpopulation. Furthermore RAPD analysis gave a good indication of the variation found in the Cape parrot population, which is important for conservation purposes. In order to maximize conservation efforts and strategies in an endangered species, determining the level of genetic diversity and variation found in the remaining individuals of the population is of great importance. This information could provide powerful insight for conservation purposes and depending on the level of diversity detected, appropriate breeding programmes could be set up in order to increase the genetic variation and thereby reduce the chance of extinction of the species.

The following important findings emerged from this investigation:

- RAPD technology, once optimised for the species of interest, is successful in producing clear and reliable DNA fingerprints, provided the same protocol is followed carefully throughout the investigation.
- An optimised protocol for fingerprinting the Cape parrot using RAPDs was established.
- Possible sex identification, population assignment and a degree of kinship determination was determined using RAPDs.
- Little variation was found within the representative Cape parrot population as a whole due to small population size and possible inbreeding.
- As expected for an avian species, little genetic sub-division or differentiation was observed between the three populations analysed.

CHAPTER 1
INTRODUCTION

The extinction of species is part of the natural and continuous process of evolution. It is only in recent times that humankind has speeded up the process with the development of technology to suit their immediate ambitions and needs (Mountford, 1988). The extinction of species as a natural process of evolution would have continued even if humans had not emerged as the world's most successful predator. However, the rate would have been far slower as environmental changes would have developed over hundreds of years, therefore allowing the majority of species to adapt and only the weaker and over-specialized kinds to become extinct (Mountfort, 1988).

There are a number of ecological mechanisms that underlie extinction and it is important to have an understanding of them if any efforts in conservation are to be considered. The two major sources of extinction are habitat loss and human persecution or introduced predators (Mountford, 1988). Not all taxa however, are equally vulnerable to extinction; this being because different taxa are threatened by different mechanisms of extinction. Owens and Bennett (2000) calculated that 70 % of the endangered species analyzed were affected by habitat loss and 35 % by human persecution. In addition, they found that it was relatively unusual for a species to be affected by both of these factors. Birds, however, seem to be one such class of animals where the majority of endangered species is affected by habitat loss as well as human persecution (Owens & Bennett, 2000).

1.1 ENDANGERED BIRD SPECIES

It is believed that approximately 100 unique species of birds have become extinct during the past 600 years, but today more than 1000 are considered as nearing

extinction. Of these, nearly half have such critically small populations that it seems unlikely that they will survive for many more years unless some drastic action is undertaken to conserve them (Mountford, 1988).

Birds suffer in the hands of humankind in many different ways. They are hunted by humans who trap them, poison them, steal their eggs, introduce new predators into their environment and most importantly change, or completely destroy their natural habitat (Owens & Bennett, 2000). All these factors result in a drastic reduction in a species' population as well as the potential for extinction if they continue to be plagued. Extinction risk is however not evenly distributed among the various avian lineages. Some families such as the parrots, cranes, pheasants, pigeons and albatrosses (to name but a few) are significantly more threatened than other species (Owens & Bennett, 2000). The reason for this is that these species are either particularly vulnerable when it comes to habitat destruction or that they are more important to humankinds' ever growing needs of wealth, status and pride. One of the most endangered families of birds in society today are the parrots (*Psittacine*) and they seem to be affected by both these factors in that they are not only loosing their natural habitat at an alarming rate, but are simultaneously being hunted and sold for monetary benefit. This possibly accounts for their drastic reduction in numbers over the last 100 years (Forshaw, 1991).

1.1.1 Parrots and humankind

Parrots are commonly used as pets, particularly in American households; however little is known about their basic biology, natural habitat, and preferred food or how they live in the wild. This is partly because most species are tropical forest dwellers and nest in elevated tree cavities that are difficult to reach (Mountford, 1988). They also have large home ranges and are generally difficult to catch and band for research (Snyder *et al.,* 1991). Parrots therefore boast relatively few published studies on them in their wild habitat in comparison to other bird families. This lack of knowledge has resulted in most ornithologists and ecologists not knowing much about

the biology of these birds and the extent of their conservation problems (Beissinger & Snyder, 1991).

At present there are 330 species of parrots found in the world with 100 of these (30 %) considered endangered or giving cause for concern (Collar & Juniper, 1991). This high proportion has mainly resulted from a combination of habitat destruction and exploitation for the pet trade (Collar & Juniper, 1991).

a. Habitat Destruction

No other factor is more detrimental to the survival of parrots than humankind's destruction of their habitat (Mountford, 1988). Parrots, like all other animals are dependent on a particular environment for their food as well as shelter. Each species has become adapted to a particular environment after years of evolution and it would therefore have serious consequences if this environment would suddenly change. Although great changes in climate and vegetation are part of the natural processes of the world, and have occurred since prehistoric times, they are usually gradual processes taking place over thousands of years (Mountford, 1998). Wildlife today is however, subjected to the sudden and often drastic modification or destruction of its habitat on a scale that is unparalleled in the world's history. This sudden environmental change is brought about by the ever increasing knowledge and developing technology of humans, and does not give a species the time to adapt to its changing surroundings (Mountfort, 1988).

The majority of parrots are found in tropical forests of the world. However these lush areas are being destroyed at an alarming rate of 28 hectares a minute worldwide (Mountford, 1988). At the beginning of the century, there was an almost continuous equatorial green belt of tropical rain forest around the world, which has today been reduced to isolated patches and if nothing is done within the next 30 years, will be completely destroyed (Collar & Juniper, 1991).

The parrot population has decreased drastically as a direct result of the rain forests' depletion. There are numerous cases and examples which demonstrate this, such as the case of the parrots found on the Caribbean Islands (Snyder *et al.*, 1987). Since the colonization of the Caribbean by Europeans, the islands have undergone extensive deforestation. At the time of their discovery, there were a minimum of 28 different parrot species on the islands, including macaws, parrots and parakeets (Butler, 1991). However, in subsequent years, this number has been reduced to half, with only four parrot species remaining in the Lesser Antilles. Of these few surviving species, including the Saint Lucia parrot (*Amazona versicolor*) and the Saint Vincent parrot (*Amazona guildingii*), all are considered endangered or 'nearly threatened' (Butler, 1991). Although many of these birds were hunted in the past for food and trapped to supply pets for overseas markets, the main threat posed to these parrots is the scarcity of a suitable habitat for them (Colar & Juniper, 1991). Habitat destruction therefore remains the most serious cause of extinction for psittacines and if nothing is done to conserve the parrot's natural habitat, within thirty years, most of the remaining species will be extinct (Collar & Juniper, 1991).

b. Human exploitation and the parrot trade

The number of threatened species is much higher in parrots than for almost any other family of birds (Collar & Juniper, 1991). This is due to the fact that the usual cause of extinction in birds through habitat destruction is coupled with direct human exploitation in parrots. Today, parrots are not necessarily hunted for food as they were in the past, but are trapped and sold as pets for large sums of money (Mountford, 1988).

The cage-bird trade has become a huge industry over the past few years with increasing popularity of owning a parrot as either a passion or a hobby. Because of their beauty, bright colours and ability to talk, parrots are found in about 10 % of American households (Beissinger & Snyder, 1991). According to a study by Thomsen and Brautigam (1991) a few years ago the estimated retail value of parrots imported

into the United States alone in 1986 was over $ 300 million. Trading has grown considerably over the years and is probably triple the value today. The parrot trade has therefore become a multi-million dollar business and source of wealth to many people including law-breakers, who make money off parrots they pass off as their own but which have been illegally removed from their natural habitat. This illegal trading has been, and still is, a major threat to countless species of parrots, particularly the endangered ones (Thomsen & Mulliken, 1991).

Trade, in particular the international trade, is responsible for the plight of many species, despite the fact that the majority of these species are protected by national or international controls and laws (Thomsen & Mulliken, 1991). The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is responsible for these trading controls; however, parrots are still being smuggled out of South America, Australia, the Caribbean and Africa in large numbers. Individuals from the CITES Appendix I and II, including the Amazon, Macaw and Conure species, are illegally removed from the wild in one country and then moved to a country where the particular species is not banned (Thomson & Mulliken, 1991). A particular trapper in the West Indies said it was very easy to avoid custom officials by using yachts to move birds from one island to another, from there they are then 'legally' exported (Butler, 1991). Bird collectors and aviculturists pay large sums of money to obtain these rare birds, thereby giving law-breakers more incentives to partake in the illegal trade.

It is difficult to assess the degree of threat represented by trade as compared with habitat loss in the endangered parrots of the world. The lack of information on wild populations of most parrot species prevents the quantitative assessment of the effects of different levels of trade (Snyder *et al.*, 1991). Furthermore, the contribution of illegal trading cannot be accurately determined. However, it is a known fact that the habitats of most species are either changing or being destroyed at rapid rates (Mountford, 1988). To use the arguments presented by some aviculturists and the pet industry: "if we don't remove the birds from the wild, they'll die anyway due to habitat

destruction" (Thomsen & Mulliken, 1991). A more recent study however, indicated that trade does play a significant part in the extinction of parrots and that if it did not take place, the number of species regarded as threatened today, would be reduced to half (Snyder *et al.*, 1991). Trade alone may therefore not result in the extinction of a species; however, coupled with the threat already posed by habitat destruction, it is a major factor to be considered in the parrots' extinction.

1.2 CONSERVATION PLANS AND STRATEGIES

Conservation is not a modern phenomenon and has been around for over a thousand years. The difference is however, that nowadays the need for it has increased drastically as many species from both the plant and animal kingdoms have already become, or are in the process of becoming extinct.

The conservation of wildlife has to be seen in the context of humankind's own survival. Where wildlife thrives, a healthy natural environment must exist and conversely, where it is declining or has been wiped out, the environment is degraded and humankind has taken another step towards its own classification as an endangered species. A very good indication of a healthy environment is the presence of birds. Whatever harms birds can eventually be considered as harmful to humans (Mountford, 1988).

1.2.1 Parrot conservation

The protection of birds on a worldwide scale was first established in 1922 by the International Council for Bird Preservation (ICBP) (Mountford, 1988). Since then numerous organisations have been established to prevent the extinction, and formulate ways to conserve endangered species. Widespread concern for the conservation of parrots is however, relatively recent. It was first established at a conference held in St. Lucia in 1981, and was termed the 'parrot crises' (Snyder *et al.*,

1991). Here, the main threats to parrots were acknowledged to be habitat destruction, international and domestic trade and killing for food and feathers. Participants urged that more areas should be set aside for reserves and that there should be better regulation of the bird trade by having all countries joining CITES. Emphasis was placed on increasing law enforcement to counteract illegal trading of parrots and urgent requests were made for researchers to gain more knowledge on the basic biology of psittacines (Snyder *et al.,* 1991). Despite all these efforts and conservation strategies, evidence presented by case studies and research in subsequent years, indicated that the conservation status of this family has continued to worsen (Collar & Juniper, 1991; Thomson & Mulliken, 1991; Wiley *et al.*, 1991). Parrots now have a larger proportion of endangered species than any other family of birds.

The two main threats posed to parrots are habitat destruction and trading (Snyder *et al.*, 1991). However, psittacines possess a number of traits that make their conservation particularly difficult and make them extremely vulnerable to human influences (Snyder *et al.*, 1991):

1. Their bright colours and loud vocalisations tend to be highly conspicuous, making them easy to trap or shoot.
2. Birds of many species are relatively large making them worthwhile as game for subsistence hunters.
3. They are often regarded as pests when they feed off orchards and plantations and are therefore a threat to agricultural farmers.
4. They are very popular pets.
5. Most species nest in natural tree cavities, which mean they are highly affected by deforestation.
6. Deliberate destruction of nesting sites whilst harvesting for trade, threatens their breeding success, as natural tree cavities are not that plentiful.
7. Most parrots are found in third world countries or countries which lack the resources to fund a conservation programme.

Despite these factors and the gloomy picture painted for conservation efforts, there has been a rapid increase of interest in continuing with the conservation of these birds (Snyder *et al.,* 1991). Habitat destruction is one of the greatest threats posed to most species of psittacines and it is therefore imperative to try and reduce this process in any possible way. There is however, no universal formula for successful habitat preservation. Conservationists will continue to be challenged to search for new ideas and solutions to protect a particular region, whether it is through ecotourism or through coordinated efforts with the government. In Saint Vincent, a small island in the Caribbean, a parrot education programme was established, comprising posters, questionnaires and fact sheets on the endangered Saint Vincent parrot. This resulted in a significant increase in public awareness and most of the public (80 %) rated it 'very important' for the government to spend time and money protecting the parrot and its natural habitat (Butler, 1991). Studies by Thomsen & Mulliken (1991) and Wiley *et al.* (1991) have however, indicated that many parrots are not truly specialized for one specific environment only, and can in fact survive in disturbed areas as long as they are not hunted and adequate nesting sites are provided. Despite this new information, habitat preservation is still very important, not only for parrots, but for wildlife in general. It therefore continues to be one of the primary concerns and focuses in conservation projects and schemes.

The other primary source of extinction for parrots is that of trading. In order to control the bird trade, stricter and clearer rules need to be established relating to local as well as international trade (Snyder *et al.*, 1991). Aviculturists who want to raise birds to sell will have to be registered and their facilities open for inspection at any time. Furthermore, the breeder must possess some form of proof to ensure that a chick is that of one of his captive pairs, and not a chick that has been illegally removed from a nest in the wild.

The new trading laws should not affect the pet industry itself, as Budgerigars and Cockatiels fulfill most of their customers' needs. The major problem however, lies with the demands of hobbyist, aviculturists and collectors of rare and exotic species,

such as parrots. Hopefully with the aid of education, only captive-bred birds will come to be accepted.

A third factor that is a very important component of, and without which conservation could not take place, is an understanding of basic biological knowledge of a species. In order to establish a successful conservation programme, biological and behavioural information about the species in question needs to be known (Snyder *et al.*, 1991). Parrots are unfortunately not very well researched and there is an urgent need for data banks to be set up, providing necessary information on a particular species. For each species the relative impacts of trade and habitat destruction need to be known, as well as the effect of other limiting factors, in order to develop a species-specific conservation programme (Snyder *et al.*, 1991).

Conservation might not have been very successful in the past few years but some success has been achieved in preventing the extinction of one or two species. The Carolina parakeet (*Conuropsis carolinensis*) of the United States and the Glaucous macaw (*Anodorhynchus glaucous)* both became extinct in the twentieth century due to failed conservation efforts (Collar & Juniper, 1991), however conservation of the Puerto Rican amazon (*Amazona vittata*) seems to be progressing positively (Wiley *et al.*, 1991). The Puerto Rican parrot was reduced to approximately 13 individuals in 1975 however, as a result of conservation efforts, the population increased to 30 wild individuals and 65 captive individuals in 1992 and has continued to increase (Brock & White, 1992).

It is very difficult to conduct conservation programmes dealing in particular with habitat destruction when the human population is expanding at such a rapid rate. However, there is no other alternative than to continue with conservation efforts and techniques that are available, to ensure the best possible effort is made to prevent the further extinction of psittacine species.

1.3 THE CAPE PARROT FAMILY

The Cape family of parrots has recently (Clancey, 1997) undergone a taxonomic re-classification. Previously it consisted of three sub-species: *Poicephalus robustus robustus, Poicephalus robustus suahelicus* and *Poicephalus robustus fuscicollus.* However, under the new classification, *Poicephalus robustus robustus* is regarded as a separate species based on morphological, biogeographical and ecological differences. It is now known as the only true Cape Parrot, with the other two sub-species referred to as the Grey-Headed Parrot and the Brown-Necked Parrot respectively (Wirminghaus *et al.*, 2000).

The Cape parrot family is believed to have once comprised a single species that was found all over Africa in woodlands and afromontane forests (Wirminghaus *et al.*, 2002). These forests and woodlands became fragmented during the ice age and the glacial maximum, resulting in the separation of populations of the species. The separate populations differentiated over the years to such a degree that the individuals of the different geographical locations were considered sub-species. The first sub-species, *Poicephalus robustus suahelicus*, (now commonly known as the Grey-Headed Parrot) is found in low-lying woodland in south-central Africa. It is believed to have retained typical characters of the ancestral super species such as wide habitat tolerances resulting in a relatively large population as compared to the other sub-species (Wirminghaus *et al.*, 2002). The second sub-species, *Poicephalus robustus fuscicollis*, (now commonly known as the Brown-Necked Parrot) is found in Gambia in western Africa, occupying a similar habitat to *Poicephalus robustus suahelicus*. It differs significantly morphologically from *Poicephalus robustus suahelicus* however, cannot easily be separated on plumage colouring (Wirminghaus *et al.*, 2002). The third sub-species to have developed is *Poicephalus robustus robustus*, (now known as the Cape Parrot) and is found in afromontane forests in South Africa. It is the smallest of the three birds and differs noticeably from them by having an olive-green head (Wirminghaus *et al.*, 2002).

1.3.1 The Cape parrot *(Poicephalus robustus)*

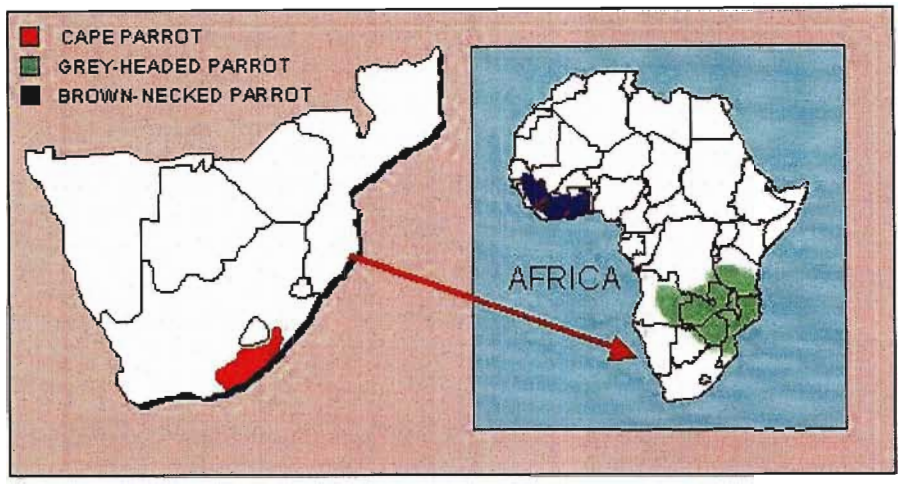
The true Cape parrot (*Poicephalus robustus)* is South Africa's only endemic parrot and is regarded as rare and endangered (See figure 1.1).

Figure 1.1 The endangered Cape parrot (*Poicephalus robustus*).

Unfortunately the plight of this species has only been realised in the last few years, thus, very little information and published research exists. Most of the research done on this species focuses on their population size (Wirminghaus *et al.*, 2000), their preferred diet (Wirminghaus *et al.*, 2002) and possible conservation strategies (Wirminghaus *et al.*, 1999). According to research conducted by Downs (2001) it is believed that there are less than 500 birds left in the wild. The rapid decline in numbers of the Cape parrots' population over the past 50 years can be attributed to several factors. The first, and possible main reason, is the loss of its natural habitat.

The Cape parrot's preferred habitat is the yellowwood (*Podocarpus*) afromontane forests, otherwise known as mist-belt forests. In South Africa, these forest patches are found in the Eastern Cape and southern KwaZulu-Natal (Figure 1.2).

Figure 1.2 Distribution map of the previously believed three sub-species of the Cape parrot family.



Parrots use these yellowwood trees as nesting sites, building nests in holes (referred to as snags) high up in dead trees (Low, 1994). Yellowwoods are common canopy trees in afromontane forests but are also valuable commercial timbers and have been logged for many years (Downs, 2001). Timber logging industries in the Eastern Cape are presently still depleting the yellowwoods that are so essential for the species' survival, both in terms of habitat and nesting holes. The Cape parrot however, not only relies on the yellowwood trees as a habitat, but also as a source of food. The fruits of these trees have a high energy and fat content and are the primary component of the parrots' diet (Wirminghaus *et al.*, 1999). Having a specialized diet, specific nesting requirements as well as habitat, which all centre on the ever decreasing yellowwood tree, is posing a great threat to the species survival. It is believed that the destruction of these mist-belt forests has greatly reduced the numbers of Cape parrots presently found in the wild (Wirminghaus *et al.*, 1999).

Another factor believed to have influenced the decreasing population is the introduced psittacine beak and feather disease virus (PBFDV) among wild birds. This virus, which affects the parrot's immune system, was first discovered in captive birds

but has since spread to the wild populations with the escaping of cage birds. PBFDV is an airborne virus that spreads rapidly within a population once an individual is infected, killing many birds, particularly the younger ones. Presently there are no available vaccines to prevent the spread of the disease (Horsfield, 2000).

A third factor affecting the parrot population size is the illegal trafficking of this species both on a national and international scale (Wirminghaus *et* al., 1999). Trafficking of rare and valuable animals has taken place for many years, especially the illegal trading of parrots. As the Cape parrot is becoming increasingly rare and therefore highly sought after, its commercial value has multiplied to the extent that illegal black market trapping for avicultural trade is on the rise. Cape parrots, particularly nestlings, appear to be poached by a discrete, small group in KwaZulu-Natal and the Eastern Cape. There have been increasing reports of young boys offering nestlings for sale on the side of the road (Wirminghaus *et al.*, 1999). It is however, not only the poaching which poses a problem, but the human disturbances in the afromontane forests that arise as a result thereof. Removal of Cape parrots for any reason should therefore be discouraged, as the population is on the decline and their afromontane habitat extremely sensitive to human disturbances (Wirminghaus *et al.*, 1999).

CITES came into force to presumably reduce and control trading, however the live-bird trade (especially in parrots) has increased irrespectfully thereof (Forshaw, 1991). Some species can withstand trade especially if their wild populations are managed with care, however, others (such as the Cape parrot) are already so rare and their wild populations difficult to manage that the natural populations are on rapid decline as a result of trading. In order to improve this management, a system of individual identification needs to be devised so that any stolen or illegally removed bird can be traced back to its original owner or habitat (Beissinger & Snyder, 1992).

Conventional methods for uniquely identifying both captive and wild animals involve ear tagging, rings, radio collars or microchips. These forms of identification are often lost, are difficult to maintain and can be removed by thieves of rare and expensive

animals who possess both the knowledge and funding to do so. DNA fingerprinting provides a way in which individual identification is permanent and reliable (Fritsch & Rieseberg, 1995). Thus, there is a need, particularly in endangered species such as the Cape parrot, to enter individuals into studbooks and databases containing DNA fingerprints in order to identify the birds. This would allow for comparisons to be made between fingerprints obtained from birds believed to have been stolen so that disputes could be settled and prosecutions, secured. DNA fingerprints are also useful in determining kinship and paternity and these additions to the studbook could prove extremely useful in cases where chicks are passed on as those of a captive breeding pair, when in fact they have been illegally removed from the wild. Another useful application of DNA fingerprinting is that it can provide an understanding of the genetic variation and diversity present in the population and among individuals. Obtaining this information is particularly important for endangered species, such as the Cape parrot, as it indicates the amount of genetic variation present in the population so that appropriate conservation strategies can be implemented. Furthermore, as is the case in most endangered species, the effects of small populations with regard to inbreeding and level of homozygosity can also be determined.

1.4 DNA FINGERPRINTING

DNA fingerprinting, identification analysis, profiling or typing all refers to the characterization of one or more relatively rare features of an individual's genome. Every organism has a characteristic phenotype or physical appearance because it possesses a unique, hereditary genotype or make-up (Kirby, 1990). The exception to this rule are identical twins, or clones, as they have the same unique genotype with only subtle differences in phenotype, brought about through complex developmental events and environmental conditions. The DNA of any individual is identical whether it is extracted from hair roots, feathers, leaves, blood cells or semen specimens (Bruford *et al.*, 1992). These principles of individual uniqueness and identical DNA structure within all tissues of an organism provide the basis of DNA fingerprinting.

The DNA present in every cell of an organism is termed the genome and it is this genome that when analyzed, produces a DNA fingerprint unique to the individual. The DNA making up the genome is predominately found in the nucleus however, a small portion can also be found in cellular organelles called mitochondria (Snustad & Simmons, 2000). The small size, relatively rapid rate of evolutionary change and maternal inheritance of mitochondrial DNA, have made it useful for examining population structure and history (Tegelstroem, 1992).

Both sources of DNA, nuclear and mitochondrial, are broadly made up of two types of sequences: (i) sequences that encode for proteins that determine phenotypic traits such as plumage, height and weight, and (ii) sequences that do not appear to encode a particular product (Snustad & Simmons, 2000). DNA fingerprinting is based on identifying polymorphic DNA that varies from individual to individual. Genetic markers, or molecular markers as they are otherwise known as, are identified through amplification of their specific sequences by short DNA probes called primers. These primers produce different sized amplification products, which may be accompanied by cleavage. The resulting products are separated in a suitable matrix and visualized as a fragment profile, called a fingerprint (Avise, 1994). The genome of most eukaryotes is so vast, that it contains many mutations in sequence composition, brought about by substitutions, insertions and deletions. The probability therefore, that two individuals contain an identical set of markers is low, providing a means to produce unique molecular fingerprints for individuals (Krawczak & Schmidtke, 1994). The probability of producing unique fingerprints depends on the type and number of molecular markers used to produce the fingerprints.

Since its development, DNA fingerprinting has found numerous successful applications in the field of biology, wildlife and conservation. Genetic analysis has gone from being relatively obscure in wildlife and conservation research, to being significantly emphasized today due to the ability of these revolutionized molecular techniques to determine relationships between individuals, populations and species (Haig, 1998). Some researchers have however questioned the relative importance of

genetic information, stating that ecological and demographic issues should be regarded as providing more important information than genetic analysis (Lande, 1988). Nowadays it has been realised that these factors each make a significant contribution to research and that combining them will give a much better and more detailed description of the individual or species in question (Haig, 1998).

DNA fingerprinting focuses mainly on intra-population studies, where there is a need to identify individuals (Congiu *et al.,* 2000; Kang *et al.,* 2002), close relatives (Heinkel *et al.,* 2000) or determine paternity (Gilbert *et al.*, 1991; Fowler *et al.,* 1998; Masello *et al.,* 2002). It has however, also been applied to population-based studies, looking at population structure, size and population-specific markers (Haig, 1998). Many different types of organisms, ranging from mammals and insects to microorganisms and plants have been analysed using molecular techniques. DNA fingerprinting has been used in paternity studies and kinship determination in lions (Gilbert *et al.,* 1991) where definite parentage, both maternal and paternal, was assessed for 78 cubs. Likewise, Fowler *et al.* (2001) successfully determined parentage in numerous koalas using DNA analysis. DNA fingerprinting also proved useful in population studies on rattlesnakes (Lougheed *et al.,* 2000), where DNA markers were claimed useful for defining broad-scale genetic structures in snake populations as well as providing important inputs in conservation initiatives. Numerous studies have also been carried out on birds boasting countless published research articles on the successful use of molecular techniques. Some studies have examined the issue of monogamy and extra-pair fertilization in certain species using fingerprinting (Delehanty *et al.,* 1998; Questiau *et al.,* 1999; Quinn *et al.,* 1999). Others have determined genetic variability in endangered species such as the Puerto Rican parrot (Brock & White, 1992) where the level of relatedness between 65 parrots was examined. Similarly, a study conducted on the endangered Imperial eagle (Padilla *et al.,* 2000) measured the genetic diversity in 25 eagles, the results of which served to establish more adequate mating in order to preserve genetic variability.

1.4.1 Types of molecular markers used for individual identification

Many molecular markers are not genes in the classical sense in that they do not encode for a particular protein product. They are however extremely useful in a number of fields of genetics such as mapping and fingerprinting as they are constant 'landmarks' in the genome which display a large amount of polymorphism. These different polymorphic types are termed 'alleles' and it is these alleles that are the essential ingredient required for the fingerprinting process (Parker *et al.*, 1998).

Prior to the development of new molecular techniques, protein assays were used to determine genetic variation between individuals. These protein assays were based on allozymes, which is the name given to different allelic forms of nuclear-encoded enzymes. This technique allowed for population structure analyses in a number of different species (including birds) for the first time (Hamrick & Allard, 1972; Selander & Johnson, 1973), and the literature on the subject has become voluminous. 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 analysis (Parker *et al.*, 1998; Smouse & Chevillon, 1998).

Since the advent of polymorphism detection at the DNA level in 1985, DNA fingerprinting has been continually refined and made more accurate with the improvement of technology by allowing the direct examination of the DNA itself (Parkin, 1991). The gene, rather than the gene product as is the case in protein assays, is investigated for variation. Much of this genetic variation may be associated with non-coding regions of the DNA and thus has no impact on phenotypic expression of variability, nor is it subject to natural selection, which acts at the level of the gene product (Cooke & Buckley, 1987). The physical aspects of DNA also offer several advantages over allozymes. DNA is found in nearly all cells of all organisms and can be recovered from both living and dead tissue. Tissues can also be stored more easily under field conditions and in many cases only nanograms of DNA are needed for analysis, when using polymerase chain reaction (PCR) for amplification (Parker *et*

al., 1998). Therefore, for inter- and intrapopulation studies, studying the DNA itself, is far more useful and accurate in studies on individual identification, paternity testing, inbreeding assessment, genetic diversity and population structure analysis.

In the investigation of genetic differences between individuals, the challenge is however, to find an appropriate method that will reliably reveal sufficient genetic variation to answer a particular question, with a minimum amount of effort and expense (Parker *et al.*, 1998). The type of genetic markers available for these investigations can be sub-divided into two groups according to the number of loci involved. The first group or class of genetic markers, known as the multi-locus markers, analyze several loci simultaneously, yielding a DNA fingerprint in one step. The single-locus markers, on the other hand, aim at one locus and therefore require the combination of several locus-specific assays to achieve a similar multi-locus type DNA profile (Krawczak & Schmidtke 1994).

At present a number of techniques are available that fall under either multi-locus methods of detection or single-locus methods. The more popular methods make use of PCR, which allows for the production of large amounts of a specific DNA fragment with a particular length and sequence, from small amounts of template DNA (Innis *et al.*, 1990). PCR, which was developed in 1985 by Kary Mullis, has revolutionized molecular biology and genetics by permitting the isolation and characterization of specific DNA fragments and has resulted in the development of diverse applications in many fields (White *et al.*, 1989). In order for PCR to function effectively, however, primers that flank the target sequence and initiate the chain reaction are required. Some of the single-locus molecular markers such as restriction fragment length polymorphisms (RFLPs), micro- and minisatellites, require specific primers to function, whereas multi-locus markers, such as random amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs), use arbitrary primers. Due to the specific nature of the single-locus markers, only the particular locus in question is amplified, whereas in the case of multi-locus markers a number of arbitrary loci are amplified.

a. Single-locus Markers

Single-locus markers require DNA primers that hybridize only once per genome allowing amplification of a specific single locus. The researcher therefore needs to have prior sequence knowledge in order to amplify such single loci. Much money and time is thus spent establishing sequence composition and designing primers to amplify these markers. However, due to their polymorphic content, they have gained popularity in a range of different investigations such as studies of complex mating systems and comparisons of genetic variation between populations (Bruford *et al.*, 1992).

The first DNA polymorphism analysis was conducted in 1981 (Nei & Tajima, 1981) using restriction fragment length polymorphisms (RFLPs). They were the first widely used molecular markers and were seen as a new tool, which would give rise to genetic linkage maps of chromosomes in a number of species (Burke *et al.,* 1991). RFLPs are based on heritable polymorphisms in the length of fragments produced by digestion of DNA with restriction enzymes. If a restriction site is present on a strand of DNA, the DNA strand will be cleaved by the presence of the corresponding restriction enzyme that would recognise the site. This would result in the strand decreasing in size and thus showing up as two different bands on a gel. Differences in the length of the fragments generated occur as a result of mutations, changes in the base sequence of the DNA through insertions, deletions and base substitutions, which are carried through to the offspring of that individual providing a means for determining paternity (Kirby, 1990).

The advantage of using RFLPs is that when a few restriction enzymes are simultaneously used in an analysis, the resulting DNA fingerprints vary from one individual to another due to the number of different sized fragments produced by the digestion (Parker *et al.*, 1998). RFLPs can thus generate sufficient variation to investigate genetic questions about between and within population genetic variation. Furthermore they are co-dominant markers, which make them very useful to

distinguish between homozygous dominant individuals and heterozygotes (Parker *et al.*, 1998).

RFLP analysis, being a single-locus marker, gives a very simple DNA profile, namely that of the locus analysed. If only one enzyme is used, the resultant gel would only contain a few bands. However, several single-locus markers can be amplified simultaneously to give a profile with the appearance of a multi-locus profile. This 'cocktail' of enzymes will result in a multi-locus fingerprint, much the same as that obtained by a single multi-locus marker (Krawczak & Schmidtke 1994).

Simple sequence repeats (SSRs), another type of single-locus marker, exhibit variation in tandem repeat numbers and are found in numerous areas in the genome of an organism (Avise, 1994). Due to the repetition of these sequences throughout the genome, a single primer set could amplify a number of loci, resulting in a multi-locus fingerprint. Genetic variation or polymorphisms are evident as the number of core sequences present in an individual. An allele is represented by a certain number of repeats of the core sequence. (Parker *et al.,* 1998).

These simple sequence repeats can be sub-divided into two classes according to the number of nucleotides making up the repeat unit (Krawczak & Schmidtke, 1994). Minisatellites, otherwise known as variable number tandem repeats (VNTR), comprise short tandem repeats of about 40 base pairs in length. (Avise, 1994). Discovered in 1985 by Alec Jeffreys and his research team (Jeffreys *et al.,* 1985), they were first used to establish what is known today as a DNA fingerprint. The minisatellites 33.6 and 33.15 are composed of several loci, and when probed simultaneously, produce a barcode-like multi-locus DNA fingerprint revealing approximately 17 highly polymorphic bands. This makes them extremely useful and still widely used in research today (Davies, 1988).

Microsatellites are simpler in that they constitute between one and four nucleotides per repeat unit. Their discovery in various animals took place a few years later (Tautz,

1989; Stallings *et al.,* 1991) and has been extremely beneficial to both population biology and parentage assessment after finding that they are ubiquitous.

SSRs have a high polymorphic content, brought about through the varying number of repeats between individuals. Due to the large number of alleles present in many SSRs, an enormous amount of variation is possible making these markers popular fingerprinting tools (Avise, 1994). Numerous studies have employed SSRs for various reasons such as, to identify individuals, as seen in a study by Amos *et al.* in 1993. Nesje *et al.* (2000) used SSRs to successfully determine parentage in a study on the genetic relationship of 24 peregrine falcons. SSRs have also been used to identify evolutionary processes among different species, seen in a study by Primmer & Ellegren (1998) in which 76 alleles in 39 species of birds were tested.

Single-locus markers, whether they are used singly or together in a cocktail, are thus powerful tools in DNA fingerprinting (Bruford *et al.,* 1992). Comparison between individuals on the same gel is simple, as only a few, usually clear bands are present in an analysis gel. Furthermore, under optimal conditions, very little DNA template is required, in most cases less than 100 ng (Krawczak & Schmidtke, 1994). However, one major drawback of these markers is that they require prior sequence information, which is more than often not available. This means that extensive and expensive research on the organism's sequence structure is necessary before the fingerprinting analysis can take place. Furthermore, for multi-locus profiling, time-consuming amplification and screening of each locus needs to be undertaken (Bruford *et al.,* 1992).

b. Multi-locus Markers

Multi-locus fingerprints are primarily viewed as fingerprints that are generated from a number of loci and viewed in single lane in a gel. They can either be generated by the amplification of several single-locus markers, using the primers specific for each of the loci, or by amplifying many loci using one or a few arbitrary primers (Krawczak &

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 high information content and can be applied for individual identification (Fowler *et al.*, 1998) and parentage analyses (Questiau *et al.*, 1999).

Williams *et al.* (1990) developed a PCR based genetic marker, which they called randomly amplified polymorphic DNA (RAPD). This marker does not require any prior knowledge of the genome of the organism and is based on random primers. The primers are arbitrary approximately 10 to 11 nucleotide bases in length and anneal to multiple sites on the template DNA due to their short nature (Williams *et al.,* 1990). If these primers anneal in the correct orientation and are a suitable distance apart, the unknown sequence between them is amplified, resulting in visualised bands on an agarose gel (Welsh & McClelland, 1990). Polymorphisms between genotypes are due to the occurrence of single nucleotide polymorphisms (SNPs) that are present in the annealing region of the primer or due to insertions or deletion of segments in the sequence between the annealing primers. By changing the annealing sequence through inserting, deleting or substituting single bases, these SNPs alter the ability of the primer to anneal at a specific location. Without primer binding, sequence amplification cannot occur, resulting in the absence of a band in the analysis. RAPD alleles are therefore seen as 'presence' or 'absence' alleles, where a fragment will be amplified if the primer anneals; or will not, if the primer is not able to anneal. Polymorphisms are seen as presence or absence of specific bands on a gel (Bowditch *et al.,* 1993). Most loci generated by RAPDs are assumed to be dominant for band presence in the absence of segregation analysis.

RAPDs are often used to identify individuals and to analyse populations. They thus require high reproducibility. Once the ideal or optimized conditions regarding primer constraints, stringency of the reaction, DNA quantity and concentration, *Taq* Polymerase concentration and PCR cycle, have been determined for a particular species, it is imperative to keep them constant in order to be able to compare results

from different reactions (Bowditch *et al.*, 1993). Careful attention to laboratory technique will result in reproducible reactions from run to run, provided the same thermocycler is used.

Most successful results are obtained when many primers are screened so that multiple polymorphisms are detected. Not all primers will yield reliable and reproducible results, therefore only those that do should be included in the analysis and used for scoring (Bowditch *et al.,* 1993; Fritsch & Rieseberg, 1995).

RAPDs have become very important genetic markers because the assay is simple, fast and relatively inexpensive, allowing many loci to be identified in a single reaction. Furthermore, only small amounts of DNA are required and no prior knowledge of the genome in question is necessary (Fritsch & Rieseberg, 1995). They do however, have a few disadvantages. RAPDs are dominant markers therefore heterozygotes cannot be distinguished from homozygous dominant individuals (Williams *et al.,* 1990). Furthermore, the results obtained can be inconsistent because PCR is performed using very short, arbitrary primers that can lead to different patterns depending on the PCR conditions used. Therefore, in order to get consistent and reliable results, RAPDs need to be carefully standardized from run to run (Bowditch *et al.,* 1993).

The advantages RAPDs offer over the other molecular techniques, has led to a broad range of applications in fields of biology such as in ecology, conservation and population biology (Haig, 1998). Although RAPD analysis is relatively new it has already proven useful for genetic mapping, species and individual identification, pedigree analysis and parentage determination (Fritsch & Rieseberg, 1995). Numerous studies have to date successfully used this genetic marker and it is becoming increasingly more popular. Fowler *et al.* (1998) demonstrated the use of RAPDs in determining parentage and individual identification in captive Koalas. They claim RAPDs have great potential to generate large amounts of genetic information with a minimal amount of effort and time. RAPDs were also used to investigate

genetic variation in the southern brown bandicoot (Cooper, 2000) and in a study by Padilla *et al.* (2000), to determine genetic variability in the endangered Iberian Imperial eagle (*Aquila adalberti*).

To date, few studies have been published on RAPD analysis used in conservation however the advantages RAPDs offer, make this technique particularly suited to the analysis of rare and endangered species, where availability of material and prior DNA sequence knowledge are often factors that hinder detailed analysis (Fritsch & Rieseberg, 1995). RAPDs are therefore becoming an increasingly popular method used by researchers interested in a species' genetic variation as well as for individual identification and kinship determination studies in conservation.

Another molecular marker, that also uses the multi-locus system, is amplified fragment length polymorphism (AFLP). It is very similar to RAPDs in that it requires no prior sequence knowledge and is based on random amplification and identification of polymorphisms at many DNA loci (Pejic *et al.*, 1999).

The AFLP technique is based on repeated amplification using PCR on a subset of restriction fragments from a total digest of genomic DNA (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. Each fragment is then ligated to adaptors that serve as a binding site for primers. Only fragments that have both restriction sites (one at either end) will be amplified by PCR. This initial amplification reduces the total number of restricted fragments present in the reaction and is therefore pre-selective (Questiau *et al.*, 1999). The PCR product is then used as a template for a second amplification (selective) using primers with three additional selective nucleotides included at the 3' end. The amplification is selective because one selective nucleotide on each primer results in the amplification of only 1 out of 16 fragments, while two selective primers will reduce this value to 1/256. Therefore, the number of resulting amplified fragments is determined by the number and composition of the selective nucleotides used. The

final product of the whole process is a multi-locus fingerprint-like pattern on a gel that can be scored with an automated sequencer (Questiau *et al.*, 1999).

Since Vos *et al.* (1995) first published this method, it has become increasingly more popular in studies on genetics. At first it was mainly used in plant mapping as seen in a study by Schondelmaier *et al.* (1996), and in studies on crop and wild plant diversity (Travis *et al.,* 1996). A study by Ziegenhagen *et al.* in 1999, showed the usefulness of AFLPs in kinship determination and paternity analysis in oaks and a study by Pejic *et al.* (1999) analyzed the genetic similarity among maize inbred lines, using AFLPs. In the following years, however, AFLP analysis found applications in other fields of genetics such as in avian research (Questiau *et al.*, 1999).

The advantages of using AFLPs are that it is PCR based and thus does not require probe hybridization, many bands (approximately 50-100) are displayed and only a small amount of DNA is needed to get an accurate result (Desmarais *et al.*, 1998). The banding patterns also seem to be more consistent than those found in RAPDs. The major disadvantage of this method however, is that it is significantly more expensive than RAPD analysis and is technically more challenging. Furthermore, like RAPDs, AFLPs cannot distinguish between heterozygotes and homozygous dominant individuals as the process is only based on the 'presence' and 'absence' of bands. They are therefore not co-dominant (Pejic *et al.*, 1999).

1.4.2 Analysis of DNA fingerprints

Molecular markers provide powerful means for analysis of variation between species, populations, groups of individuals within populations, and between individuals both related and unrelated. This variation is visible in the DNA fingerprints produced by the various markers, however, it needs to be transformed into a numerical format from which statistical tests and measures can be calculated in order to accurately answer the questions stimulated by the investigation.

Through the years various tests, comparative indices and formulae have been developed to assess molecular variation (Avise, 1994). These mathematical and statistical formulae include measuring the genetic distance between two entities (be they individuals or populations) such as Nei's (1972 and 1978) and Roger's genetic distance measures (1972), calculating indices of nucleotide diversity, calculating heterozygosity (Lynch & Milligan, 1994) as well as using Bayesian estimators as suggested by Zhivotovsky (1999). All of these methods, although different each with their own advantages and disadvantages, calculate or estimate numerical values that can be used to determine the relationship between two entities and allow for comparisons to be made.

a. Heterozygosity and proportion polymorphism

The simplest measures of variation commonly used in research today are estimates of the level of heterozygosity and the proportion of polymorphic loci in a population. Cavalli-Sforza and Bodmer in 1971 defined a genetic polymorphism as the occurrence of two or more alleles at one locus, with appreciable frequency, in the same population (Cooke & Buckley, 1987). A locus is considered polymorphic if the frequency of the most common allele is less than 0.99 or in some cases less than 0.95.

Determining the proportion of polymorphic loci is simply undertaken by counting the number of polymorphic loci and then dividing by the total number of loci examined. The accuracy of this measure however, depends on the number of loci examined and the number of individuals examined in a population. For the latter, a recommended minimum of 30 individuals is accepted over a minimum of 14 loci however, larger values are favoured (Avise, 1994).

This type of genetic variation analysis is not satisfactory on its own, being both arbitrary and imprecise in that slightly polymorphic loci are treated in much the same way as very polymorphic loci. A more suitable measure of genetic variation is that of

the average frequency of the heterozygous individuals per locus present in the population (Cooke & Buckley, 1987). This measure is called genetic heterozygosity and can be calculated in a number of different ways, depending on whether variation within individuals, populations or loci is being examined. The values can also be determined using either observed heterozygote frequencies, such as in co-dominant markers, expected frequencies, assuming Hardy-Weinberg equilibrium, or from allele frequency data such as in dominant marker analysis (Cooke & Buckley, 1987). Hardy-Weinberg Equilibrium is only achieved if certain assumptions are made about a population. The assumptions of the Hardy-Weinberg Equilibrium are listed below:

- The population analysed must be infinitely large
- All individuals must partake in mating of random nature
- No emigration or immigration should take place
- No mutations should occur
- No forces of natural selection should be acting on the population
- No genetic drift should occur.

In dominant marker analysis, the difference between homozygous dominant individuals and heterozygotes cannot be distinguished on a gel and their frequencies therefore not determined. However, the frequency of these genotypes can be estimated once the frequency of the recessive homozygotes has been calculated. The frequency of the number of bands absent at a particular locus is the frequency of the recessive genotype (q^2). From this value, the frequency of the null-allele (q) can be calculated by taking the square root of the observed proportion of individuals displaying this recessive genotype (Stewart & Excoffier, 1996). This square-root transformation can however lead to statistically biased estimates, which is why Lynch and Milligan (1994) introduced a procedure to reduce this bias that is still widely used in dominant marker analysis today.

For RAPDs and other dominant markers, Lynch and Milligan (1994) advised that accurate estimates of genetic entities, such as allele frequencies, are best achieved

by only including loci in the analysis where the frequency of the null-phenotype exceeds a value of 3/n (where n is the number of individuals in the population sampled). When dominant marker analysis is used, two to ten times more individuals need to be sampled per locus to achieve the same degree of statistical power as compared to co-dominant analysis. For this reason, in studies using dominant markers with limited population sizes, the unbiased estimator is used to achieve a high statistical power despite the smaller sample size and the associated bias.

Following the proposed unbiased estimator of gene diversity by Lynch and Milligan (1994) involved making assumptions about the data and its analysis:

1. All RAPD loci showed complete dominance
2. All loci had two alleles
3. The marker alleles from different loci did not co-migrate to the same position on the gel
4. The population was in Hardy-Weinberg equilibrium

Once the frequency of q has been calculated from the frequency of the recessive genotype, the dominant allele frequency (p) can be determined and an estimate of the frequency of heterozygotes (2pq), obtained. It is however important to mention that Hardy-Weinberg equilibrium can not be empirically obtained using dominant markers, therefore this calculated heterozygosity represents the expected heterozygosity given Hardy-Weinberg equilibrium (Lougheed *et al.*, 2000). The mean heterozygosity for the population is obtained by summing the values obtained for each individual and then dividing this total by the total number of individuals examined.

b. Diversity indices

Numerous indices can be used to give an indication of the diversity of a population or the potential for diversity of an individual. The value of an index usually represents the amount of 'information' per individual or population. The absolute magnitude of an

index is not particularly meaningful on its own and it is therefore used as a relative index for comparing between individuals or populations.

Shannon's diversity index is one of the most popular diversity indices used in research. It is scaled from a value of zero to one, where a value of one equals the maximum diversity attainable. Shannon's index can be calculated using the following formula (Hwang, 2001):

- $H_0 = -\sum P_i \log_2 P_i$
  (where P_i is the frequency of the presence or absence amplified fragment)

As Shannon's index is based on information theory it is sensitive to the presence of rare types or individuals. Another diversity index known as Simpson's index is less sensitive to rare types and the interpretation thereof is more intuitive than Shannon's index (Szczepaniak *et al.*, 2002).

Simpson's diversity index can be calculated using the formula:

- $D = 1 - \sum n_i (n_i - 1)/ N (N - 1)$
  (where n_i is the number of individuals with the phenotype i and N the sample size)

Similar to Shannon's index, a value of zero indicates no diversity and a value of one the maximum diversity.

Originally, Simpson's index represented the probability that any two individuals, selected at random would be different. Pielou's (1975) modification of Simpson's index however, transformed this probability-based index into the class of indices to which Shannon's index belongs. For this reason these two indices are similar in many aspects and have the same applicability.

c. Genetic distance measures

The concept of genetic distance is fundamental to all fields of population analysis and molecular systematics (Avise, 1994). To date there are many different formulae available, but essentially, they all measure the amount of genetic differentiation between two entities (individuals or populations).

In 1972, Rogers and Nei independently derived ways of calculating genetic distance and similarity between taxa (Nei, 1972; Rogers, 1972). Although both measures use allelic frequencies to estimate genetic distance, their distance parameters have different properties. Rogers' method calculates an index of genetic distance (D), which is the mean geometric distance between the entities, whereas Nei's measure estimates the genetic identity (I) of allelic frequencies for alleles by a pair of taxa at a locus and is not metric. From this value (I), the genetic distance (D) is calculated as the negative natural logarithm of (I) (Cooke & Buckley, 1987).

Rogers' genetic distance is given by the formula (Rogers, 1972):

- $D = [0.5 \sum (x_i - y_i)^2]0.5$
  (when x_i and y_i are the frequencies of the *i*th alleles in populations X and Y)

Nei's two measures can be calculated according to the following formulae (Nei, 1972):

- I (for multiple loci) = $J_{xy} / \sqrt{(J_x J_y)}$
  (where J_{xy}, J_x and J_y represent the arithmetic 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)

- $D = -\ln (I)$

Genetic identity estimates the proportion of genes that are identical in the two populations being compared, whereas genetic distance estimates the accumulated number of differences per locus (Avise, 1994). The values for genetic identity range between zero and one, where a value of zero indicates that the two individuals or populations 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 & Buckley, 1987). Table 1.1 provides an idea of the results expected for an analysis on closely related populations or individuals as well as divergent or separate populations or individuals

Table 1.1 Expected values for genetic identity and genetic distance.

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

One advantage of these measures of genetic differentiation is that they can be applied to any population, whether it is haploid, diploid or selfing in nature. This is due to the fact that the definitions of genetic identity and distance depend solely on the gene frequencies and not on genotype frequencies (Cooke & Buckley, 1987). For this reason they can also be used for dominant markers such as in RAPD and AFLP analysis.

In 1978, Nei corrected his original 1972 genetic distance measures to reduce the bias produced when sampling a small population. According to Nei (1978), a small sample size can be used if many loci are studied and the average heterozygosity is low, or if the genetic distance between populations is large and the average heterozygosity is low. If this is not the case, the unbiased measure of genetic distance should used.

Nei's genetic distance, corrected for sampling bias:

- $D = -\ln [(2n - 1) J_{xy} / \sqrt{(2n - 1(J_x) . 2n - 1(J_y))}]$
  (where J_{xy}, J_x and J_y represent the arithmetic 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)

Other measures of genetic distance such as Hillis' genetic distance (Hillis, 1984) and Cavalli-Sforza and Edwards genetic distance (Cavalli-Sforza & Edwards, 1967) have been used in genetic research and investigations but are not as common. Euclidian distance measures however, are widely used, especially in studies making use of Analysis of Molecular Variance (AMOVA) measures (Excoffier *et al.*, 1992). A Euclidian distance is a mathematical concept and is simply defined as the shortest distance between two points. If p_j and p_k are two points in an n-dimensional plane, then the Euclidian distance is the scalar between them as defined by the formula:

- Euclidian distance = $(p_j - p_k)$

Euclidian distances form the basis of AMOVA and are thus an important genetic distance measure.

d. Analysis of molecular variance (AMOVA)

Analysis of molecular variance (AMOVA) is a method of estimating population differentiation and testing various hypotheses about the differentiation, directly from molecular data (Excoffier *et al.*, 1992). It treats any raw molecular data, consisting of ones and zeros, where one indicates the presence of a marker and a zero its absence, as a Boolean vector. From this information Euclidean distances are calculated by subtracting the Boolean vectors of one entity from another. A matrix consisting of all pair-wise squared Euclidian distances between all entities is

constructed and used in the analysis (Excoffier *et al.*, 1992). Squared Euclidian distances are calculated according to the following formula:

- $\theta^2_{jk} = (p_j - p_k)' W (p_j - p_k)$
  (where W is a matrix of differential weights for the various sites)

Using the constructed matrix, a hierarchical analysis of variance is performed to determine the subdivision. Usually, in the simple cases, the total variance is partitioned into between populations and within populations to give an idea as to how much of the genetic diversity can be attributed to each of these components. Variance can however be further subdivided into within individual differences, between individuals within populations, between populations within groups and between groups.

e. Wright's fixation index (F_{ST})

To analyse the inbreeding effect in population substructure, Wright developed an index known as the fixation index. This measures the reduction in heterozygosity expected with random mating at any level of population substructure relative to another (Cooke and Buckley, 1987). Three different values can be calculated depending on the levels of the population hierarchy being compared, however the most commonly used of the three is F_{ST}. F_{ST} is concerned with subpopulations relative to the population as a whole and therefore gives a measure of the extent to which a species is organized into subpopulations with restricted gene flow (Balding *et al.*, 2001).

The values for F_{ST} range from a theoretical minimum of zero, indicating no genetic differentiation between the populations, to a theoretical maximum of one, indicating fixation of alternate alleles in the different populations (Balding *et al.*, 2001). The observed maximum in most cases however, is much lower than 1. Wright proposed the following guidelines for the interpretation of F_{ST}:

- A value between 0 and 0.05 indicates little genetic differentiation.
- A value between 0.05 and 0.15 indicates moderate differentiation.
- A value between 0.15 and 0.25 indicates great genetic differentiation.
- A value above 0.25 indicates very great genetic differentiation.

Wright also mentioned that a value less than 0.05, although indicating little differentiation, was by no means negligible.

1.4.3 DNA fingerprinting in avian genetics

The DNA content in birds is relatively constant across species and is about one third of that found in mammals (Cooke & Buckley, 1987). Only 20 to 60 different proteins can easily be analysed for polymorphisms in aves and within a single species, only a few of these are likely to be polymorphic. Direct DNA analysis therefore allows for the examination of sequences not previously accessible in studies on protein polymorphism such as allozyme analysis (Cooke & Buckley, 1987).

DNA fingerprinting has found many applications in avian genetics with the advancement of technology over the last few years. Studies have been conducted on genetic variation within a species, such as in the African Grey parrot (Shi *et al.,* 2000), where seven single nucleotide polymorphisms (SNPs) were identified. Primmer *et al.* (2002) conducted a study on the genetic diversity in birds and found that the avian genome has higher nucleotide diversity as compared to the human genome. Other studies have tried to determine whether or not monogamy exists in various species using molecular markers such as minisatellites (Delehanty *et al.,* 1998; Quinn *et al.,* 1999) and microsatellites (Thusius *et al.,* 2001; Masello *et al.,* 2002) to establish paternity. All of these cases demonstrated that DNA fingerprinting, using either micro- or minisatellites, provided sufficient variability to unambiguously determine parentage in the various bird species analysed. Demographic and population studies have also been conducted in birds. Wetton *et al.* (1987) suggested that the minisatellite probes discovered in humans by Jeffreys *et al.* in 1985, are equally variable in other species,

such as in the house sparrow, and will be a powerful tool in demographic population genetics. Studies on the differences between species of birds based on DNA fingerprint analysis, using either micro- or minisatellites have also been done by Primmer and Ellegren in 1998 and Papangelou *et al.* in 1998. These studies also examined the patterns of evolution between different bird species and how closely related they are by analysing the DNA and looking at sequence similarities.

RAPD analysis on birds is a fairly recent development, however numerous successful studies have already been conducted using this technique. Garland (1998) used RAPDs to study polymorphisms in the house finch and found this method successful in obtaining differences between individuals. A study by Cooper (2000) on the southern brown bandicoot of Western Australia, used RAPD markers to successfully analyze genetic variation between and within populations. The RAPDs displayed substantial genetic variation with all the individuals possessing unique phenotypes and producing 39 polymorphic bands from three primers. Kulikova *et al.* (2002) conducted a study on Manchurian pheasants, where RAPD analysis showed high average genetic polymorphisms (P = 79.4 %) in the species. Using five arbitrary primers genetic polymorphism of the population, as well as estimated genetic distances between individuals, were assessed. From the obtained results, a Neighbour-Joining (NJ) phylogenetic tree and Unweighted Pair-Group Method using Arithmetic mean (UPGMA) dendrogram of genetic similarity were constructed. RAPD analysis also proved useful in a study by Nusser *et al.* (1996) where genetic variation in the endangered light-footed clapper rail of southern California was established. Using a total of 325 RAPD primers, very little polymorphism was detected (1 % of the analysed bands were found to be polymorphic), and it is believed that this is due to inbreeding depression in the species because of the reduced numbers.

DNA fingerprinting has, however also gained considerable interest in the fields of conservation of numerous endangered bird species such as the Iberian imperial eagle (Padilla *et al.*, 2000) and the Puerto Rican parrot (Brock & White, 1992). In the study on the Iberian imperial eagle, RAPD analysis was used to estimate the genetic

diversity within the species so that more adequate mating can be conducted to preserve genetic variation. 59.7 % of the bands obtained were polymorphic, revealing a high level of heterozygosity in the species. Polymorphism analysis and genetic variation of a species is an important factor to consider when trying to prevent extinction and implement conservation strategies, as can be seen in the study on the Iberian imperial eagle. However, kinship determination and individual identification are equally important in conservation, particularly in species where individuals are extremely sought after and valuable (Fritsch & Rieseberg, 1995). DNA fingerprinting provides a means for identifying individuals in cases where they have been stolen or illegally removed from the wild, so that they can be returned. However in order to do this, studbooks containing the fingerprints of all captive individuals need to established. This would enable the individuals believed to have been stolen, to be compared to the studbook in order to possibly identify them and determine from where they have been stolen. Although studbooks have been created for many species, most of them do not contain any genetic information, and only have microchip and ring or band information. These methods of identification are not permanent and can easily be lost or removed. There is thus a need to develop molecular techniques for individual identification of endangered species, so that permanent identification is available, particularly in those species, which are prone to theft and illegal removal from the wild.

1.5 AIMS

The industry involved in breeding and conservation of endangered bird species, has a need for the proper establishment of studbooks, containing all available information on captive as well as tagged birds. Most of the information found in studbooks is based on morphological attributes of individual birds. Although this is useful, there is a need to add molecular information in order for complete identification of individuals, particularly in a species that is threatened by illegal trading and theft, such as the Cape parrot. One of the aims of this investigation was therefore to assess the

usefulness of RAPD analysis to produce fingerprints of individuals, which could be added to the current data found in the Cape parrot studbook.

A further aim was to assess the level of genetic variation for a representative sample of the Cape parrot population. In order to maximize conservation efforts and strategies in an endangered species, determining the level of genetic diversity and variation found in the remaining individuals of the population is of great importance. This information could provide powerful insight for conservation purposes and depending on the level of diversity detected, appropriate breeding programmes could be set up in order to increase the genetic variation of subpopulations and thereby reduce the chance of extinction of the species

RAPD analysis was the obvious method of choice as it provides sufficient information for the proposed aims and is extremely rapid and cheap to use. As one of the aims in this investigation was to develop molecular data that could be added to the studbook, the methodology has to be simple and cheap so that bird breeders around the country can make use of it. The polymorphisms detected during the RAPD analysis will form the basis of individual fingerprints that can subsequently be used for studbook purposes. In order to do this the technique needs to be optimized for this particular species and a pilot investigation conducted, to determine the level of polymorphism displayed by each primer. The most polymorphic primers will then be tested on various complete families to assess the polymorphic abilities of the primers between closely related individuals.

The individual aims of this research were therefore:

- To optimize RAPD conditions for the Cape parrot species.
- To assess the level of RAPD polymorphisms displayed by the different primers tested.
- To determine the discriminating power of the polymorphic primers in families studies.

- To analyse the observed polymorphisms for genetic diversity and variation using statistical methods and formulae.

CHAPTER 2
MATERIALS AND METHODS

2.1 INTRODUCTION

A randomly amplified polymorphic DNA (RAPD) analysis, using a range of random primers, was conducted on the Cape parrot (*Poicephalus robustus*) in order to obtain a better understanding of its inherent genetic variation. Before the analysis was conducted, however, the RAPD protocol had to be optimized for the species. This ensured that optimal results were obtained from which reliable conclusions about the variation present in the sample population could be made.

2.2 MATERIALS

2.2.1 Selection of parrots

As the Cape parrot is an endangered species, not many samples are available for study purposes. The captive population of the species also tends to be relatively inbred and birds from different breeders are often related in one way or another, through selling and exchanging of chicks. This made sample collection particularly difficult as for the purposes of this investigation, unrelated individuals, as well as families were needed. Therefore, to ensure that one breeder's birds were not related to another's the Cape parrot studbook, which contains background information about birds, was consulted. The information contained in the studbook made it possible to select birds that where either unrelated or that belonged to a family.

The studbook keeper Elaine Whitman's contact details are: Whitman@venturenet.co.za.

The criteria used in the selection of birds were as follows:

1. Parrots selected between breeders should be unrelated.
2. Parrots selected from one breeder should either be a member of a bird family which consisted of a breeding male, a breeding female and their chicks, or be unrelated
3. Selected birds should be healthy and free of PBFDV.

To conduct this investigation a minimum of 30 individuals were selected from various bird breeders in South Africa. Of the thirty birds, 25 were members of families and the remaining 5, unrelated individuals. Twelve of the birds were selected from William Horsfield's birds at his farm, Amazona, in Assagay (KwaZulu Natal) and were members of three different families. Nine birds were selected from the flock of Ralph Correia at his farm, Rehoboth, in Dargle (KwaZulu Natal midlands), and belonged to a single large family that comprised of the main breeding pair, three of their chicks and four second generation birds. The remaining birds were obtained from Gill Thompson in the Eastern Cape and consisted of four full sibs of a breeding pair that at the time of this investigation were about to nest and were thus not included in the study. Five unrelated individuals were therefore included.

Sample collection proved to be more problematic than initially anticipated, with numerous breeders refusing to participate. For this reason, birds obtained from Gill Thompson were included, despite the knowledge that some of the individuals originated from Amazona and could possibly be related to individuals obtained from William Horsfield.

Information regarding sample collection for all individual parrots is presented in table 2.1

Table 2.1 Collection information of the Cape parrots included in this investigation.

CODE	OWNER'S IDENTIFICATION	LOCATION	COLLECTOR	COLLECTION DATE
1 a	3.2 (000124A357)	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
1 b	3.2 (000102FFF7)	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
1ab i	3.2 02 WAH 11	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
1 ab ii	3.2 02 WAH 20	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
1 ab iii	3.2 WAH 15	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
2 a	3.7 (4356475761)	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
2 b	3.7 (500C596124)	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
2 ab i	3.7 WAH 32	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
3 a	3.9 (0001245B22)	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
3 b	3.9 (0001D23107)	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
3 ab i	3.9 WAH 39	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
3 ab ii	3.9 WAH 40	Amazona (Assagay)	Dr C. Kingsley	10/04/2003
4 a	Breeding male	Rehoboth (Dargle)	Dr C. Kingsley	11/04/2003
4 b	Breeding female	Rehoboth (Dargle)	Dr C. Kingsley	11/04/2003
4 ab i	RV01 80	Rehoboth (Dargle)	Dr C. Kingsley	11/04/2003
4 ab ii	RV01 81 (engraved)	Rehoboth (Dargle)	Dr C. Kingsley	11/04/2003
4 ab iii	RV01 82 (engraved)	Rehoboth (Dargle)	Dr C. Kingsley	11/04/2003
4 w	RV01 77	Rehoboth (Dargle)	Dr C. Kingsley	11/04/2003
4 x	RV01 81	Rehoboth (Dargle)	Dr C. Kingsley	11/04/2003
4 y	RV01 82	Rehoboth (Dargle)	Dr C. Kingsley	11/04/2003
4 z	RV01 83	Rehoboth (Dargle)	Dr C. Kingsley	11/04/2003

5 ab i	A	Eastern Cape	Dr P. Woods	23/07/2003
5 ab ii	B	Eastern Cape	Dr P. Woods	23/07/2003
5 ab iii	C	Eastern Cape	Dr P. Woods	23/07/2003
5 ab iv	D	Eastern Cape	Dr P. Woods	23/07/2003
6	G: William No 37	Eastern Cape	Dr P. Woods	23/07/2003
7	E: William No 52	Eastern Cape	Dr P. Woods	23/07/2003
8	H: William No 54	Eastern Cape	Dr P. Woods	23/07/2003
9	I: Parents deceased	Eastern Cape	Dr P. Woods	23/07/2003
10	F: Parents deceased	Eastern Cape	Dr P. Woods	23/07/2003

2.2.2 Collection of blood sample

Blood is a convenient and popular source of DNA used in many genetic studies (Bruford *et al.*, 1992). The nucleated red blood cells of birds make it relatively simple to extract concentrated DNA from this tissue. Other sources of DNA, such as feather pulp require more meticulous technology to extract DNA effectively (Walsch *et al.*, 1991). For this investigation, blood was used as a source of DNA as it was readily available because veterinarians regularly visit the breeders to assess the flocks for disease, especially PBFD, and for other reasons such as sexing.

Blood was taken during the routine check ups of the birds. During these visits additional blood was taken for this investigation as the birds were already anaesthetized. Each bird's head was carefully placed in a small chamber containing gas to anaesthetize them. After 20 seconds, once anaesthetized, the feathers around the neck of the bird were gently pulled aside to reveal the jugular vein. The area was then cleaned using an ethanol swab and the vein pierced with a 10 ml sterile syringe. On average 1 ml of blood was drawn up and collected in sterile tubes containing EDTA to prevent coagulation. The syringe was then removed and the area wiped with the ethanol swab before applying pressure to cease blood flow. The sterile tubes

were then labelled appropriately and transported on ice before being stored in the fridge at 4 °C for further use.

The samples were labelled and coded according to family structure and relatedness, where the numbers from 1 to 5 designated the particular family to which an individual belonged. The families obtained from Amazona were labeled 1, 2 and 3 and the family from Rehoboth, which consisted of the immediate family as well as extended family members, 4. The siblings from the Eastern Cape were all designated the number 5 and the other individuals, a number from 6 to 10 to denote the fact that they are not members of a family.

To distinguish between the breeding male (or father) and the breeding female (or mother), an 'a' was allocated to the male and a 'b' to the female. Their respective chicks were labelled ab i, ab ii or ab iii depending on the number of offspring produced.

Key to the identification code of the birds:

- Numbers 1 – 5 indicate the different families.
- Numbers 6 – 10 indicate unrelated birds.
- Letters a and b indicate breeding male and breeding female respectively
- Letter combination ab indicates progeny of breeding male (a) and breeding female (b)
- Letters w, x, y and z indicate second generation birds

2.3 METHODS

The protocols used in this research are all standard protocols with minor adjustments for avian blood. The recipes for the various reagents described in the different protocols below are taken up in appendix A.

2.3.1 DNA extraction

The generation of fingerprints requires clean, unsheared, high molecular weight DNA. Degraded DNA results in a compromised fingerprint, which does not reflect the true nature of the individual. It is therefore important to produce high quality DNA by following the extraction procedure meticulously.

Many different protocols are used in various laboratories to extract double stranded high molecular weight DNA. Of these protocols, a number employ phenol, which is toxic and where possible should be avoided. In this investigation, a safer and quicker method, known as the salting-out technique was employed (Bruford *et al.*, 1992). This method involves precipitating the denatured proteins using high salt concentrations, resulting in relatively protein-free DNA, which is suitable to use in PCR reactions to generate DNA fingerprints.

The salting-out method of DNA extraction takes two days to complete. After removing the samples from storage at 4°C, 15 µl of blood from each bird was added to 500 µl 1×TNE Lysis Buffer, 50 µl 1 M Tris-HCl pH 8, 1 µl Proteinase K, 7.5 µl 25 % SDS and 7.5 µl 10 % Triton X-100 in separate tubes. The samples were incubated overnight at 37°C. The following day, a half to a third of the volume in the tubes (approximately 300 µl) of $NaCl_2$ was added to the samples. These were then shaken for 15 seconds and then centrifuged at 1 700 g (5 000 rpm) for 15 minutes. The resulting supernatant was removed to a fresh eppendorf tube and the procedure was repeated as before starting with the 15 second shake. This was done 5 times to ensure that maximum DNA was extracted from the supernatant. Two volumes (approximately 1 ml) of 100 % ethanol were then added to facilitate with DNA precipitation. The samples were mixed by inversion to obtain visible DNA strands. These strands were pelleted by centrifugation at 13 000 rpm for 15 minutes after which the supernatant was discarded. The pelleted DNA was washed in one volume (approximately 300 µl) 70 % ethanol and centrifuged at 13 000 rpm for 10 minutes. This washing procedure was repeated three more times to clean the DNA thoroughly and remove impurities. The

clean DNA was then resuspended in 20-100 µl 10 mM Tris-HCl pH 8 (depending on the size of the pellet, however in most cases 50 µl was added) overnight at 37 °C (Bruford *et al.*, 1992).

2.3.2 DNA verification and quantification

To verify the presence of DNA and to ensure that is was of high molecular weight, a diagnostic 0.8 % agarose gel was run. The gel was prepared with 0.4 g agarose in 50 ml 1 X TAE and 1.25 µl ethidium bromide (20 mg/ ml). Each well was loaded with 5 µl sample DNA, 5 µl distilled water and 2 µl loading buffer. A molecular weight marker was not included as the gel served only to verify the presence of DNA.

A Beckman DU 640 Spectrophotometer was used to determine the concentration and purity of the DNA in each sample. Samples were blanked and diluted in 10 mM Tris, with a dilution factor of 100 times, consisting of 5 µl sample in 495 µl 10 mM Tris. The purity was calculated using the formula A_{260} / A_{280} and the concentration calculated with the formula: A_{260} × dilution factor × 50 µg/ ml.

2.3.3 Generation of RAPD fingerprints

RAPD technology, which is based on PCR, follows the standard PCR protocol, with some minor adjustments. In PCR it is important that the DNA concentration in each reaction is the same so that polymorphic bands can be compared. Working stock solutions of 100 ng/ µl template DNA were prepared for all the samples, according to the formula $V_1C_1 = V_2C_2$ as seen in table 2.2.

Table 2.2 Volumes of working stock solutions of 100 ng/ μl.

SAMPLE	C1 (μg/μl)	V1 (μl)	C2 (ng/μl)	V2 (μl)	VOL 10 Mm TRIS
1 a	0.222	10	100	22.2	12.2
1 b	0.579	10	100	57.9	47.9
1 ab i	0.418	10	100	41.8	31.8
1 ab ii	0.263	10	100	26.3	16.3
1 ab iii	0.373	10	100	37.3	27.3
2 a	0.571	10	100	57.1	47.1
2 b	0.378	10	100	37.8	27.8
2 ab i	0.251	10	100	25.1	15.1
3 a	0.328	10	100	32.8	22.8
3 b	0.066	10	100	06.6	-3.4
3 ab i	0.331	10	100	33.1	23.1
3 ab ii	0.241	10	100	24.1	14.1
4 a	0.604	10	100	60.4	50.4
4 b	0.364	10	100	36.4	26.4
4 ab i	1.194	10	100	119.4	109.4
4 ab ii	0.834	10	100	83.4	73.4
4 ab iii	0.594	10	100	59.4	49.4
4 w	0.878	10	100	87.8	77.8
4 x	0.845	10	100	84.5	74.5
4 y	0.429	10	100	42.9	32.9
4 z	0.451	10	100	45.1	35.1
5 ab i	0.592	10	100	59.2	49.2
5 ab ii	1.208	10	100	120.8	110.8
5 ab iii	0.927	10	100	92.7	82.7
5 ab iv	1.051	10	100	105.1	95.1

6	1.359	10	100	135.9	125.9
7	1.108	10	100	110.8	100.8
8	1.287	10	100	128.7	118.7
9	1.061	10	100	106.1	96.1
10	1.423	10	100	142.3	132.3
4 w *	1.353	10	100	135.3	125.3
4 x *	1.522	10	100	152.2	142.2
4 z *	1.572	10	100	157.2	147.2

* denotes the samples that were used to test for reproducibility

a. Primers

A total of fifteen primers were assessed for polymorphisms. Two arbitrary primers were designed and 13 selected from the Operon Technologies RAPD 10-mer primers Set A and Set C. All 15 primers were synthesized by the Molecular and Cell Biology Synthetic DNA Laboratory at the University of Cape Town. Working stock solutions of 100 μM were made with 1 X TE for all the primers used. Table 2.3 provides information on the 15 chosen primers.

Table 2.3 Primer information of working stock solutions.

REFERENCE NUMBER	SEQUENCE	OD*	C_1 (μM)	V_1 (μl)	C_2 (μM)	V_2 (μl)	Vol 1 X TE*
Arbitrary primers							
17: 03 1251	aaa cgg gcg g	171.5	1923	10	100	192.3	182.3
25: 03 1250	tcg gcg agc c	182.1	2042	10	100	204.2	194.2
Operon Set A primers							
A-01: 03 0096	cag gcc ctt c	459.5	5152	10	100	515.2	505.2
A-02: 03 0997	tgc cga gct g	507.6	5692	10	100	569.2	559.2
A-04: 03 0998	aat cgg gct g	443.7	4975	10	100	497.5	487.5
A-06: 03 0999	ggt ccc tga c	473.4	5308	10	100	530.8	520.8

A-09: 03 1253	ggg taa cgc c	143.9	2735	10	100	273.5	263.5
A-10: 03 1000	gtg atc gca g	439.1	4923	10	100	492.3	482.3
A-12: 03 1001	tcg gcg ata g	479.1	5372	10	100	537.2	527.2
A-13: 03 1002	cag cac cca c	465.5	5219	10	100	521.9	511.9
A-17: 03 1003	gac cgc ttg t	501.6	5624	10	100	562.4	552.4
A-19: 03 1254	caa acg tcg g	144.6	1621	10	100	162.1	152.1
A-20: 03 1004	gtt gcc atc c	470.0	5269	10	100	526.9	516.9
Operon Set C primers							
C-05: 03 1252	gat gac cgc c	195.8	195.	10	100	219.5	209.5
C-06: 03 1005	gaa cgg act c	401.1	4496	10	100	449.6	439.6

* OD = optical density given by the laboratory involved in the synthesis of the primers
* VOL 1 X TE = amount of TE added to 10 μl of primer to obtain 100 μM concentration

b. PCR conditions

RAPD-PCR reaction conditions were set up using a PCR Core Kit from Roche Diagnostics. Optimized volumes of reagents were carried out in a 25 μl reaction and consisted of: 2.5 μl 10×Buffer (with $MgCl_2$), 0.5 μl of 200 μM dNTPs, 1 μl of 1 mM $MgCl_2$, 0.25 μl of 1 μM primer, 18.75 μl distilled H_2O, 1 μl DNA (100 ng/μl) and 1 μl (1 unit) *Taq* polymerase. *Taq* polymerase purchased at a later stage of the investigation had a greater concentration (5 units per μl) thus, in this case only 0.2 μl was added to the PCR reaction. The volume of distilled water was adjusted accordingly to obtain a final volume of 25 μl in each PCR tube. Master mixes, consisting of all reagents excluding the template DNA, were used to speed up the process and make it more accurate. The PCR reagents, excluding the DNA template, were pipetted into an eppendorf tube and gently shaken in order to equally distribute the reagents within the tube. The *Taq* polymerase was added last so that minimal hybridization would take place between the primer sequences. 24 μl of the master mix was then added to each PCR tube on ice, containing 1 μl of template DNA. The PCR tubes were then placed in the GENE AMP PCR 9700 machine and left to run their course.

Before any primers were screened and analysed, the PCR protocol was optimized for the Cape parrot. This involved testing a number of different combinations of the PCR reagents using different concentrations and volumes, as well as conditions such as temperatures. The values and combinations that gave the best result were then selected as the optimum setting for that reagent as illustrated in table 2.4.

Table 2.4 Optimized PCR reaction.

REAGENTS	SUPPLIER'S CONCENTRATION	OPTIMAL CONCENTRATION	VOLUME
10 x PCR Buffer	10 x	1 x	2.5 µl
dNTPs	10mM	200µM	0.5 µl
$MgCl_2$	25 mM	1 mM	1 µl
Taq polymerase *	2 U/ µl	1 U/ µl	1 µl
Primer	100 µM	1 µM	0.25 µl
dH_2O	-	-	18.75 µl
DNA template	100 ng/ µl	100 ng	1 µl

* If *Taq* polymerase 5 U/ µl was used, the volume added to each reaction decreased to 0.2 / µl. As a result the dH_2O was increased to 19.55 µl.

A range of values, between 1 mM and 5 mM were tested in order to determine the optimum concentration of magnesium chloride of 2.5 mM. Similarly, five annealing temperatures, ranging between 34 °C and 42 °C, were tested to obtain the optimum annealing temperature of 40 °C. The optimum DNA concentration was found to be 100 ng using 40 cycles in the PCR reaction. This combination of reagents and settings were found to be the optimum PCR conditions for the Cape parrot and produced the brightest and most reliable bands.

The optimized PCR cycle conditions started with an initial denaturation step at 94 °C for 3 minutes followed by 40 cycles with the cycle profile: 1 minute at 94 °C, 1 minute at 40 °C and 2 minutes at 72 °C. The cycle was concluded with 10 minutes at 72 °C after which the sample tubes were stored at 4 °C until use.

2.3.4 Determination and screening of polymorphic primers

An investigation of the genetic variation in the Cape parrot has not been conducted before. There is thus no information available about which primers are polymorphic and most suited for an investigation of this nature. Therefore, a pilot investigation was initially conducted on a limited number of individuals in order to determine which of the 15 chosen primers were most suited to continue with the investigation. The 15 primers were tested on a few individuals that represented each of the sources of the birds as well as both sexes. Once the polymorphic primers were identified, they were employed to analyse all 30 individuals.

RAPD-PCR analysis was performed on all primers and the results analyzed on a 2 % agarose slab gel (250 ml 0.5 X TBE) to which 6.25 µl ethidium bromide (20 mg/ml) was added. The samples were then run at 100 V for approximately 5.5 hours in 1 litre 0.5 X TBE and 25 µl ethidium bromide (20 mg/ ml). Thereafter, the gel was placed on an UV transilluminator to visualize the bands.

During the pilot investigation it was found that TBE, as compared to other buffers such as TAE, had a better buffering capacity for smaller fragments, such as those produced in RAPD reactions, particularly in gels running over longer periods of time. Furthermore, a 1 % agarose gel separated the RAPD fragments more effectively than gels of a higher concentration agarose.

2.3.5 Reproducibility

Reproducibility is one of the main concerns when employing the RAPD assay. With population investigations such as this, it is very important that the reproducibility is high so that comparisons can be conducted accurately (Bowditch *et al.*, 1993). In this investigation, the reproducibility was tested in several ways through the use of internal standards, repeated analyses and reproducibility tests.

A complete test for reproducibility using separate DNA extractions from the same sample was conducted. The extractions were performed on three samples (4w, 4x and 4z) in the same manner as in the previous extractions. The DNA purity and concentration of these samples was determined and can be seen_in Table 2.2 (denoted *). PCR reactions, using primers 17 and A-06 were then performed on these samples (4w*, 4x* and 4z*) as well as the on the same samples (4w, 4x and 4z) from the previous DNA extraction. The PCR products were analysed on a 1 % agarose gel and compared.

In order to maintain reproducibility and equivalence from one primer and gel to another, an internal standard was employed. This internal standard consisted of DNA sample (2a) amplified by primer A-06, and was run on every gel. The known profile of the individual using this particular primer was compared between experiments and if the profile deviated from the expected, the PCR reaction was excluded from the investigation and thus not scored.

As a final test for reproducibility, the RAPD reactions for each of the determined polymorphic primers was repeated twice, to ensure the resulting gels were identical and thus included in the investigation.

2.4 STATISTICAL ANALYSIS

There are numerous statistical methods available to analyze molecular data. It is therefore important that before software is selected to analyze the data, knowledge of the types of analyses that can be conducted is obtained so that suitable software can be selected.

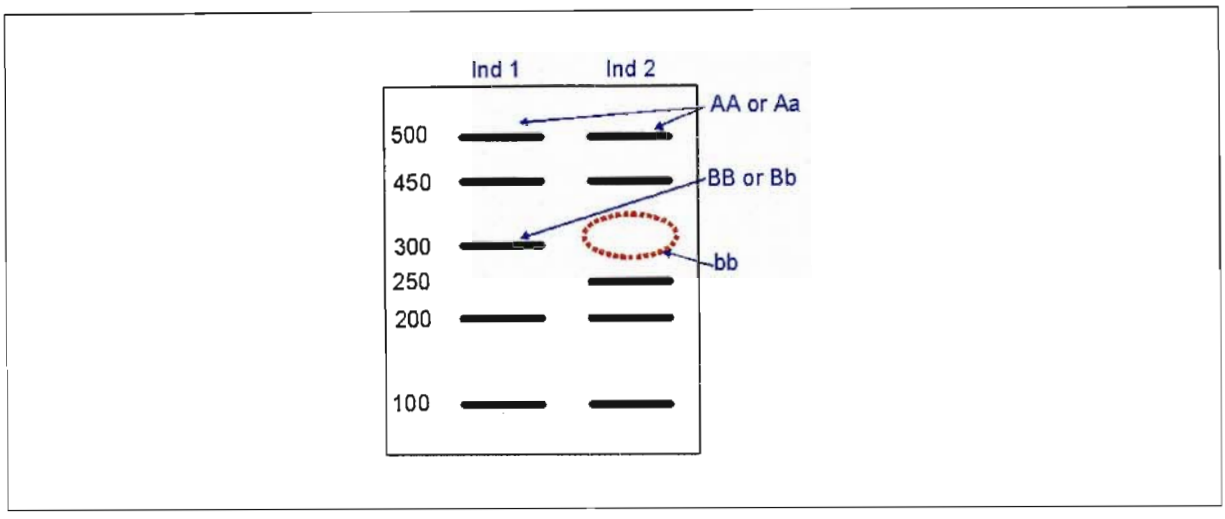
Broadly the steps to follow when analyzing molecular data can be summarized as follows:

- Assess which types of analyses will be undertaken such as inter- or intra-population analyses.
- Select appropriate software that will be used to do the various analyses and calculations.
- Convert the gel fingerprint data into a digital fingerprint according to the specifications of the particular software chosen.
- Import data into software package and do the analyses.
- Interpret results.

2.4.1 Scoring RAPD gels and individual genotyping

In order to analyze molecular fingerprints, it is necessary to convert the gel data into a digital format. The fingerprints in the case of a RAPD are considered to be the phenotypes of a set of loci generated with a particular primer. The phenotypes of a particular locus are the fragments of a particular size produced by a particular primer and can have only two different alleles, namely, a *presence* allele and an *absence* allele, where the *presence* allele produces a fragment of the particular size, while the *absence* allele does not, due to mutation(s) in the annealing site(s) of the primer. As RAPD markers are dominant in nature, a genotype homozygous (AA) for the *presence* allele cannot be distinguished from a genotype that is heterozygous (Aa) for the *presence* allele. A fragment at a particular size therefore indicates a dominant homozygote (AA) or heterozygote (Aa), while its absence, indicates the recessive genotype (aa). Figure 2.1 illustrates the presence or absence of bands in homozygous and heterozygous individuals for RAPDs.

Figure 2.1 The different genotypes associated with band presence and band absence in dominant marker analysis.



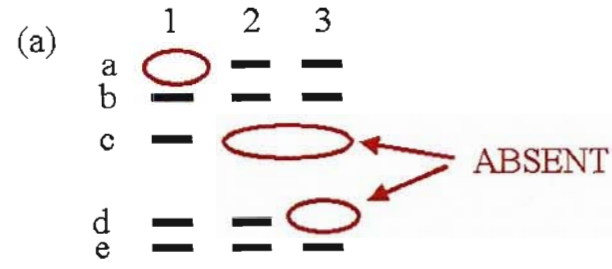
Before scoring the agarose gels, suitable loci for scoring, were identified. A suitable locus in this investigation was defined as:

- consisting of a single band,
- being perfectly reproducible,
- clearly and distinctly present in individuals given a value of 1, and
- being between the sizes of 3 054 bp and 298 bp.

The last criterion was included as most individuals only produced reliable and reproducible bands within this range. Bands larger than 3 054 bp and smaller than 298 bp were generally faint and their presence inconsistent when comparing profiles between repeated gels.

Once suitable loci were identified, reproducible RAPD gels displaying polymorphisms were scored according to the binary number system where a value of 1 was given to a present band at a particular locus and a value of 0 when it was absent. The resulting profiles for an individual using a particular primer therefore consisted of a series of ones and zeros ranging from as many as ten digits to as few as five as illustrated in figure 2.2.

Figure 2.2 **Example of how a RAPD gel was scored. (a) Showing an agarose fingerprint representation and (b) the corresponding profiles of the presence and absence of bands a – e for three individuals 1, 2 and 3.**



(b)

	a	b	c	d	e
1	0	1	1	1	1
2	1	1	0	1	1
3	1	1	0	0	1

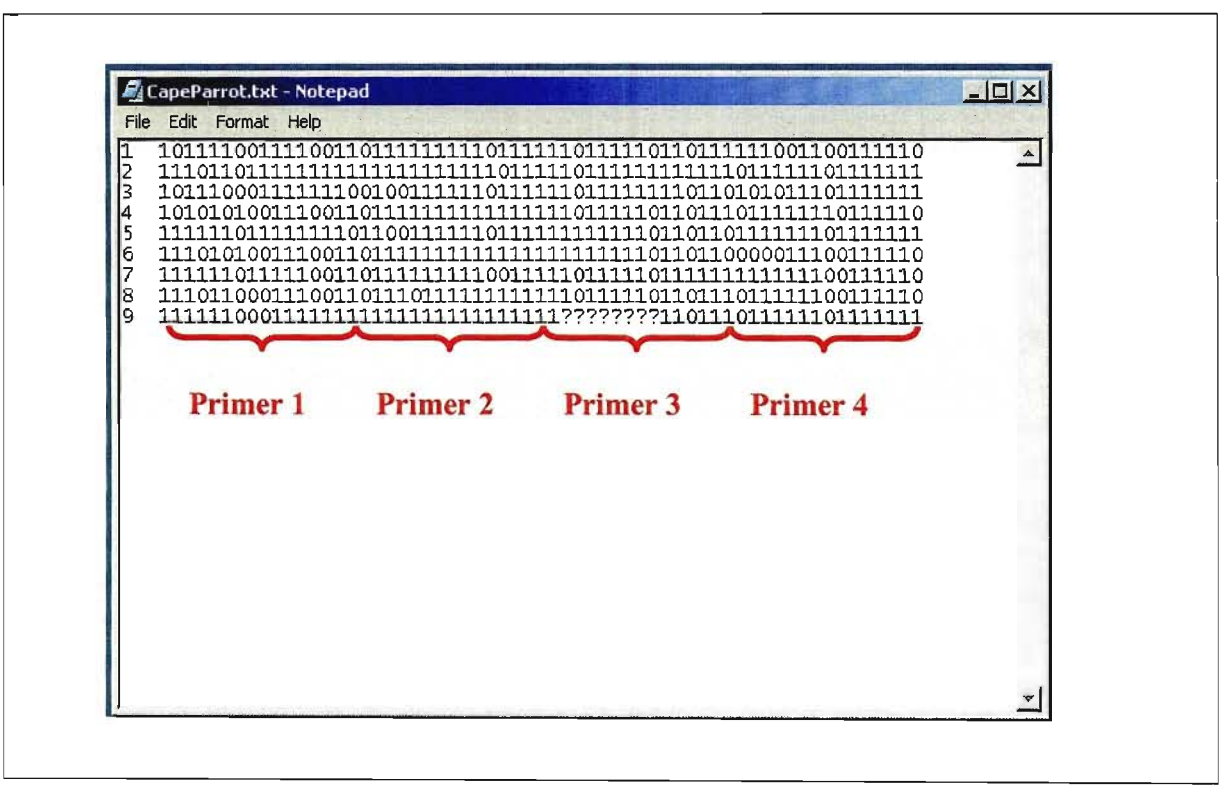
The red circles represent the absence of bands in an individual's profile.

In some cases, where bands were faint or not clearly visible, it was difficult to determine whether or not a band should be considered present in an individual. Whether or not these bands should be scored as 'present' is solely up to the researcher. The researcher should however try and standardize what he or she decides these fainter bands be scored as, and maintain this throughout the scoring process so as to minimize errors. Today computer programmes and software used to score the RAPD data are available, however they were not employed in this investigation. The repetition of all the reactions provided a way of 'double-checking' the results and coming up with a final decision in the borderline cases.

In the few individuals where both gels had produced faint bands for a particular locus, a value of zero was given to indicate band absence.

The numerical series of zeros and ones, termed a profile or fingerprint, resulting from the scoring, was the raw data used in statistical tests and analyses. Once these profiles were constructed for each individual, they were entered into a text file as a data set. The photos of the gels used for scoring were therefore no longer required. An example of a text file (CapeParrot.txt) containing the digital fingerprint data of nine arbitrary individuals prepared with Notepad, Windows' internal word processor, is represented in figure 2.3.

Figure 2.3 Digital fingerprint of nine arbitrary individuals prepared with notepad.



In total, 85 loci were scored for all 30 individuals across the 15 primers. In some cases, no data was available due to variable banding in an individual using the same primer. In these cases the missing binary data was substituted with either '?' or '.' depending on the programme used for the analysis, as seen in figure 2.3. The individual scores for the polymorphic primers are taken up in Appendix B.

2.4.2 Identification of polymorphic primers and calculation of percentage polymorphism

The preliminary analyses of the scored data involved counting the number of bands produced by all 15 primers and then calculating the percentage of bands displaying polymorphisms. Other calculations such as calculating the proportion of polymorphic loci and identifying the average number of bands produced by primers were also included as an indication of the amount of variation present. After the general analysis on all the primers was complete, those primers that produced reliable and reproducible polymorphic bands were identified. Polymorphic primers were identified as possessing loci with varying values of one and zero, depicting the presence or absence of a band in an individual. If the band was present in all thirty individuals, the locus was termed monomorphic and conversely if it was absent in some individuals, it was termed polymorphic. Once the polymorphic primers were identified, the monomorphic primers were ignored and the analysis continued on the selected polymorphic primers.

Each of the polymorphic primers was analysed separately and the number of polymorphisms evident in each primer in relation to the number of bands produced calculated and given as a percentage polymorphism. This served to indicate the variation produced by each individual primer and thus their individual merit.

2.4.3 Correlation between percentage GC content of primers and number of amplified bands

A correlation analysis was conducted to determine whether the percentage GC content of the primers affected the number of amplified bands produced. Statistical Processing for the Social Sciences (SPSS), version 11.0 (LEAD technologies, Inc) was used to perform the calculations. SPSS is a powerful data management and statistical analysis programme, specifically designed for the social sciences, which can however perform statistical procedures for many other fields provided the data is in numerical format. The data was entered as two columns, one containing values for the percentage GC content of the primers and the other, the values of the corresponding number of bands produced by each primer. From this information Pearson's correlation co-efficient was calculated and a corresponding scatter plot illustrating the relationship between the two entities, constructed. SPSS was obtained from www.spss.com.

2.4.4 Calculation of genetic variation using heterozygosity

One way to measure the genetic variation present in a population or between populations is to calculate heterozygosity. As the analysis was conducted on dominant markers, the assumptions, conditions and suggestions proposed by Lynch & Milligan (1994) were followed.

Heterozygosity can be calculated in a number of different ways, each method giving a value indicative of the genetic variation in the population. This can however only be achieved if the population is in Hardy-Weinberg Equilibrium and not affected by any evolutionary changes such as mutation and selection and that the mating is random with no inbreeding. The frequencies obtained from the different methods, are not identical but should be similar. In this investigation the heterozygosity was calculated using two different methods to ensure correct results. Both methods followed the conditions proposed by Lynch & Milligan (1994), which suggest that in order to obtain

an unbiased and accurate estimate of the allele frequency (q), only those loci where the frequency of the null-phenotype exceeds a value of 3/n (where n is the number of individuals in the population sampled) should be included in the analysis. In this investigation, the number of individuals sampled was 30 therefore loci with recessive phenotypic values of less than 0.1 were regarded as monomorphic and therefore not included in the analysis.

In the first method the heterozygosity was calculated for each of the polymorphic primers. The number of null alleles (given a value of zero) for each of the loci in all of the polymorphic primers was counted. Recessive allele frequencies (q) were only calculated for polymorphic loci with a null-phenotypic frequency (q^2) more than 0.1. From this value the dominant allele frequency (p) was determined and an estimate of the frequency of heterozygotes (2pq) was obtained for each of the polymorphic loci. These values were averaged for each individual primer from which a mean heterozygosity value could be determined for the Cape parrots in this investigation.

The second method involved calculating the average heterozygosity for each individual and thus obtaining values for each of the three subpopulations as well as for the population as a whole. The number of null alleles present in each individual across all primers were determined and used to estimate the frequencies of (p) and (q). As before the heterozygosity was calculated using the formula (2pq). The values obtained for the individuals in one subpopulation were averaged and compared to the values obtained from the other two subpopulations, as well as to the population as a whole.

2.4.5 Calculation of genetic variation using diversity indices and genetic distance measures

The genetic diversity was evaluated using POPGENE version 1.31 (Yeh *et al.*, 1999), which is a free, user-friendly software programme. It is a Microsoft Window-based computer package with graphical user interfaces and simple menus and dialog boxes.

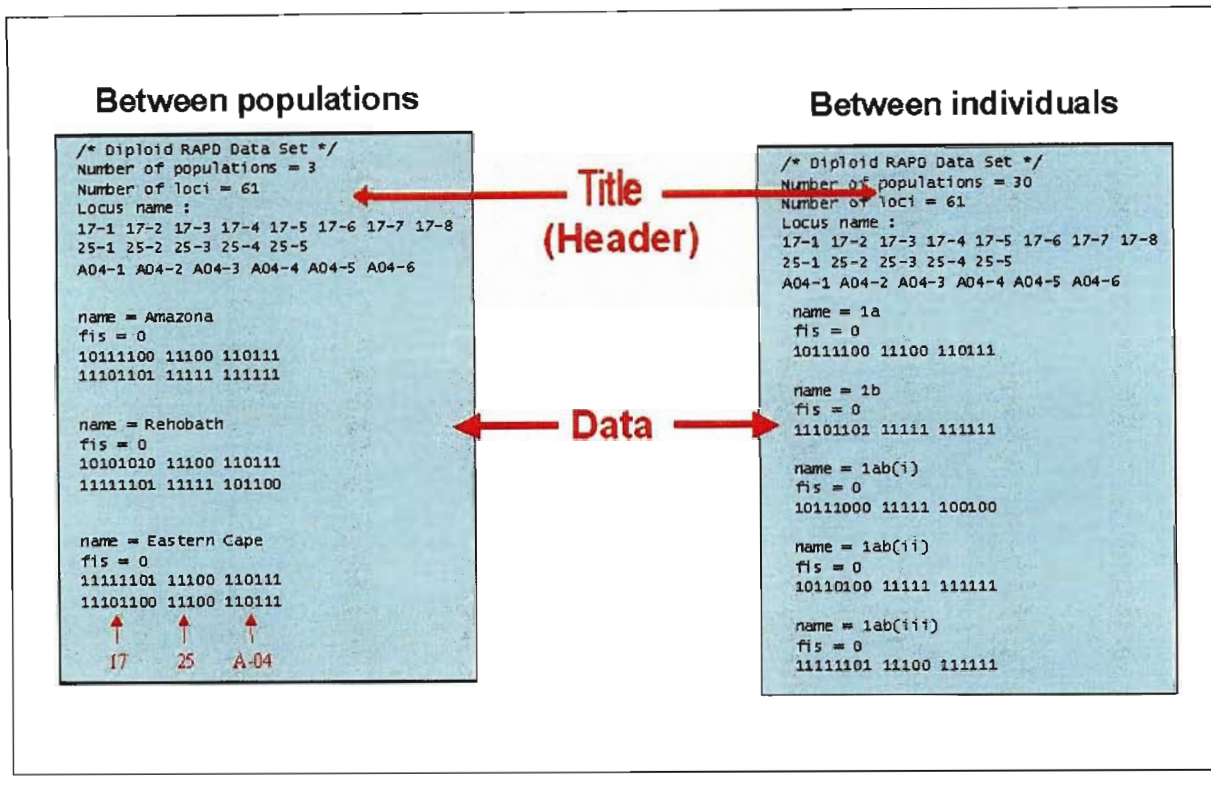
POPGENE was specifically designed for the population genetic analysis of co-dominant as well as dominant markers, using either haploid or diploid data. Most types of population genetic measures, such as allele and genotypic frequencies, diversity indices, neutrality tests and genetic distances could be computed using POPGENE. POPGENE was obtained from www.ualberta.ca/~fyeh/.

a. Creating digital fingerprints for POPGENE

The RAPD scores were entered into the programme as two separate input files, one that would be used to evaluate between population diversity and the other to compare the diversity between all individuals as well as between members of a single family as illustrated in figure 2.4. In both cases the file consisted of a header section (specifying the title, number of populations, number of loci and the locus names) as well as a data section. The data was entered in columns representing the profiles obtained by each primer for each individual.

In the case of the between population input file, the data was grouped into three sections representing the different populations and labelled accordingly. The three populations were labelled: 'Amazona' for those 12 individuals originating from Assagay, 'Rehoboth' for the nine individuals obtained from Dargle and 'Eastern Cape' for the remaining 9 samples. In the case of the between individuals and between members of a family, each individual was labelled as a separate population, the data thus consisting of 30 sections. In both of the input files, each population was given a F_{IS} value of zero to denote Hardy-Weinberg equilibrium. Examples of the input files for POPGENE are taken up in Appendix C.

Figure 2.4 Example of the two POPGENE input files created for analysis between populations and between individuals within a population.



b. Within and between population analysis

Once the data was loaded into the programme the analysis on the between population input file was conducted. This was performed using dominant marker analysis on diploid data assuming Hardy-Weinberg equilibrium (F_{IS} = 0) for the separate populations. For the intra-population analysis Shannon's Index of phenotypic diversity was used as an indication of the amount of variation in each population compared to the population as a whole. Values were obtained for each individual across all loci in the separate populations. The values were then averaged for each primer in the three populations and from these values a mean was calculated for the individual populations as well as for the population as a whole.

Using the same input file, Nei's (1972) genetic identity and genetic distance, as well as Nei's (1978) unbiased genetic identity and genetic distance were calculated for the three populations. This served to determine the genetic distance as well as the genetic identity between the populations. From the results of the genetic identity and genetic distance measures, a dendrogram depicting the relationship between the three populations of Cape parrots was constructed by POPGENE. This programme used UPGMA, which is an adoption of programme NEIGHBOR of PHYLIP version 3.5c by Joe Felsenstein (1993), to construct the dendrogram.

c. Between individuals and within families

Using the second data set, where each individual was labelled as a separate population, the genetic identity and genetic distance values between all the individuals were computed. After the previous analysis between the populations, and observing the insignificant difference between Nei's original and unbiased methods, it was decided to compute only the original genetic distance and genetic identity. A dendrogram depicting the relationship between all 30 individuals was constructed using UPGMA function in POPGENE.

Using the same input file, but excluding all individuals other than the family to be analysed, Nei's genetic identity and genetic distance were computed for the three families from Amazona, the family from the Rehoboth and the four siblings from the Eastern Cape population. A further analysis was conducted on the determined related individuals from Amazona and the Eastern Cape. A dendrogram was constructed for all of the above analyses to illustrate the relationship between family members.

2.4.6 Calculation of genetic variation using Analysis of Molecular Variance (AMOVA)

Analysis of Molecular Variance (AMOVA) is a method of estimating population differentiation and testing various hypotheses about the differentiation, directly from

molecular data. It is a widely used method of analysis that is included in almost every investigation in order to determine the contribution of the between and within population or group variation to the total variation.

The ARLEQUIN software package ver. 2.000 (Schneider *et al.*, 2000), the preferred software for these analyses, was used to compute these values of variation segregation. It consists of a Java graphical interface that allows the user to rapidly select the analyses required to perform with the data. Many different types of raw data can be handled by ARLEQUIN, ranging from DNA sequences to microsatellite data and RFLP to allele frequency data. This package does, however, not deal with dominant marker analysis directly, because the functions originally written for RFLPs are directly applicable for dominant markers such as RAPDs and AFLPs, which also require binary data (values of 0 and 1). The ARLEQUIN software can be obtained from www.unige.ch/arlequin/.

ARLEQUIN offers a wide range of statistical tests and measures that can be applied to data, very similar and in some cases identical to those computed by POPGENE. Due to POPGENE being more user-friendly and less challenging to the casual computer user, ARLEQUIN was only used to conduct an AMOVA and calculate a Euclidian distance matrix.

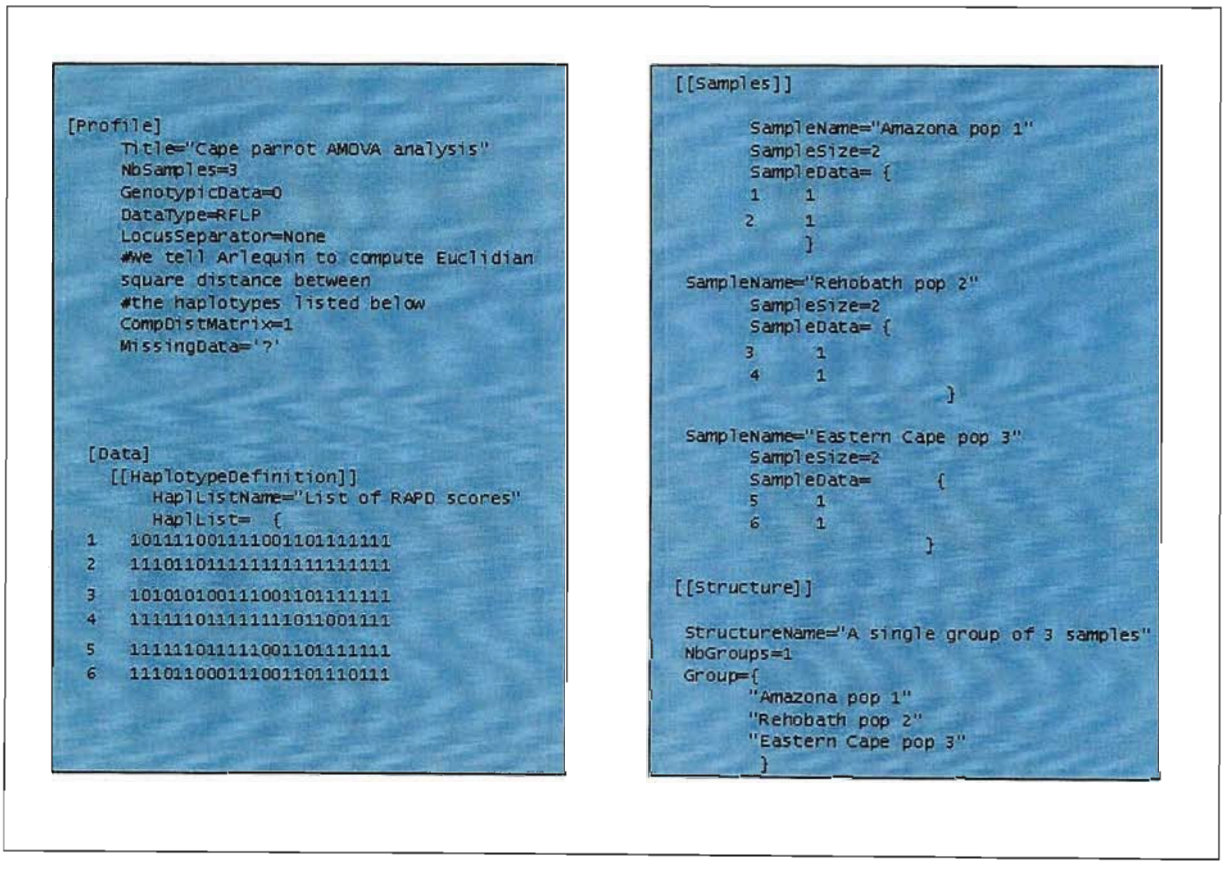
To conduct an AMOVA with the data requires the following steps:

- Convert the molecular fingerprint data into digital data according to the specifications of ARLEQUIN,
- Launch ARLEQUIN and create an analysis profile, namely, the Project file,
- Perform the specified calculations on the Project file, and
- Interpret the resulting output.

a. Creating digital fingerprints for ARLEQUIN

An input file, meeting the specific requirements for the ARLEQUIN package, was constructed using the raw score data saved in the text file created earlier. Similarly to POPGENE, the input file consisted of a profile section specifying the properties of the data such as the title of the project, type of data, number of samples and other specifications, as well as a data section containing the raw data from the text file. Following the raw data was a section detailing the structure of the data. Here the frequency of each specified profile in each population was given as well as an idea as to how the populations were compiled and grouped. An example of an ARLEQUIN input file is shown in figure 2.5, the original input file constructed for ARLEQUIN is taken up in Appendix E.

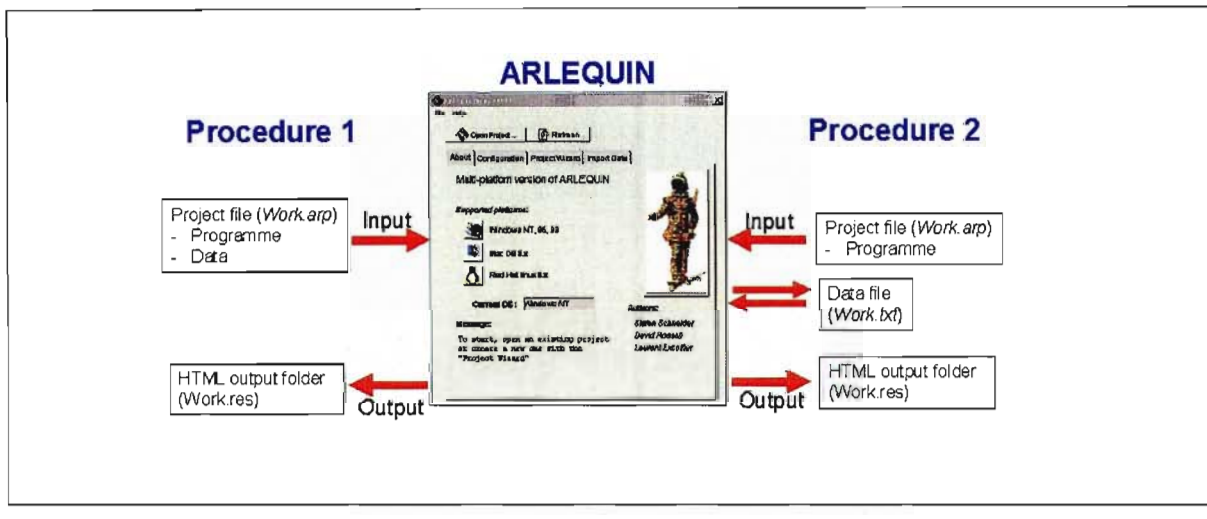
Figure 2.5 An example of an ARLEQUIN input file showing the three sections.



b. Working with ARLEQUIN

ARLEQUIN analyses are undertaken by creating a Project file with the extension 'arp.'. This file contains the input file with all the instructions for the analyses and may contain the data section (Procedure 1) or may have the data section in an external file, in which case it is simply referred to (Procedure 2). Figure 2.6 illustrates the possible routes that can be followed using the procedures mentioned to create a project file.

Figure 2.6 Showing the two possible routes that can be followed in order to create a project file.



In this investigation, procedure 1 was followed and a project file, containing the data section, was created using the 'project wizard'. Project wizard allows the user to create an 'arp' folder in which the completed input file can be pasted. A 'mock' folder, whose contents and specifications needed not be accurate due to editing later, was thus created using project wizard. The input file was copied into this folder and the original contents, deleted. This resulted in a project file (with an 'arp' extension) containing the input file for the analysis that would be recognised by the ARLEQUIN programme.

Once the project file was saved, it was opened by ARLEQUIN using the 'Open Project' option and loaded into the programme. If it was successfully loaded, an outline of the project file and its contents were displayed in the programme window. By selecting the 'Calculation Settings' option the various statistical tests offered by ARLEQUIN were presented in the interface. In this investigation, the 'Genetic structure' calculation containing the AMOVA analysis was selected and the appropriate settings, including whether or not a distance matrix should be computed and displayed, specified. An AMOVA analysis was conducted on the data from the specified project file, by constructing a Euclidean distance matrix, using 1 023 permutations. Wright's fixation index (F_{ST}) was also calculated by the programme. The 'Run' function was then selected, after which the specified computations were undertaken by the programme. The results were viewed in the AMOVA folder as a separate folder, with the extension '.res', and interpreted.

CHAPTER 3
RESULTS

3.1 INTRODUCTION

In this investigation the suitability of RAPD analysis was undertaken in the endangered Cape parrot species, *Poicephalus robustus,* to assess the extent of genetic variation in the captive population.

As this was the first investigation of its kind, the PCR protocol and conditions had to be optimised for this particular species before the analysis could be conducted.

Once the optimum conditions were established, the 15 chosen primers were screened using all 30 samples in order to identify the polymorphic primers. Further analyses were carried out on the identified polymorphic primers with regard to individual, within subpopulations as well as between subpopulation analysis.

The results of this investigation are presented in the following order:

- Optimization of RAPD-PCR protocol for the Cape parrot.
- Screening of 15 primers on all individuals.
- Analysis of the polymorphic primers at the individual level.
- Analysis of the polymorphic primers at the population level.
- Analysis of the polymorphic primers between sub populations.
- Proposal for the genotyping of a species for the first time.

The actual print outs of the results by the various statistical programmes are taken up in Appendix B, D and F.

3.2 DNA EXTRACTION

DNA extraction was performed using the salting-out technique as suggested by Bruford *et al.* (1992). This method produced high quality DNA suitable for RAPD analysis. The concentrations and purities of the DNA are displayed in Table 3.1.

Table 3.1 Quality and quantity of DNA extractions from all samples.

INDIVIDUALS	ABSORPTION (260nm)	ABSORPTION (280nm)	PURTIY (260nm/280nm)	CONCENTRATION (µg/ µl)
1 a	0.0443	0.0222	1.99	0.222
1 b	0.1157	0.0614	1.88	0.579
1 ab i	0.0835	0.0430	1.94	0.418
1 ab ii	0.0526	0.0290	1.81	0.263
1 ab iii	0.0746	0.0397	1.87	0.373
2 a	0.1141	0.0614	1.85	0.571
2 b	0.0756	0.0382	1.97	0.378
2 ab i	0.0502	0.0232	2.16	0.251
3 a	0.0655	0.0351	1.86	0.328
3 b	0.0132	0.0061	2.16	0.066
3 ab i	0.0661	0.0356	1.85	0.331
3 ab ii	0.0481	0.0269	1.79	0.241
4 a	0.1208	0.0630	1.92	0.604
4 b	0.0727	0.0373	1.95	0.364
4 ab i	0.2387	0.1382	1.73	1.194
4 ab ii	0.1668	0.0908	1.84	0.834
4 ab iii	0.1187	0.0635	1.87	0.594
4 w	0.1756	0.0962	1.83	0.878
4 x	0.1689	0.0938	1.80	0.845
4 y	0.0857	0.0435	1.97	0.429
4 z	0.0902	0.0482	1.87	0.451
5 ab i	0.1183	0.0584	2.03	0.592
5 ab ii	0.2416	0.1353	1.79	1.208
5 ab iii	0.1854	0.1069	1.73	0.927

5 ab iv	0.2102	0.1132	1.86	1.051
6	0.2718	0.1581	1.72	1.359
7	0.2215	0.1190	1.86	1.108
8	0.2573	0.1374	1.87	1.287
9	0.2122	0.1176	1.80	1.061
10	0.2845	0.1530	1.85	1.423
4 w *	0.2705	0.1464	1.85	1.353
4 x *	0.3043	0.1610	1.89	1.522
4 z *	0.3144	0.2083	1.51	1.572

* denotes the samples that were used to test for reproducibility

3.3 OPTIMIZATION OF RAPD PROTOCOL FOR FINGERPRINTING

Successful PCR analysis is species specific and is dependent on obtaining the correct combination of reagents and conditions. It is therefore important to optimize these reagent concentrations and conditions when analyzing a species for the first time so as to obtain consistent and reliable results. Common reagents requiring optimization include DNA, primer and magnesium chloride concentrations. These entities were determined by varying the concentrations of one particular reagent, whilst keeping all others in the reaction constant. Optimum values for the entity in question could therefore be identified by comparing the results of the varying concentrations and identifying which one produced the best fingerprint.

3.3.1 Optimization of reaction conditions

Several values of DNA concentrations, ranging from 25 ng/ µl to 150 ng/ µl were tested. It is very important to determine the correct amount of template, as too little DNA can result in very faint or no bands, whereas too much DNA can produce smeared bands. It was found that 100 ng/ µl of genomic DNA gave the most consistent and brightest DNA fingerprints for this particular species. When comparing this value to investigations in other bird species it was found that they used similar amounts of template DNA (Padilla *et al.*, 2000).

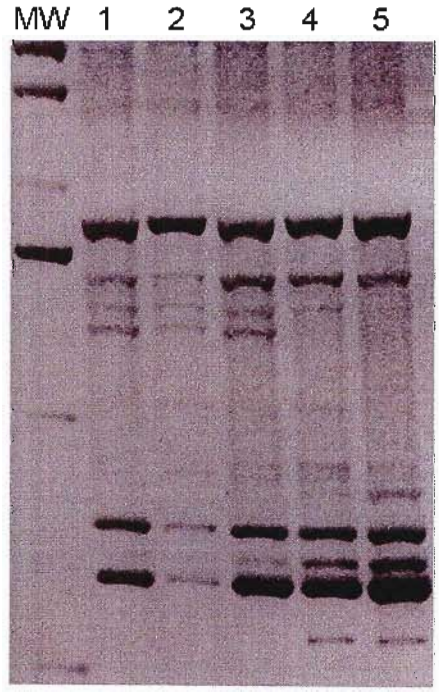
The optimal $MgCl_2$ concentration was found to be between 2 mM and 2.5 mM when testing a range of values from 1 mM to 5 mM concentrations as suggested by protocols. When comparing this value to the literature it was again in agreement with other RAPD analyses performed on birds (Cooper, 2000; Petrie *et al.*, 1998; Padilla *et al.*, 2000). As the PCR buffer already contained $MgCl_2$, it was calculated that only 1 mM extra $MgCl_2$ needed to be added to the reaction.

The optimum annealing temperature was found to be 40 °C, when values from 34 °C to 42 °C were analysed at 2 °C intervals. Figure 3.1 illustrates the various temperatures tested in order to determine the optimum temperature.

Figure 3.1 Gel showing various annealing temperatures used to determine the optimum temperature.

KEY:

MW = molecular weight marker X
1 = 34 °C
2 = 36 °C
3 = 38 °C
4 = 40 °C
5 = 42 °C

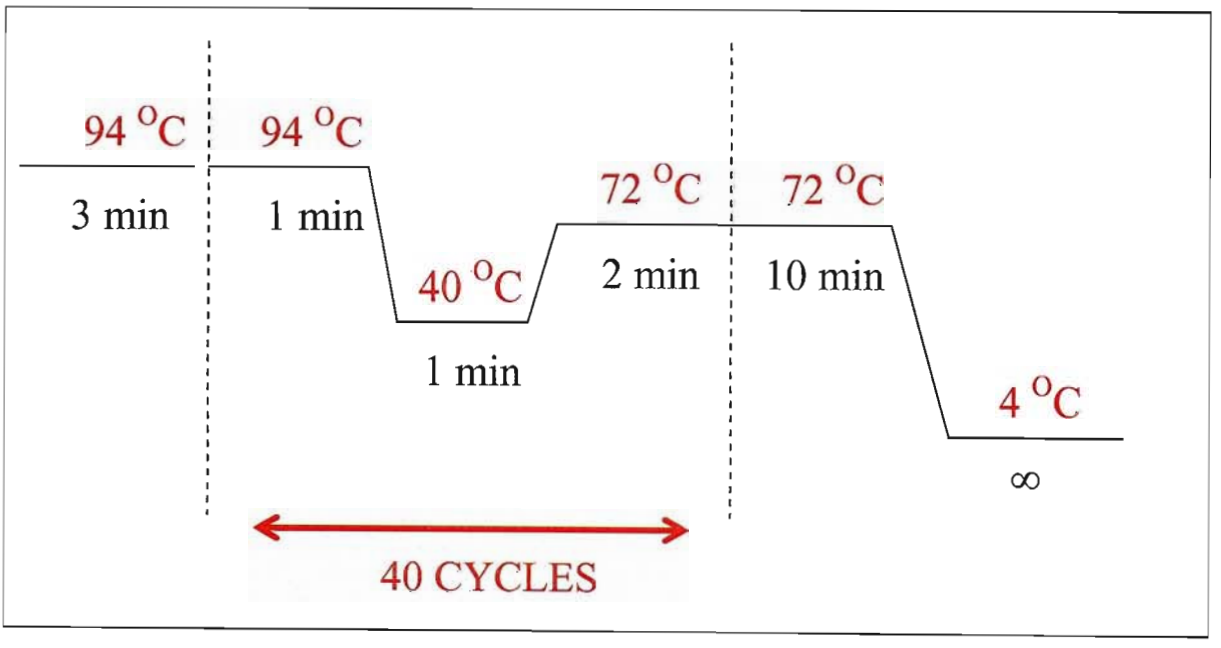


Lower temperatures amplified fainter bands that were inconsistent and although amplification at 42 °C produced good results, there were fewer bands present than at 40 °C. It was therefore decided that 40 °C was the optimal annealing temperature and was used throughout the investigation.

Initially the optimal primer concentration was believed to be 2 μM, however after viewing primer-dimer formations in numerous gels (which usually indicates an excess of primer), this concentration was reduced to 1 μM. This reduced concentration produced a good fingerprint and prevented primer-dimer formations from occurring.

It was found that 40 cycles gave the best and most consistent DNA profiles. Fewer cycles, such as 35, resulted in unreliable fainter profiles due to a lower product yield, whereas more cycles increased the amount of non-specific background products. Figure 3.2 illustrates the optimal PCR profile and settings determined for this investigation

Figure 3.2 Showing optimised PCR settings and cycle numbers.



The optimised reaction conditions and reagent concentrations used in this investigation are summarised in table 3.2.

Table 3.2 Optimised PCR reaction.

REAGENTS	SUPPLIER'S CONCENTRATION	OPTIMAL CONCENTRATION	VOLUME
10 x PCR Buffer	10 x	1 x	2.5 µl
dNTPs	10mM	200µM	0.5 µl
$MgCl_2$	25 mM	1 mM	1 µl
Taq polymerase *	2 U/ µl	1 U/ µl	1 µl
Primer	100 µM	1 µM	0.25 µl
dH_2O	-	-	18.75 µl
DNA template	100 ng/ µl	100 ng	1 µl

* If *Taq* polymerase 5 U/ µl was used, the volume added to each reaction decreased to 0.2 / µl. As a result the dH_2O was increased to 19.55 µl.

3.3.2 Reproducibility

Reproducibility is one of the major concerns when performing a RAPD analysis and has been the reason why some researchers have opted for other methods of analysis in order to ensure reproducibility. As this investigation was one of the first of its kind in this particular species, it was important to determine if the RAPD-PCR, once optimised, could actually be repeated and produce identical results.

In order to do this, RAPD-PCR analysis was conducted on two sets of samples from 3 individuals that had undergone identical but separate DNA extraction procedures. If this technique was reproducible, the 2 samples from each individual should have produced identical fingerprints. Figure 3.3 illustrates the DNA fingerprints produced by the separate extractions of the same samples.

Figure 3.3 Gel showing reproducibility between separate extractions of the same DNA samples.

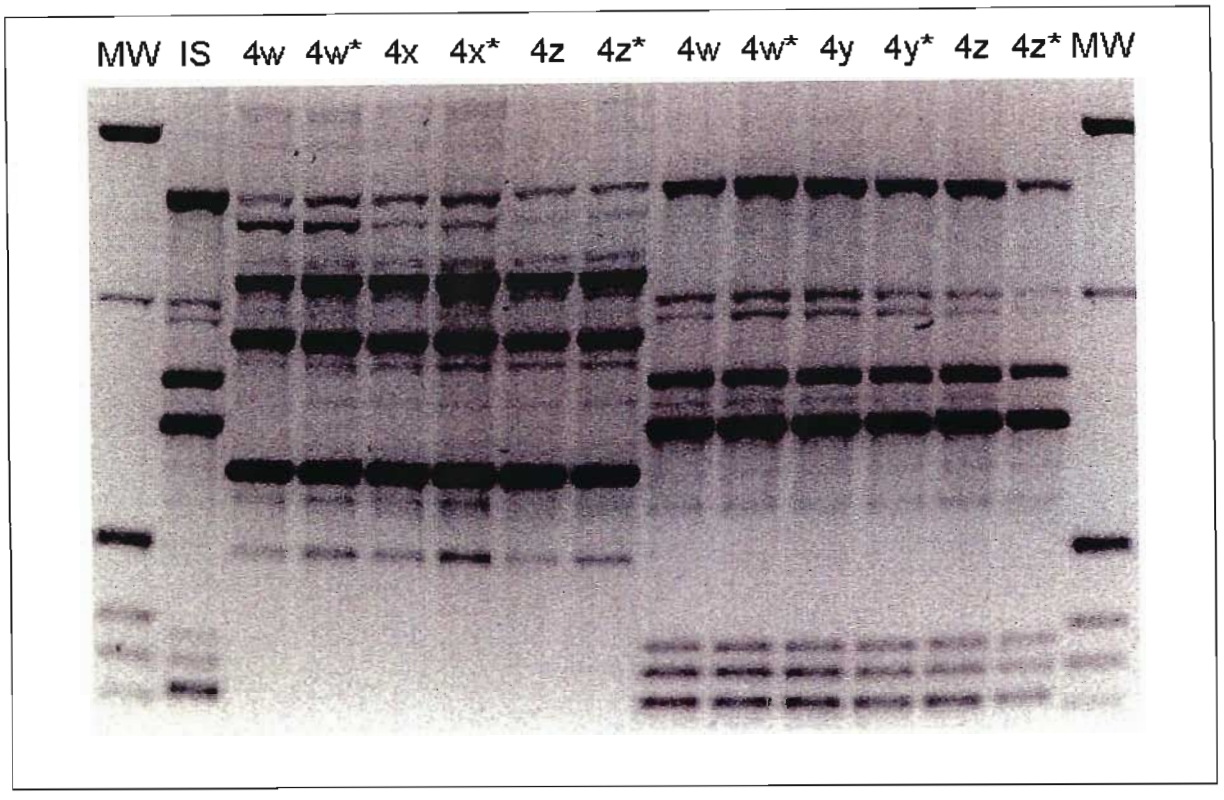


Figure 3.3 shows that the DNA fingerprints of the 2 samples from the same individuals (adjacent to one another on the gel) were completely identical despite one set of DNAs being extracted a few months after the others.

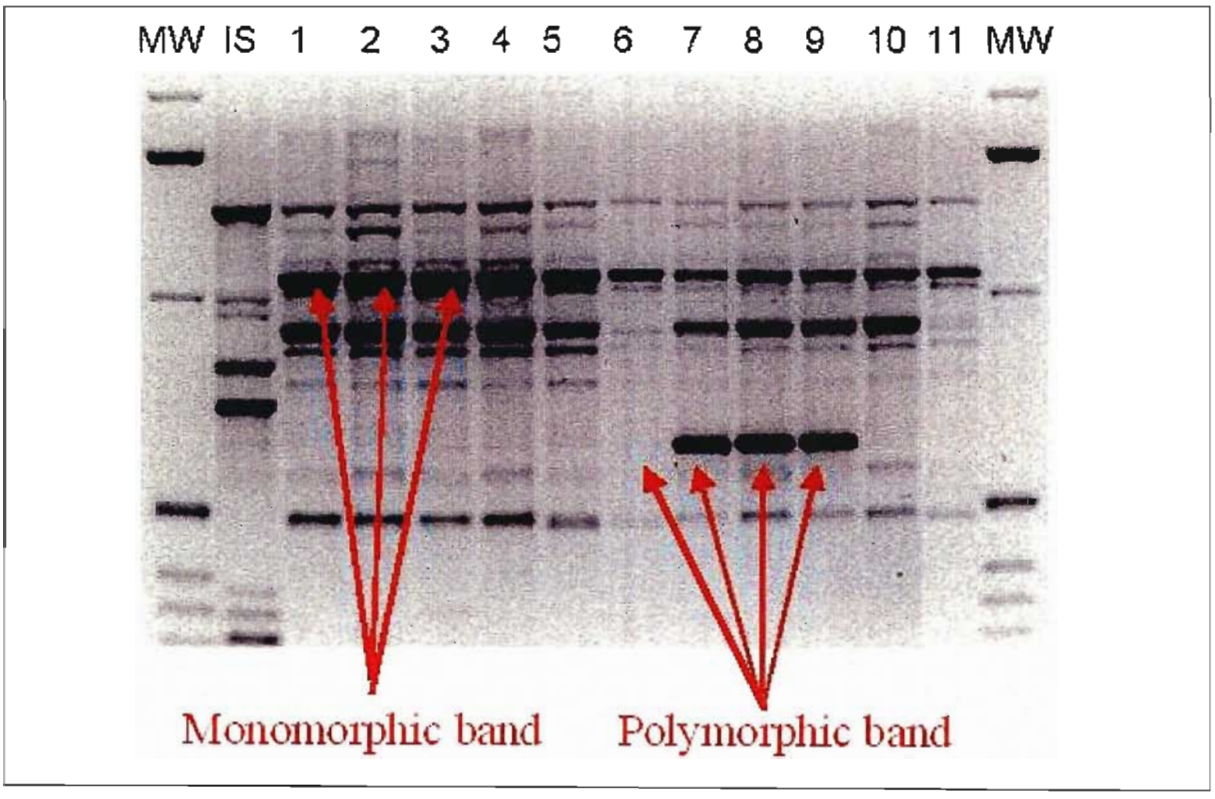
As a further test of reproducibility, all of the reactions on the polymorphic primers were repeated and run on separate gels. The profiles produced between repeated reactions were identical in all cases. Furthermore, the internal standard provided by the sample (2 a) and amplified by primer A-06 produced the identical profile on all the gels.

From these results it was therefore concluded that provided the same protocol, conditions and laboratory are used, RAPD technology is 100 % reproducible. It is important to stress however, that complete reproducibility is only guaranteed once the protocol for the species in question has been optimized and the exact same standard protocol is followed for each reaction within the same laboratory. If these conditions are met, the produced DNA fingerprints will be reproducible.

3.4 SCREENING OF PRIMERS

The 15 chosen primers were screened using all 30 individuals. The results of this screening process were in the form DNA fingerprints seen as bands on a gel. Figure 3.4 illustrates what a typical gel looked like. In the extreme left and right lanes, molecular weight markers were run to indicate the band sizes of the samples. To ensure reproducibility the internal standard was run in the second lane followed by the samples. Both monomorphic as well as polymorphic bands were evident on most gels.

Figure 3.4 Example of a typical gel used for scoring in the analysis, showing both monomorphic as well as polymorphic bands.



The presence or absence of a band at a specific position represented a specific locus with two possible alleles; the *presence* allele (indicated by the presence of a band) and the *absence* allele (indicated by the absence of a band). The information obtained from the numerous DNA profiles regarding the *presence* and *absence* alleles was converted into digital data using the binary number system. A

value of '1' indicated the *presence* allele at a particular locus and a value of '0', the *absence* allele. Table 3.3 contains the molecular data corresponding to each analysed individual in digital format. Two primers, primer A-09 and A-12, were not included as they produced bands that varied between individuals and repeats, and thus failed to produce consistent results.

Table 3.3 Digital representation of the genotypes of each individual showing the *presence* and *absence* alleles at each locus.

	17	25	A-01	A-02	A-04	A-06	A-10	A-13	A-17	A-19	A-20	C-05	C-06
1 a	101111001	11100	1111111111	1111111	1101111111	11011111	11	111	10111110	11	11011	111100110	0111110
1 b	111011011	11111	1111111111	1111111	1111111111	11101111	11	111	10111111	11	11111	101111110	1111111
1 ab i	101110001	11111	1111111111	1111111	1001001111	11011111	11	111	10111111	11	11011	010101110	1111111
1 ab ii	101101001	11111	1111111111	1111111	1111110111	11111111	11	111	10111111	11	11111	101110110	1111111
1 ab iii	111111011	11100	1111111111	1111111	1111111111	11111111	11	111	10111110	11	11111	111111110	0111110
2 a	111111011	11100	1111111111	1111111	1101001100	11111111	11	111	11111110	11	11011	100011111	0111110
2 b	101110001	11111	1111111111	1111111	1011001100	11101111	11	111	10111111	11	11011	111111110	1111111
2 ab i	101101001	11111	1111111111	1111111	1111011111	11111111	11	111	11111111	11	11011	111111111	1111111
3 a	111111011	11100	1111111111	1111111	1101011111	11011111	11	111	10111110	11	11011	101111111	0111110
3 b	101110001	11111	1111111111	1111111	1101010111	11111111	11	111	00011101	11	11011	110011110	1111111
3 ab i	111111011	11100	1111111111	1111111	1101111111	11111111	11	111	10111110	11	11011	101111110	0111110
3 ab ii	111111001	11100	1111111111	1111111	1101011100	11011111	11	111	10111110	11	11011	111111110	0111110
4 a	101010100	11100	1111111111	1111111	1101111111	11111111	11	111	10111110	11	11011	101111111	0111110
4 b	111111011	11111	1111111111	1111111	1011001111	11011111	11	111	11111110	11	11011	011111110	1111111
4 ab i	111010100	11100	1111111111	1111111	1101111111	11111111	11	111	11111110	11	11011	000001110	0111110
4 ab ii	111011011	11100	1111111111	1111111	1101111111	11111111	11	111	11111110	11	11011	111111110	0111110
4 ab iii	101010100	11100	1111111111	1111111	1101011111	11111111	11	111	11111110	11	11011	101111110	0111110
4 w	111010111	11100	1111111111	1111111	1101111111	11011111	11	111	10111110	11	11011	101111111	0111110
4 x	111010111	11100	1111111111	1111111	1101111111	11011111	11	111	11111110	11	11011	101001111	0111110
4 y	111110001	11100	1111111111	1111111	1001001111	11011111	11	111	11111110	11	11011	101101111	0111110
4 z	111111111	11111	1111111111	1111111	1111001101	11111111	11	111	10111111	11	11011	111111110	1111111
5 ab i	111111011	11100	1111111111	1111111	1101111111	11001111	11	111	10111110	11	11111	111111110	0111110
5 ab ii	111011000	11100	1111111111	1111111	1101110111	11111111	11	111	10111110	11	11011	101111110	0111110
5 ab iii	111111000	11111	1111111111	1111111	1111111111	11111111	11	111	---	11	11011	101111110	1111111
5 ab iv	111011011	11100	1111111111	1111111	1101111111	11101111	11	111	10111110	11	11111	111111110	0111110
6	111011011	11100	1111111111	1111111	1101001100	11011111	11	111	10111110	11	11011	101111110	0111110
7	111110011	11111	1111111111	1111111	1111111111	11011111	11	111	10111111	11	11011	111111111	1111111
8	111111011	11111	1111111111	1111111	1111111111	11111111	11	111	10111111	11	11111	---	1111111
9	111010011	11111	1111111111	1111111	1111111101	11111111	11	111	10111111	11	11011	101010111	1111111
10	111111011	11100	1111111111	1111111	1001001111	11011111	11	111	10111110	11	11011	101110110	0111110

In total, 85 reproducible loci were amplified whilst screening the primers. Of the 85 loci analysed, 28 (33 %) were found to be polymorphic. This value however, included the results obtained from the polymorphic primers as well as the monomorphic primers. Excluding the bands obtained from the monomorphic primers produced a total of 61 bands therefore increasing the percentage polymorphic loci to 46 %. Table 3.4 presents a summary of the findings.

Table 3.4 Summary of the findings while screening all primers.

PRIMER	SEQUENCE	VISIBLE BANDS	POLYMORPHIC BANDS
17	aaa cgg gcg g	9	6
25	tcg gcg agc c	5	2
A-01	cag ggc ctt c	10	-
A-02	tgc cga gct g	7	-
A-04	aat cgg gct g	10	6
A-06	ggt ccc tga c	8	2
A-09	ggg taa cgc c	Variable	-
A-10	gtg act gca g	2	-
A-12	tcg gcg ata g	Variable	-
A-13	cag cac cca c	3	-
A-17	gac cgc ttg t	8	2
A-19	caa acg tcg g	2	-
A-20	gtt gcc atc c	5	1
C-05	gat gac cgc c	9	7
C-06	gaa cgg act c	7	2
TOTAL	**---**	**85***	**28**

* the total number of amplified bands included bands from monomorphic primers however not those from the two unreliable primers with varying number of bands.

3.4.1 Identification of polymorphic primers

Primers were termed polymorphic if they produced bands that were present in some individuals and absent in others. Of the 15 primers screened, eight (53.3 %) were found to be polymorphic. The eight polymorphic primers were primer 17, primer 25, primer A-04, primer A-06, primer A-17, primer A-20, primer C-05 and primer C-06. Of the remaining seven primers, 5 were found

to be completely monomorphic and in some cases displayed very few bands. Monomorphic primers included primer A-01, primer A-02, primer A10, primer A-13 and primer A-19. The last two primers, primer A-09 and primer A-12 (as mentioned before), were generally poorly amplified and inconsistent and thus not reliable

The eight polymorphic primers produced 61 amplified loci, 28 (46 %) of which were polymorphic. The individual primers produced between one and seven polymorphic bands, with the percentage polymorphisms ranging between 20 % and 78 %. A summary of the findings of the individual polymorphic primers is presented in table 3.5.

Table 3.5 Number of polymorphic loci and percentage polymorphism in the polymorphic primers.

PRIMER	SEQUENCE	NUMBER SCORED MARKERS	POLY BANDS *	% POLY*
17	aaa cgg gcg g	9	6	67
25	tcg gcg agc c	5	2	40
A-04	aat cgg gct g	10	6	60
A-06	ggt ccc tga c	8	2	25
A-17	gac cgc ttg t	8	2	25
A-20	gtt gcc atc c	5	1	20
C-05	gat gac cgc c	9	7	78
C-06	gaa cgg act c	7	2	29
TOTAL	---	**61**	**28**	**46**

* POLY BANDS = polymorphic bands and % POLY = percentage polymorphism

The individual scores for the polymorphic primers in each of the individuals are taken up in Appendix B.

3.4.2 Percentage GC content versus number of amplified bands

In order to establish whether or not the percentage GC content of the primers affected the number of amplified bands in a fingerprint, a correlation analysis using

SPSS was undertaken. Table 3.6 presents the information that was used to conduct the analysis.

Table 3.6 Percentage GC content of primers and the respective number of bands produced.

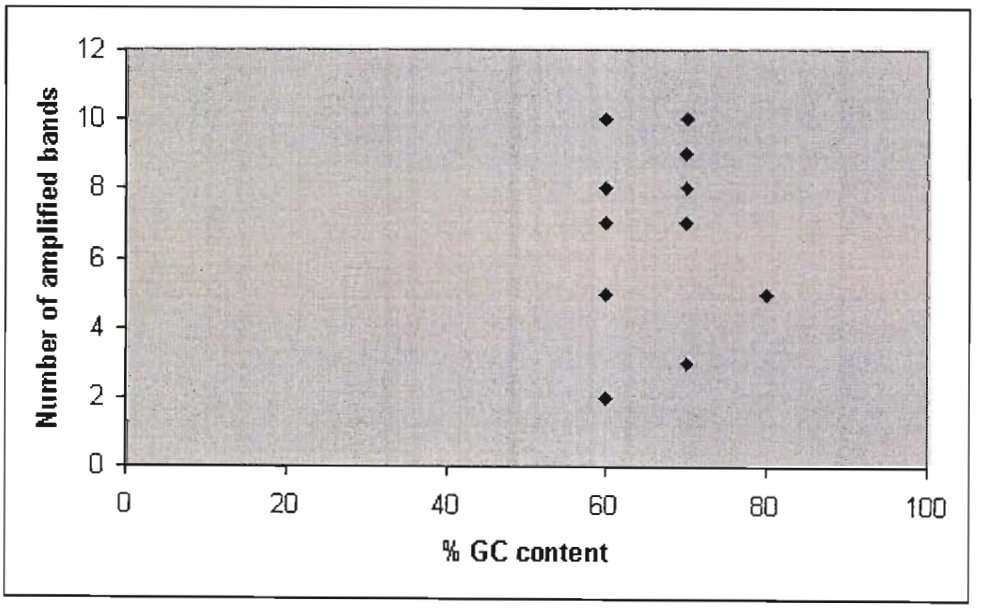
PRIMER	% GC CONTENT	NUMBER OF BANDS
17	70	9
25	80	5
A-01	70	10
A-02	70	7
A-04	60	10
A-06	70	8
A-10	60	2
A-13	70	3
A-17	60	8
A-19	60	2
A-20	60	5
C-05	70	9
C-06	60	7

Primers A-09 and A-12 were not included in the analysis as they produced variable numbers of bands.

From this information Pearson's correlation coefficient was calculated to be 0.165, suggesting a very slight positive relationship between the variables. According to informal interpretation, a value of less than 0.2 indicates almost no relationship, therefore it was concluded that there was no significant correlation between the percentage GC content of the primers and the number of bands produced. A scatter plot, depicting the relationship between the variables, suggested the same. A significant correlation is viewed as a diagonal line between points in a scatter plot. The line can either be sloped in a positive direction or in a negative one depending on the type of correlation evident between the two variables. Zero correlation is observed when the points form a circular structure when connecting them.

In the scatter plot presented in figure 3.5, no line is evident, indicating that no relationship exists between the percentage GC content of the primers and the number of bands produced. The primers were however, chosen on the basis of having a relatively high GC content (above and including 60 %). In order to establish whether there was in fact a significant positive relationship between these two variables, primers possessing low GC contents should be included in an analysis.

Figure 3.5 Scatter plot depicting the relationship between the percentage GC content of the primers and the respective number of bands produced.



3.5 INDIVIDUAL ANALYSIS

Using the eight polymorphic primers, a genotypic analysis was conducted for all the individuals. Each locus in the polymorphic primers was analysed and the number of individuals possessing alleles for band presence (*presence* allele), noted.

Loci were labelled polymorphic if they displayed the *absence* allele in some individuals and the *presence* allele, in others, provided the null phenotype (*absence* allele) exceeded a value greater than 3/n (where n = the number of individuals in the analysis) as suggested by Lynch and Milligan (1994). As there were 30 individuals in this investigation, the frequency of the null allele (q) had to exceed a value of 0.1 in order to be termed polymorphic. Without this constraint, five more bands would have been termed polymorphic and added to the existing total of 28, but as the null phenotype was only present in three or less individuals they were classified as monomorphic. Table 3.7 presents the number of individuals displaying the *presence* allele at each locus analysed.

Table 3.7 Number of individuals displaying the *presence* allele at a locus for a particular primer.

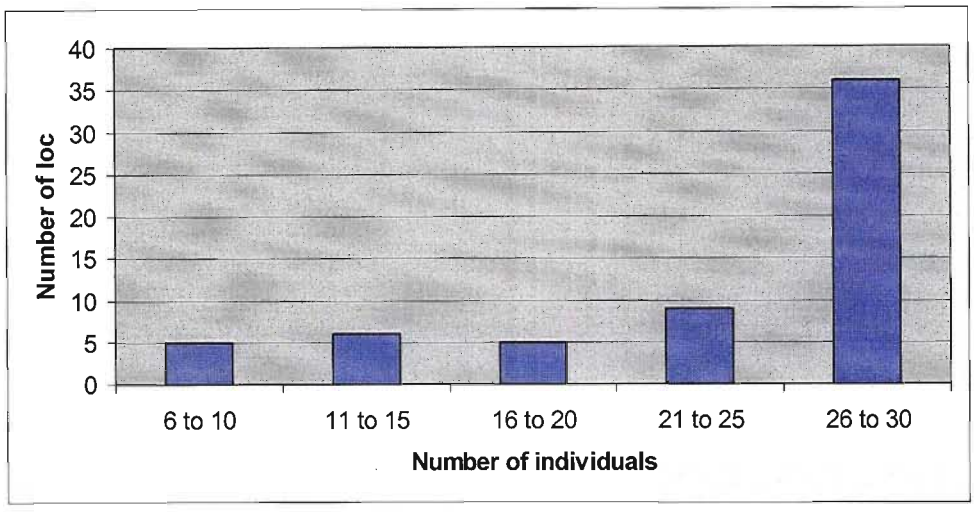
	Locus 1	Locus 2	Locus 3	Locus 4	Locus 5	Locus 6	Locus 7	Locus 8	Locus 9	Locus 10
Primer 17	30	22	30	19	28*	20	6	17	25	-
Primer 25	30	30	30	12	12	-	-	-	-	-
Primer A-04	30	25	11	30	16	21	27*	30	24	26
Primer A-06	30	30	18	26	30	30	30	30	-	-
Primer A-17	29*	8	28*	30	30	30	28*	10	-	-
Primer A-20	30	30	6	30	30	-	-	-	-	-
Primer C-05	26	13	25	24	24	25	30	30	9	-
Primer C-06	12	30	30	30	30	30	12	-	-	-

* Indicates loci regarded as monomorphic due to the Lynch and Milligan (1994) constraint despite bands being absent in a few individuals.

A value of 30, as well as values denoted with a '*' in the above table, indicates that a locus is monomorphic for the *presence* allele. Conversely, very low values are

indicative of almost all individuals having the *absence* allele other than those few represented by the value. Figure 3.6 summarizes the findings of the above table.

Figure 3.6 Bar chart showing the number of loci for which individuals possessed the *presence* allele.

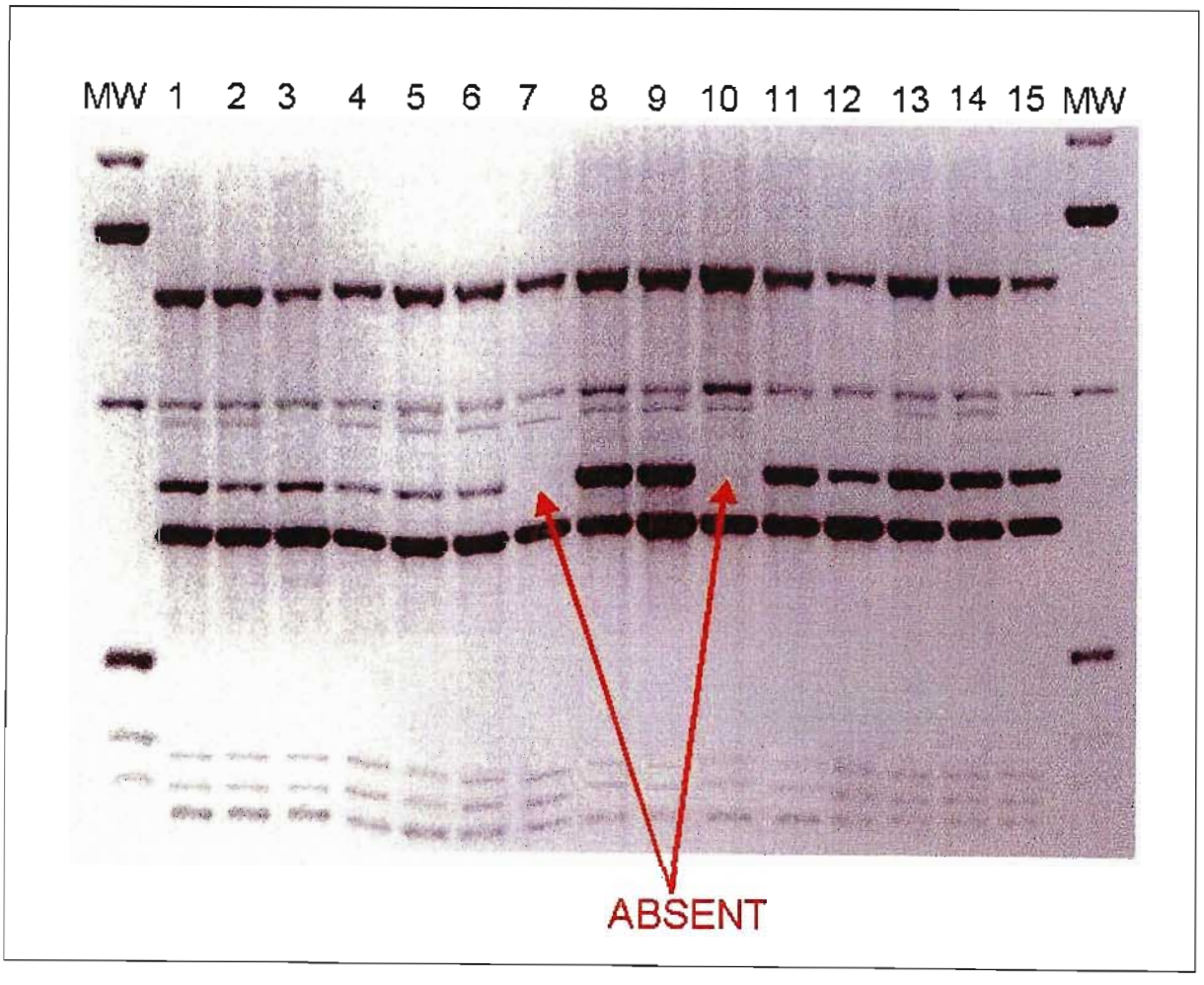


The bar chart shows that the majority of loci (36), were found in most of the individuals and were thus regarded as monomorphic. Nine loci were found in 21 to 25 individuals therefore, again, not displaying much variation. In a highly polymorphic population, the majority of loci would fall in the lower categories of individuals indicating the presence of many different loci in only one or two individuals. This would result in unique individual fingerprints and genotypes suitable for individual identification. In this investigation however, the opposite was observed. The number of loci decreased in the lower categories of individuals, resulting in very similar fingerprints and genotypes with little variation between individuals. It was therefore, not possible to identify each individual using these primers or combinations thereof, however a few genotypic similarities were observed. These similarities allowed for the discovery of relationships between certain individuals previously regarded as unrelated.

3.5.1 Genotyping individuals

In the genotypic analysis of the profiles produced by each individual, it was noted that many genotypes were identical. It was therefore not possible to identify most individuals based on unique DNA fingerprints. Some genotypic similarities were however, observed between various individuals and prompted further research into their relationship. After careful examination of the profiles in question and with the help of the breeders concerned, relationships between individuals previously regarded as unrelated, were established. This was evident with one locus observed using primer A-06, where the *presence* allele was present in all but four individuals, as illustrated in figure 3.7.

Figure 3.7 Individual profiles produced by primer A-06 clearly showing the absence of band number four in two individuals.

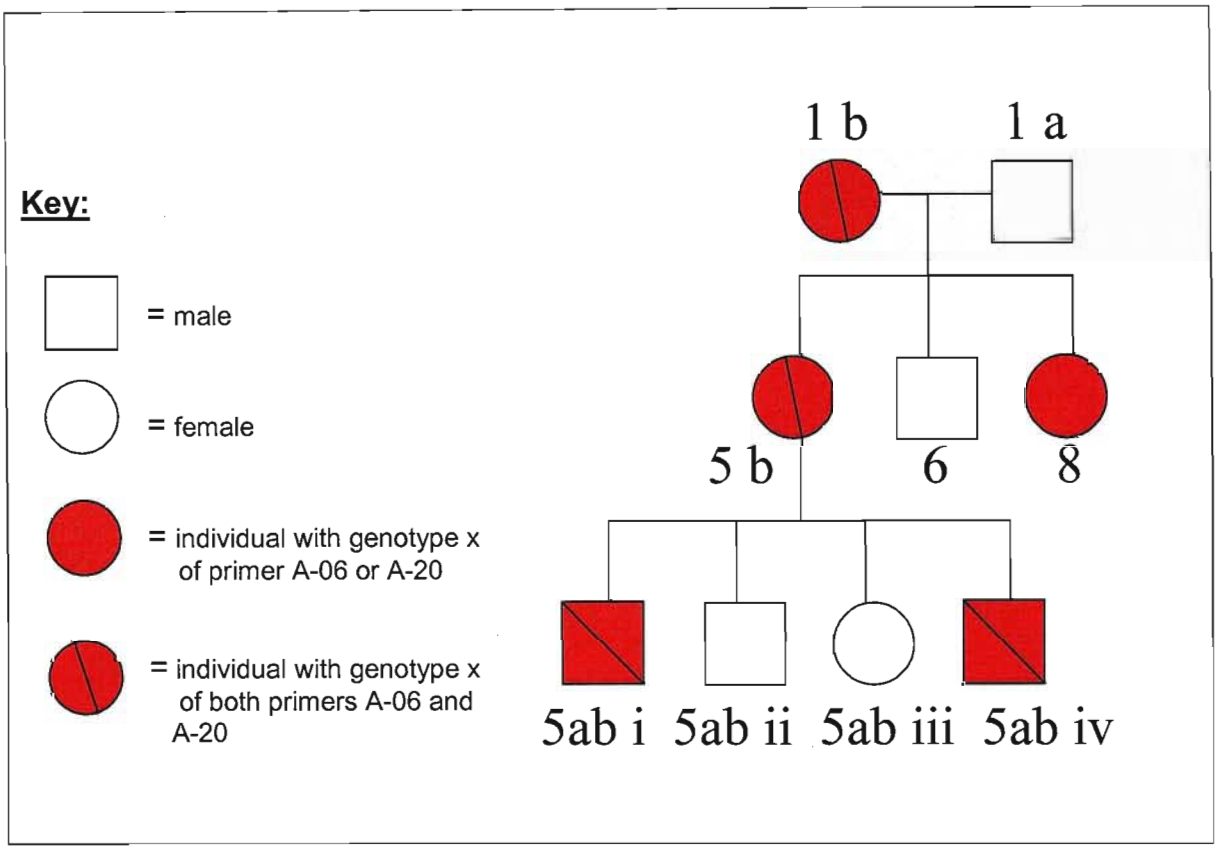


One of the criteria regarding sample collection was that the samples collected from different breeders should not be related. However this criterion had to be over looked (due to a lack of available samples) resulting in some of the individuals from the Eastern Cape population originating from the Amazona population in Assagay. From the information given at sample collection it was noted that individuals 6, 7 and 8 were originally from Assagay. Nothing was however mentioned about the siblings 5 ab i, 5 ab ii, 5 ab iii and 5 ab iv other than that they were bred in the Eastern Cape. It was therefore interesting to find that band number four (in figure 3.7), one of the brightest bands in the profile, was distinctly absent in individuals 1 b, 2 b, 5 ab i and 5 ab iv, possibly suggesting that these individuals could be related.

Inquiring about the possibility of relatedness between these individuals revealed that the breeding female had in fact come from Assagay and been mated with a confiscated wild caught male to produce chicks 5 ab i – 5 ab iv. The true identity and relationship between the siblings in the Eastern Cape and individuals 1 b and 2b was therefore established from the genotypes produced by primer A-06.

Further proof of the relationship between these individuals was obtained in the genotypic analysis of primer A-20. Band three was absent in the majority of the individuals tested, however was present in some (specifically family one) from the Amazona population and three individuals (5 ab i, 5 ab iv and 8) from the Eastern Cape population. The presence of band three in individual 8 can be explained by the fact that it originated from Amazona and the *presence* allele in the siblings from the Eastern Cape by the fact that their maternal parent was originally from Amazona. Figure 3.8 illustrates the discovered relatedness between the individuals from the two populations.

Figure 3.8 Pedigree showing the determined relationship between individuals displaying similar genotypes for primer A-06 and A-20 from two separate populations.



The results from the analysis on primer A-06 suggested that the breeding female in the Eastern Cape population (mother of the siblings 5 ab i – 5 ab iv), must have been related to either or both individuals 1 b or 2 b due to the shared, rare absence of band four. With the added results of primer A-20 however, the breeding female seemed more closely related to individual 1 b as both possessed band three when using primer A-20 (whereas 2 b did not) and displayed the null phenotype for band four using primer A-06 (figure 3.8).

Further investigation and consultations with the breeders from Amazona and the Eastern Cape population, revealed that the maternal parent of siblings 5 ab i – 5 ab iv, as well as individuals 6 and 8, were the offspring of individuals 1 a and 1 b from Amazona (figure 3.8). This finding explained why the four siblings seemed to

be more closely related to individual 1 b and that the assumption that they were, based on the produced fingerprints, was correct.

An interesting observation made while studying the produced fingerprints of primer C-05 was that individuals 1 b, 5 ab ii, 5 ab iii and 6 all produced identical profiles. This finding matches the assumption made about the relationship of these individuals using primers A-06 and primer A-20. Another observation using primer C-05, was that individuals 5 ab i and 5 ab iv shared the same profile as 1 ab iii, which according to the given information, is the 'uncle' of these two siblings. Looking at the profiles of the other primers (Appendix B), it was noted that these two siblings again shared the same profile with 1 ab iii for primers A-20 and A-17, indicating that these three birds were genetically very similar.

3.5.2 Nei's genetic identity and genetic distance between individuals

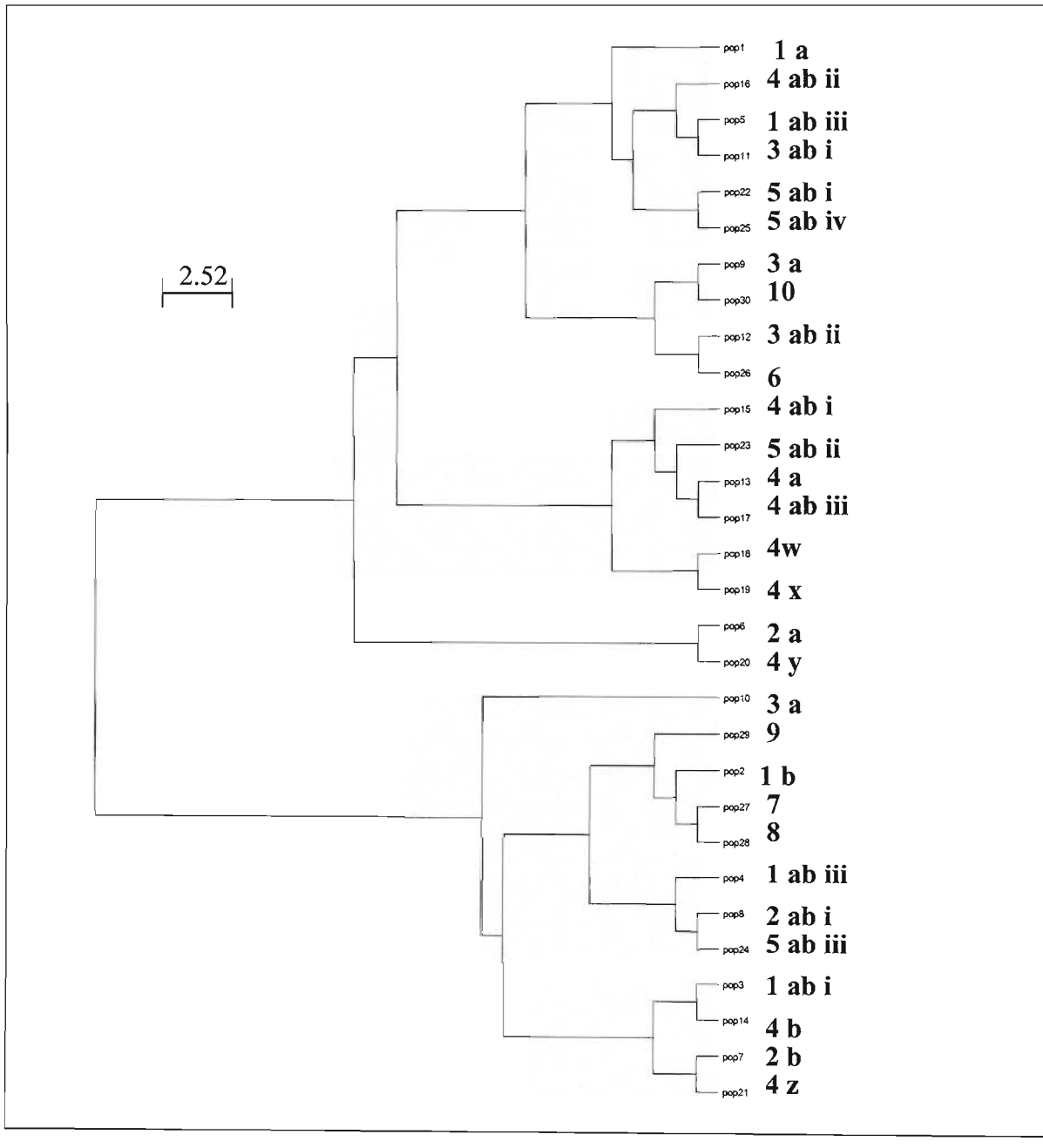
Nei's genetic identity and genetic distance was calculated using the statistical software programme, POPGENE (Yeh *et al.,* 1999). From the values presented as distance matrices, dendrograms were constructed depicting the various relationships between individuals. The POPGENE printout including Nei's measures of identity, are taken up in Appendix D.

Nei's original and unbiased measures were used. The unbiased measures were included in the analysis comparing the three subpopulations to observe the effect a small sample size would have on the results. The difference between Nei's original and Nei's unbiased measures were minimal and did not affect the general observations and trends. For this reason only the original measures were used in this analysis. The values are taken up in Appendix D.

The dendrogram in figure 3.9 clearly illustrates which individuals have similar genotypes, according to the digital data produced using the various primers. It was expected that individuals within the same family would be grouped together as they should share more DNA than unrelated individuals. In some cases, this relatedness was evident such as with siblings 5 ab i and 5 ab iv and parent 4a and offspring 4 ab iii. However, in most cases, individuals with no apparent relatedness

and stemming from separate subpopulations, were grouped together such as 2 a from Assagay and 4 y from Dargle.

Figure 3.9 Dendrogram depicting the genetic similarity between the 30 individual Cape parrots used in this investigation.



Individual identity and kinship determination using RAPDs was therefore not possible in all cases.

3.5.3 Nei's genetic identity and genetic distance between members of a family

Nei's genetic identity and distance were calculated between members of each family in the three populations. As there was very little difference between the values obtained using Nei's original and Nei's unbiased estimates, it was decided to only calculate values for Nei's original measures.

a. Family 1 (Amazona)

Table 3.8 presents Nei's genetic identity and genetic distance for each of the members in family 1 from Amazona. It was evident that each parent had a sibling that was more similar to itself, as illustrated in the dendrogram in figure 3.10.

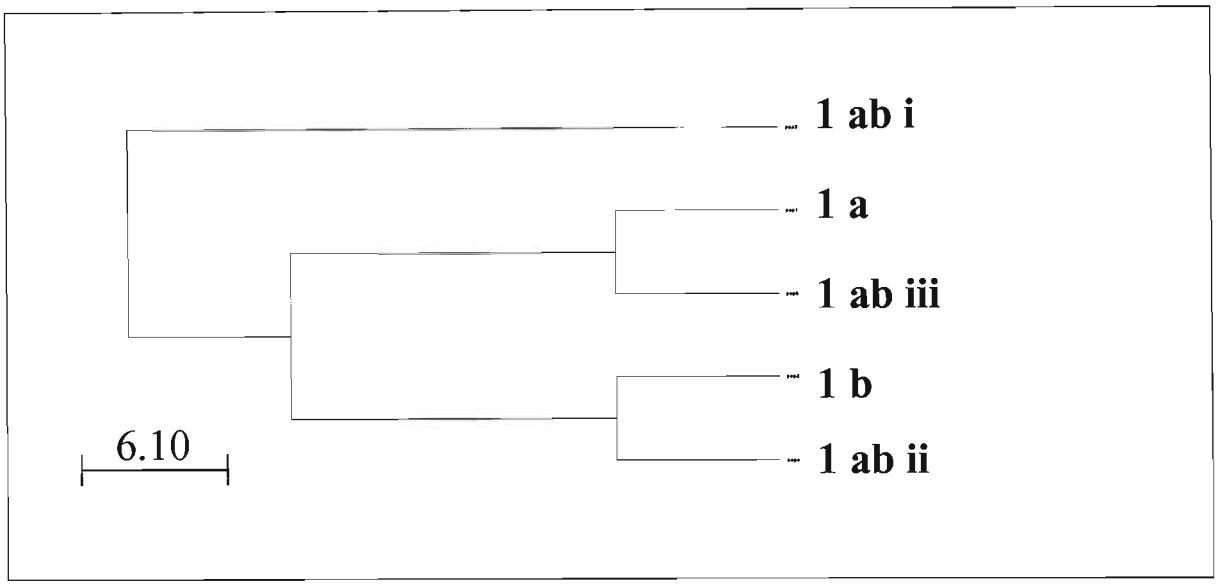
Table 3. 8 Nei's original measures for family 1 of the Amazona population.

INDIVIDUALS	1 a	1 b	1 ab (i)	1 ab (ii)	1 ab (iii)
1 a	---	0.754	0.803	0.803	0.885
1 b	0.282	---	0.754	0.885	0.869
1 ab (i)	0.219	0.282	---	0.771	0.721
1 ab (ii)	0.219	0.122	0.261	---	0.820
1 ab (iii)	0.122	0.141	0.327	0.199	---

Nei's genetic identity (above diagonal) and Nei's genetic distance (below diagonal)

Chick 1 ab iii was more genetically similar to the father and chick 1 ab ii more to the mother. Chick 1 ab i seemed to be the least similar to either parent as well as the least similar to either one of its siblings.

Figure 3.10 Dendrogram depicting the relationship between members of family 1 from Amazona.



b. Family 2 (Amazona)

In table 3.9, Nei's genetic identity and genetic distance for family 2 of Amazona are presented. It could be concluded that the chick produced by the following breeding pair was genetically more similar to the mother than to the father.

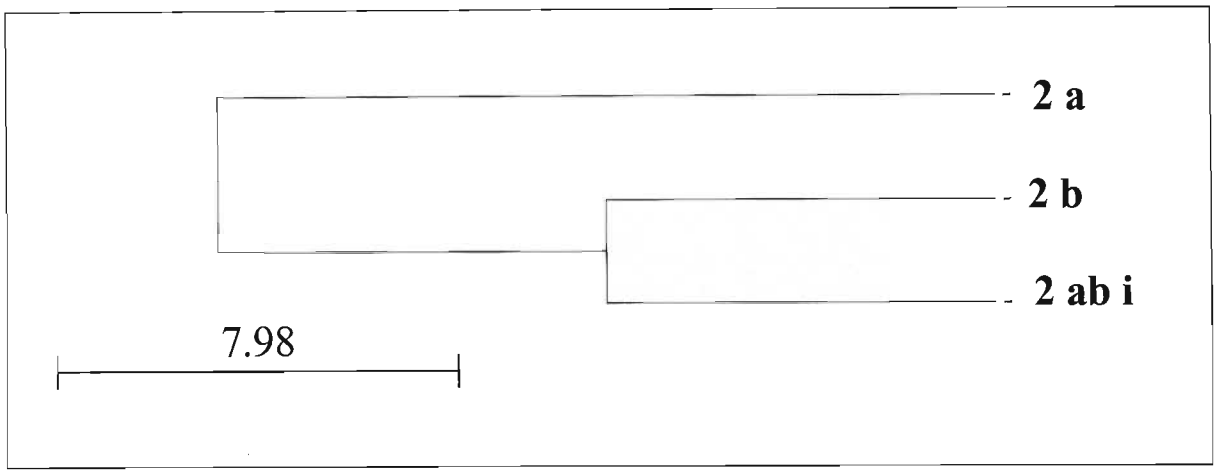
Table 3.9 Nei's original measures for family 2 of the Amazona population.

INDIVIDUALS	2 a	2 b	2 ab (i)
2 a	---	0.738	0.754
2 b	0.304	---	0.853
2 ab (i)	0.282	0.160	---

Nei's genetic identity (above diagonal) and Nei's genetic distance (below diagonal)

The small genetic distance of 0.16 between the mother and the chick indicative of a high similarity was also illustrated in the dendrogram presented in figure 3.11.

Figure 3.11 Dendrogram depicting the relationship between members of family 2 from Amazona.



c. Family 3 (Amazona)

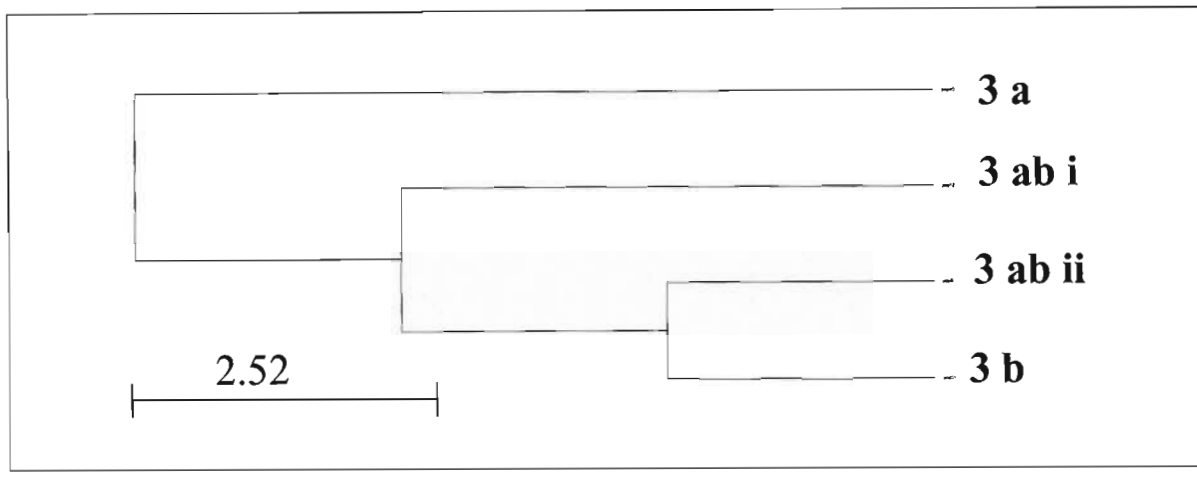
Table 3.10 presents the values obtained using Nei's genetic identity and genetic distance for family 3 from Amazona. Both chicks were genetically more similar to the father, with chick 3 ab (i) being particularly similar displaying a very low distance measure of 0.05 from the father. The mother was slightly more genetically similar to the chicks than to its male partner, which was expected, however still the most distantly related as is illustrated in figure 3.12.

Table 3.10 Nei's original measures for family 3 of the Amazona population.

INDIVIDUALS	3 a	3 b	3 ab (i)	3 ab (ii)
3 a	---	0.721	0.951	0.918
3 b	0.327	---	0.738	0.738
3 ab (i)	0.050	0.304	---	0.902
3 ab (ii)	0.086	0.304	0.104	---

Nei's genetic identity (above diagonal) and Nei's genetic distance (below diagonal)

Figure 3.12 Dendrogram depicting the relationship between members of family 3 from Amazona.



d. Family 4 (Rehoboth)

The family from Rehoboth was made up of the immediate family, consisting of the father (4a), mother (4b) and three chicks (4ab i, 4ab ii and 4ab iii), as well as the extended family, which included four second generation chicks (4w, 4x, 4y, and 4z). From table 3.11 and figure 3.13, it was clear that the three chicks seemed to be more genetically similar to the father than to the mother. Of the three chicks, individual 4 ab iii was the most similar to the father, displaying a genetic distance of only 0.05. Chick 4 ab ii seemed to be the least similar to either of its siblings. The maternal parent was relatively distant from the family as a whole, although she was more genetically similar to her chicks than to her mate, which was expected.

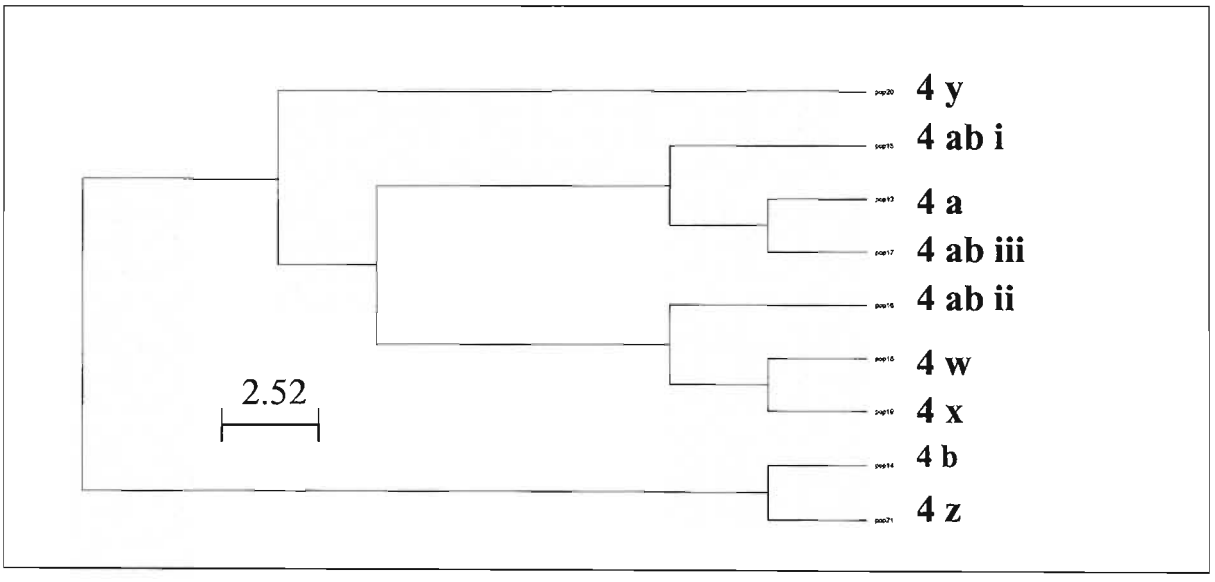
The extended family members 4 w and 4 x were very genetically similar, displaying a low genetic distance of 0.05. The three second generation chicks 4 w, 4 x and 4 y were all more genetically similar to their second generation paternal parent (4 a), whereas 4 z was more similar to the second generation maternal parent (4 b), with a distance value of 0.122 between them. Based on these results, 4 z was more similar to its second generation maternal parent than to its parents.

Table 3.11 Nei's original measures for family 4 from the Rehoboth population.

	4 a	4 b	4ab(i)	4ab(ii)	4ab(iii)	4 w	4 x	4 y	4 z
4 a	---	0.689	0.885	0.869	0.951	0.934	0.885	0.836	0.738
4 b	0.373	---	0.705	0.820	0.738	0.754	0.738	0.820	0.885
4ab(i)	0.122	0.350	---	0.853	0.902	0.853	0.902	0.820	0.689
4ab(ii)	0.141	0.199	0.160	---	0.885	0.902	0.885	0.836	0.803
4ab(iii)	0.050	0.304	0.104	0.122	---	0.885	0.869	0.853	0.754
4w	0.068	0.282	0.160	0.104	0.122	---	0.951	0.869	0.771
4x	0.122	0.304	0.104	0.122	0.141	0.050	---	0.885	0.721
4y	0.179	0.199	0.199	0.180	0.160	0.141	0.122	---	0.738
4z	0.304	0.122	0.373	0.219	0.282	0.261	0.327	0.304	---

Nei's genetic identity (above diagonal) and Nei's genetic distance (below diagonal)

Figure 3.13 Dendrogram depicting the relationship between members of family 4 from Rehoboth.



e. Siblings 5 (Eastern Cape)

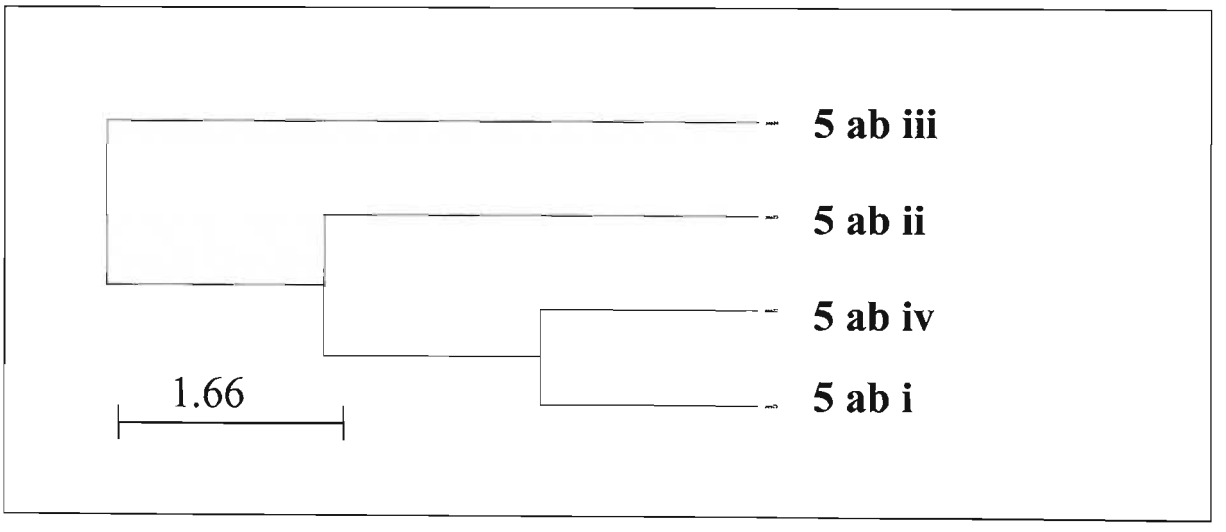
From the results in the table 3.12 and dendrogram in figure 3.14, it was concluded that the siblings 5 ab i and 5 ab iv were more genetically similar than any of the other siblings. Nei's genetic distance value between them was measured as 0.033, which was very low. Sibling 5 ab iii was the least similar to the other siblings, as was evident in the dendrogram.

Table 3.12 Nei's original measures for the siblings from the Eastern Cape population.

INDIVIDUALS	5 ab (i)	5 ab (ii)	5 ab (iii)	5 ab (iv)
5 ab (i)	---	0.869	0.787	0.967
5 ab (ii)	0.141	---	0.853	0.902
5 ab (iii)	0.240	0.160	---	0.787
5 ab (iv)	0.033	0.104	0.240	---

Nei's genetic identity (above diagonal) and Nei's genetic distance (below diagonal)

Figure 3.14 Dendrogram depicting the relationship between the siblings (group 5) from the Eastern Cape.



f. Comparison between family 1 (Amazona) and siblings 5 (Eastern Cape)

To establish the genetic similarities determined with genotyping between the individuals in the Eastern Cape population related to those from the Amazona population, Nei's original genetic measures were used. The maternal parent of the siblings 5 ab i – 5 ab iv, as well as individuals 6, 7 and 8, were originally bred by individuals 1 a and 1 b from Amazona. Family 1 was therefore compared to these individuals from the Eastern Cape population and the results presented in table 3.13 and illustrated as a dendrogram in figure 3.15.

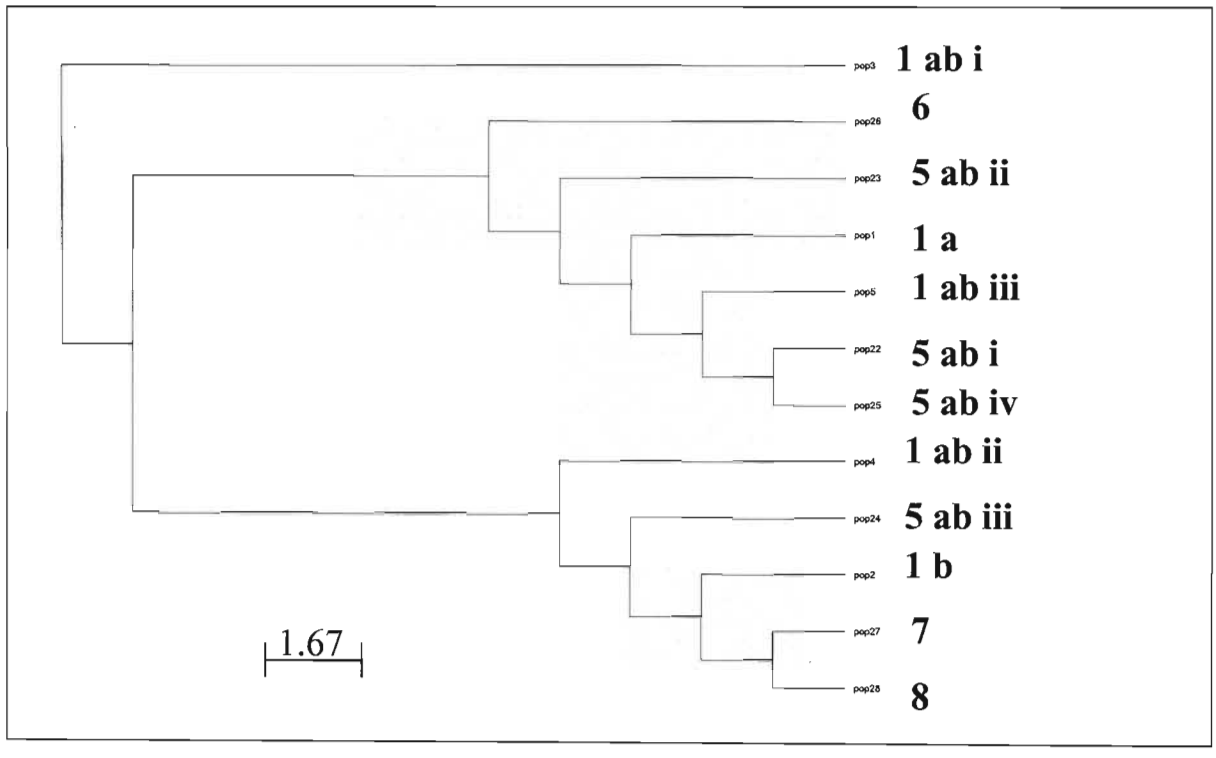
Table 3.13 Nei's original measures for the related individuals from the Amazona and Eastern Cape population.

	1 a	1 b	1(i)	1(ii)	1(iii)	5 (i)	5(ii)	5(iii)	5(iv)	6	7	8
1 a	---	0.75	0.80	0.80	0.89	0.90	0.87	0.79	0.87	0.84	0.80	0.79
1 b	0.28	---	0.75	0.89	0.87	0.85	0.82	0.90	0.89	0.79	0.86	0.93
1(i)	0.22	0.28	---	0.77	0.72	0.74	0.70	0.79	0.70	0.74	0.84	0.79
1(ii)	0.22	0.12	0.26	---	0.82	0.77	0.80	0.89	0.77	0.71	0.84	0.89
1(iii)	0.12	0.14	0.33	0.20	---	0.95	0.89	0.84	0.95	0.85	0.85	0.90
5(i)	0.10	0.16	0.30	0.26	0.05	---	0.87	0.79	0.97	0.87	0.84	0.85
5(ii)	0.14	0.20	0.35	0.22	0.12	0.14	---	0.85	0.90	0.87	0.77	0.79
5(iii)	0.24	0.10	0.24	0.12	0.18	0.24	0.16	---	0.79	0.75	0.89	0.90
5(iv)	0.14	0.12	0.35	0.26	0.05	0.03	0.10	0.24	---	0.87	0.80	0.85
6	0.18	0.24	0.30	0.35	0.16	0.14	0.14	0.28	0.14	---	0.77	0.75
7	0.22	0.12	0.18	0.18	0.16	0.18	0.26	0.12	0.22	0.26	---	0.95
8	0.24	0.07	0.24	0.12	0.10	0.16	0.24	0.10	0.16	0.28	0.05	---

Nei's genetic identity (above diagonal) and Nei's genetic distance (below diagonal). The samples from 1(i) to 5(iv) excluded their 'ab' components denoting them as progeny of parents (a) and (b), due to lack of space in the table

Siblings 5 ab i, 5 ab ii and 5 ab iv were more genetically similar to their second generation paternal parent (1 a) from Amazona, whereas sibling 5 ab iii, which was the least similar of the siblings (figure 3.14), was more similar to the second generation parent 1 b. 5ab i and 5 ab iv were regarded as more similar than any other siblings, displaying a genetic distance of 0.03. Individuals 7 and 8 were also similar displaying a genetic distance of 0.05 between them. They were also more genetically similar to their maternal parent (1 b) than to their paternal parent, (1 a).

Figure 3.15 Dendrogram depicting the relationship between the related individuals from Amazona and the Eastern Cape.



3.6 POPULATION ANALYSIS

In the previous section the level of analysis was focussed on the individuals and their specific genotypes. In this section, however, although the basic analysis will be based on the individual genotypes, the results will relate to the population as a whole.

3.6.1 Allele frequencies and heterozygosities

The allele frequencies (p) and (q) were estimated for each locus analysed in this investigation across all individuals. The recessive allele (q) was estimated from the recessive genotypes using the Lynch and Milligan (1994) constraint. Once this value was obtained, (p) was calculated accordingly.

The values obtained for each locus were averaged for a particular primer, resulting in an average (q) value for each primer. From these values the average heterozygosity for each primer was determined, using the formula 2pq. The average heterozygosity for the population as a whole was estimated by averaging the values obtained for each primer.

The table 3.14 gives the (q) values estimated for each locus in the investigation as well as the average value for each primer. The highest (q) value (or lowest p value) was observed in primers A-20 and A-17 and the lowest (q) value (or highest p value) in primer C-05. Interestingly, the primer with the highest percentage polymorphism was primer C-05 and the lowest, primers A-20 and A-17.

Table 3.14 Table showing the (q) values estimated for each locus in all the polymorphic primers.

	Locus 1	Locus 2	Locus 3	Locus 4	Locus 5	Locus 6	Locus 7	Locus 8	Locus 9	Locus 10	AV*
Primer 17	0.00	0.51	0.00	0.61	0.00	0.58	0.89	0.66	0.41	-	0.61
Primer 25	0.00	0.00	0.00	0.77	0.77	-	-	-	-	-	0.77
Primer A-04	0.00	0.41	0.80	0.00	0.68	0.55	0.00	0.00	0.45	0.37	0.54
Primer A-06	0.00	0.00	0.63	0.36	0.00	0.00	0.00	0.00	-	-	0.50
Primer A-17	0.00	0.85	0.00	0.00	0.00	0.00	0.00	0.81	-	-	0.83
Primer A-20	0.00	0.00	0.89	0.00	0.00	-	-	-	-	-	0.89
Primer C-05	0.33	0.74	0.37	0.42	0.42	0.37	0.00	0.00	0.83	-	0.49
Primer C-06	0.77	0.00	0.00	0.00	0.00	0.00	0.77	-	-	-	0.77

* AV = average (q) value for each primer

From the values in table 3.14, the estimated heterozygosities were determined for each locus and averaged for the primers. These estimated heterozygosities are presented in table 3.15.

Table 3.15 Estimated heterozygosities for each locus analysed in the polymorphic primers.

	Locus 1	Locus 2	Locus 3	Locus 4	Locus 5	Locus 6	Locus 7	Locus 8	Locus 9	Locus 10	AV*
Primer 17	0.00	0.50	0.00	0.48	0.00	0.49	0.20	0.45	0.48	-	0.43
Primer 25	0.00	0.00	0.00	0.35	0.35	-	-	-	-	-	0.35
Primer A-04	0.00	0.48	0.32	0.00	0.44	0.50	0.00	0.00	0.50	0.47	0.45
Primer A-06	0.00	0.00	0.47	0.46	0.00	0.00	0.00	0.00	-	-	0.47
Primer A-17	0.00	0.26	0.00	0.00	0.00	0.00	0.00	0.31	-	-	0.29
Primer A-20	0.00	0.00	0.2	0.00	0.00	-	-	-	-	-	0.20
Primer C-05	0.44	0.38	0.47	0.49	0.49	0.47	0.00	0.00	0.28	-	0.43
Primer C-06	0.35	0.00	0.00	0.00	0.00	0.00	0.35	-	-	-	0.35

* AV = average (q) value for each primer

The total heterozygosity (H_0) for the population in this investigation was estimated to be 0.37. This was the mean value calculated across all loci for each primer (table 3.15). The primer displaying the most heterozygosity was Primer A-06, with a mean value of 0.47. Primer A-20 displayed the least heterozygosity with a value of 0.20.

Heterozygosity can be calculated in a number of different ways, resulting in slightly different values. The results obtained using a second method are presented in table 3.16. This table gives the average heterozygosity estimated for each individual across all loci, as well as the average heterozygosities for the three subpopulations. The total population heterozygosity was calculated to be 0.45, slightly higher than the heterozygosity estimated using the first method.

Table 3.16 Heterozygosities estimated for each individual and population.

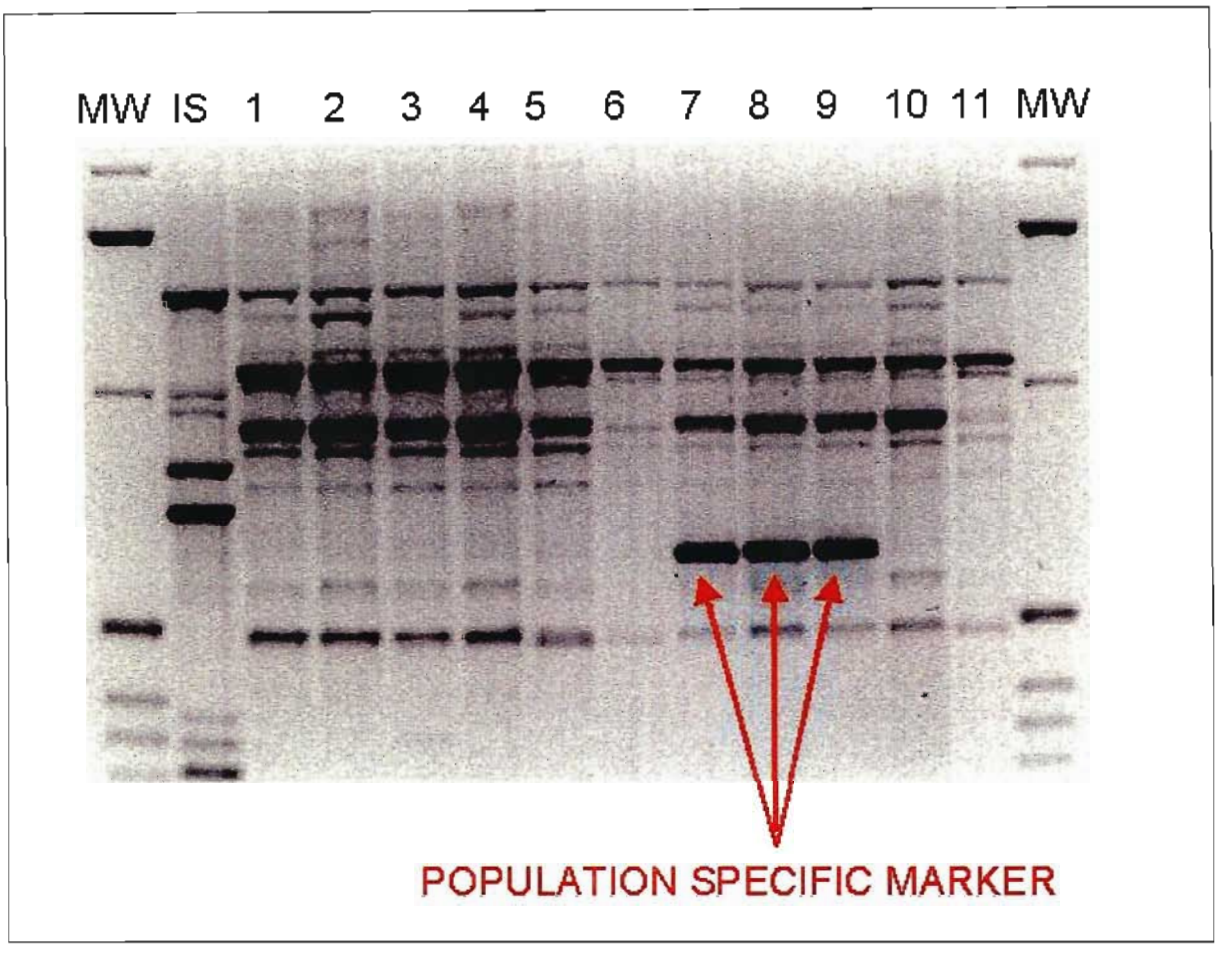
INDIVIDUALS	HETEROZYGOSITY	
1 a	0.49	
1 b	0.36	
1 ab i	0.49	
1 ab ii	0.44	
1 ab iii	0.43	
2 a	0.49	
2 b	0.48	AMAZONA = 0.46
2 ab i	0.39	
3 a	0.47	
3 b	0.49	
3 ab i	0.46	
3 ab ii	0.49	
4 a	0.48	
4 b	0.44	
4 ab i	0.50	
4 ab ii	0.45	
4 ab iii	0.49	REHOBOTH = 0.47
4 w	0.47	
4 x	0.48	
4 y	0.49	
4 z	0.39	
5 ab i	0.46	
5 ab ii	0.49	
5 ab iii	0.40	
5 ab iv	0.46	
6	0.50	EASTERN CAPE = 0.43
7	0.36	
8	0.27	
9	0.44	
10	0.49	

3.6.2 Population specific markers

An interesting finding in this investigation was the detection of a population specific marker, distinctively present in one population. This marker (band number 7 in the

fingerprints produced by primer 17 in figure 3.16) was only present in individuals from the population in Dargle at Rehoboth farm and appeared in neither the Eastern Cape population nor the population from Amazona in Assagay.

Figure 3.16 Population specific marker in the individuals from Rehoboth.



Upon examination of the individuals displaying the band, it was noted that they all belong to a single family, which could suggest that this marker is family specific and not population specific as previously assumed. The marker was present in the breeding male (4 a) and two chicks (4 ab i and 4 ab iii) but absent in the female (4 b) and the third chick (4 ab ii). From this we can deduce that the male must have been heterozygous due to one of the chicks displaying the null phenotype (if it is assumed that the absence of a band indicates a homozygous recessive genotype). Further more, three of the second generation offspring (4 w, 4 x and 4 z) also displayed the band. Whether this marker is in fact population specific,

would require further analysis of the individuals from Rehoboth, including more unrelated samples.

3.6.3 Sex specific markers

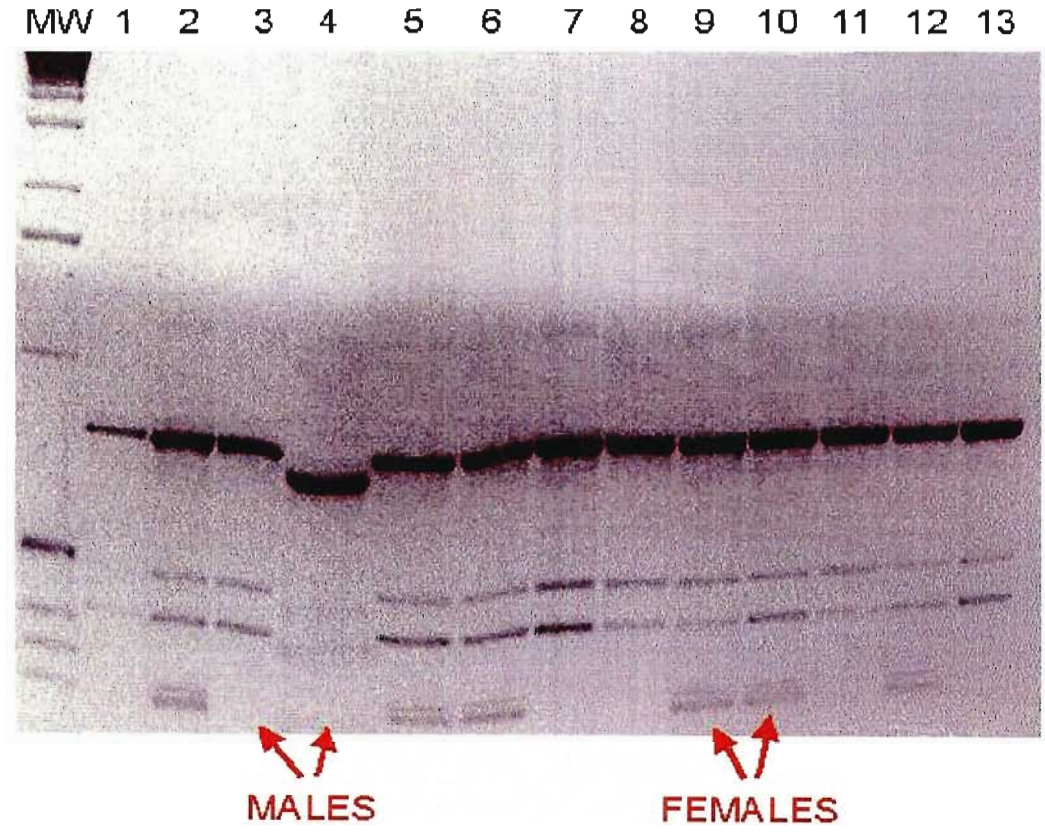
Another interesting finding in this investigation was the identification of several sex specific loci. Careful analysis of the profiles on the gels as well as the individual genotypes, revealed that two primers, primer 25 and primer C-06, produced polymorphic loci with *present* alleles in the exact same individuals. In both cases, the polymorphic bands were present in 40 % of the individuals.

From the information supplied by William Horsfield upon collection of the blood, it was established that the *presence* allele was only present in female individuals. The loci produced by these two primers were therefore able to determine the sex of individual parrots.

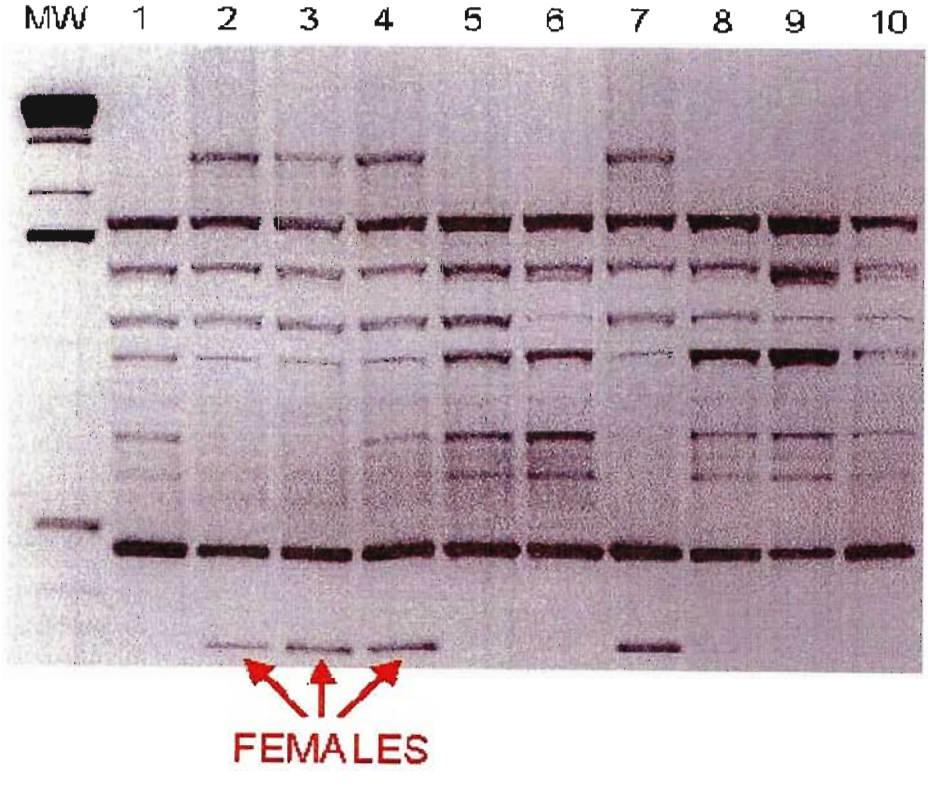
The breeders from the other two populations had not given out information regarding the sexes of the parrots. After contacting them and establishing the sex of each individual bird, it was 100 % confirmed that the presence of the bands in a parrot was indicative of the female sex. Primer 25 and C-06 could therefore be used to reliably identify the sex of individual parrots using the methods employed in this investigation. Figure 3.17 illustrates the sex specific markers present in females for primer 25 and primer C-06.

Figure 3.17 DNA profiles of the two primers, primer 25 and primer C-06, showing the sex specific markers present in females only.

PRIMER 25



PRIMER C-06



3.7 POPULATION COMPARISON

Various statistical tests and measures were used to analyse the results obtained from the eight polymorphic primers to allow for comparison between the three populations. These tests were implemented with the help of statistical software programmes such as POPGENE (Yeh *et al.,* 1999) and ARLEQUIN (Schneider *et al.,* 2000). POPGENE was used to assess Shannon's index of phenotypic diversity for all primers across all individuals, as well as Nei's genetic identity and genetic distance. The ARLEQUIN programme was used to assess the within and between population variation by performing an AMOVA on the data. Wright's F_{ST} was also determined by ARLEQUIN.

3.7.1 Shannon's index of phenotypic diversity

Shannon's index was calculated for all three populations (labelled according to the place of the sample's collection) as well as for the populations as a whole.

The diversity of the population of Cape parrots (as a whole) sampled for this investigation was low, scoring a value of 0.276 across all primers. Comparing the individual populations, Amazona seemed to have the highest diversity with a value of 0.286 as compared to the Rehoboth population with a phenotypic index of 0.195 and the Eastern Cape population with 0.215. It was observed that the Amazona population had a higher diversity than the population as a whole. This was expected, as the inclusion of the lesser diverse populations, would reduce this value for the total population.

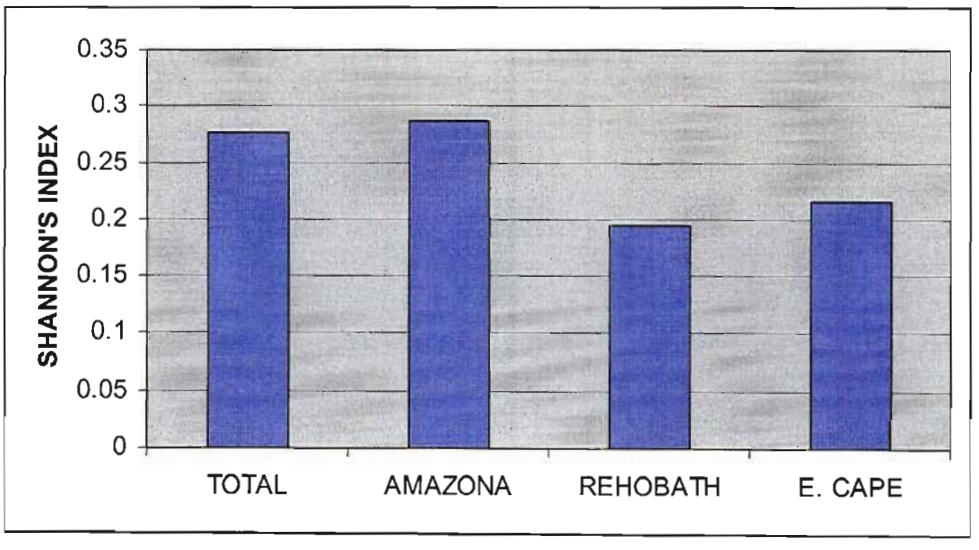
Table 3.17 presents the individual values calculated for each primer in each of the three populations as well as for the population as a whole. Individual Shannon's index values calculated for each locus in all of the primers are taken up in Appendix D.

Table 3.17 Shannon's Index of Phenotypic Diversity calculated for each primer in the three populations as well as for the population as a whole.

PRIMER	TOTAL POPULATION	AMAZONA	REHOBOTH	EASTERN CAPE
17	0.460	0.347	0.405	0.301
25	0.215	0.242	0.145	0.227
A-04	0.434	0.453	0.301	0.455
A-06	0.159	0.170	0.080	0.166
A-17	0.256	0.338	0.113	0.064
A-20	0.069	0.079	0.000	0.095
C-05	0.464	0.483	0.416	0.250
C-06	0.154	0.173	0.104	0.162
MEAN	**0.276**	**0.286**	**0.195**	**0.215**

A graph was created for visual comparison and is presented in figure 3.18.

Figure 3.18 Graph comparing Shannon's index of phenotypic diversity between the three subpopulations as well as the total population.



Referring back to Table 3.17, RAPD diversity also varied among primers. Certain primers such as A-06 and A-20 generally detected very low levels of diversity in all populations whereas primers 17, A-04 and C-05 detected fairly high values. Variation was also evident between primers within individual populations. In the Amazona population, primers A-04 and C-05 displayed high values, whereas in the Rehoboth population, primers C-05 and 17 produced high values. Primer A-20 failed to display any diversity in the Rehoboth population.

The sex-specific primers, primer 25 and C-06 were included for comparison's sake. These primers generally scored low diversity values, which were expected as there are only two possible variations: male and female. However, the population with the least amount of sex variation, and thus the lowest phenotypic index for both primers was the Rehoboth population. This result was confirmed with the information obtained from the breeders as the population consisted of seven males and two females. The other two populations were fairly even with respect to the number of males versus females, thus producing a higher diversity index

3.7.2 Nei's genetic identity and genetic distance between subpopulations

To establish the genetic similarities, as well as the genetic distance between the three populations, Nei's original and unbiased measures were calculated. The unbiased measures were included to observe the effect a small sample size would have on the results. Table 3.18 presents Nei's original measures on the data including and excluding the sex-specific primers. and Table 3.19 presents Nei's unbiased measures on both types of data. The actual printout of the results produced by POPGENE is taken up in Appendix D.

Table 3.18 Nei's original measures of genetic identity and genetic distance.

INCLUDING SEX-SPECIFIC PRIMERS				EXCLUDING SEX-SPECIFIC PRIMERS			
POP	1	2	3	POP	1	2	3
1	---	0.948	0.959	1	---	0.937	0.949
2	0.054	---	0.954	2	0.066	---	0.943
3	0.042	0.048	---	3	0.053	0.059	---

Nei's genetic identity (above diagonal) and Nei's genetic distance (below diagonal). 1= Amazona population, 2 = Rehoboth population and 3 = Eastern Cape population.

Table 3.19 Nei's unbiased measures of genetic identity and genetic distance.

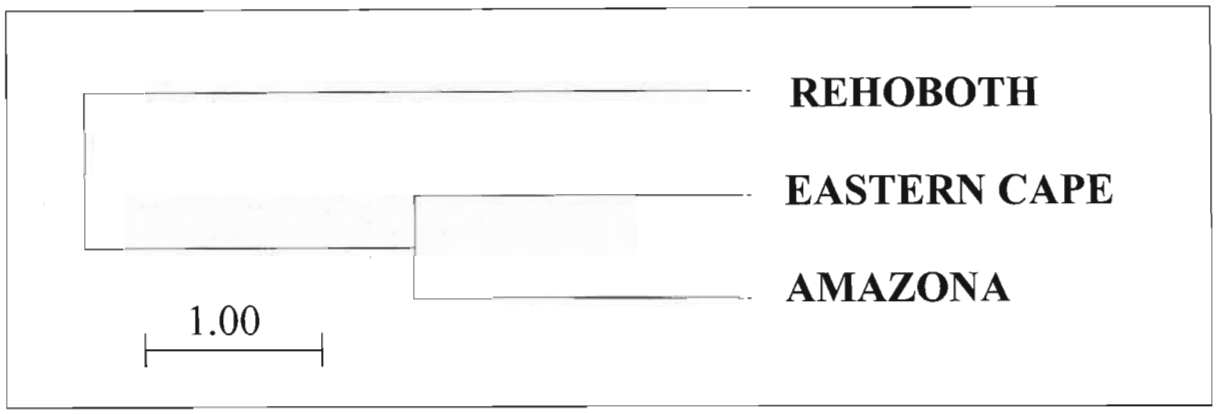
INCLUDING SEX-SPECIFIC PRIMERS				EXCLUDING SEX-SPECIFIC PRIMERS			
POP	1	2	3	POP	1	2	3
1	---	0.959	0.970	1	---	0.949	0.961
2	0.042	---	0.964	2	0.053	---	0.956
3	0.030	0.037	---	3	0.040	0.047	---

Nei's genetic identity (above diagonal) and Nei's genetic distance (below diagonal). 1= Amazona population, 2 = Rehoboth population and 3 = Eastern Cape population.

The above results indicate that in all cases the population from Amazona and the population from the Eastern Cape were most similar and displayed the smallest genetic distance value and conversely the highest genetic identity value. The two populations with the least similarity and greatest genetic distance between them were the individuals from Rehoboth and those from Amazona. Generally speaking, the genetic distance values were all less than 0.1 and the genetic identity values all above 0.9, which is indicative of very closely related populations and individuals.

From the information and values calculated using Nei's genetic distance, a UPGMA dendrogram was constructed and is presented in Figure 3.19.

Figure 3.19 Dendrogram constructed from Nei's Genetic Distance measures.



The difference between Nei's original and Nei's unbiased measures, were minimal and did not affect the general observations and trends. The unbiased measures were however, slightly higher for genetic identity and slightly lower for genetic distance indicating that the small sample size did reduce the genetic identity and increase the genetic distance by a marginal amount. The average difference (taking into account all values for both sex-including and sex-excluding data) between the two measures was 0.012. As the general 'cut-off' values to determine whether or not populations are closely related or diverging is 0.1 for genetic distance and 0.9 for genetic identity, this small difference between the original and unbiased measures, hardly had an effect in this case.

A separate analysis was conducted on data containing the sex-specific primers 25 and C-06 and compared with the results obtained from data excluding the values for these two primers. It was thought the inclusion of sex-specific primers would affect Nei's measures by implying that two populations were more similar due to them both possessing similar female to male ratios as compared to a population, such as the one from Rehoboth, which consisted mainly of males. This was however not observed as, although the inclusion of these sex-specific primers did increase the genetic identity and decrease the genetic distance between populations, it did so equally between all population comparisons. Furthermore,

the difference was so slight, as with the difference between original and unbiased measures, it did not have a significant affect.

3.7.3 Analysis of molecular variance (AMOVA)

To calculate and assess the molecular variance between and within the subpopulations, the software programme ARLEQUIN ver 2.000 (Schneider *et al.*, 2000) was used. A Euclidian distance matrix was constructed from which the between population and within population variances were calculated. The results of the AMOVA and the complete Euclidian distance matrix are taken up in Appendix F.

AMOVA analysis enabled partitioning of the overall RAPD variation between the within and between variance components as seen in Table 3.20. Random permutations (1023) were used to test for the significance of the variance components produced.

Table 3.20 Summary of analysis of molecular variance.

SOURCE OF VARIATION	d.f.*	SUMS OF SQUARES	VARIANCE COMP. *	% VARIATION	P*
Between	2	17.09	0.32	5.59	0.0488
Within	27	145.44	5.39	94.41	0.0488
Total	29	162.53	5.71		

*P = Levels of significance were based on 1023 random permutations.
*d.f. = Degrees of freedom
* VARIANCE COMP. = variance components

Significant variation was found both between and within populations, with a P value of less than 0.05 (P = 0.0488). The results were thus not highly significant (P < 0.01) but significant never the less. Most of the variation (94.41 %) was found

within the different subpopulations and very little variation (5.59 %) was found between them. The three subpopulations were thus very similar, displaying little differentiation between them.

3.7.4 Wright's fixation index (F_{ST})

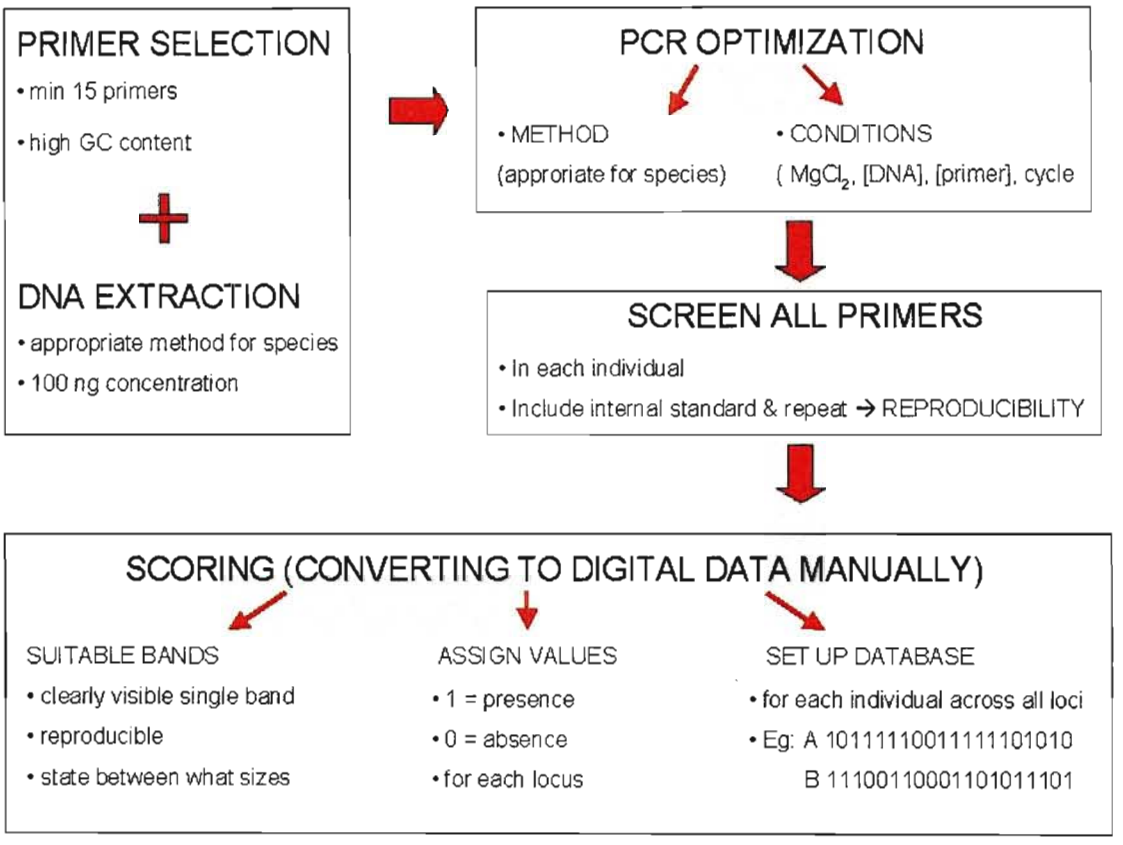
The calculation of Wright's fixation index (F_{ST}), confirmed the results displayed in the AMOVA analysis. In this investigation a significant F_{ST} value of 0.056 was obtained. According to Wright's guidelines, a value of 0.05 or less accounts for little genetic differentiation between subpopulations. The F_{ST} calculated in this analysis was only slightly above this value, thus indicating that a small amount of differentiation was evident between the populations.

3.8 PROPOSAL FOR FIRST TIME GENOTYPING OF A SPECIES

One of the aims of this investigation was to develop a technique for genotyping this particular species using RAPD analysis. As this has never been done before, experimenting with various procedures and determining which steps need to be followed, was necessary. The determined steps could be applied to any species requiring first time genotyping using RAPD analysis. A proposal for genotyping a species for the first time was therefore established and is presented in figure 3.20.

The guidelines as to what steps should be followed to fingerprint a species for the first time (presented in figure 3.20) includes information starting from DNA extraction and primer selection to the final step of setting up a database for the genotypes of each individual. How to go about analysing the data once in digital format has not been included, as the method of analysis will depend directly upon the research question.

Figure 3.20 Flow diagram illustrating the first time genotyping of a species.



CHAPTER 4
DISCUSSION AND CONCLUSION

Birds have been well studied taxonomically, behaviourally and ecologically as they offer certain advantages to a researcher in that they are easily visible, largely diurnal, easy to count and their growth rates are deterministic. However, compared to other classes of animals, both invertebrates and vertebrates, much remains unknown about the genetics of birds. The discovery of the existence of avian sex chromosomes, Z and W, was only shown in the 1960s (Ohno, 1967) and a recent examination of the GenBank sequence database, revealed that a mere 0.4 % of vertebrate sequence entries are derived from avian species, two-thirds of which are entries from a single species, the domestic chicken *Gallus gallus* (Benson *et al.*, 2000).

This lack of empirical data is a major problem confronting avian genetics in all fields, from population and paternity analyses to conservation genetics. There is thus a need to develop and gain more information regarding the genetics of this class. This is particularly important in threatened and endangered species, such as the Cape parrot, where information regarding the genetic variation within the population and determining methods for individual identification, would be of great value to the conservation programme.

4.1 DNA FINGERPRINTING AND GENETIC VARIATION

The Cape parrot (*Poicephalus robustus*) is a threatened species and it is believed that there are less than 500 birds left in the wild (Downs, 2001). For conservation purposes it is useful to determine the amount of genetic variation present in the remaining population in order to establish effective breeding programmes and conservation plans to increase the population size.

One of the main generalisations, made from genetic studies of wild populations of birds, is that genetic variation and variability is widespread and universal in this group. All the different techniques used, suggest that birds have a wide store of genetic variation upon which natural selection can act. From the results of this investigation however, a low genetic variation was detected in the species, with the majority of loci tested being monomorphic. The number of polymorphic loci out of all the loci tested was 33 % across the eight polymorphic primers. The majority displayed *presence* alleles in most individuals, with only a few *absence* alleles specific to a select group of individuals. In contrast, in a population displaying high genetic variation, the majority of *presence* alleles would be individual specific or present in only a select group of individuals.

In comparison to studies of other threatened bird species, such as in the Iberian imperial eagle where the determined level of polymorphism was 59.7 % (Padilla *et al.,* 2000), the level of polymorphism detected in this species was low. In an endangered species, a relatively low genetic variation is expected due to the small population size, however in the Cape parrot, the genetic variation has possibly been further reduced as a result of inbreeding within the subpopulations. A similar deduction was seen in a study on the endangered light-footed clapper rail (Nusser *et al.*, 1996), where the low genetic variation was mainly attributed to inbreeding depression.

Another measure used to assess the genetic variation in a population is to calculate the average heterozygosity. The heterozygosity observed in this investigation (H = 0.37) was higher than the values calculated in most avian studies, where the mean heterozygosity for birds was estimated to be 0.145 (Purves *et al.*, 1995). Upon examination of a list of values for different species it was noted that the majority displayed heterozygosities of less than 0.1, however one or two species, such as the *Tachyeres patachonicus,* displayed higher values of 0.31 (Cooke & Buckley, 1987).

The heterozygosity in an endangered species is expected to be substantially lower than in non-threatened species due to the reduction in genetic variability. It was therefore surprising to obtain this high heterozygosity for a threatened species with

low genetic variation. However, the heterozygosities observed by RAPD studies in other threatened avian species (Bowditch *et al.*, 1993, Haig *et al.,* 1994; Nusser *et al.*, 1996), were also relatively high, such as in the endangered Iberian imperial eagle, where a significantly high heterozygosity of 0.267 was observed (Padilla *et al.*, 2000). A possible explanation for observing high heterozygosity values in these investigations could be related to the GC content of the primers. RAPD analysis detects variation in both coding and non-coding DNA however, most of the informative polymorphic sequences are found in the repetitive DNA, part of the non-coding DNA regions. Repetitive DNA is rich in GC base pairs and as the primers were selected on the basis of a high GC content, they could be targeting these repetitive sequences exclusively. This combined with the fact that non-coding DNA has a higher mutation rate, could account for the high heterozygosity. In support of this theory, Stephens *et al.* (1992) found substantially higher heterozygosities in endangered species when using minisatellite analysis, which detects the non-coding DNA exclusively.

Another possible explanation for the high level of heterozygosity present in this investigation has to do with population bottlenecks. Bottlenecks occur when the population size of a species is reduced due to various factors such as poaching, climatic changes and diseases. The most characteristic effect of a population bottleneck is that the expected number of alleles in a sample is reduced more rapidly than expected heterozygosity (Nei *et al.*, 1975). Rare alleles do not contribute significantly to the heterozygosity and are more likely to be lost. Therefore, depending on the number of alleles observed in the analysis, the heterozygosity is expected to be greater than what would be expected in a normal population under mutation-drift equilibrium. The heterozygosity would however only be greater directly after the population bottleneck so this would only be evident for a short space of time before the heterozygosity would return to normal. It is important to remember that if a population is not in Hardy-Weinberg equilibrium, the observed heterozygosity may not be a good indication of the amount of genetic variation present in the population. This could be the case where mating between relatives, inbreeding, is common, as is the case in this species.

The lack of genetic variation in this species did prevent the ability to identify individual birds on the basis of unique DNA fingerprints. In various other studies (Fowler *et al.*, 1998; Petrie *et al.*, 1998; Cooper, 2000), RAPDs have successfully and accurately determined paternity and produced DNA fingerprints unique to each individual, however, due to the lack of genetic variation present in this investigation, it was not possible for all individuals. Identification was however, possible on a number of levels with regard to a specific subpopulation, the sex of individuals as well as determination of kinship and relatedness to a certain extent. In particular, identification of the sex of individuals was 100 % accurate and successful. The two primers able to discriminate between the sexes (primer 25 and primer C-06) could possibly be developed into sex specific probes allowing for simple, cheap and accurate sex determination in the species. Furthermore, Nei's genetic identity and genetic distance measures based on the RAPD data were able to accurately determine relatedness between some individuals. RAPD analysis was therefore successful in producing reproducible DNA fingerprints in the species that were able to determine relatedness to some extent, determine the sex of individuals and identify individuals from a particular subpopulation, as well as in determining the genetic variation of the species.

4.2 POPULATION ANALYSIS, STRUCTURE AND COMPARISONS

Birds, in general, have been found to possess a large amount of genetic variation, primarily maintained by selection or as more recent studies suggest, the consequences of recombination and mutations (Primmer *et al.*, 2002). However, differentiation among and between different taxonomic levels of this group, is far less than in other classes of vertebrates (Cooke & Buckley, 1987). Numerous explanations for this, such as the class having a more recent evolutionary divergence, have been given; however, the most plausible seems to be simply the ability of birds to cover large distances as a result of flight and thus having a high dispersal rate and consequent potential for gene flow.

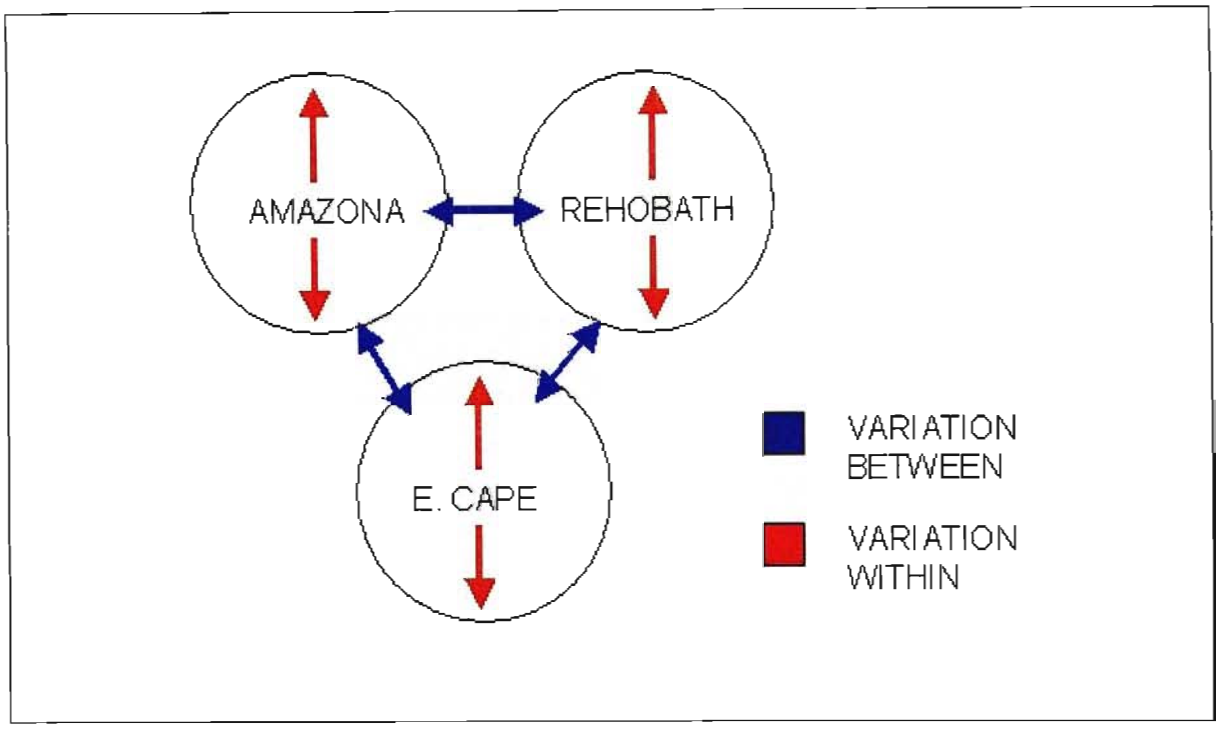
One way to measure gene flow is to analyse the fixation index, F_{ST}. This value can vary from one to zero. If a species is organized into many strongly differentiated

subpopulations, as a result of limited gene flow, the F_{ST} value will be large. Conversely if the subpopulations within a species are similar and undifferentiated owing to extensive gene flow between them, this value will be small. A study by Barrowclough (1983) compared F_{ST} values for a range of avian populations as well as from other vertebrates. Most of the avian species had F_{ST} values of 0.1 or less. In comparison, other taxa had values as high as 0.9, as was seen in salamanders. From Barrowclough's study, it can therefore be concluded that less than 10 % of the variation found in birds can be attributed to locality, indicating that substantial gene flow is found among subpopulations of a species. Recently, numerous studies on various different species of birds (Nusser *et al.,* 1996, Cooper, 2000, Padilla *et al.* 2000) have demonstrated similar findings and confirmed the large amount of gene flow between populations.

In this investigation an estimated F_{ST} value of 0.056 was obtained between the three subpopulations analysed. This value, although very low, falls within the range of values expected for avian species (less than 0.1). A value of less than 0.05 would have indicated almost no differentiation among the subpopulations, however, this value indicates that a small amount of variation was evident.

The AMOVA analysis supports the findings of the fixation index F_{ST}. Significant variation was found between and within the subpopulations, although the results were not highly significant, with $P < 0.5$ and not $P < 0.1$. The majority of the variation was found within each subpopulation and little variation was observed between them. Figure 4.1 clearly illustrates the difference in contribution of the two types of variances, where the within subpopulation variation was larger than the between subpopulation variation. This confirms that little differentiation was observed between the subpopulations and that significant gene flow was acting between them.

Figure 4.1 Illustration of the subdivision of variance between and within the three subpopulations.



In other studies such as Cooper (2000), the subpopulations analysed were wild and in relatively close proximity, thus allowing for the free movement of individuals between them. However, in this investigation, the individuals were captive and therefore not able to move freely between the populations. Gene flow would therefore not have been the probable cause of the low F_{ST} value. What was however discovered was the regular exchanging of chicks between breeders of the various subpopulations, thus imitating the natural process of gene flow. In this way, birds from different populations and gene pools were introduced, preventing differentiation between them and possibly increasing the genetic variation within a population.

The variation experienced within each subpopulation was measured using Shannon's index of phenotypic diversity. The subpopulation displaying the most variation and significantly more than either of the others was the Amazona population from Assagay. The population from the Eastern Cape followed, with the Rehoboth population displaying the least amount of variation. These findings were

expected as the Rehoboth population consisted of one family including extended family members and was thus not expected to display much variation as compared to the Amazona population, which consisted of three separate families. A distance analysis between the three populations using Nei's genetic identity and genetic distance measures, indicated that the Amazona and Eastern Cape populations were more closely related than either of them to the Rehoboth population. This result was again expected due to the exchanging of chicks between these two populations.

4.3 CONCLUDING REMARKS

In this investigation, RAPD analyses proved successful in the first time analysis of an endangered species at both the intra- and inter population level. Successful optimisation of the technique resulted in reliable and reproducible DNA fingerprints of all the individuals tested, suggesting that RAPDs are extremely useful in situations where relatively inexpensive first approximations of the genetic variation are needed, such as in rare and endangered species.

RAPDs have been known to produce unique individual profiles (Fowler *et al.*, 1998, Cooper, 2000), however due to the lack of genetic variation within this species, individual identification to a certain extent was not possible. The lack of genetic variation observed in the Cape parrot can be attributed to the endangered status of the species and the resulting small population. However, compared to other endangered birds, such as the Iberian imperial eagle (Padilla *et al.*, 2000), the variation in the Cape parrot is still significantly lower. This could be attributed to the added effect of inbreeding within the populations, resulting in a further reduction in genetic variation. Genotyping, identification of sex as well as determining the population of origin of an individual, was however possible and successfully determined with this technique.

Comparisons between subpopulations, determination of subdivisions of variance as well as determining the degree of differentiation between populations was also successful. It was found that this species followed similar trends in subdivision and

population structure as many avian species. Differentiation between subpopulations was found to be marginal due to large amounts of gene flow between the populations as a result of exchanging of chicks between breeders, and only a small portion of the overall variation was attributed to between population variance.

4.4 CONSERVATION STRATEGIES AND FUTURE PROSPECTS

Low genetic diversity in a species does not necessarily mean that it is imperilled and doomed, however it does indicate that action needs to be taken to increase variation, particularly if the species is threatened. Careful planning and genetic modelling in conservation programmes can successfully improve the status of an endangered species and possibly increase the numbers. In order to ensure the survival of the Cape parrot and increase its genetic variation, a number of proposed steps, based on this investigation, should be taken, as listed below.

- Breeders should avoid breeding between related individuals to prevent genetic uniformity and possible fixation of harmful alleles.
- Exchanging of chicks between different populations should be encouraged to introduce new genetic material and increase variation.
- Samples of blood should be taken from all individuals and stored for research and allow for updates of genetic variation within a population.
- A programme for the introduction of captive individuals into the wild should be established.
- Research should focus on developing methods of individual identification based on this study, using more individual specific molecular markers such as AFLPs and micro- and minisatellites to prevent illegal trafficking and stealing of the species.

This investigation, although not fully able to identify individuals based on unique DNA profiles itself, was important in laying the foundations for individual identification. Further research should focus on finding a suitable marker that will

be sensitive enough to pick up individual differences between such a genetically uniform species. A good indication of the variation found in the Cape parrot population has however been determined for conservation purposes and the sequence of events required to fingerprint an individual from this particular species, established. The results of this genetic investigation, coupled with the findings of previous research in terms of morphological, behavioural and ecological data of the Cape parrot, should thus ensure that a successful conservation programme is set up to preserve this indigenous species and prevent its extinction.

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APPENDIX

A. LABORATORY SOLUTION RECIPES

EDTA (0.5 M)

- 73.0625 g EDTA
- 500 ml dH_2O

Adjust to pH 8 with concentrated NaOH (saturated solution). To dissolve the salt the pH must be around 8 (Autoclave)

ETHANOL (70 %)

- 350 ml absolute ethanol
- 150 ml dH_2O

ETHIDIUM BROMIDE (20 mg/ ml)

- 0.4 g EtBr
- 20 ml dH_2O

LOADING BUFFER

- 0.0125 g Bromophenol blue
- 0.0125 g Xylene cyanol
- 1.5 g glycerol
- 5 ml dH_2O

NaCl (5 M)

- 146.1 g NaCl
- 500 ml dH_2O (Autoclave)

PROTEINASE K

- 0.5 g Proteinase K
- 20 ml dH_2O

SDS (25 %)

- 5 g SDS
- 20 ml dH_2O

TAE (10 X)

- 48.46 g Tris
- 41.1 g anhydrous NaAc
- 3.72 g Na_2EDTA

Adjust to pH 8.5 with glacil acetic acid. Make up to 1 litre with dH_2O (Autoclave) (A working solution of 1 x TAE is commonly used therefore 1: 10 dilution of 10 x TAE)

TBE (5 X)

- 54 g Tris Base
- 27.5 g Boric Acid
- 20 ml 0.5 M EDTA (pH 8)

Make up to 1 litre with dH_2O (Autoclave) A working solution of 0.5 x TBE provides more than enough buffering power therefore 1: 10 dilution of 5 x TBE

TNE (1 X)

- 25 ml 1 M Tris (50 mM)
- 10 ml 5 M NaCl (100 mM)
- 5 ml 0.5 M EDTA (5 mM)
- 460 ml dH_2O

Adjust to pH 7.5 with concentrated HCl (Autoclave)

TRIS (1 M)

- 121.1 g Tris
- 1 litre dH_2O

Adjust to pH 8 with concentrated HCl (Autoclave)

TRIS (10 mM)

- 1.211 g Tris
- 1 litre dH_2O

Adjust to pH 8.8 with concentrated HCl (Autoclave)

TRITON X-100 (10 %)

- 2 ml Triton X-100
- 18 ml dH_2O

B. INDIVIDUAL SCORES FOR POLYMORPHIC PRIMERS

SAMPLES	PRIMER 17	PRIMER 25	PRIMER A-04	PRIMER A-06	PRIMER A-17	PRIMER A-20	PRIMER C-05	PRIMER C-06
1 a	101111001	11100	1101111111	11011111	10111110	11011	111100110	0111110
1 b	111011011	11111	1111111111	11101111	10111111	11111	101111110	1111111
1 ab i	101110001	11111	1001001111	11011111	10111111	11011	010101110	1111111
1 ab ii	101101001	11111	1111110111	11111111	10111111	11111	101110110	1111111
1 ab iii	111111011	11100	1111111111	11111111	10111110	11111	111111110	0111110
2 a	111111011	11100	1101001100	11111111	11111110	11011	100011111	0111110
2 b	101110001	11111	1011001100	11101111	10111111	11011	111111110	1111111
2 ab i	101101001	11111	1111011111	11111111	11111111	11011	111111111	1111111
3 a	111111011	11100	1101011111	11011111	10111110	11011	101111111	0111110
3 b	101110001	11111	1101010111	11111111	00011101	11011	110011110	1111111
3 ab i	111111011	11100	1101111111	11111111	10111110	11011	101111110	0111110
3 ab ii	111111001	11100	1101011100	11011111	10111110	11011	111111110	0111110
4 a	101010100	11100	1101111111	11111111	10111110	11011	101111111	0111110
4 b	111111011	11111	1011001111	11011111	11111110	11011	011111110	1111111
4 ab i	111010100	11100	1101111111	11111111	11111110	11011	000001110	0111110
4 ab ii	111011011	11100	1101111111	11111111	11111110	11011	111111110	0111110
4 ab iii	101010100	11100	1101011111	11111111	11111110	11011	101111110	0111110
4 w	111010111	11100	1101111111	11011111	10111110	11011	101111111	0111110
4x	111010111	11100	1101111111	11011111	11111110	11011	101001111	0111110
4y	111110001	11100	1001001111	11011111	11111110	11011	101101111	0111110
4z	111111111	11111	1111001101	11111111	10111111	11011	111111110	1111111
5 i	111111011	11100	1101111111	11001111	10111110	11111	111111110	0111110
5 ii	111011000	11100	1101110111	11111111	10111110	11011	101111110	0111110
5 iii	111111000	11111	1111111111	11111111	---	11011	101111110	1111111
5 iv	111011011	11100	1101111111	11101111	10111110	11111	111111110	0111110
6	111011011	11100	1101001100	11011111	10111110	11011	101111110	0111110
7	111110011	11111	1111111111	11011111	10111111	11011	111111111	1111111
8	111111011	11111	1111111111	11111111	10111111	11111	---	1111111
9	111010011	11111	1111111101	11111111	10111111	11011	101010111	1111111
10	111111011	11100	1001001111	11011111	10111110	11011	101110110	0111110

C. POPGENE INPUT FILE

1. FOR BETWEEN POPULATION COMPARISONS

```
/* Diploid RAPD Data Set */
Number of populations = 3
Number of loci = 61
Locus name :
17-1 17-2 17-3 17-4 17-5 17-6 17-7 17-8 17-9
25-1 25-2 25-3 25-4 25-5
A04-1 A04-2 A04-3 A04-4 A04-5 A04-6 A04-7 A04-8 A04-9 A04-10
A06-1 A06-2 A06-3 A06-4 A06-5 A06-6 A06-7 A06-8
A17-1 A17-2 A17-3 A17-4 A17-5 A17-6 A17-7 A17-8
A20-1 A20-2 A20-3 A20-4 A20-5
C05-1 C05-2 C05-3 C05-4 C05-5 C05-6 C05-7 C05-8 C05-9
C06-1 C06-2 C06-3 C06-4 C06-5 C06-6 C06-7

name = Amazona
fis = 0
101111001 11100 1101111111 11011111 10111110 11011 111100110 0111110
111011011 11111 1111111111 11101111 10111111 11111 101111110 1111111
101110001 11111 1001001111 11011111 10111111 11011 010101110 1111111
101101001 11111 1111110111 11111111 10111111 11111 101110110 1111111
111111011 11100 1111111111 11111111 10111110 11111 111111110 0111110
111111011 11100 1101001100 11111111 11111110 11011 100011111 0111110
101110001 11111 1011001100 11101111 10111111 11011 111111110 1111111
101101001 11111 1111011111 11111111 11111111 11011 111111111 1111111
111111011 11100 1101011111 11011111 10111110 11011 101111111 0111110
101110001 11111 1101010111 11111111 00011101 11011 110011110 1111111
111111011 11100 1101111111 11111111 10111110 11011 101111110 0111110
111111001 11100 1101011100 11011111 10111110 11011 111111110 0111110

name = Rehoboth
fis = 0
101010100 11100 1101111111 11111111 10111110 11011 101111111 0111110
111111011 11111 1011001111 11011111 11111110 11011 011111110 1111111
111010100 11100 1101111111 11111111 11111110 11011 000001110 0111110
111011011 11100 1101111111 11111111 11111110 11011 111111110 0111110
101010100 11100 1101011111 11111111 11111110 11011 101111110 0111110
111010111 11100 1101111111 11011111 10111110 11011 101111111 0111110
111010111 11100 1101111111 11011111 11111110 11011 101001111 0111110
111110001 11100 1001001111 11011111 11111110 11011 101101111 0111110
111111111 11111 1111001101 11111111 10111111 11011 111111110 1111111

name = Eastern Cape
fis = 0
111111011 11100 1101111111 11001111 10111110 11111 111111110 0111110
111011000 11100 1101110111 11111111 10111110 11011 101111110 0111110
111111000 11111 1111111111 11111111 ........ 11011 101111110 1111111
111011011 11100 1101111111 11101111 10111110 11111 111111110 0111110
111011011 11100 1101001100 11011111 10111110 11011 101111110 0111110
111110011 11111 1111111111 11011111 10111111 11011 111111111 1111111
111111011 11111 1111111111 11111111 10111111 11111 ......... 1111111
111010011 11111 1111111101 11111111 10111111 11011 101010111 1111111
111111011 11100 1001001111 11011111 10111110 11011 101110110 0111110
```

E. ARLEQUIN INPUT FILE

```
[Profile]
     Title="Cape parrot AMOVA analysis"
     NbSamples=3
     GenotypicData=0
     DataType=RFLP
     LocusSeparator=None
     #We tell Arlequin to compute Euclidian square distance between
     #the haplotypes listed below
     CompDistMatrix=1
     MissingData='?'

[Data]
  [[HaplotypeDefinition]]
      HaplListName="List of RAPD scores"
      HaplList=  {
 1   10111100111100110111111111101111110111110110111111001100111110
 2   11101101111111111111111111101111101111111111110111111101111111
 3   10111000111111100100111111011111101111111110110101011101111111
 4   10110100111111111111011111111111101111111111110111011011111111
 5   11111101111100111111111111111111101111101111111111111100111110
 6   11111101111100110100110011111111111111110110111000111110111110
 7   10111000111111101100110011101111101111111110111111111101111111
 8   10110100111111111101111111111111111111111110111111111111111111
 9   11111101111100110101111111011111101111101101110111111110111110
10   10111000111111110101011111111111000111011101111001111011111111
11   11111101111100110111111111111111101111101101110111111100111110
12   11111100111100110101110011011111101111101101111111111100111110
13   10101010011100110111111111111111101111101101110111111110111110
14   11111101111111101100111111011111111111110110110111111101111111
15   11101010011100110111111111111111111111110110110000011100111110
16   11101101111100110111111111111111111111110110111111111100111110
17   10101010011100110101111111111111111111110110111011111100111110
18   11101011111100110111111111011111101111101101110111111110111110
19   11101011111100110111111111011111111111101101110100111110111110
20   11111000111100100100111111011111111111101101110110111110111110
21   11111111111111111100110111111111101111111101111111111101111111
22   11111101111100110111111111001111101111101111111111111100111110
23   11101100011100110111011111111111101111101101110111111100111110
24   11111100011111111111111111111111????????110111011111101111111
25   11101101111100110111111111101111101111101111111111111100111110
26   11101101111100110100110011011111101111101101110111111100111110
27   11111001111111111111111111011111101111111101111111111111111111
28   11111101111111111111111111111111101111111111?????????1111111
29   11101001111111111111111011111111101111111101110101011111111111
30   11111101111100100100111111011111101111101101110111011001111110
}

   [[Samples]]

      SampleName="Amazona pop 1"
      SampleSize=12
      SampleData=      {
    1     1
    2     1
    3     1
    4     1
    5     1
```

```
6      1
7      1
8      1
9      1
10     1
11     1
12     1
     }

    SampleName="Rehoboth pop 2"
    SampleSize=9
    SampleData=       {
13     1
14     1
15     1
16     1
17     1
18     1
19     1
20     1
21     1
     }

    SampleName="Eastern Cape pop 3"
    SampleSize=9
    SampleData=       {
22     1
23     1
24     1
25     1
26     1
27     1
28     1
29     1
30     1
     }

[[Structure]]

    StructureName="A single group of 3 samples"
    NbGroups=1
    Group={
 "Amazona pop 1"
 "Rehoboth pop 2"
 "Eastern Cape pop 3"
    }
```

F. ARLEQUIN RESULTS

```
//////////////////////////////////////////////////////////////////////
RUN NUMBER 1 (21/10/03 at 09:21:09)
//////////////////////////////////////////////////////////////////////

Project information:
--------------------
      NbSamples      =  3
      DataType       =  RFLP
      GenotypicData  =  0

==============================
Settings used for Calculations
==============================

 General settings:
 -----------------
        Deletion Weight                 = 1
        Transition Weight Weight        = 1
        Tranversion Weight Weight       = 1
        Epsilon Value                   = 1e-07
        Significant digits for output   = 5
        Use original haplotype definition
        Alllowed level of missing data = 0.05

 Active Tasks:
 -------------

  Analysis of Molecular Variance:

            No. of Permutations = 1000

  Distance matrix:

            Compute distance matrix
            Molecular distance : Pairwise difference
            Gamma a value      = 0

=============================================================================
GENETIC STRUCTURE ANALYSIS
=============================================================================


Number of usable loci for distance computation : 61
Allowed level of missing data                  : 0.05

List of usable loci :

   1     2     3     4     5     6     7     8     9    10    11
12    13    14    15
  16    17    18    19    20    21    22    23    24    25    26
27    28    29    30
  31    32    33    34    35    36    37    38    39    40    41
42    43    44    45
  46    47    48    49    50    51    52    53    54    55    56
57    58    59    60
  61

List of loci with too much missing data :

NONE
```

```
================================================================
  AMOVA ANALYSIS
================================================================

---------------------------
Genetic structure to test :
---------------------------

No. of Groups = 1

      [[Structure]]

            StructureName = "A single group of 3 samples"
            NbGroups = 1
            IndividualLevel = 0
            DistMatLabel = ""
            Group={
            "Amazona pop 1"
            "Rehoboth pop 2"
            "Eastern Cape pop 3"
            }

---------------------------

Distance method: Pairwise difference

-------------------------
AMOVA design and results :
-------------------------

Reference: Weir, B.S. and Cockerham, C.C. 1984.
           Excoffier, L., Smouse, P., and Quattro, J. 1992.
           Weir, B. S., 1996.
-------------------------------------------------------------------
 Source of                  Sum of      Variance         Percentage
 variation      d.f.        squares     components       variation
-------------------------------------------------------------------
 Among
 populations      2         17.089       0.31895 Va           5.59

 Within
 populations     27        145.444       5.38683 Vb          94.41
-------------------------------------------------------------------
 Total           29        162.533       5.70578
-------------------------------------------------------------------
 Fixation Index      FST :      0.05590
-------------------------------------------------------------------


Significance tests (1023 permutations)
------------------

Va and FST : P(rand. value > obs. value)  =  0.04301
             P(rand. value = obs. value)  =  0.00098
             P(rand. value >= obs. value) =  0.04399+-0.00700


Histogram of variance components null distributions
```

```
================================================

                    Va
  -0.35172           7
  -0.27060          50
  -0.18948         148
  -0.10836         221
  -0.02725         195
   0.05387         143
   0.13499         118
   0.21611          68
   0.29723          33
   0.37835          17
   0.45946           5
   0.54058           9
   0.62170           3
   0.70282           3
   0.78394           1
   0.86505           0
   0.94617           1
   1.02729           1
   1.10841           0
   1.18953           0

Observed  values:
-----------------
Va : 0.31895

////////////////////////////////////////////////////////////////////
END OF RUN NUMBER 1 (21/10/03 at 09:21:09))
Total computing time for this run : 0h 0m 0s 172 ms
////////////////////////////////////////////////////////////////////
```

D. POPGENE RESULTS

1. POPULATION COMPARISONS

Dendrogram

* File Name: dgram1.plt

Between	And	Length
2	1	0.43105
1	pop1	2.09565
1	pop3	2.09565
2	pop2	2.52670

Summary of Genic Variation Statistics for All Loci
[See Nei (1987) Molecular Evolutionary Genetics (p. 176-187)]
****

Locus	Sample Size	na*	I*
17-1	30	1.0000	0.0000
17-2	30	2.0000	0.6816
17-3	30	1.0000	0.0000
17-4	30	2.0000	0.6858
17-5	30	2.0000	0.4451
17-6	30	2.0000	0.6782
17-7	30	2.0000	0.3802
17-8	30	2.0000	0.6493
17-9	30	2.0000	0.6228
25-1	30	1.0000	0.0000
25-2	30	1.0000	0.0000
25-3	30	1.0000	0.0000
25-4	30	2.0000	0.5380
25-5	30	2.0000	0.5380
A04-1	30	1.0000	0.0000
A04-2	30	2.0000	0.6749
A04-3	30	2.0000	0.5090
A04-4	30	1.0000	0.0000
A04-5	30	2.0000	0.6493
A04-6	30	2.0000	0.6927
A04-7	30	2.0000	0.5765
A04-8	30	1.0000	0.0000
A04-9	30	2.0000	0.6863
A04-10	30	2.0000	0.6109
A06-1	30	1.0000	0.0000
A06-2	30	1.0000	0.0000
A06-3	30	2.0000	0.6585
A06-4	30	2.0000	0.6148
A06-5	30	1.0000	0.0000

A06-6	30	1.0000	0.0000
A06-7	30	1.0000	0.0000
A06-8	30	1.0000	0.0000
A17-1	29	2.0000	0.3658
A17-2	29	2.0000	0.4515
A17-3	29	2.0000	0.3658
A17-4	29	1.0000	0.0000
A17-5	29	1.0000	0.0000
A17-6	29	1.0000	0.0000
A17-7	29	2.0000	0.3658
A17-8	29	2.0000	0.4958
A20-1	30	1.0000	0.0000
A20-2	30	1.0000	0.0000
A20-3	30	2.0000	0.3437
A20-4	30	1.0000	0.0000
A20-5	30	1.0000	0.0000
C05-1	29	2.0000	0.5790
C05-2	29	2.0000	0.5745
C05-3	29	2.0000	0.6194
C05-4	29	2.0000	0.6778
C05-5	29	2.0000	0.6463
C05-6	29	2.0000	0.6166
C05-7	29	1.0000	0.0000
C05-8	29	1.0000	0.0000
C05-9	29	2.0000	0.4581
C06-1	30	2.0000	0.5380
C06-2	30	1.0000	0.0000
C06-3	30	1.0000	0.0000
C06-4	30	1.0000	0.0000
C06-5	30	1.0000	0.0000
C06-6	30	1.0000	0.0000
C06-7	30	2.0000	0.5380
Mean	30	1.5410	0.3037
St. Dev		0.5025	0.2934

* na = Observed number of alleles
* I = Shannon's Information index [Lewontin (1972)]

he number of polymorphic loci is : 33
The percentage of polymorphic loci is : 54.10

****
Nei's Original Measures of Genetic Identity and Genetic distance
[See Nei (1972) Am. Nat. 106:283-292)] **
****

INCLUDING PRIMERS 25 AND C-06 (sex-specific)

pop ID	1	2	3
1	****	0.9479	0.9590
2	0.0535	****	0.9535
3	0.0419	0.0476	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

```
*******************************************************************************
Nei's Unbiased Measures of Genetic Identity and Genetic distance
[See Nei (1978)  Genetics 89:583-590]
*******************************************************************************

INCLUDING PRIMERS 25 AND C-06 (sex-specific)

=======================================
pop ID    1         2         3
=======================================
1           ****    0.9585    0.9703
2         0.0423      ****    0.9640
3         0.0302    0.0366      ****
=======================================

Nei's genetic identity (above diagonal) and genetic distance (below
diagonal).


*******************************************************************************
Nei's Original Measures of Genetic Identity and Genetic distance
[See Nei (1972) Am. Nat. 106:283-292)]                          **
*******************************************************************************

EXCLUDING PRIMERS 25 AND C-06 (sex-specific)

=======================================
pop ID    1         2         3
=======================================
1           ****    0.9365    0.9483
2         0.0656      ****    0.9430
3         0.0531    0.0587      ****
=======================================

Nei's genetic identity (above diagonal) and genetic distance (below
diagonal).


*******************************************************************************
Nei's Unbiased Measures of Genetic Identity and Genetic distance
[See Nei (1978)  Genetics 89:583-590]                           **
*******************************************************************************

EXCLUDING PRIMERS 25 AND C-06 (sex-specific)

=======================================
pop ID    1         2         3
=======================================
1           ****    0.9485    0.9605
2         0.0529      ****    0.9545
3         0.0403    0.0466      ****
=======================================

Nei's genetic identity (above diagonal) and genetic distance (below
diagonal).
```

Summary Statistics : POPULATION 1 AMAZONA

Locus	Sample Size	na*	ne*	h*	I*
17-1	12	1.0000	1.0000	0.0000	0.0000
17-2	12	2.0000	1.7071	0.4142	0.6047
17-3	12	1.0000	1.0000	0.0000	0.0000
17-4	12	2.0000	1.6969	0.4107	0.6010
17-5	12	2.0000	1.9348	0.4832	0.6762
17-6	12	2.0000	2.0000	0.5000	0.6931
17-7	12	1.0000	1.0000	0.0000	0.0000
17-8	12	2.0000	1.5646	0.3609	0.5467
17-9	12	1.0000	1.0000	0.0000	0.0000
25-1	12	1.0000	1.0000	0.0000	0.0000
25-2	12	1.0000	1.0000	0.0000	0.0000
25-3	12	1.0000	1.0000	0.0000	0.0000
25-4	12	2.0000	1.7071	0.4142	0.6047
25-5	12	2.0000	1.7071	0.4142	0.6047
A04-1	12	1.0000	1.0000	0.0000	0.0000
A04-2	12	2.0000	1.9348	0.4832	0.6762
A04-3	12	2.0000	1.5646	0.3609	0.5467
A04-4	12	1.0000	1.0000	0.0000	0.0000
A04-5	12	2.0000	1.5646	0.3609	0.5467
A04-6	12	2.0000	2.0000	0.5000	0.6931
A04-7	12	2.0000	1.9348	0.4832	0.6762
A04-8	12	1.0000	1.0000	0.0000	0.0000
A04-9	12	2.0000	2.0000	0.5000	0.6931
A04-10	12	2.0000	2.0000	0.5000	0.6931
A06-1	12	1.0000	1.0000	0.0000	0.0000
A06-2	12	1.0000	1.0000	0.0000	0.0000
A06-3	12	2.0000	1.9533	0.4880	0.6811
A06-4	12	2.0000	1.9348	0.4832	0.6762
A06-5	12	1.0000	1.0000	0.0000	0.0000
A06-6	12	1.0000	1.0000	0.0000	0.0000
A06-7	12	1.0000	1.0000	0.0000	0.0000
A06-8	12	1.0000	1.0000	0.0000	0.0000
A17-1	12	2.0000	1.6969	0.4107	0.6010
A17-2	12	2.0000	1.1892	0.1591	0.2958
A17-3	12	2.0000	1.6969	0.4107	0.6010
A17-4	12	1.0000	1.0000	0.0000	0.0000
A17-5	12	1.0000	1.0000	0.0000	0.0000
A17-6	12	1.0000	1.0000	0.0000	0.0000
A17-7	12	2.0000	1.6969	0.4107	0.6010
A17-8	12	2.0000	1.7071	0.4142	0.6047
A20-1	12	1.0000	1.0000	0.0000	0.0000
A20-2	12	1.0000	1.0000	0.0000	0.0000
A20-3	12	2.0000	1.3022	0.2321	0.3939
A20-4	12	1.0000	1.0000	0.0000	0.0000
A20-5	12	1.0000	1.0000	0.0000	0.0000
C05-1	12	2.0000	1.6969	0.4107	0.6010
C05-2	12	2.0000	1.8439	0.4577	0.6502
C05-3	12	2.0000	2.0000	0.5000	0.6931
C05-4	12	2.0000	1.9348	0.4832	0.6762
C05-5	12	2.0000	1.9348	0.4832	0.6762
C05-6	12	2.0000	1.9348	0.4832	0.6762
C05-7	12	1.0000	1.0000	0.0000	0.0000
C05-8	12	1.0000	1.0000	0.0000	0.0000
C05-9	12	2.0000	1.3022	0.2321	0.3939
C06-1	12	2.0000	1.7071	0.4142	0.6047
C06-2	12	1.0000	1.0000	0.0000	0.0000
C06-3	12	1.0000	1.0000	0.0000	0.0000
C06-4	12	1.0000	1.0000	0.0000	0.0000

C06-5	12	1.0000	1.0000	0.0000	0.0000
C06-6	12	1.0000	1.0000	0.0000	0.0000
C06-7	12	2.0000	1.7071	0.4142	0.6047
Mean	12	1.5082	1.3862	0.2143	0.3096
St. Dev		0.5041	0.4132	0.2209	0.3145

* na = Observed number of alleles
* ne = Effective number of alleles [Kimura and Crow (1964)]
* h = Nei's (1973) gene diversity
* I = Shannon's Information index [Lewontin (1972)]

The number of polymorphic loci is : 31
The percentage of polymorphic loci is : 50.82 %

****

SUMMARY STATISTICS: POPULATION 2 REHOBOTH

Locus	Sample Size	na*	ne*	h*	I*
17-1	9	1.0000	1.0000	0.0000	0.0000
17-2	9	2.0000	1.9935	0.4984	0.6915
17-3	9	1.0000	1.0000	0.0000	0.0000
17-4	9	2.0000	1.4279	0.2997	0.4767
17-5	9	1.0000	1.0000	0.0000	0.0000
17-6	9	2.0000	1.4279	0.2997	0.4767
17-7	9	2.0000	1.9533	0.4880	0.6811
17-8	9	2.0000	1.8000	0.4444	0.6365
17-9	9	2.0000	1.9533	0.4880	0.6811
25-1	9	1.0000	1.0000	0.0000	0.0000
25-2	9	1.0000	1.0000	0.0000	0.0000
25-3	9	1.0000	1.0000	0.0000	0.0000
25-4	9	2.0000	1.2631	0.2083	0.3631
25-5	9	2.0000	1.2631	0.2083	0.3631
A04-1	9	1.0000	1.0000	0.0000	0.0000
A04-2	9	2.0000	1.9935	0.4984	0.6915
A04-3	9	2.0000	1.2631	0.2083	0.3631
A04-4	9	1.0000	1.0000	0.0000	0.0000
A04-5	9	2.0000	1.8000	0.4444	0.6365
A04-6	9	2.0000	1.9533	0.4880	0.6811
A04-7	9	1.0000	1.0000	0.0000	0.0000
A04-8	9	1.0000	1.0000	0.0000	0.0000
A04-9	9	2.0000	1.8000	0.4444	0.6365
A04-10	9	1.0000	1.0000	0.0000	0.0000
A06-1	9	1.0000	1.0000	0.0000	0.0000
A06-2	9	1.0000	1.0000	0.0000	0.0000
A06-3	9	2.0000	1.8000	0.4444	0.6365
A06-4	9	1.0000	1.0000	0.0000	0.0000
A06-5	9	1.0000	1.0000	0.0000	0.0000
A06-6	9	1.0000	1.0000	0.0000	0.0000
A06-7	9	1.0000	1.0000	0.0000	0.0000
A06-8	9	1.0000	1.0000	0.0000	0.0000
A17-1	9	1.0000	1.0000	0.0000	0.0000
A17-2	9	2.0000	1.9533	0.4880	0.6811
A17-3	9	1.0000	1.0000	0.0000	0.0000
A17-4	9	1.0000	1.0000	0.0000	0.0000
A17-5	9	1.0000	1.0000	0.0000	0.0000
A17-6	9	1.0000	1.0000	0.0000	0.0000
A17-7	9	1.0000	1.0000	0.0000	0.0000
A17-8	9	2.0000	1.1209	0.1078	0.2192
A20-1	9	1.0000	1.0000	0.0000	0.0000

A20-2	9	1.0000	1.0000	0.0000	0.0000
A20-3	9	1.0000	1.0000	0.0000	0.0000
A20-4	9	1.0000	1.0000	0.0000	0.0000
A20-5	9	1.0000	1.0000	0.0000	0.0000
C05-1	9	2.0000	1.9935	0.4984	0.6915
C05-2	9	2.0000	1.4279	0.2997	0.4767
C05-3	9	2.0000	1.8000	0.4444	0.6365
C05-4	9	2.0000	1.9935	0.4984	0.6915
C05-5	9	2.0000	1.9533	0.4880	0.6811
C05-6	9	1.0000	1.0000	0.0000	0.0000
C05-7	9	1.0000	1.0000	0.0000	0.0000
C05-8	9	1.0000	1.0000	0.0000	0.0000
C05-9	9	2.0000	1.6119	0.3796	0.5674
C06-1	9	2.0000	1.2631	0.2083	0.3631
C06-2	9	1.0000	1.0000	0.0000	0.0000
C06-3	9	1.0000	1.0000	0.0000	0.0000
C06-4	9	1.0000	1.0000	0.0000	0.0000
C06-5	9	1.0000	1.0000	0.0000	0.0000
C06-6	9	1.0000	1.0000	0.0000	0.0000
C06-7	9	2.0000	1.2631	0.2083	0.3631
Mean	9	1.3934	1.2635	0.1489	0.2194
St. Dev		0.4926	0.3817	0.2025	0.2896

* na = Observed number of alleles
* ne = Effective number of alleles [Kimura and Crow (1964)]
* h = Nei's (1973) gene diversity
* I = Shannon's Information index [Lewontin (1972)]

The number of polymorphic loci is : 24
The percentage of polymorphic loci is : 39.34 %

****

SUMMARY STATISTICS: POPULATION 3 EASTERN CAPE

Locus	Sample Size	na*	ne*	h*	I*
17-1	9	1.0000	1.0000	0.0000	0.0000
17-2	9	1.0000	1.0000	0.0000	0.0000
17-3	9	1.0000	1.0000	0.0000	0.0000
17-4	9	2.0000	1.8000	0.4444	0.6365
17-5	9	1.0000	1.0000	0.0000	0.0000
17-6	9	2.0000	1.9935	0.4984	0.6915
17-7	9	1.0000	1.0000	0.0000	0.0000
17-8	9	2.0000	1.9935	0.4984	0.6915
17-9	9	2.0000	1.9935	0.4984	0.6915
25-1	9	1.0000	1.0000	0.0000	0.0000
25-2	9	1.0000	1.0000	0.0000	0.0000
25-3	9	1.0000	1.0000	0.0000	0.0000
25-4	9	2.0000	1.6119	0.3796	0.5674
25-5	9	2.0000	1.6119	0.3796	0.5674
A04-1	9	1.0000	1.0000	0.0000	0.0000
A04-2	9	2.0000	1.8000	0.4444	0.6365
A04-3	9	2.0000	1.6119	0.3796	0.5674
A04-4	9	1.0000	1.0000	0.0000	0.0000
A04-5	9	2.0000	1.9935	0.4984	0.6915
A04-6	9	2.0000	1.9935	0.4984	0.6915
A04-7	9	2.0000	1.8000	0.4444	0.6365
A04-8	9	1.0000	1.0000	0.0000	0.0000
A04-9	9	2.0000	1.9935	0.4984	0.6915
A04-10	9	2.0000	1.8000	0.4444	0.6365
A06-1	9	1.0000	1.0000	0.0000	0.0000

A06-2	9	1.0000	1.0000	0.0000	0.0000
A06-3	9	2.0000	1.8000	0.4444	0.6365
A06-4	9	2.0000	1.9935	0.4984	0.6915
A06-5	9	1.0000	1.0000	0.0000	0.0000
A06-6	9	1.0000	1.0000	0.0000	0.0000
A06-7	9	1.0000	1.0000	0.0000	0.0000
A06-8	9	1.0000	1.0000	0.0000	0.0000
A17-1	8	1.0000	1.0000	0.0000	0.0000
A17-2	8	1.0000	1.0000	0.0000	0.0000
A17-3	8	1.0000	1.0000	0.0000	0.0000
A17-4	8	1.0000	1.0000	0.0000	0.0000
A17-5	8	1.0000	1.0000	0.0000	0.0000
A17-6	8	1.0000	1.0000	0.0000	0.0000
A17-7	8	1.0000	1.0000	0.0000	0.0000
A17-8	8	2.0000	1.4951	0.3311	0.5132
A20-1	9	1.0000	1.0000	0.0000	0.0000
A20-2	9	1.0000	1.0000	0.0000	0.0000
A20-3	9	2.0000	1.4279	0.2997	0.4767
A20-4	9	1.0000	1.0000	0.0000	0.0000
A20-5	9	1.0000	1.0000	0.0000	0.0000
C05-1	8	1.0000	1.0000	0.0000	0.0000
C05-2	8	2.0000	1.4951	0.3311	0.5132
C05-3	8	1.0000	1.0000	0.0000	0.0000
C05-4	8	2.0000	1.8420	0.4571	0.6496
C05-5	8	1.0000	1.0000	0.0000	0.0000
C05-6	8	2.0000	2.0000	0.5000	0.6931
C05-7	8	1.0000	1.0000	0.0000	0.0000
C05-8	8	1.0000	1.0000	0.0000	0.0000
C05-9	8	2.0000	1.3022	0.2321	0.3939
C06-1	9	2.0000	1.6119	0.3796	0.5674
C06-2	9	1.0000	1.0000	0.0000	0.0000
C06-3	9	1.0000	1.0000	0.0000	0.0000
C06-4	9	1.0000	1.0000	0.0000	0.0000
C06-5	9	1.0000	1.0000	0.0000	0.0000
C06-6	9	1.0000	1.0000	0.0000	0.0000
C06-7	9	2.0000	1.6119	0.3796	0.5674
Mean	9	1.3770	1.2881	0.1600	0.2311
St. Dev		0.4887	0.3956	0.2124	0.3037

==========

* na = Observed number of alleles
* ne = Effective number of alleles [Kimura and Crow (1964)]
* h = Nei's (1973) gene diversity
* I = Shannon's Information index [Lewontin (1972)]

The number of polymorphic loci is : 23
The percentage of polymorphic loci is : 37.70 %

****

2. WITHIN FAMILY ANALYSIS

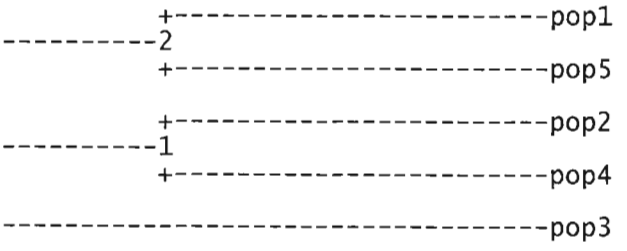
Family 1
Population: 1(1a) 2(1b) 3(1abi) 4(1abii) 5(1abiii)

****
Nei's Original Measures of Genetic Identity and Genetic distance
[See Nei (1972) Am. Nat. 106:283-292)]
****

pop ID	1	2	3	4	5
1	****	0.7541	0.8033	0.8033	0.8852
2	0.2822	****	0.7541	0.8852	0.8689
3	0.2191	0.2822	****	0.7705	0.7213
4	0.2191	0.1219	0.2607	****	0.8197
5	0.1219	0.1406	0.3267	0.1989	****

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
4	3	3.09972
3	2	4.41449
2	pop1	6.09449
2	pop5	6.09449
3	1	4.41449
1	pop2	6.09449
1	pop4	6.09449
4	pop3	13.60871

Family 2
Population: 6(2a) 7(2b) 8(2abi)

```
*******************************************************************************
Nei's Original Measures of Genetic Identity and Genetic distance
[See Nei (1972) Am. Nat. 106:283-292)]
*******************************************************************************

=======================================
pop ID    6         7         8
=======================================
6           ****    0.7377    0.7541
7         0.3042      ****    0.8525
8         0.2822    0.1596      ****
=======================================

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
*******************************************************************************


  +-------------------------------------------------------pop6
--2
  !                          +------------------------------pop7
  +--------------------------1
                             +------------------------------pop8


* File Name: dgram1.plt

Between        And             Length
-------        ---             ------
   2           pop6           14.66110
   2              1            6.67959
   1           pop7            7.98151
   1           pop8            7.98151
```

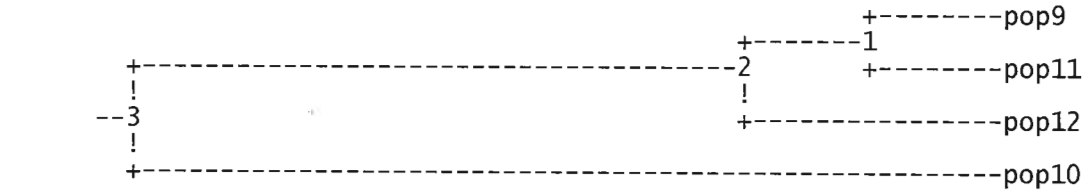
Family 3
Population: 9(3a) 10(3b) 11(3abi) 12(3abii)

****
Nei's Original Measures of Genetic Identity and Genetic distance
[See Nei (1972) Am. Nat. 106:283-292)]
****

pop ID	9	10	11	12
9	****	0.7213	0.9508	0.9180
10	0.3267	****	0.7377	0.7377
11	0.0504	0.3042	****	0.9016
12	0.0855	0.3042	0.1035	****

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
3	2	10.85854
2	1	2.20503
1	pop9	2.52154
1	pop11	2.52154
2	pop12	4.72657
3	pop10	15.58512

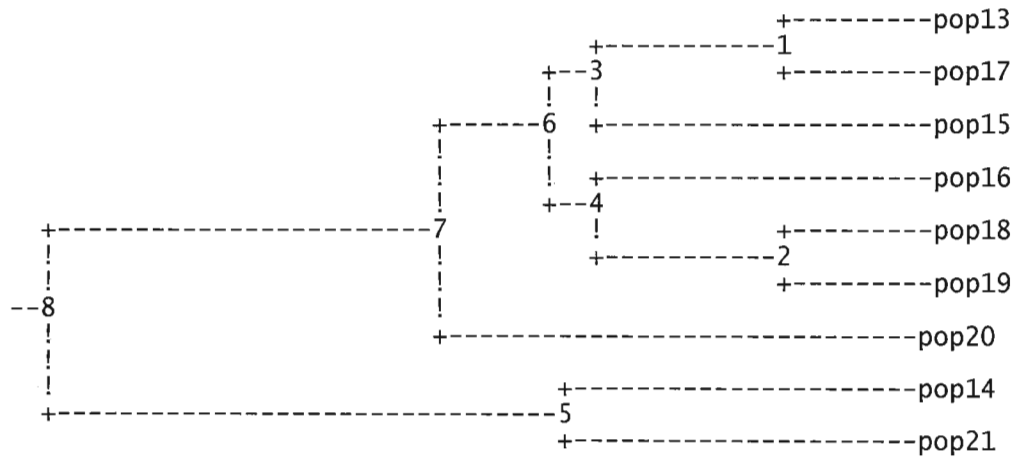
Family 4
Population: 13(4a) 14(4b) 15(4abi) 16(4abii) 17(4abiii) 18(4w) 19(4x)
20(4y) 21(4z)

****
Nei's Original Measures of Genetic Identity and Genetic distance
[See Nei (1972) Am. Nat. 106:283-292)]
****

pop ID	13	14	15	16	17	18	19
20	21						
13	****	0.6885	0.8852	0.8689	0.9508	0.9344	0.8852
0.8361	0.7377						
14	0.3732	****	0.7049	0.8197	0.7377	0.7541	0.7377
0.8197	0.8852						
15	0.1219	0.3497	****	0.8525	0.9016	0.8525	0.9016
0.8197	0.6885						
16	0.1406	0.1989	0.1596	****	0.8852	0.9016	0.8852
0.8361	0.8033						
17	0.0504	0.3042	0.1035	0.1219	****	0.8852	0.8689
0.8525	0.7541						
18	0.0678	0.2822	0.1596	0.1035	0.1219	****	0.9508
0.8689	0.7705						
19	0.1219	0.3042	0.1035	0.1219	0.1406	0.0504	****
0.8852	0.7213						
20	0.1790	0.1989	0.1989	0.1790	0.1596	0.1406	0.1219
****	0.7377						
21	0.3042	0.1219	0.3732	0.2191	0.2822	0.2607	0.3267
0.3042	****						

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
8	7	6.41825
7	6	1.83954
6	3	0.68344
3	1	3.11422
1	pop13	2.52154
1	pop17	2.52154
3	pop15	5.63576
6	4	0.68344
4	pop16	5.63576
4	2	3.11422
2	pop18	2.52154
2	pop19	2.52154
7	pop20	8.15874
8	5	8.48250
5	pop14	6.09449
5	pop21	6.09449

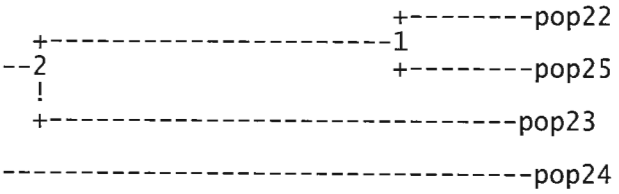
Siblings 5
Population: 22(5i) 23(5ii) 24(5iii) 25(5iv)

****
Nei's Original Measures of Genetic Identity and Genetic distance
[See Nei (1972) Am. Nat. 106:283-292)]
****

pop ID	22	23	24	25
22	****	0.8689	0.7869	0.9672
23	0.1406	****	0.8525	0.9016
24	0.2397	0.1596	****	0.7869
25	0.0333	0.1035	0.2397	****

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
3	2	4.54653
2	1	4.43624
1	pop22	1.66682
1	pop25	1.66682
2	pop23	6.10307
3	pop24	10.64960

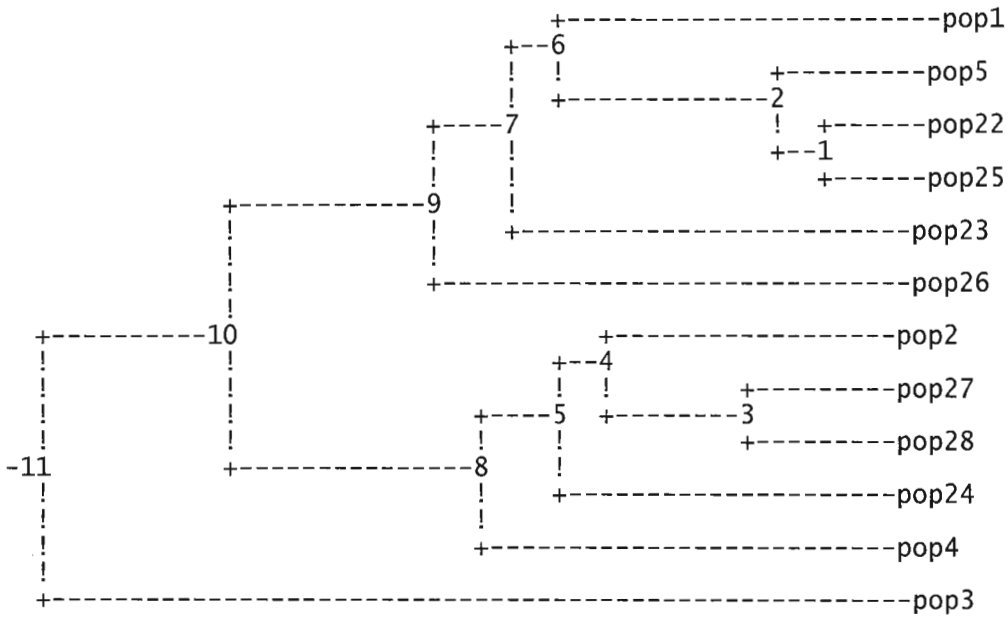
Greater Family (Family 1 and Siblings 5)
Population: 1(1a) 2(1b) 3(1abi) 4(1abii) 5(1abiii) 22(5i) 23(5ii)
24(5iii) 25(5iv) 26(6) 27(7) 28(8)

****
Nei's Original Measures of Genetic Identity and Genetic distance
[See Nei (1972) Am. Nat. 106:283-292)]
****

pop ID	1	2	3	4	5	22	23
24	25	26	27	28			
1	****	0.7541	0.8033	0.8033	0.8852	0.9016	0.8689
0.7869	0.8689	0.8361	0.8033	0.7869			
2	0.2822	****	0.7541	0.8852	0.8689	0.8525	0.8197
0.9016	0.8852	0.7869	0.8852	0.9344			
3	0.2191	0.2822	****	0.7705	0.7213	0.7377	0.7049
0.7869	0.7049	0.7377	0.8361	0.7869			
4	0.2191	0.1219	0.2607	****	0.8197	0.7705	0.8033
0.8852	0.7705	0.7049	0.8361	0.8852			
5	0.1219	0.1406	0.3267	0.1989	****	0.9508	0.8852
0.8361	0.9508	0.8525	0.8525	0.9016			
22	0.1035	0.1596	0.3042	0.2607	0.0504	****	0.8689
0.7869	0.9672	0.8689	0.8361	0.8525			
23	0.1406	0.1989	0.3497	0.2191	0.1219	0.1406	****
0.8525	0.9016	0.8689	0.7705	0.7869			
24	0.2397	0.1035	0.2397	0.1219	0.1790	0.2397	0.1596
****	0.7869	0.7541	0.8852	0.9016			
25	0.1406	0.1219	0.3497	0.2607	0.0504	0.0333	0.1035
0.2397	****	0.8689	0.8033	0.8525			
26	0.1790	0.2397	0.3042	0.3497	0.1596	0.1406	0.1406
0.2822	0.1406	****	0.7705	0.7541			
27	0.2191	0.1219	0.1790	0.1790	0.1596	0.1790	0.2607
0.1219	0.2191	0.2607	****	0.9508			
28	0.2397	0.0678	0.2397	0.1219	0.1035	0.1596	0.2397
0.1035	0.1596	0.2822	0.0504	****			

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
*****************************************************************************
```

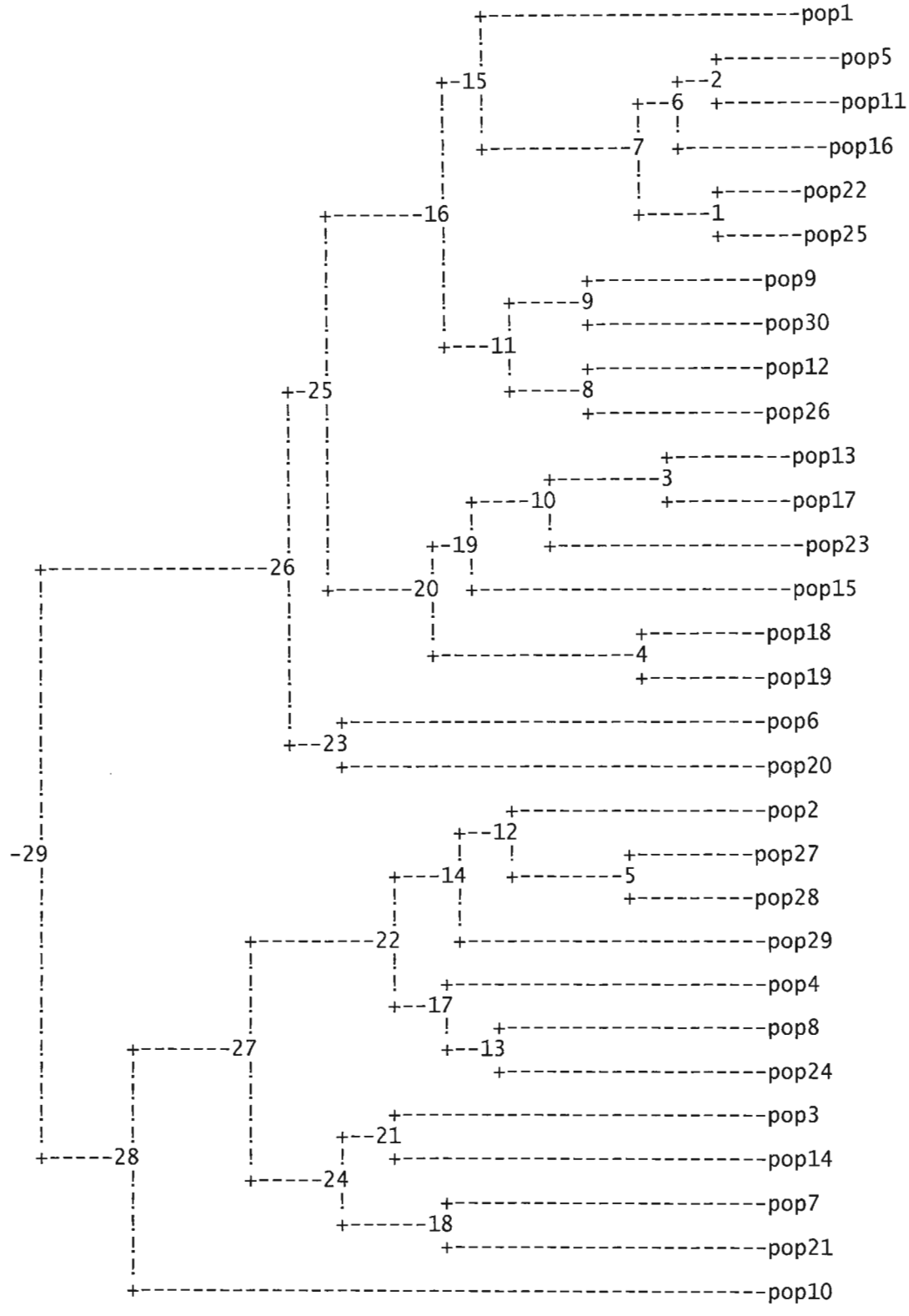


* File Name: dgram1.plt

Between	And	Length
11	10	3.09658
10	9	3.18491
9	7	1.27181
7	6	0.23222
6	pop1	6.10021
6	2	3.57866
2	pop5	2.52154
2	1	0.85472
1	pop22	1.66682
1	pop25	1.66682
7	pop23	6.33243
9	pop26	7.60424
10	8	3.98018
8	5	1.32612
5	4	0.74004
4	pop2	4.74281
4	3	2.22127
3	pop27	2.52154
3	pop28	2.52154
5	pop24	5.48285
8	pop4	6.80897
11	pop3	13.88573

3. INDIVIDUAL ANALYSIS

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
29	26	4.60648
26	25	0.57776
25	16	2.26046
16	15	0.12133
15	pop1	5.91443
15	7	2.81060
7	6	0.14750
6	2	0.43479
2	pop5	2.52154
2	pop11	2.52154
6	pop16	2.95634
7	1	1.43701
1	pop22	1.66682
1	pop25	1.66682
16	11	1.30506
11	9	1.33957
9	pop9	3.39113
9	pop30	3.39113
11	8	1.33957
8	pop12	3.39113
8	pop26	3.39113
25	20	1.94440
20	19	0.25162
19	10	1.37364
10	3	2.20503
3	pop13	2.52154
3	pop17	2.52154
10	pop23	4.72657
19	pop15	6.10021
20	4	3.83029
4	pop18	2.52154
4	pop19	2.52154
26	23	0.89248
23	pop6	7.98151
23	pop20	7.98151
29	28	1.65661
28	27	2.13960
27	22	2.62673
22	14	1.26886
14	12	1.04586
12	pop2	4.74281
12	5	2.22127
5	pop27	2.52154
5	pop28	2.52154
14	pop29	5.78867
22	17	0.96304
17	pop4	6.09449
17	13	0.91746
13	pop8	5.17703
13	pop24	5.17703
27	24	1.67963
24	21	0.97554
21	pop3	7.02910
21	pop14	7.02910
24	18	1.91014
18	pop7	6.09449
18	pop21	6.09449
28	pop10	11.82386

Nei's Original Measures of Genetic Identity and Genetic distance
[See Nei (1972) Am. Nat. 106:283-292)]

pop ID	1	2	3	4	5	6	7	8	9	10	11
12	13	14	15	16	17	18	19	20	21	22	23
24	25	26	27	28	29	30					
1	****	0.7541	0.8033	0.8033	0.8852	0.7705	0.7377	0.7869	0.8852	0.7377	0.9016
0.9016	0.8525	0.7705	0.8033	0.8852	0.8361	0.8525	0.8361	0.8525	0.7541	0.9016	0.8689
0.7869	0.8689	0.8361	0.8033	0.7869	0.7377	0.8852					
2	0.2822	****	0.7541	0.8852	0.8689	0.7213	0.8197	0.8361	0.8033	0.7541	0.8525
0.7541	0.7705	0.8197	0.7213	0.8361	0.7541	0.8033	0.7541	0.7049	0.8689	0.8525	0.8197
0.9016	0.8852	0.7869	0.8852	0.9344	0.8852	0.7705					
3	0.2191	0.2822	****	0.7705	0.7213	0.7049	0.8689	0.8197	0.7541	0.8361	0.7377
0.7705	0.7213	0.8689	0.7377	0.7213	0.7377	0.7213	0.7049	0.8197	0.8197	0.7377	0.7049
0.7869	0.7049	0.7377	0.8361	0.7869	0.7377	0.7869					
4	0.2191	0.1219	0.2607	****	0.8197	0.6721	0.8033	0.8852	0.7541	0.8033	0.8033
0.7377	0.7541	0.7705	0.6721	0.7541	0.7377	0.7213	0.6721	0.6885	0.8197	0.7705	0.8033
0.8852	0.7705	0.7049	0.8361	0.8852	0.8361	0.7541					
5	0.1219	0.1406	0.3267	0.1989	****	0.8197	0.7541	0.8033	0.9016	0.7213	0.9508
0.8852	0.8361	0.8197	0.7869	0.9344	0.8197	0.8689	0.8197	0.8033	0.8361	0.9508	0.8852
0.8361	0.9508	0.8525	0.8525	0.9016	0.7869	0.8689					
6	0.2607	0.3267	0.3497	0.3973	0.1989	****	0.7377	0.7541	0.8852	0.7049	0.8689
0.8689	0.7869	0.7705	0.8033	0.8525	0.8033	0.8197	0.8361	0.8525	0.7869	0.8033	0.8033
0.7541	0.8033	0.9016	0.7377	0.7541	0.7705	0.8525					
7	0.3042	0.1989	0.1406	0.2191	0.2822	0.3042	****	0.8525	0.7213	0.8033	0.7377
0.8033	0.7213	0.8361	0.6393	0.7213	0.7377	0.6885	0.6393	0.7541	0.8852	0.7377	0.7049
0.8197	0.7377	0.7705	0.8361	0.8197	0.8033	0.7541					
8	0.2397	0.1790	0.1989	0.1219	0.2191	0.2822	0.1596	****	0.8033	0.8197	0.7869
0.7869	0.7705	0.8525	0.6885	0.8033	0.7869	0.7377	0.7213	0.7705	0.8689	0.7541	0.7541
0.9016	0.7541	0.7213	0.8852	0.9016	0.8197	0.7377					
9	0.1219	0.2191	0.2822	0.2822	0.1035	0.1219	0.3267	0.2191	****	0.7213	0.9508
0.9180	0.8689	0.8197	0.7869	0.9016	0.8525	0.9344	0.8852	0.9016	0.8033	0.9180	0.8852
0.8033	0.8852	0.9180	0.8525	0.8361	0.7869	0.9344					
10	0.3042	0.2822	0.1790	0.2191	0.3267	0.3497	0.2191	0.1989	0.3267	****	0.7377
0.7377	0.7213	0.7377	0.7049	0.7213	0.7377	0.6885	0.6721	0.6885	0.7869	0.7049	0.7377
0.7869	0.7049	0.6721	0.8033	0.7869	0.7705	0.6885					
11	0.1035	0.1596	0.3042	0.2191	0.0504	0.1406	0.3042	0.2397	0.0504	0.3042	****
0.9016	0.8852	0.8033	0.8361	0.9508	0.8689	0.9180	0.8689	0.8525	0.8197	0.9344	0.9344
0.8525	0.9344	0.9016	0.8361	0.8525	0.8033	0.9180					

12	0.1035	0.2822	0.2607	0.3042	0.1219	0.1406	0.2191	0.2397	0.0855	0.3042	0.1035
****	0.8197	0.8033	0.7705	0.8852	0.8361	0.8525	0.8033	0.8525	0.8197	0.9016	0.8689
0.7869	0.8689	0.9344	0.8033	0.7869	0.7377	0.8852					
13	0.1596	0.2607	0.3267	0.2822	0.1790	0.2397	0.3267	0.2607	0.1406	0.3267	0.1219
0.1989	****	0.6885	0.8852	0.8689	0.9508	0.9344	0.8852	0.8361	0.7377	0.8197	0.9180
0.8033	0.8525	0.8197	0.7869	0.7705	0.7869	0.8033					
14	0.2607	0.1989	0.1406	0.2607	0.1989	0.2607	0.1790	0.1596	0.1989	0.3042	0.2191
0.2191	0.3732	****	0.7049	0.8197	0.7377	0.7541	0.7377	0.8197	0.8852	0.8033	0.7377
0.8525	0.7705	0.8033	0.8689	0.8525	0.7705	0.8525					
15	0.2191	0.3267	0.3042	0.3973	0.2397	0.2191	0.4473	0.3732	0.2397	0.3497	0.1790
0.2607	0.1219	0.3497	****	0.8525	0.9016	0.8525	0.9016	0.8197	0.6885	0.7705	0.8689
0.7869	0.8033	0.7705	0.7049	0.6885	0.7377	0.7541					
16	0.1219	0.1790	0.3267	0.2822	0.0678	0.1596	0.3267	0.2191	0.1035	0.3267	0.0504
0.1219	0.1406	0.1989	0.1596	****	0.8852	0.9016	0.8852	0.8361	0.8033	0.9180	0.9180
0.8361	0.9508	0.8852	0.8197	0.8361	0.7869	0.8689					
17	0.1790	0.2822	0.3042	0.3042	0.1989	0.2191	0.3042	0.2397	0.1596	0.3042	0.1406
0.1790	0.0504	0.3042	0.1035	0.1219	****	0.8852	0.8689	0.8525	0.7541	0.8033	0.9016
0.8197	0.8361	0.8361	0.7377	0.7213	0.7377	0.8197					
18	0.1596	0.2191	0.3267	0.3267	0.1406	0.1989	0.3732	0.3042	0.0678	0.3732	0.0855
0.1596	0.0678	0.2822	0.1596	0.1035	0.1219	****	0.9508	0.8689	0.7705	0.8852	0.8852
0.7705	0.8852	0.8852	0.8525	0.8033	0.8197	0.8689					
19	0.1790	0.2822	0.3497	0.3973	0.1989	0.1790	0.4473	0.3267	0.1219	0.3973	0.1406
0.2191	0.1219	0.3042	0.1035	0.1219	0.1406	0.0504	****	0.8852	0.7213	0.8361	0.8361
0.7541	0.8361	0.8361	0.8033	0.7541	0.8033	0.8197					
20	0.1596	0.3497	0.1989	0.3732	0.2191	0.1596	0.2822	0.2607	0.1035	0.3732	0.1596
0.1596	0.1790	0.1989	0.1989	0.1790	0.1596	0.1406	0.1219	****	0.7377	0.8197	0.8197
0.7705	0.7869	0.8525	0.7869	0.7377	0.7213	0.9016					
21	0.2822	0.1406	0.1989	0.1989	0.1790	0.2397	0.1219	0.1406	0.2191	0.2397	0.1989
0.1989	0.3042	0.1219	0.3732	0.2191	0.2822	0.2607	0.3267	0.3042	****	0.7869	0.7541
0.8689	0.7869	0.8197	0.8852	0.9016	0.8525	0.8033					
22	0.1035	0.1596	0.3042	0.2607	0.0504	0.2191	0.3042	0.2822	0.0855	0.3497	0.0678
0.1035	0.1989	0.2191	0.2607	0.0855	0.2191	0.1219	0.1790	0.1989	0.2397	****	0.8689
0.7869	0.9672	0.8689	0.8361	0.8525	0.7377	0.8852					
23	0.1406	0.1989	0.3497	0.2191	0.1219	0.2191	0.3497	0.2822	0.1219	0.3042	0.0678
0.1406	0.0855	0.3042	0.1406	0.0855	0.1035	0.1219	0.1790	0.1989	0.2822	0.1406	****
0.8525	0.9016	0.8689	0.7705	0.7869	0.7705	0.8525					
24	0.2397	0.1035	0.2397	0.1219	0.1790	0.2822	0.1989	0.1035	0.2191	0.2397	0.1596
0.2397	0.2191	0.1596	0.2397	0.1790	0.1989	0.2607	0.2822	0.2607	0.1406	0.2397	0.1596
****	0.7869	0.7541	0.8852	0.9016	0.8525	0.7705					

25	0.1406	0.1219	0.3497	0.2607	0.0504	0.2191	0.3042	0.2822	0.1219	0.3497	0.0678
0.1406	0.1596	0.2607	0.2191	0.0504	0.1790	0.1219	0.1790	0.2397	0.2397	0.0333	0.1035
0.2397	****	0.8689	0.8033	0.8525	0.7705	0.8525					
26	0.1790	0.2397	0.3042	0.3497	0.1596	0.1035	0.2607	0.3267	0.0855	0.3973	0.1035
0.0678	0.1989	0.2191	0.2607	0.1219	0.1790	0.1219	0.1790	0.1596	0.1989	0.1406	0.1406
0.2822	0.1406	****	0.7705	0.7541	0.7705	0.9180					
27	0.2191	0.1219	0.1790	0.1790	0.1596	0.3042	0.1790	0.1219	0.1596	0.2191	0.1790
0.2191	0.2397	0.1406	0.3497	0.1989	0.3042	0.1596	0.2191	0.2397	0.1219	0.1790	0.2607
0.1219	0.2191	0.2607	****	0.9508	0.9016	0.7869					
28	0.2397	0.0678	0.2397	0.1219	0.1035	0.2822	0.1989	0.1035	0.1790	0.2397	0.1596
0.2397	0.2607	0.1596	0.3732	0.1790	0.3267	0.2191	0.2822	0.3042	0.1035	0.1596	0.2397
0.1035	0.1596	0.2822	0.0504	****	0.8852	0.7705					
29	0.3042	0.1219	0.3042	0.1790	0.2397	0.2607	0.2191	0.1989	0.2397	0.2607	0.2191
0.3042	0.2397	0.2607	0.3042	0.2397	0.3042	0.1989	0.2191	0.3267	0.1596	0.3042	0.2607
0.1596	0.2607	0.2607	0.1035	0.1219	****	0.7541					
30	0.1219	0.2607	0.2397	0.2822	0.1406	0.1596	0.2822	0.3042	0.0678	0.3732	0.0855
0.1219	0.2191	0.1596	0.2822	0.1406	0.1989	0.1406	0.1989	0.1035	0.2191	0.1219	0.1596
0.2607	0.1596	0.0855	0.2397	0.2607	0.2822	****					

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

2. FOR WITHIN FAMILY AND BETWEEN INDIVIDUAL ANALYSIS

```
/* Diploid RAPD Data Set */
Number of populations = 30
Number of loci = 61
Locus name :
17-1 17-2 17-3 17-4 17-5 17-6 17-7 17-8 17-9
25-1 25-2 25-3 25-4 25-5
A04-1 A04-2 A04-3 A04-4 A04-5 A04-6 A04-7 A04-8 A04-9 A04-10
A06-1 A06-2 A06-3 A06-4 A06-5 A06-6 A06-7 A06-8
A17-1 A17-2 A17-3 A17-4 A17-5 A17-6 A17-7 A17-8
A20-1 A20-2 A20-3 A20-4 A20-5
C05-1 C05-2 C05-3 C05-4 C05-5 C05-6 C05-7 C05-8 C05-9
C06-1 C06-2 C06-3 C06-4 C06-5 C06-6 C06-7

name = 1a
fis = 0
101111001 11100 1101111111 11011111 10111110 11011 111100110 0111110

name = 1b
fis = 0
111011011 11111 1111111111 11101111 10111111 11111 101111110 1111111

name = 1ab(i)
fis = 0
101110001 11111 1001001111 11011111 10111111 11011 010101110 1111111

name = 1ab(ii)
fis = 0
101101001 11111 1111110111 11111111 10111111 11111 101110110 1111111

name = 1ab(iii)
fis = 0
111111011 11100 1111111111 11111111 10111110 11111 111111110 0111110

name = 2a
fis = 0
111111011 11100 1101001100 11111111 11111110 11011 100011111 0111110

name = 2b
fis = 0
101110001 11111 1011001100 11101111 10111111 11011 111111110 1111111

name = 2ab(i)
fis = 0
101101001 11111 1111011111 11111111 11111111 11011 111111111 1111111

name = 3a
fis = 0
111111011 11100 1101011111 11011111 10111110 11011 101111111 0111110

name = 3b
fis = 0
101110001 11111 1101010111 11111111 00011101 11011 110011110 1111111

name = 3ab(i)
fis = 0
111111011 11100 1101111111 11111111 10111110 11011 101111110 0111110

name = 3ab(ii)
fis = 0
111111001 11100 1101011100 11011111 10111110 11011 111111110 0111110

name = 4a
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```
fis = 0
101010100 11100 1101111111 11111111 10111110 11011 101111111 0111110

name = 4b
fis = 0
111111011 11111 1011001111 11011111 11111110 11011 011111110 1111111

name = 4ab(i)
fis = 0
111010100 11100 1101111111 11111111 11111110 11011 000001110 0111110

name = 4ab(ii)
fis = 0
111011011 11100 1101111111 11111111 11111110 11011 111111110 0111110

name = 4ab(iii)
fis = 0
101010100 11100 1101011111 11111111 11111110 11011 101111110 0111110

name = 4w
fis = 0
111010111 11100 1101111111 11011111 10111110 11011 101111111 0111110

name = 4x
fis = 0
111010111 11100 1101111111 11011111 11111110 11011 101001111 0111110

name = 4y
fis = 0
111110001 11100 1001001111 11011111 11111110 11011 101101111 0111110

name = 4z
fis = 0
111111111 11111 1111001101 11111111 10111111 11011 111111110 1111111

name = 5(i)
fis = 0
111111011 11100 1101111111 11001111 10111110 11111 111111110 0111110

name = 5(ii)
fis = 0
111011000 11100 1101110111 11111111 10111110 11011 101111110 0111110

name = 5(iii)
fis = 0
111111000 11111 1111111111 11111111 ........ 11011 101111110 1111111

name = 5(iv)
fis = 0
111011011 11100 1101111111 11101111 10111110 11111 111111110 0111110

name = 6
fis = 0
111011011 11100 1101001100 11011111 10111110 11011 101111110 0111110

name = 7
fis = 0
111110011 11111 1111111111 11011111 10111111 11011 111111111 1111111

name = 8
fis = 0
111111011 11111 1111111111 11111111 10111111 11111 ......... 1111111

name = 9
```

```
fis = 0
111010011 11111 1111111101 11111111 10111111 11011 101010111 1111111

name = 10
fis = 0
111111011 11100 1001001111 11011111 10111110 11011 101110110 0111110
```