STUDIES OF THE POPULATION STRUCTURE AND GENETIC DIVERSITY OF DOMESTICATED AND "WILD" OSTRICHES (*Struthio camelus*)

A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

of

RHODES UNIVERSITY

by

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DECEMBER 1999

ABSTRACT

DNA sequencing and restriction fragment length polymorphism analysis (RFLP) of polymerase chain reaction (PCR) amplified mitochondrial DNA fragments, and random amplified polymorphic DNA sequence (RAPD) analysis were techniques evaluated in this study for applicability in the investigation various aspects of genetic diversity within the ostrich (*Struthio camelus*). The genetic aspects that were investigated were (i) relationships between ostrich subspecies, (ii) genetic variability between and within domesticated populations of southern African ostriches (*Struthio camelus australis*), (iii) linking egg production in domesticated ostriches to RAPD profiles, and (iv) determining the zygosity of twin ostriches.

In the first part of this study DNA sequencing and the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) methods were evaluated for resolving genetic differences in the small mtDNA fragments of the ostrich. DNA sequencing of PCR amplified 450 bp 12S rRNA gene fragments of representatives from the southern African population ostrich (*S.c. australis*) did not reveal any differences between the populations from different geographical areas, representing ostrich lineages with different breeding histories. The PCR-RFLP analysis of mtDNA fragments (450 bp 12S rRNA gene fragment and 550 bp D-loop region) also did not reveal any genetic variability between the domesticated *S.c. australis* populations included in this study.

PCR-RFLP analysis of a 450 bp 12S rRNA gene fragment, however, showed differences between the subspecies *S.c. australis* and *S.c. molybdophanes*. The proportion of shared fragments (F) between these two subspecies was 0.286 and nucleotide sequence divergence estimated at 8.9 %. Divergence time between these two subspecies was estimated at 4.5 million

years ago. The data presented from this study are comparable to the data from a previous study in which the entire mitochondrial genome and a larger number of restriction enzymes were used.

The PCR-RFLP method thus demonstrated its usefulness for genetic studies of ostriches at the subspecies level. The sequences used in this study could not reveal any markers that were useful for genetic studies of ostriches at the population level.

In the second part of the study the RAPD method was evaluated for application in the genetic studies of ostriches. RAPD profiles, based on three RAPD primers, revealed differences between three subspecies of ostriches and indicated relationships between these subspecies that are consistent with observations from other studies. The numerical analysis of pooled and individual primer data demonstrated that the subspecies *S.c. australis* is more closely related to *S.c. massaicus* than to *S.c. molybdophanes*. RAPD marker differences between *S.c. molybdophanes* on the one hand, and *S.c. massaicus* and *S.c. australis* on the other is also consistent with observations from studies that proposed separate species status for *S.c. molybdophanes*.

RAPD analysis by five primers revealed geographic variation between *S.c. australis* populations. The clustering patterns observed in the dendrograms and Neighbour Joining Trees generated by computer programs showed trends of separating ostrich populations into geographical groups, possibly reflecting their different breeding histories. In the RAPD profiles of the inbred population, band-sharing was generally greater than in the outbreeding group. RAPD analysis thus showed that it may be a useful method in the population studies of domesticated *S.c. australis*.

RAPDs also generated data that grouped ostriches according to trends in egg production capabilities. Analysis of RAPD profiles by computer software showed a Neighbour Joining Tree and a dendrogram that predominantly grouped ostriches into clusters associated with either good or poor egg production. Evidence supporting the suitability of RAPDs as a tool in breeding programmes of ostriches was thus provided by this study.

RAPDs also provided data, demonstrating that two sets of ostrich twins were non-identical twins.

It was demonstrated by this study that RAPDs analysis may be a useful technique for applying to (1) systematic (2) population (3) breeding and (4) twin studies of ostriches (*Struthio camelus*).

Table of Contents

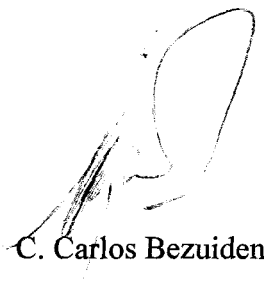
Chapter 4

List of Figures

List of Tables

DECLARATION

This dissertation reports the results of original research which I carried out in the Faculty of Science, Rhodes University, Grahamstown. None of it has been submitted for a degree at any other university.



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ACKNOWLEDGEMENTS

I am most grateful to Dr Nigel Barker of the Department of Botany, Rhodes University, for supervising corrections to this thesis. I would like to thank him for the enthusiasm, encouragement and patience displayed at all times.

I am thankful for the supervision of Professor Ralph Kirby, for arranging all the samples and for his advice.

To Mr Joseph Slingers, thank you very much for proof reading drafts of this thesis and for your invaluable comments.

Thanks to the staff and fellow post graduate students of the Department of Biochemistry and Microbiology, for encouragement and advice.

I would like to thank all my colleagues for technical assistance and encouragement. Thanks go to Clifford D. Nxomani and Michael J. Naidoo, for all the advice, constructive discussions and sharing of ideas and skills. Thanks to Dr. Patsy Goetsch and Prof. Don Henry for the valuable constructive comments and for proof reading drafts of this thesis.

Special thanks to the staff of the Electron Microscope Unit, Rhodes University, especially Neil Cannon for taking and reproducing the photographs of the gels.

Thanks to Dr. Siva Pillay for providing the facility to print various drafts of this thesis.

The effort of Dr Ernesto Almira of the Interdisciplinary Centre for Biotechnology Research at

the University of Florida, in sequencing the mtDNA fragments at very short notice, was appreciated.

To my family, Sharon, Angeron, Krisdan and Joshua-Paul, I sincerely appreciate your love, care, support and encouragement. Thank you for all the sacrifices that you made without complaining. Without you this would not be possible. I would like to express my sincere thanks to my parents, in-laws, brothers and sisters for their encouragement.

Thank you to all my friends and the staff and Principal of Uitenhage High School. I sincerely appreciate your support.

I would like to thank the following individuals and institutions for providing the samples that were used in this study: Prof. T. Robinson and Ms S. Freitag (University of Pretoria), Dr. C. Brown, (Rhodes University), University of Cape Town Medical School, Kalani Investments Company and Domesticated Ostrich Products (PTY) LTD.

The Foundation of Research and Development (FRD) partly funded this research. The financial support of the Deutscher Akademischer Austauschdienst (Daad) is also acknowledged.

Chapter 1

General Introduction

1.1 THE OSTRICH AND THE OSTRICH INDUSTRY IN SOUTHERN AFRICA

1.1.1 INTRODUCTION

The African continent and the adjoining parts of Arabia, Palestine and Asia Minor is the natural home of ostriches (Suborder Struthiones, Family STRUTHIONIDAE, species *Struthio camelus*; (Duerden, 1919; Grzimek, 1970; Brown *et al.*, 1982; Mindell *et al.*, 1997: Figure 1.1). They are the only living representatives of an avian family with unique characteristics that distinguish them from all other living birds. These characteristics are feet that consist of two toes, the absence of a keel, the presence of a penis (in the males), necks that are largely naked or covered with downy feathers and thighs which are almost naked. In males the tarsus is large and covered with brightly coloured scutes or shields. Ostriches are the largest of all living birds. The mature males are black and white and the females and immature males dull-grey (Grzimek, 1970; Brown *et al.*, 1982).

1.1.2 TAXONOMY OF OSTRICHES *Struthio camelus*

Ostriches belong to the monophyletic ratite group (Cooper *et al.*, 1992; Lee *et al.*, 1997). Ratites can be further divided into 8 subgroups: tinamous (Tinamidae), kiwi (Apterygidae), moas (Dinornithidae), elephant-birds (Aepyornithidae), cassowaries (Casuariidae), emus (Dromiceidae), rheas (Rheidae) and ostriches (Struthionidae). The evolution and relationships of the ratites has been the subject of several recent studies (Cooper *et al.*, 1992; Hedges *et al.*, 1995; Härlid *et al.*, 1997, 1998; Lee *et al.*, 1997; Mindell *et al.*, 1997; van Tuinen *et al.*, 1998; Härlid and Arnason, 1999) as has the relationship of birds to reptiles and mammals (Hedges and Sibley, 1994; Seutin *et al.*, 1994; Härlid *et al.*, 1997, 1998; Van Tuinen *et al.*, 1998).

Freitag and Robinson (1993) and Baker *et al.* (1995) examined the taxonomy and genetic structure of populations of different ratites. Molecular evidence, based on mtDNA sequences,

shows that ratites diverged from other extant birds about 90 million years ago (m.y.a), and that the rhea-ostrich divergence was 51 m.y.a. (Härlid *et al.*, 1997, 1998; Mindell *et al.*, 1997; van Tuinen *et al.*, 1998). Divergence times of between 200 000 and 900 000 years were proposed for kiwi subspecies (Baker *et al.*, 1995), and a divergence time of between 4.1 million years and 30 000 years was proposed for ostrich subspecies (Freitag and Robinson, 1993).

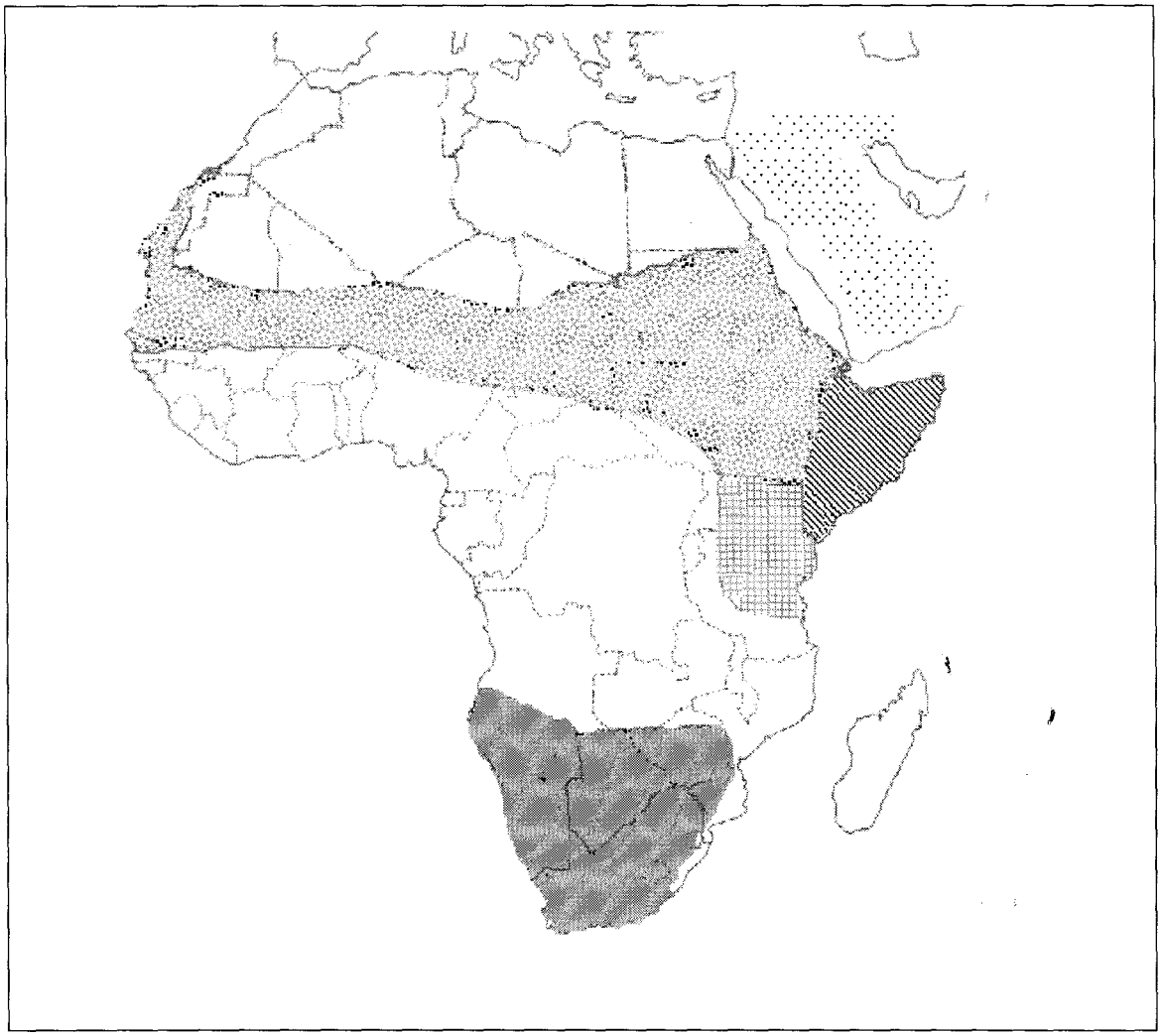


Figure 1.1: A distribution map of ostriches (*Struthio camelus*) across Africa and Arabia (From: Grzimek, 1970; Brown *et al.*, 1982; Freitag and Robinson, 1993). The following key indicates the distribution of the different subspecies:

S.c. australis, *S.c. camelus*

S.c. massaicus, *S.c. molybdophanes*,

S.c. syriacus.

The ostrich, *Struthio camelus*, is divided into six subspecies based on morphological characters of the adult birds. The morphological characteristics are based on factors such as size and colour differences, presence of a bald patch on the crown, size of the egg and nature of the egg pores (Duerden, 1919; Brown *et al.*, 1982). Molecular studies have elucidated the relationships of the extant subspecies (Table 1.1; Figure 1.2: Freitag and Robinson, 1993; Kimwele *et al.*, 1998; Kumari and Kemp, 1998). A brief description of each subspecies appears below:

Struthio camelus australis Gurney (*S. c. australis*): These birds are native to Africa south of the Zambezi River (Zimbabwe to the Cape Province: Figure 1.1) and once inhabited the whole of the southern sub-continent of Africa (Grzimek, 1970). The southern African *S.c. australis* is smaller, more slender and has shorter legs than the North African ostrich (*S.c. camelus*). A claw is rarely present on the small toe and scales on the tarsus and third toe are usually continuous. The skin of the neck and body is dark grey in mature hens and dark blue in mature cocks. In sexually mature male ostriches the beak, front part of the head, the naked skin around the eyes and the tarsal scales are bright scarlet. The crown of the head is without a bald patch. The eggs are deeply pitted and are more oval than the eggs of North African ostriches.

Struthio camelus camelus Linnaeus (*S. c. camelus*): These birds are native to North and West Africa, with their range stretching to Sudan and Uganda (Figure 1.1: Duerden, 1919; Grzimek, 1970; Brown *et al.*, 1982). A claw is present on the small toe and there are scales over the large toe. The body colour of immature birds and that of chicks is creamy yellow. Mature cocks become bright red or scarlet on the legs, neck and head during the breeding season. The crown of their heads has a single or partly divided bald patch. A collar of white feathers is found between the lower and naked upper neck. The females are dull-brown. In the breeding season, the neck, bill and scutes of females become bright red. Their eggs are smooth and free from pittings

and pores and also larger than those of the southern African ostrich (Brown *et al.*, 1982).

Struthio camelus molybdophanes Reichenow (*S. c. molybdophanes*): The range of this very distinct ostrich race is Kenya, Somalia and Ethiopia (Figure 1.1). The crown is bald, like *S.c. camelus*, but the neck and thighs are blue-grey. The body plumage of the male individuals is strikingly black. The tail feathers are white and they have broad white neck rings. These birds are larger than the other subspecies (Duerden, 1919; Brown *et al.*, 1982).

Struthio camelus massaicus Neumann (*S. c. massaicus*): The natural range of the massai ostrich is East Kenya to Tanzania (Figure 1.1). The crown of these birds may be partially bald. Males have a narrow white neck ring. Both wing and tail feathers are white. The neck and thighs of both sexes are pinkish grey and flush bright red in the breeding season. These birds are similar in size to the other subspecies (Brown *et al.*, 1982).

The Arabian ostrich, *S. c. syriacus*, became extinct in 1968, while *S.c. spatzi* from North West Africa became completely hybridized with *S.c. camelus* (Brown *et al.*, 1982). The subspecies *S.c. camelus*, *S.c. massaicus, S.c. molybdophanes* and *S.c. australis* are only found in the 'pure' form in populations that have been protected from hybridization for extended periods (Brown *et al.*, 1982). Although the northern subspecies (*S.c. camelus, S.c. massaicus, S.c. molybdophanes*) have overlapping ranges, the *S.c. molybdophanes* has clear morphological, behavioural and molecular differences from both *S.c. camelus* and *S.c. massaicus* (Brown *et al.*, 1982; Freitag and Robinson, 1993; Kumari and Kemp, 1998). Figure 1.2 shows phylogenetic relationships between the different extant ostrich subspecies. These relationships were revealed by mtDNA analysis (Freitag and Robinson, 1993). The divergence times, as estimated by mtDNA analysis, are shown in Table 1.1 (Freitag and Robinson, 1993). From Figure 1.2 and Table 1.1 it is evident that that *S.c.*

molybdophanes is the most divergent of the subspecies. The estimated divergence time of *S.c. molybdophanes* from the other subspecies is more than 3.6 m.y. bp. *S.c. massaicus* and *S.c. australis* are more closely related to each other than to any of the other two subspecies and divergence between the latter two subspecies was estimated at 30 000 years.

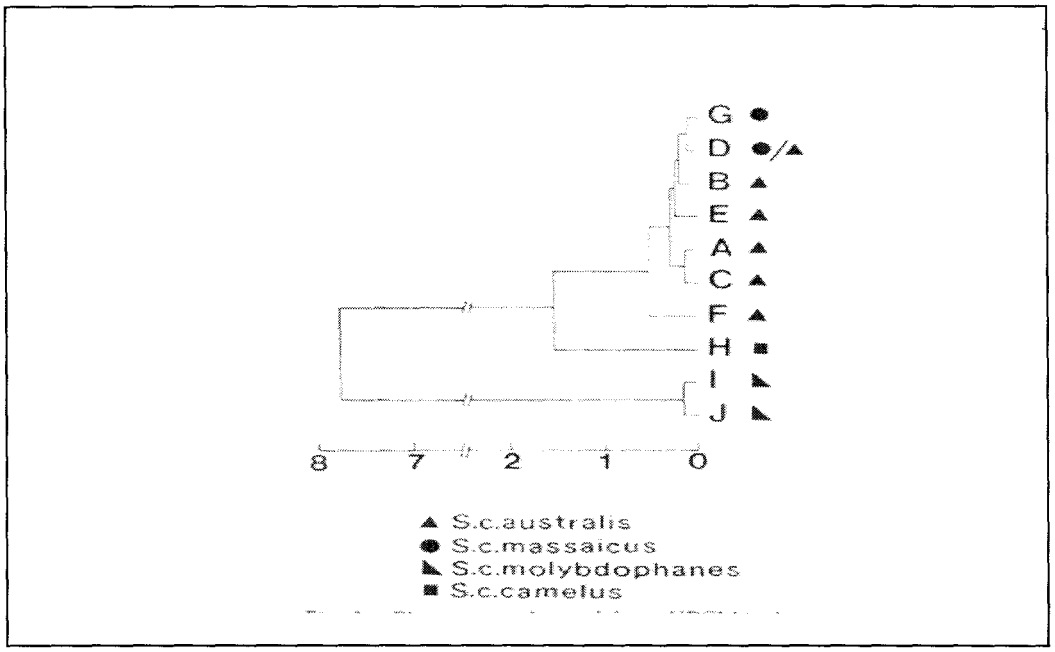


Figure 1.2: A tree representing phylogenetic relationship between the different ostrich subspecies. The relationships were derived from RFLP analysis of mtDNA (Freitag and Robinson, 1993).

Table 1.1: A summary of the percentage sequence divergence and time of divergence between the ostrich subspecies. The divergence times and percentage sequence were derived from RFLP analysis of mtDNA (Freitag, 1992).

Subspecies	**Divergence Times** (m.y. = million year)	**Percentage Sequence Divergence**
S.c. camelus vs *S.c. molybdophanes*	4.1 m.y. bp	8.23
S.c. massaicus vs *S.c. molybdophanes*	3.6 m.y. bp	7.25
S.c. australis vs *S.c. molybdophanes*	3.6 m.y. bp	7.16
S.c. camelus vs *S.c. massaicus*	635 000 year bp	1.27
S.c. camelus vs *S.c. australis*	605 000 year bp	1.21
S.c. massaicus vs *S.c. australis*	30 000 year bp	0.06

Ancestral ostriches probably freely roamed Africa, the Middle East and southern Asia (Maclean, 1990). In time, natural geographical barriers such as dense forests and constantly flowing rivers and lakes separated the areas where the different populations of these ancestral ostriches were

established (Freitag, 1992; Freitag and Robinson, 1993). The Ethiopian system of the Great Rift Valley may have caused a very effective barrier resulting in the isolation of *S.c. molybdophanes* from the rest of the subspecies (Freitag and Robinson, 1993). This geographical separation probably resulted in the efficient disruption of gene flow and the development of the latter subspecies to morphological and genetic distinctiveness. Freitag and Robinson (1993) proposed separate species status for *S.c. molybdophanes* based on genetic divergence calculated from mtDNA data. This proposal was supported by microsatellite data of Kumari and Kemp (1998).

1.1.3 HISTORICAL PERSPECTIVE ON THE OSTRICH INDUSTRY IN SOUTHERN AFRICA

The ostrich industry developed in the 1800's as a result of the demand for ostrich feathers that were sold at prices equalling their weight in gold. The feathers were obtained by hunting wild ostriches. The decline in the number of wild ostriches in South Africa during the first half of the 1800's resulted in the domestication of these birds and, at the same time, saved them from extinction (Douglass, 1881; Layard, 1884; Duerden, 1908, 1909, 1910, 1911a; Grzimek, 1970; Brown *et al.*, 1982). Farmers in the Cape Province, South Africa, feared that continued persecution of wild ostriches would eventually exhaust their income from ostrich feathers. Despite extensive criticism, some farmers started collecting orphaned chicks and hand-raising them. These were the first domesticated ostriches (Douglass, 1881; Layard, 1884).

Despite fresh genetic stock constantly being introduced into newly established domesticated ostrich populations, breeding stock already had a narrow genetic base. The selective breeding programs by these early ostrich breeders caused a further decrease in the genetic variability of the domesticated populations (Duerden, 1910, 1920). When domestication appeared successful, other farmers followed suit or purchased chicks from the breeders (Douglass, 1881, Layard, 1884,

Schreiner, 1898, Duerden, 1908, 1909). The number of breeding ostriches increased from the initial 80 to close to 25 000 by 1875 (Wagner, 1986). By the turn of the century ostriches were exported to European countries such as Germany (Duerden, 1909). By 1912 breeding populations were established in the USA (Wagner, 1986).

The ostrich farming industry has a history of challenges. Firstly, there was the ostrich feather boom periods (±1800 - 1920) and a later revival around 1947 (Wagner, 1986) in which world ostrich numbers first declined and then soared. In 1914 South Africa alone had over a million domesticated ostriches (Duerden, 1919). This period was followed by a collapse in the ostrich feather industry. The First World War (1914 - 1918) initiated a slump in the world price of ostrich feathers. A change in fashions in the period subsequent to the war (1920 - 1925) caused a further decrease in the feather price. The world depression (1930 - 1935) resulted in the price of once valuable feathers hitting rock bottom (Grzimek, 1970; Wagner, 1986). Over-supply of ostrich feathers, with no prospect of a recovery in the market, made ostrich farming an almost obsolete agricultural activity (Grzimek, 1970; Wagner, 1986). A brief recovery in the industry was experienced around 1942, but the socio-economic effects of World War II (1939 - 1945) also had devastating implications for the ostrich industry (Wagner, 1986).

Since the beginning of the ostrich industry ostrich agricultural activity was maintained in the little Karoo area of South Africa, which included Oudtshoorn (Agricultural Information Sheet, 1989). This became the heartland of domesticated southern African ostriches and Oudtshoorn became synonymous with good quality breeding ostriches (Brown *et al.*, 1982; Wagner, 1986). Isolated populations existed elsewhere (Eastern Cape- Grahamstown area, Transvaal, Great Karoo, Northern and Western Cape Province) but these populations were small and breeding strategies were almost non-existent (Harrison, 1990, 1991; Coetsee, 1993). Prior to 1991 stringent

regulations and laws favouring the Little Karoo Agricultural Co-operation (LKAC) were not conducive to large-scale ostrich production elsewhere in the country (Harrison, 1990, 1991).

1.1.4 THE PRESENT AND FUTURE OF THE OSTRICH INDUSTRY IN SOUTHERN AFRICA

The revival of the modern ostrich industry started around 1945 and was once again triggered by interest in feathers (Wagner, 1986). Emphasis has, however, shifted from feather production to hide and meat production. This triggered new global interest in developments in the ostrich industry (Billet, 1984, 1991; Wagner, 1986; Swart *et al.*, 1987; Bell, 1991).

The "modern" period in the ostrich industry could be regarded as the period after World War II to the present time (Wagner, 1986). It is based on a new set of principles aimed at fulfilling different needs such as breeding ostriches that are superior in so far as quantity and quality of meat and size and quality of the hides are concerned. The long term survival of the modern ostrich industry depends on achieving a high level of production of ostrich products. The emphasis thus needs to be on scientific breeding and rearing programs as well as production of high quality hides and low fat meat (Billet, 1984, 1991; Swart *et al.*, 1987; Bell, 1991; Lynn, 1991; Jarvis, 1993).

Ostrich meat is a health food and a delicacy. It is a red meat that is low in cholesterol and has a unique taste (Bell, 1991). Ostrich meat thus has a unique niche in the local and international red meat markets (van Rooyen, 1995). Ostrich hides are tanned into expensive leather and their feathers are used for decorations and other functional tools (Billet, 1984, 1991; Wagner, 1986; Swart *et al.*, 1987; Bell, 1991; Lynn, 1991; Jarvis, 1993).

Southern Africa has several advantages over other continents that enable it to build and expand its ostrich industry into one of its most valuable key industries (Billet, 1984, 1991; Coetsee, 1993; van Rooyen, 1995). It has the necessary geographic and climatic conditions, and more than a century of experience in domesticating and farming these birds (Douglass, 1881; Schreiner, 1898; Duerden, 1919; Grzimek, 1970; Brown *et al.*, 1982; Billet, 1984, 1991; Wagner, 1986; Swart *et al.*, 1987; Jarvis, 1993). The fast turnover rate of this industry and the insatiable market mean that ostrich farming has the potential of bringing prosperity to several of the present war-stricken and impoverished countries of southern Africa (Harrison, 1990, 1991; Billet, 1991; Coetsee, 1993). However, profitable ostrich farming can only materialise if effective management strategies are followed, as initial inputs are very expensive (Billet, 1991; Coetsee, 1993; Correspondent, Grocotts Mail, August 31, 1993).

Domesticated ostrich populations are presently found in many countries throughout the world where they are used for breeding or raised for slaughtering purposes. New ostrich farms are constantly being established all over South Africa and its neighbouring countries, as farmers change to ostrich production (Bell, 1991; Lynn, 1991; Coetsee, 1993, van Rooyen, 1995). Countries such as Wales, England, France, Germany, Italy, Canada, Israel, Australia, America (USA as well as Central and South America), the Netherlands and Spain have established their own breeding stocks, with the intention of becoming self-sufficient (Bell, 1991; Correspondent, Business Day, May 13, 1991; Lynn, 1991; Correspondent, Farmers Weekly, April 2, 1993).

The change by farmers from their traditional crop production to ostrich farming was initiated by factors such as a decrease in mohair and meat prices and the low rainfall in most parts of southern Africa over the period (1980 - 1993). Ostrich farming offers lucrative advantages that are unique to the industry (Stuart, 1982; Viljoen, 1991; Coetsee, 1993; van Rooyen, 1995). There is thus a

steady increase in the total number of ostriches as well as new breeding populations in South Africa. This upswing in the industry is also greatly influenced by relaxation in the original stringent laws that had ruled the industry since 1950. The relaxation and subsequent repeal of these laws effectively destroyed the monopoly of the LKAC on the ostrich industry (Correspondent, Grocotts Mail, October 29, 1993).

New associations such as the East Cape Ostrich Producers' Association (ECOPA) and the Transvaal Ostrich Producers' Association (TOPA) were formed during 1993 to protect and represent the interest of ostrich farmers (Correspondent, Grocotts Mail, October 29, 1993).

Ostrich farming has the potential to provide a large turnover within a short period of time. Less than 2% of a mature ostrich is wasted. A single breeding pair could produce 40 - 60 eggs per season. The hatchability and survival rate (to slaughtering or breeding) is 50 - 70 %, meaning that 20 to 40 individuals are produced per pair per season (Viljoen, 1991; Correspondent, Farmers Weekly, April 2, 1993; van Rooyen, 1995). Chicks could, by feedlotting, be slaughter-ready within a year after hatching (Billet, 1984; Jarvis, 1993). In 1993, profits were in the order of R2 500 to R3 000 per bird (Correspondent, Farmers Weekly, April 2, 1993).

In the absence of alternatives, most ostrich breeders are still using breeding conditions and methods determined almost 90 years ago. This approach uses phenotypic characteristics (morphological and physiological) as criteria for selecting breeding stock (Swart *et al.*, 1987; Jarvis, 1993; van Rooyen, 1995; van Schalkwyk *et al.*, 1996). This selection process is subjective, tedious and costly, as it requires several generations before effective decisions can be made. As ostriches are polygamous using a communal nest (Bertram, 1978, 1992; Maclean, 1990), fertility and fidelity of individuals included in the breeding stock are not always known, and great effort

is required to isolate these individuals. In some cases, instead of a breeding pair, a breeding trio (one cock and two hens) is used (Billet, 1984; Correspondent, Farmers Weekly, April 2, 1993).

Breeding pairs can fetch up to $100 000 in the USA (Lynn, 1991; Correspondent, Farmers Weekly, April 2, 1993; Webpage: http//www.islandnet.co./~ski/ostrich/usa.html, 1999). Great care is thus exercised in selecting these individuals. Parameters that influence the value of breeding individuals include the overall egg production rate, fecundity, mortality/survival rate, size, food conversion rate and fat/muscle ratio of the population from which these individuals originated. There should be a proven record of these phenotypic characteristics being stablely inherited by the offspring (van Schalkwyk *et al.*, 1996). It is therefore important to investigate and establish the basis on which these characteristics are inherited. The influence of factors such as genetic variability on the general fitness of ostrich breeding populations is very important. In birds, limited genetic variability has been linked to low egg production capabilities, hatching failure as well as the general fitness of the offspring (Greenwood, 1987; Oldfield, 1989; Bensch *et al.*, 1994).

An unacceptably high mortality rate has been observed in ostrich chicks for the period from hatching to the age of six months (Billet, 1984, 1991). This has been formally linked to feed types and feeding habits of newly hatched chicks as well as the absence of shelters (Swart *et al.*, 1987; Jarvis, 1993). The influence of genetic factors on the high mortality rate in chicks has not been established, although it is suspected that genetics could have an influence on the breeding capacity of the present ostrich populations (van Rooyen, 1995).

The important concerns and uncertainties raised here need to be addressed for the southern African ostrich, *Struthio camelus australis* (*S.c. australis*). The effects of hybridization may still

be prevalent within the population but may be undetermined and breeding populations may be highly inbred. Furthermore, the possibility exists that different kinds of ostrich agricultural activities in various areas of southern Africa may have resulted in the artificial evolution of geographic lineages.

1.1.5 PROBLEMS FACING THE OSTRICH INDUSTRY

A lack of awareness of developments within this industry, as well as advances made in animal husbandry, could jeopardise the advantages inherent in South Africa's ostrich industry (Stuart, 1982; Harrison, 1990,1991; Correspondent, *Business Day*, May 13, 1991; Coetsee, 1993; Correspondent, *Grocotts Mail*, August 31, 1993). There are already reports of the largest ostrich farm in the world and new ostrich lineages that have been developed in America (Correspondent, *Business Day*, May 13, 1991; Webpage: http://www.newberryfarms.com, 1998).

Problems facing the industry are related to controversies over the taxonomy of ostriches, whether hybridization of the different subspecies should be allowed, and what the level of hybridization within existing subspecies is (Grzimek, 1970; Brown *et al.*, 1982; Freitag and Robinson, 1993; Kumari and Kemp, 1998). The effects of hybridization experiments carried out in the previous and present century are still undetermined, although mtDNA evidence (Freitag and Robinson, 1993) proposed that "*camelus* genes of the 1912 experiments were diluted out of the *australis* population."

The aim of the 1912 breeding experiments was to determine to what degree the quality of the plumage of the southern African (*S.c. australis*) birds could be improved by hybridisation with the North African birds (*S.c. camelus*). The experiments were embarked on to maintain the leading edge that South African feathers, at the time, had on the world feather markets (Wagner,

1986). In these experiments the progeny with the desired traits (ideal plumage characters - greatest length, width, density, richest gloss and perfect shape at the highest stage of development) were selected. Attempts were made to "fix" these traits in unique lineages by inbreeding (Duerden, 1912, 1917, 1919).

The first generation hybrid birds were phenotypic intermediates between the North African ostrich and the South African ostrich. The crosses of these (the first generation hybrids) produced progeny that had the phenotypic appearance of their South African grandparents, suggesting that the characteristic for distinctive sizes underwent segregation in the second (F_2) generation (Duerden, 1919). The hybrid progeny were translocated to various South African farms.

The progress of these experiments was slow, due to long periods of maturity between generations and several other problems such as undesirable traits surfacing in the progeny. Subsequent to World War I (1914 - 1918), ostrich feathers became very cheap and did not warrant further research (Wagner, 1986).

Similar experiments are still being conducted and subspecies are hybridized in search of superior strains, similar to the production of poultry strains. The Newberry farms in the USA claims that they have produced a unique lineage, which they call the Newberry Black Ostrich. This lineage is the result of breeding ostriches from Oudtshoorn (*S.c. australis*) and ostriches from Israel. The subspecies to which the Israeli ostriches belong is unknown since true Israeli ostriches belonged to the extinct subspecies *Struthio camelus syriacus* (Brown *et al.*, 1982). The lineage, however, boasts "all the characteristics (faster maturing rates, larger body and less aggressiveness than other ostriches) that are needed for the present agricultural market" (Webpage: http://www.newberryfarms.com, 1998).

Hybridization of subspecies or species, be it deliberate, unintentional or natural, could have serious genetic implications on the resultant populations. It could influence breeding capabilities and general fitness of organisms (Ballou and Cooper, 1992). However, available data sets suggest that hybrids may not always be less fit than the parent species (For an overview see Rieseberg, 1995). They can have fitness equivalent or intermediate to both parents. The percentage extreme characters observed in hybrids was shown to increase in later generation hybrids (Rieseberg, 1995). In ostriches, Duerden (1920) observed that hybrids of *S.c. camelus* and *S.c. australis* displayed intermediate characteristics. The reproductive maturity of these hybrids was, however, slower than the parent subspecies. The second generation hybrids however showed the characteristics of their *S.c. australis* grandparents, indicating segregation of the characteristics in the F_2 generation. Some of the effects of hybridization may be present within domesticated ostrich populations but these may be obscured by other phenomena such as inappropriate nutritional and unfavorable environmental conditions (Billet, 1984, 1991; Jarvis, 1993; van Rooyen, 1995). The genetic implications of hybridisation on ostriches are still undetermined and should be investigated. In such a study novel or extreme characteristics should be determined. Rieseberg (1995) listed some of the novel or extreme characteristics observed in hybrids as follows:

1. An increased mutation rate may be observed.
2. Complementary action due to new combinations of normal alleles.
3. Placement of alleles under new regulatory patterns may lead to different expression rates in the hybrids when compared to parent species.
4. The fixation of recessive genes that were present in the parent species in heterozygous forms.
5. Reduced developmental stability.

A possible genetic skin disorder has been observed in ostriches (identified as *Struthio camelus* var *domesticus*: Perelman *et al.*, 1995). The disease was found to be non-transmissible, suggesting a genetic related phenomenon. Unfortunately no further information regarding the forces that might have resulted in this phenomenon are available.

One subspecies (*S.c. spatzi*) has already become completely hybridised (Brown *et al.*, 1982). No genetic data of this subspecies in pure form will thus ever be available for comparison.

Another major problem facing the industry is the limited knowledge about the genetic diversity within and between domesticated populations. Studies by Freitag and Robinson (1993) showed low levels of interpopulation genetic diversity of mitochondrial DNA (mtDNA) of southern African ostriches (*S.c.australis*). To maintain a leading edge in ostrich production and the preservation of possible genetic diversity that the present ostrich lineages may contain, it is important that information about the determinants of genetic structuring of populations such as mating systems, selection, evolutionary history, life-history characteristics and mechanisms of gene flow be determined. Appropriate methodologies should be investigated that could resolve these inter and intrapopulation genetic issues. The studies of Freitag and Robinson (1993) could not resolve genetic questions of ostriches at the intrapopulational level. Additional methods are thus needed to generate data that will resolve issues such as genetic variability between and within populations and the effects of domestication, hybridization and inbreeding on ostriches.

Molecular methods have proven track records in taxonomy as well as various applications of animal and plant breeding questions (Smith and Simpson, 1986; Georges *et al.*, 1990; Dowling *et al.*, 1990; Haley, 1991; Kreitman, 1991; Cushwa and Medrano, 1996; Dodgson *et al.*, 1997; Hoelzel, 1998). Molecular techniques that assay the genotype rather than the phenotype have

significant advantages (Kreitman, 1991; Dodgson *et al.*, 1997). DNA is a relatively stable molecule that can be extracted from small amounts of tissue (Jeffreys *et al.*, 1988; Li *et al.*, 1988). DNA can be obtained using non-destructive methods (Grimberg *et al.*, 1989; Kawasaki, 1990; Shrikant and Subramanian 1992; Kimwele *et al.*, 1998), and suitably preserved tissue can be used as a source of DNA from extinct taxa (Dowling *et al.*, 1990; Ellegren, 1991; Kreitman 1991; Onadim and Cowell, 1991; Parkin and Wetton, 1991; Cooper *et al.*, 1992; Härlid *et al.*, 1997, 1998; Lee *et al.*, 1997; Mindell *et al.*, 1997; van Tuinen *et al.*, 1998). Methods such as restriction fragment length polymorphism analysis (Kessler and Avise, 1985; Soller and Beckmann, 1986; Freitag and Robinson, 1993), DNA sequencing (Hillis and Moritz, 1990; Hillis *et al.*, 1990; Mindell *et al.*, 1997) and DNA fingerprinting (Jeffreys *et al.*, 1985a,b; Burke and Burford, 1987; Haley, 1991) are very useful in phylogenetic studies.

1.2 OBJECTIVES OF THIS STUDY

The aim of this study was to determine the degree of genetic variability between and within different domesticated ostrich (*S.c. australis*) populations. Information about genetic variability of ostriches is essential for the effective management of these birds to avoid breeding strategies that could lead to inbreeding or outbreeding depression. The latter phenomena can lead to decreased reproductive fitness. The observed reduced reproductive fitness in ostrich breeding populations is mostly attributed to nutritional and physical factors. Genetic causes are acknowledged but rarely fully investigated. This could be ascribed to the lack of available testing methods.

It was thus the intention to evaluate different molecular methods for their ability to generate characters that could be applied to various intraspecific questions of ostrich biology. These applications ranged from differentiating ostrich subspecies, determining inter- and intrapopulation

genetic diversity, identifying ostriches within a population that has certain qualitative characters and determining whether twin ostriches are identical or non-identical twins. One approach was to investigate genetic diversity in cytoplasmic gene fragments (mitochondrial genome) and a second method investigated the genetic diversity at the genomic level.

The methods and aims can be detailed as follows:

1. To evaluate sequencing of a 12S rRNA gene fragment for population studies of ostriches.
2. To develop and evaluate a PCR-RFLP method of a short mitochondrial sequence (450 bp 12S rRNA) for taxonomic and population studies of ostriches.
3. To develop and evaluate a PCR-RFLP method of a mitochondrial sequences (550 bp Displacement loop) for taxonomic and population studies of ostriches.
4. To evaluate the RAPD technique for its ability to:
   (a) distinguish different ostrich subspecies
   (b) distinguish different geographic populations and to determine genetic variability within these populations.
   (c) Provide information that may be useful for breeding and management decisions
   (d) Determine whether twin ostriches are identical or non-identical twins.

Chapter 2

Sequencing and RFLP Analysis of Polymerase Chain Reaction Amplified Mitochondrial DNA Fragments

2.1 GENERAL INTRODUCTION

In this study the mitochondrial DNA was targeted as a potential source of genetic diversity within the domesticated southern African ostrich *(Struthio camelus australis)* gene pool. Mitochondria and chloroplasts are cellular organelles of eukaryotes that contain their own DNA. The mitochondrial genome has been utilized in a variety of systematic and evolutionary questions in both plants and animals. Numerous studies have demonstrated the usefulness of this molecule as an indicator of past evolutionary events within and across a variety of taxa. Examples include Glaus *et al.* (1980), Anderson *et al.* (1981,1982), Clary and Wolstenholme, (1985), Roe *et al.* (1985), Avise *et al.* (1986), Becker (1987), Shields and Helm-Bychowski (1988), Desjardins and Morais (1990), Mindell *et al.* (1991, 1997), Cooper *et al.* (1992), Freitag and Robinson (1993), Quinn and Wilson (1993), Avise *et al.* (1994a,b), Garcia and Davis (1994), Klein and Brown (1994), Seutin *et al.* (1994), Baker *et al.* (1995), Hedges *et al.* (1996), Sorenson and Fleischer (1996), Härlid *et al.* (1997, 1998), Mindell *et al.* (1998) and Saville *et al.* (1998). The maternal inheritance pattern of mtDNA in animals suggests that it is unaffected by recombination. Although mitochondrial genomes have rapid average evolutionary rates they also demonstrate a tendency to become homogenous within populations (Dowling *et al.*, 1990). These are some of the characteristics which make mtDNA particularly useful for a range of molecular systematic and phylogenetic applications (Dowling *et al.*, 1990). The most useful aspect of this molecule is that it contains highly conserved as well as highly variable regions (Dowling *et al.*, 1990; Simon, 1991; Härlid *et al.*, 1997, 1998).

The genome of the mitochondrion is a closed, circular, superhelical molecule which, unlike nuclear DNA, is present in multiple-copies in cells, although only contributing about 1 % to the total cellular DNA (Smith *et al.,* 1971; Shields and Helm-Bychowski, 1988; Hammans, 1994). Eukaryote life depends on mitochondrial functions and activities as mitochondria

generate adenosine triphosphate (ATP), the energy currency of the cell. The subunits of the enzymes involved in ATP production are coded, at least in part, by the mitochondrial genome while the rest are coded for by the nuclear genome (Hammans, 1994). The mitochondrial genome consists of several coding regions (13 protein coding, 2 ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) genes: Table 2.2). The protein coding regions are not interspersed with introns as is the case in nuclear genes, but there is a non-coding region; the displacement loop (D-loop/control region), which is involved in replication of the mtDNA genome.

The "prokaryotic-origin" hypothesis of mitochondria implies that the genome was reduced early in eukaryotic evolution to a minimum size that is still compatible with function (De Robertis and De Robertis, 1980; Küntzel and Köchel, 1981; Schwarz and Kössel, 1981; Spencer *et al.*, 1984; Watson *et al.*, 1987; Lake, 1988; Hammans, 1994; Blackstone, 1995; Sorenson and Fleischer, 1996). This explains the absence of introns. Since the mitochondrial functions are highly constrained it is expected that the mitochondrial genome structure would share these constraints (Kreitman, 1991; Sorenson and Fleischer, 1996). Evidence indicates that vertebrate mitochondrial genomes evolve 5 to 10 times faster than single copy nuclear DNA (Sorenson and Fleischer, 1996). This implies a 0.5 to 2.0 % nucleotide substitution per lineage per million years (Brown *et al.*, 1979; Kreitman, 1991; Sorenson and Fleischer, 1996).

The rate of evolution between and within the coding and non-coding regions was found to vary considerably within and amongst vertebrate species (Shields and Helm-Bychowski, 1988; Kreitman, 1991; Sorenson and Fleischer, 1996; Mindell *et al.*, 1998). These characteristics provide this molecule with the potential for use in a range of studies; from below the population level to the level of order and above (Shields and Helm-Bychowski, 1988; Kreitman, 1991; Simon, 1991; Hedges and Sibley, 1994; Hedges *et al.*, 1995; Sorenson and Fleischer, 1996; Mindell *et al.*, 1998; van Tuinen *et al.*, 1998). Studies of avian

mitochondrial genomes has shown these features of mtDNA hold true for birds (Kessler and Avise, 1985; Shields and Helm-Bychowski, 1988; Avise and Ball, 1992; Hedges and Sibley, 1994; Klein and Brown, 1994; Hedges *et al.*, 1995; Sorenson and Fleischer, 1996; Härlid *et al.*, 1997, 1998; Mindell *et al.*, 1998; van Tuinen *et al.*, 1998).

2.1.1 STRUCTURE AND SIZE OF mtDNA

Electron microscopy, restriction fragment analysis, restriction site mapping and direct sequencing of the entire or partial mitochondrial genomes have provided an understanding of the genome size, gene order and function, nucleotide and sequence divergence (Brown and Vinograd, 1974; Watson *et al.*, 1987; Shields and Helm-Bychowski, 1988). Comparisons of complete and partial mitochondrial nucleotide sequences of fish, amphibians, lizards, birds and mammals revealed a great amount of stability with regard to size, gene content and genomic organisation within vertebrates (Anderson *et al.*, 1981, 1982; Roe *et al.*, 1985; Becker, 1987; Shields and Helm-Bychowski, 1988; Sorenson and Fleischer, 1996; Mindell *et al.*, 1998). Initially indirect estimates and recently direct sequencing provided evidence that avian mitochondrial genomes are uniform in size, ranging from about 16.3 kb to 17.3 kb (Shields and Helm-Bychowski, 1988; Desjardins and Morais, 1990; Härlid *et al.*, 1997, 1998) (Table 2.1).

Table 2.1: Genome sizes (kb) of mitochondria of birds and selected vertebrates. ([a]Shields and Helm-Bychowski, 1988; [b]Desjardins and Morais, 1990; [c]Härlid *et al.*, 1997; [d]Härlid *et al.*, 1998).

TAXON	GENOME SIZE	ANALYSIS METHOD
Peking Duck	16.7 ± 0.3 kb	Contour Length in Electron Microscopy[a]
	16.6 ± 1.0 kb	Restriction mapping[a]
Duck	16.5 ± 0.2 kb	Restriction fragments[a]
Sparrows	17.3 kb	Restriction fragments[a]
Galliformes	16.3 ± 0.2 kb	Restriction mapping [a]
	16.4 kb	Restriction fragments[a]
Chickens	16.741 kb	Sequencing[b]
Ostrich	16.591 kb	Sequencing[c]
Rhea	16.710 kb	Sequencing[d]
Human	16.569 kb	Sequencing[a]
Cow	16.338 kb	Sequencing[a]
Xenopus sp.	17.553 kb	Sequencing[a]

2.1.2 ORGANIZATION OF VERTEBRATE MITOCHONDRIAL GENES

The positions of the different long open reading frames (ORFs), the rRNA genes, the tRNA genes and the D-loop (control region) of humans, *Xenopus* and birds are shown in Figure 2.1. On the mitochondrial genome of higher vertebrates, the 13 ORFs are organized such that ten of the ORFs are found on the heavy chain (H-chain) and three on the light (L-chain)(Table 2.2; Figure 2.1). Both the rRNA genes are on the H-chain. The tRNAs are arranged such that 8 are on the L-chain and 14 on the H-chain.

Table 2.2: Enzyme complexes of the respiratory chain (Adapted from Hammans, 1994). Those marked with "*" indicate the open reading frames found on the light chain of the mitochondrial genome.

	COMPLEX	NO OF SUBUNITS	ENCODED BY mtDNA
I	NADH ubiquinone reductase (ND)	± 41	7 (ND1, ND2, ND3, ND4, ND4L*, ND5, ND6*
II	Succinate dehydrogenase	4	0
III	Ubiquinone-cytochrome-c reductase	11	1 (cytochrome *b*)
IV	Cytochrome-c oxidase (CO)	13	3 (COI, COII, COII)
V	ATP synthetase (ATPase)	14	2 (subunits 6, 8*)
	TOTAL	83	13

The order of genes on the mitochondrial genome is remarkably conserved in animals (Figure 2.1) and differences in gene order have been reported to occur between phyla (Roberts *et al.*, 1983; De la Cruz *et al.*, 1984; Wolstenholme *et al.*, 1987) and between avian species (Mindell *et al.*, 1998). Northern blot hybridization analysis (Glaus *et al.*, 1980) and restriction map analysis suggested that the organisation of chicken mtDNA is similar to that of all higher vertebrates. This was initially extrapolated to all the avian groups.

However, direct sequencing of the whole mitochondrial genome of the chicken (*Gallus gallus domesticus*) showed that a unique order of mitochondrial genes exits in chickens compared to vertebrates as diverse as humans and *Xenopus* (Desjardins and Morais, 1990). The difference is found in the order in which the NADH ubiquinone reductase subunit 6 (ND6), $tRNA^{-E}$ and the cytochrome *b* genes are arranged with respect to the control region. For many avian species the ND6 and $tRNA^{-E}$ are positioned immediately upstream of the control region. In the other vertebrates the cytochrome *b* is immediately upstream (Figure 2.1; inversion is indicated by arrows). This gene order was also observed in Anseriformes (Quinn and Wilson, 1993), *Struthio camelus* (Härlid *et al.*, 1997) and *Rhea americana* (Härlid *et al.*, 1998).

Mindell *et al.* (1998) first observed a novel gene order in *Smithornis sharpei* and *Falco peregrinus* when they sequenced the entire mitochondrial genomes of these two bird species. This gene order was different from the order observed in other bird species, but it also involved the orientation of the ND6 and $tRNA^{-E}$ to the control region (Figure 2.1). In this rearrangement the $tRNA^{-P}$ orientation in also affected. The mitochondrial gene order of non-avian vertebrates indicate that the $tRNA^{-P}$ is situated adjacent to the control region. In mitochondrial gene order of *Gallus gallus domesticus* (chickens) and several other bird species the $tRNA^{-P}$ is upstream from the ND6 gene. The novel gene arrangement observed by Mindell *et al.* (1998) show $tRNA^{-P}$ immediately downstream from the control region, i.e.

between the control region and the ND6 gene, suggesting that during the gene rearrangement events that ND6 and $tRNA^{-P}$ could have been linked. This study by Mindell *et al.* (1998) showed that the novel gene order was present in another representative of Falconiformes, and representatives of Cuculiformes (Duculidae), Piciformes (Picidae) and Passeriformes (Suboscines). Passerifomes (Oscines), however, had the same mitochondrial gene order as *Gallus gallus domesticus*. The difference in gene order between the Oscine and Suboscine clades of Passiformes appeared to be diagnostic for these clades, suggesting phylogenetic usefulness of gene order characters (Mindell *et al.*, 1998).

The observations about different gene orders in mitochondrial genomes of vertebrates, particularly between non-avian vertebrates and avian species, suggested several mechanisms describing the origin of this phenomenon. These mechanisms include events linked to duplication of segments and subsequent gradual deletions as well as possible recombination events (Desjardins and Morais, 1990; Sorenson and Fleischer, 1996; Mindell *et al.*, 1998; Saville *et al.*, 1998).

Despite this unique order of genes, the evidence still suggested that many features (L or H-strand sence, size and number of genes) of the mitochondrial genome were fairly conserved (Desjardins and Morais, 1990, Quinn and Wilson, 1993; Härlid *et al.*, 1997, 1998; Mindell *et al.*, 1998).

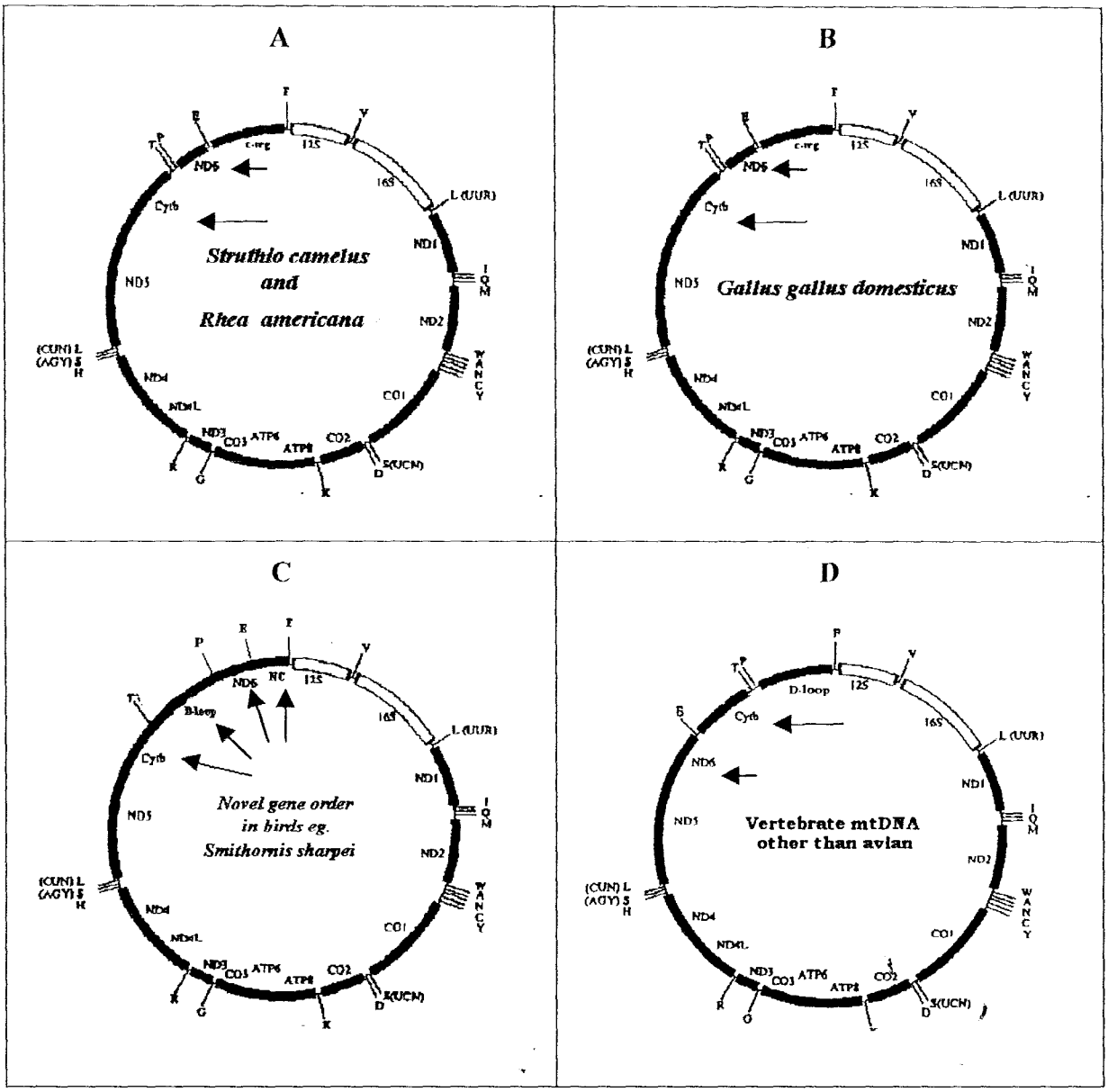


Figure 2.1: The genetic map of (A) the ostrich (*Struthio camelus*) mitochondrial genome and rhea (*Rhea americana*), compared to that of (B) chicken (*Gallus gallus domesticus*) (C) grey headed broad-bill (*Smithornis sharpei*) and (D) from other vertebrates. The tRNA genes are identified by a single letter amino acid code. The genomes represented by A were adapted from Härlid *et al.* (1997, 1998), and genomes B and D adapted from Dejardins and Morais, 1990. Genome C was adapted from Mindell *et al.* (1998). The arrows (◄—) indicate the regions of difference between avian mtDNA and mtDNA of the other vertebrates as well as the novel gene order in some birds.

2.1.3 INHERITANCE OF mtDNA

Early studies indicated that offspring receive all their mtDNA from their maternal parent (Giles *et al.*, 1980). Paternal mtDNA contributions to the zygote were estimated at one per

thousand maternal mtDNA (Giles *et al.,* 1980; Gyllensten *et al.*, 1991). Giles *et al.* (1980) found that their data sets only supported the maternal inheritance of mtDNA, and that paternal mtDNA might only be detected if it was present at a level of at least 4 %. Studies by Ferris *et al.*, (1982) confirmed maternal inheritance of mtDNA. They showed that in old inbred mice no variation in the mtDNA genotypes existed and concluded that this phenomenon was due to the fact that only one female lineage contributed to the formation of all old inbred lines. The genetics of this molecule would thus be unaffected by independent assortment and recombination (Shields and Helm-Bychowski, 1988). These estimates and hypothesis were based on relatively low resolution experiments which involved the restriction analysis of complete mitochondrial genomes. Explanations for the very low levels of paternal mtDNA contributions to zygotes were that mitochondria are carried in the midpiece of the sperm and that they do not penetrate the egg at fertilization. When sperm mitochondria do enter the ovum, they are changed in such a manner that they cannot be used (Watson *et al.*, 1987).

Gyllensten *et al.* (1991) determined that paternally inherited mtDNA molecules may be present at a frequency of 10^{-4} relative to the contributions of the maternally inherited mtDNA. Using backcrossed mice lineages and high resolution PCR-based experiments the biparental inheritance mechanism of mtDNA was shown (Gyllensten *et al.*, 1991). This raised many questions regarding the usefulness of mtDNA as an evolutionary clock but also provided explanations for mitochondrial disorders in which transmission of mtDNA by both parents had been observed.

The usefulness of mtDNA in evolutionary studies had also been questioned as a result of observations of the transferral of mtDNA across species boundaries of wild and laboratory mice (Ferris *et al.*, 1983) and recombination in natural and laboratory strains of the fungus

Armillaria gallica (Saville *et al.*, 1998). Phenomena such as these may obscure evolutionary histories of diverging populations (Becker, 1987).

Although transposition events for D-loop sequences between then mitochondrial and nuclear genomes were described for some bird species the nuclear sequences were found to be reflecting ancestral states and the mtDNA demonstrating high mutation rates reflecting recent evolutionary events (Sorenson and Fleischer, 1996). The hypothesis that mtDNAs are clonally inherited is thus relevant for birds and implies that gene trees can be constructed using mtDNA information (Sorenson and Fleischer, 1996). A particular application of within-species mitochondrial phylogenies is in the analysis of population structure, the pattern of migration, determining absolute dates for certain evolutionary events such as speciation and detecting population bottleneck events (Shields and Helm-Bychowski, 1988; Simon, 1991; Chapco *et al.*, 1992a).

2.1.4 SIZE VARIATION OF mtDNA

Size variations of mtDNA have been observed between and within several vertebrate species (Table 2.1). Length variability in the D-loop region is probably responsible for the observed length-polymorphisms (Martin *et al.*, 1992). Direct sequencing evidence showed that the D-loop region of chickens (1227 bp) was slightly larger than that of human (1122 bp), mouse (879 bp) and cow (910 bp) but shorter than that of *Xenopus* (2134 bp) (Desjardins and Morais, 1990), providing an explanation for the size difference observed between mtDNA of vertebrates species. Härlid *et al.*, (1997, 1998) determined that the D-loop region of ostrich and rhea was 1 028 bp and 1168 bp respectively. Initial evidence suggested that all length mutations were confined to the D-loop (Brown and Wright, 1979) but when complete mtDNA sequences of additional species became available, it was found that length mutations also occurred outside the D-loop region (Anderson *et al.,* 1982). Cann and Wilson (1983) found

that only 21 % of the length mutations observed in human mtDNA were in the D-loop region. Even though an additional non-coding region (seemly without functional constraints preventing mutations) has been reported for some bird species no size variations for this sequence had been observed (Mindell *et al.*, 1998). However, length variations in coding regions such as tRNA, NADH3 have been reported for some bird species (*Gallus gallus domesticus*: Desjardins and Morais, 1990; *Struthio camelus*: Härlid *et al.*, 1997).

The length-polymorphisms observed within the different regions of vertebrate mitochondrial genome suggest these sequences might be suitable for molecular systematic exploitation.

2.1.5 SYSTEMATIC USEFULNESS OF THE CODING REGIONS

The coding regions include the protein coding, tRNA and rRNA genes. The systematic usefulness of these regions has been extensively reviewed by Simon (1991) and Mindell *et al.* (1997). Coding regions are generally regarded as structurally conserved due to functional constraints, and they evolve at a much slower rate than the D-loop (Watson *et al.*, 1987; Simon, 1991; Hammans, 1994). The conserved nature of these genes allows for the alignment of sequence data from distantly related taxa (Watson *et al.*, 1987; Hammans, 1994; Mindell *et al.*, 1997).

The coding genes of avian mitochondrial genomes are generally of similar sizes although some differences due to single or few nucleotide deletions or insertions were observed in NADH ubiquinone reductase (ND) and tRNA genes (Desjardins and Morais, 1990; Seutin *et al.*, 1994; Härlid *et al.*, 1998). These differences are useful as evolutionary markers. Phylogenetic analysis of ND and tRNA sequences of the WANCY region, for example, was used to show crocodilian-bird relationships (Seutin *et al.*, 1994).

2.1.5.1 rRNA GENES

The variable regions of rRNA genes are those coding for the single stranded loops. The most conserved segments are found within the gene coding for the small ribosomal subunit (12S rRNA gene), which is the gene coding for the tRNA attachment site (Simon, 1991). The other conserved region is involved in maintaining the characteristic secondary and tertiary structure of rRNA molecules (Simon, 1991).

In recent studies sequence data from rRNA genes have been extensively used to answer questions about avian systematics and to test hypotheses regarding phylogeny (Cooper *et al.*, 1992; Quinn and Wilson, 1993; Hedges and Sibley, 1994; Hedges *et al.*, 1995; Hedges *et al.*, 1996; van Tuinen *et al.*, 1998).

The results presented by Cooper *et al.* (1992) based on 380 bp of the 12S rRNA gene showed evidence of intraspecific variation of ratites (kiwis and moas). Recent analysis of this fragment from different ostrich subspecies, by different laboratories, showed that 11 to 12 nucleotide differences existed between the sequences (Cooper *et al.*, 1992; Härlid *et al.*, 1997; Lee *et al.*, 1997; van Tuinen *et al.*, 1998). The subspecific identity of the specimens that were used by these researchers was unknown. These findings show that the 12S rRNA gene fragment may be useful for distinguishing different subspecies of ostriches.

Previous avian genetic studies utilising 12S rRNA data addressed issues at or above the species level (Quinn and Wilson, 1993; Liu *et al.*, 1996; Mindell *et al.*, 1997). Van Tuinen *et al.* (1998) found that the 400 bp 12S rRNA fragment that was used by Cooper *et al.* (1992) to determine the basal group of birds and to provide evidence for the evolution of kiwis and moas in New Zealand may have had limited resolving power. Lee *et al.* (1997) and van Tuinen *et al.* (1998) reanalysed the results of Cooper *et al.* (1992) using longer sequences and

found that new data sets did not agree with the double colonization theory proposed by Cooper *et al.* (1992). Lee *et al.* (1997) showed that morphological data supporting the evolutionary relationship of moas and kiwis contradicted Cooper's finding. Van Tuinen *et al.* (1998), supporting the findings of Lee *et al* (1997), argues for the use of multiple marker approaches that include nuclear markers and mitochondrial gene sequences.

Sequence data from the mitochondrial 12S rRNA gene and the mitochondrial 16S rRNA gene were also used (augmented by data from nuclear genes) to indicate the phylogenetic position of the hoatzin (*Opisthocomus hoazin*) and to show that they shared a recent common ancestor with cuckoos (Hedges *et al.*, 1995).

2.1.5.2 PROTEIN CODING GENES

In the thirteen protein coding genes of the mtDNA it has been observed that silent substitutions evolve faster than nucleotide substitutions that cause amino acid replacement (Bibb *et al.*, 1981; Anderson *et al.*, 1982; Simon, 1991).

The best known mitochondrial protein coding gene is the cytochrome *b* gene. It was regarded as an "industry standard" and some of the first universal primers for gene amplification by means of the polymerase chain reaction (PCR) were synthesized for this gene. These primers could amplify cytochrome *b* sequences from a wide spectrum of taxa (Kocher *et al.*, 1989). This gene (partial sequences or entire gene) has been used in a variety of evolutionary studies of birds (Kornegay *et al.*, 1993; Quinn and Wilson, 1993; Avise *et al.*, 1994 a,b; Baker *et al.*, 1995; Härlid *et al.*, 1998). Cytochrome *b* data sets were verified using information that was obtained by methods such as allozymes (Baker *et al.*, 1995), DNA-DNA hybridization (Kornegay *et al.*, 1993), sequence data sets from other mitochondrial genes (Quinn and Wilson, 1993; Härlid *et al.*, 1998) and data sets from nuclear genes (Kornegay *et al.*, 1993).

Some of the first observations were used to describe how spatial heterogeneity in the mitochondrial cytochrome *b* genes of vertebrates fit predictions based on protein structure and function models (Kocher *et al.*, 1989).

Meyer (1994) reviewed the phylogenetic usefulness of the cytochrome *b* gene and pointed out that the expectation of this gene as an evolutionary molecular marker may have been too high. He stated that the limited variation in the first and second codon positions results in insufficient phylogenetic information to answer deep evolutionary questions. This latter observation was also made by Avise *et al.* (1994a).

Despite this, several studies regarding avian systematics and evolution tested hypotheses using cytochrome *b* genes. Kornegay and co-workers (1993) compared the cytochrome *b* sequences from 10 different bird species and showed that New World quails were not part of the order Galliformes although morphology suggested the contrary. This observation was in agreement with DNA-DNA hybridization data sets (Kornegay *et al.*, 1993). Cytochrome *b* data sets also showed a closer phylogenetic relationship between New World vultures and some storks (Avise *et al.*, 1994a). DNA-DNA hybridization as well as morphological characters supported this conclusion. Baker *et al.* (1995), on the other hand, used cytochrome *b* sequence information to show very high levels of genetic structuring in the brown kiwi populations. These sequences proved to be useful diagnostic markers for the different populations sampled. In a recent study Härlid *et al.* (1997) used complete cytochrome *b* gene data sets to show that the order Passeriformes is the basal avian order. Their finding was supported by data sets from complete mtDNAs (Härlid *et al.*, 1998; Härlid and Arnason, 1999). The examples cited showed that although cytochrome *b* data sets may have certain drawbacks, the usefulness of the information cannot be underestimated.

The evidence presented in this section thus show the usefulness of 12 S rRNA gene sequences and cytochrome *b* gene in the genetic studies of birds. For the purposes of this study the 12S rRNA gene fragment was selected because its sequence was already known for the ostrich (Cooper *et al.* 1992). Data that was generated could thus be compared the sequences that had already been published.

2.1.6 SYSTEMATIC USEFULNESS OF THE NON-CODING REGIONS

In addition to the D-loop another non-coding regions has been reported for some avian species (Mindell *et al.*, 1998). These authors observed that the additional non-coding region (844 bp for *Falco peregrinus* and 301 bp for *Smithornis sharpei*) was situated between the $tRNA^{-E}$ and $tRNA^{-F}$ (Figure 2.1). The sequences of this region of *Smithornis sharpei* showed 66 % similarity to the D-loop sequences of this species. No similarity was, however, observed between the control region and the non-coding region of *Falco peregrinus* (Mindell *et al.*, 1998)

Although the D-loop region of vertebrates was shown to be associated with the hypervariability of the mitochondrial genome, conserved regions within the D-loop were also discovered. Three such conserved sequence blocks (CSB-1, 2 and 3) have been identified in vertebrate D-loop regions that may serve as a control for mtDNA synthesis, thus refuting the notion that the D-loop is void of functional and structural constraints. In the domestic chicken, Japanese quail and snow goose sequences similar to CSB-1 were observed (Desjardins and Morais, 1990; Quinn and Wilson, 1993). Härlid *et al.* (1997, 1998) found sequences similar to CSB-1 and CSB-2 in both ostriches and rheas. Sequences corresponding to CSB-3 sequences were identified in ostriches (Härlid *et al.*, 1997) but were absent in the D-loop of the domestic chicken (Desjardins and Morais, 1990), snow goose (Quinn and Wilson, 1993) and rheas (Härlid *et al.*, 1998). Repetitive sequences were also observed in the D-loop of

birds. The length of the D-loop varies by 12 bp in within ostriches (Härlid *et al.*, 1997) to 27 to 29 bp in chickens and geese (Quinn and Wilson, 1993).

These characteristics of the D-loop (length variability, conserved and variable regions) make this molecule ideal for studies at and below the population level (Meyer, 1994). The conserved regions indicate decreased evolutionary change. It is estimated that the avarage mutation rate of this region of the mitochondrion is 3 to 5 times faster than that of the cytochrome *b* gene, providing large amounts of information at population level (Meyer, 1994; Sorenson and Fleischer, 1996) but it may also be informative at higher levels of taxonomy (Martin *et al.*, 1992; Quinn and Wilson, 1993).

The observation that nuclear sequences homologous to the D-loop region were observed in some bird species and that the nuclear sequences were preferentially amplified by universal polymerase chain reaction (PCR) primers, indicates that phylogenetic analysis data from these sequences should be carefully scrutinized (Sorenson and Fleischer, 1996). When ambiguous results are obtained and the purpose of the study was to use mtDNA sequences for evolutionary analysis then results can be corroborated by repeating experiments using tissues that contain higher quantities of mtDNA than nuclear DNA and employing methods such as ceasium chloride ($CsCl_2$) ultracentrifugation to obtain purified mtDNA. PCR-Primers specific for mtDNA should be selected for the amplification of these sequences.

2.1.7 METHODS FOR DETECTING mtDNA VARIATION

A variety of molecular techniques that detect variation within and amongst taxa are available (See Hillis and Moritz, 1990; Kreitman, 1991; Moore *et al.*, 1992; Dodgson *et al.*, 1997 for reviews). Table 2.3 lists mostly ratites but also other selected bird orders, families, genera and populations and some of the techniques that have been used to analyse mtDNA variation.

Table 2.3: Summary of some of the recently used methods for analysis of mtDNA variation in ostrich (*Struthio camelus*) and other selected avian taxa. The table indicates the taxon, the evolutionary questions that were addressed by the study, the method and the reference. The symbols [1] and [2] indicate that the entire mitochondrion[1] or selected sequences[2] were, respectively used for the analysis.

EVOLUTIONARY QUESTION ADDRESSED	TAXON	METHOD	REFERENCE
Basal position of birds and relationships to other vertebrates	*Struthio camelus* (Ostrich)	Sequencing[2]	Van Tuinen *et al.*, 1998; Cooper *et al.*, 1992
	Struthio camelus (Ostrich)	Sequencing[1]	Härlid *et al.*, 1997
	Rhea americana (Rhea)	Sequencing[1]	Härlid *et al.*, 1998
	Avian and other vertebrates	Sequencing[2]	Hedges and Sibley, 1994; Seutin *et al.*, 1994
Taxonomic and systematic relationships at and above the species level	*Struthio camelus* (Ostrich)	Sequencing[2]	Van Tuinen *et al.*, 1998
	Struthio camelus (Ostrich)	Morphology & Sequencing[2]	Lee *et al.*, 1997
	Ratites	Sequencing[2]	Cooper *et al.*, 1992
	Several avian taxa	Sequencing[2,1]	Mindell *et al.*, 1997; Mindell *et al.*, 1998
	Stercorarius pomarinus (Skua)	Sequencing[2] & RFLP	Cohen *et al.*, 1997
	Opisthocomus hoazin (Hoatzin)	Sequencing[2]	Hedges *et al.*, 1995
Taxonomic and systemetic relationships below species level	*Struthio camelus* (Ostrich)	RFLP	Freitag and Robinson, 1993
	Struthio camelus (Ostrich)	Sequencing[2] & PCR-RFLP	This study
	Apteryx australis (Kiwi)	Sequencing[2]	Baker *et al.*, 1995
Inter- and intrapopulation relationships	*Struthio camelus* (Ostrich)	RFLP	Freitag and Robinson, 1993
	Struthio camelus (Ostrich)	Sequencing[2] & PCR-RFLP	This study
	Apteryx australis (Kiwi)	Sequencing[2]	Baker *et al.*, 1995

Restriction enzyme analysis of purified mitochondrial genomes is one of the powerful tools that has been extensively used for analysis of avian mtDNA (Kessler and Avise, 1985; Avise *et al.*, 1988; Shield and Helm-Bychowski, 1988; Freitag, 1992; Cohen *et al.*, 1997). The

method results in restriction fragment length polymorphisms (RFLP) and the information provides quantitative estimates of the amount of mtDNA sequence variation between individuals. This indirect method of measuring nucleotide evolution is regarded as having lower resolving power than direct sequencing (Gyllensten *et al.*, 1991). Its use has, however, led to several hypotheses and conclusions regarding the evolution of mtDNA and mechanisms that may have caused different levels of variability of this molecule within and amongst taxa (Kessler and Avise, 1985; Gyllensten *et al.*, 1991; Sorenson and Fleischer, 1996; Mindell *et al.*, 1998; Saville *et al.*, 1998). Of particular relevance to avian systematics are the studies by Kessler and Avise (1985) who concluded that: (1) Individuals appear to be homoplasmic but sequence heterogeneity may be extensive among conspecifics; (2) Amongst conspecifics and closely related individuals, polymorphisms take the form of silent base substitutions and minor insertions/deletions (Kessler and Avise, 1985). Results from high resolution sequencing methods, in which partial or entire genes as well as complete mitochondrial genomes were analysed for variability, agree with these conclusions (Table 2.3).

Another technique which combines RFLP analysis with PCR can be used for determining variation within short sequences of nuclear as well as mtDNA origin (400 - 2400 bp) (Saperstein and Nickerson, 1991; Akopyanz *et al.*, 1992; Martin *et al.*, 1992). The usefulness of this approach for studies on fish populations was first reported by Martin *et al.* (1992). Once variant genotypes were identified by restriction analysis, the fragments were directly sequenced to determine the exact sequence divergence. The time and cost saving advantages make the PCR-RFLP method very attractive when large sample sizes are involved (Martin *et al.*, 1992).

The above overview shows that mtDNA polymorphism can be detected using several different methods, independently or in tandem. Presently, the general approach is to use RFLP

and PCR based sequencing methods to study evolutionary phenomena within and between taxa. These methods had been applied to avian phylogenetic and systematic studies with varying success rates. RFLP and sequencing methods were successfully applied in genetic studies of ratites (Cooper *et al.*, 1992; Baker *et al.*, 1995), and in particular, ostrich population genetic studies (Freitag and Robinson, 1993; Härlid *et al.*, 1998; van Tuinen *et al.*, 1998). However, only RFLP analysis information is available for ostrich population studies (Freitag, 1992; Freitag and Robinson, 1993). No published studies applying this technique to other avian populations have been located. This study could thus be the first attempt using PCR-RFLP in avian genetic studies.

The history of the ostrich domestication process has been well recorded (Duerden, 1908 - 1920; Wagner, 1986), clearly highlighting the events that could have had an impact on the genetic fitness of present domesticated ostrich populations (See Chapter 1). Events during the slump periods in the ostrich industry were vaguely recorded. It would thus be of interest to use high resolution PCR based methods to determine genetic variability within and between ostrich populations to determine the effect, if any, these events have had on ostrich populations, and to compare these results to previous studies.

2.1.8 AIMS OF THIS STUDY

Two regions of the mitochondrial genome, a 450 bp 12S rRNA gene fragment and a 550 bp D-loop fragment, were identified as sequences that could reveal valuable information regarding the genetic diversity of ostriches.

The objectives were as follows:

1. To develop and evaluate the effectiveness of PCR-RFLP methods of short mitochondrial sequences for population studies of domesticated ostriches.

2. To determine the genetic diversity within the present domesticated ostrich population of southern Africa.
3. To determine the effects of historical events such as domestication, hybridization and genetic bottle-necks on the genetic diversity of these populations.

PCR-RFLP of a short (450 bp) mitochondrial coding sequence (12S rRNA) was used to resolve taxonomic and population questions of domesticated ostriches (*Struthio camelus*). The 12S rRNA gene fragment was also sequenced and compared to the sequence published by Cooper *et al.* (1992). Additional population level variability was assessed by PCR-RFLP of a short (550 bp) mitochondrial non-coding sequence (D-loop).

Each of these methods (sequencing and PCR-RFLP of a coding region and the PCR-RFLP analysis of a non-coding region) are treated separately and are discussed in detail below.

2.2 PARTIAL SEQUENCING OF A 12S rRNA GENE FRAGMENT

2.2.1 INTRODUCTION

Direct comparisons of sequences yield extremely high resolution and provides character information that can be converted to estimates of sequence divergence (Cracraft and Mindell, 1989; Mindell *et al.*, 1997). Depending on the sequences selected, it can highly informative at the intra-populational level (Dowling *et al.*, 1990; Baker *et al.*, 1995; Mindell *et al.*, 1997). The 12S rRNA gene fragment was selected for this study because its sequence was known for the ostrich (Cooper *et al.*, 1992) and it consists of conserved and variable regions which are useful in systematic studies (Simon, 1991).

The aim of this part of the study was to sequence a 12S rRNA gene fragment of ostriches representatives from different geographical regions of southern Africa. The sequences were then compared to the sequences published by Cooper *et al.* (1992).

2.2.2 MATERIALS AND METHODS

2.2.2.1 SAMPLES AND SAMPLE COLLECTION

This study included representatives of ostriches (*S.c. australis*) from Swaziland, the Republic of South Africa, the Little Karoo (Oudtshoorn) and representatives of crosses between individuals from Namibia and Oudtshoorn. (See Appendix B).

Blood samples individuals from Oudtshoorn and Namibian/Oudtshoorn crosses were supplied by the Zoology Department, Rhodes University, Grahamstown, South Africa. Blood samples of the Swaziland ostriches were supplied by a commercial company (Domesticated Ostrich Products).

A technician collected blood samples by venipuncture, in sterile vac-u-test tubes. EDTA (final concentration 50 mM) was used as anticoagulant. EDTA-whole blood samples were stored at 4°C until required for further analysis. Sample collection was carried out under sterile conditions to prevent contamination by micro-organisms and samples were collected and handled separately to prevent cross contamination.

2.2.2.2 DNA ISOLATION

Genomic DNA was isolated from whole blood samples, using a modified form of the isolation procedure from the Life Codes Corporation (Grimberg *et al.,* 1989). The Life Codes Corporation method was generally used for isolation of DNA from human blood samples.

In this procedure, 100 µl whole blood was mixed with 500 µl of a cell lysis solution [0.32 M sucrose, 10 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$ and 1 % (v/v) Triton X-100] and incubated on ice for 10 minutes. The mixture was centrifuged (12 000g for 30 seconds), the supernatant discarded and the pellet resuspended in cell lysis solution. After again centrifuging at 12 000g for 30 seconds, the supernatant was removed and the pellet resuspended in pellet lysis solution [10 mM Tris-HCl (pH 8.0), 10 mM NaCl and 10 mM EDTA]. Following centrifugation (12 000g for 30 seconds), the pellet was resuspended in 300 µl pellet lysis solution containing proteinase K at 10 µg/ml (Boehringer Mannheim, Germany) and incubated for 1 hour at 56°C. After incubation, an equal volume of chloroform:isoamyl alcohol (24:1) was added and the mixture incubated for 10 minutes at room temperature, with occasional agitation. The mixture was then centrifuged (12 000g for 5 minutes at 4°C). The aqueous phase was removed to a fresh sterile 1.5 ml microfuge tube and the DNA precipitated from the aqueous phase with 100 % ice-cold ethanol. The precipitating DNA mixtures were stored at -20°C overnight. Subsequent to this, the DNA was centrifuged (12 000g for 5

minutes at 4°C), the supernatant discarded and the pellet vacuum dried and resuspended in 100 µl pellet lysis solution.

DNA concentrations were determined using a spectrophotometric method. A Shimadzu (160A) spectrophotometer was used to determine the absorbance of the DNA solutions at 260 and 280 nm. The apparatus also calculated the 260/280 ratios. Ratios of 1.5 - 1.8 would indicate that DNA samples are sufficiently purified for digestion by restriction enzymes (Kirby, 1990). Such samples would thus be suitable for enzymic amplification by PCR. DNA was diluted in sterile Milli-Q H_20 to final working concentrations of 50 ng/µl. One hundred ng of DNA was used in each PCR reaction.

The integrity and quality of DNA from blood samples was also checked by agarose electrophoresis. DNA samples (10 µl) were resolved on 0.7 % (w/v) agarose gels, in 1 X TBE electrophoresis buffer. The molecular weight marker on these gels was Lambda DNA digested with *Hind*III. Electrophoresis was at room temperature for 1.5 hours at 80 V. Large flat-bed electrophoresis gels and running chambers were used.

2.2.2.3 PCR AMPLIFICATION OF A 12S rRNA GENE FRAGMENT FOR SEQUENCE ANALYSIS

The 25µl PCR mixtures contained 100 ng of genomic DNA, 100µM dNTPs (Boehringer Mannheim, Germany), 50 picomole of each primer, 1 unit *Taq* polymerase (Biolabs, New England), 1 X *Taq* reaction buffer [50 mM Tris·HCl (pH 9.0), 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100) (Promega, USA or Buffer V, Biolabs, New England)]. Additional $MgCl_2$ (Promega, USA or BioLabs, New England) was added to a final concentration of 3.5 mM per reaction. One percent Acetamide (Sigma, USA) was included in the reactions. The reactions were overlaid with 25 µl mineral oil (Sigma, USA) to prevent evaporation during the thermal

cycling. The addition of $MgCl_2$ to a final concentration of 3.5 mM (Innes and Gelfand, 1990) and 1 % (w/v) acetamide increased the specificity of the enzyme. The denaturant (acetamide) eliminated PCR problems that could occur as a result of DNA denaturation and nonspecific priming (Reysenbach *et al.*, 1992).

Extreme care was taken to prevent carry-over and contamination during all stages of the lab work (Heinrich, 1991). Work areas were separated into DNA isolation, PCR preparation and PCR analysis areas. The bench tops in all the work areas were cleaned with 70 % ethanol immediately before and after use. Microfuge tubes were capped immediately after the transfer of reagents during the preparation of PCR products. Negative controls that were included in the routine PCR runs did not show any amplification, indicating no carry-over of DNA or contamination of reagents.

A Hybaid air-cooled thermal cycler (Omnigene) was used for PCR amplifications. The reaction profile was 94°C for 5 minutes initial denaturation, followed by 35 cycles of 94°C for 30 seconds, annealing 60°C for 30 seconds, and chain extension at 72°C for 60 seconds. After the final cycle the samples were held at 72°C for 5 minutes.

The 12S rRNA gene fragment from the different individuals were amplified using the primer pair:

1. L1091 (5' -AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3') and
2. H 1478 (5'-TGACTGCAGAGGGTGACGGGCGGTGTGT-3') (Kocher *et al.*, 1989).

2.2.2.4 AGAROSE ELECTROPHORESIS

Agarose electrophoresis was used to check whether the PCRs had worked and to determine the sizes of the fragments. The agarose matrix is suited to the resolution of larger DNA

fragments. The sensitivity of ethidium bromide is, however, confined to 0.25 - 1 μg DNA that could be detected on agarose gels (Ausubel *et al.*, 1988; Maniatis *et al.*, 1989). The resolution of small restriction fragments could be negatively affected by this type of electrophoresis when the DNA concentration is low.

After each PCR run, samples were checked for amplification by electrophoresis on 1 % (w/v) agarose gels which contained 20 μg ethidium bromide (Sigma, USA) for each 50 ml of agarose. Three μl PCR product was mixed with 2 μl loading buffer [0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF and 50 % (v/v) glycerol in Milli-Q water] and loaded onto the gel wells. Electrophoresis was at 100 V for 60 minutes. A Hoefer (HE 33) Minnie flat bed submarine gel system was used for casting and running the gels. A DNA standard (Boehringer Mannheim, DNA molecular weight marker VI) was included on each gel. The products were visualized using an Ultra Violet Transilluminator that was linked to a 80386SX computer running computer software UVP version 2.0 for DOS system (UVP analysis system, UVP Gel Documentation System, GDS 2000). This software was used to photograph, manipulate and analyse the gels.

2.2.2.5 PREPARATION OF THE 12S rRNA GENE FRAGMENTS FOR SEQUENCING

Purified double-stranded 12S rRNA gene fragments were sequenced by the DNA Sequencing Core Laboratory of the Interdisciplinary Centre for Biotechnology Research at the University of Florida. The following equipment was used by the laboratory to analyse and sequence the DNA fragments: automated DNA sequencers (ABI 373a), a Perkin Elmer-Cetus PEC 9600 thermocycler, a Robotic Workstation (ABI Catalyst 800), a Savant SpeedVac System, a Beckman DU Spectrophotometer and a Hoefer Scientific TKO 100 Minifluorometer.

Duplicate DNA samples from individuals of the Oudtshoorn (ODT), Oudtshoorn/Namibian hybrids (O/N) and the Royal Swazi (SWA) populations were used as templates for amplification. The 12S rRNA gene fragments were amplified using conditions described in Section 2.2.2.3. The success of the amplification reactions and the integrity of the fragments were checked by agarose electrophoresis (Section 2.2.2.4).

The DNA was separated from the mineral oil using a chloroform:isoamyl procedure. This step was followed by precipitation with absolute ethanol, vacuum drying and resuspending in sterile Milli-Q water (Maniatis *et al.*, 1989). The DNA fragments of duplicate samples were then pooled.

DNA concentrations were determined by measuring absorbances at 260 nm on a Shimadzu (160A) spectrophotometer. The DNA concentrations were calculated using the Beer-Lambert equation (Plummer, 1978). The purified DNA samples were then dispatched to the service provider at the University of Florida via a courier service (DHL).

The sequences were provided as hard copies (printouts, Appendix C) as well as stored on computer discs. The sequences were visually aligned against published sequences.

2.2.3 RESULTS AND DISCUSSION

2.2.3.1 ANALYSIS OF ISOLATED AND PCR AMPLIFIED DNA

The modified Life Codes Corporation DNA extraction method (Grimberg *et al.*, 1989) yielded DNA of high quality and integrity (500 ng to 1500 ng per 100 µl whole blood and 260/280 ratios between 1.2 and 1.5). The genomic DNA consisted of a mixture of mitochondrial and nuclear DNA and is depicted in Figure 2.2. Evidence of DNA degradation

was minimal and the DNA samples were of a quality that was suitable for amplification by PCR.

Figure 2.3 depicts 1 % (w/v) agarose gels stained with ethidium bromide showing resolved PCR amplified 12S rRNA gene. The PCR amplified DNA fragments were consistently of uniform size estimated at 450 bp. The fragment sizes were estimated using the UVP analysis system (Section 2.2.6). The molecular weight standard was the DNA molecular weight marker VI from Boehringer Mannheim (Germany).

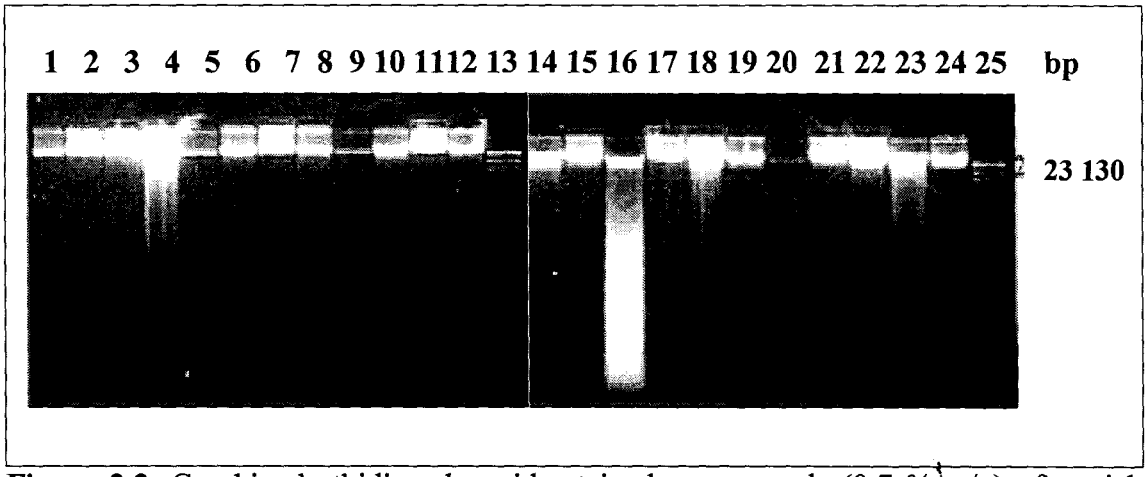


Figure 2.2: Combined ethidium bromide stained agarose gels (0.7 % w/v) of ostrich genomic DNA. Lanes 1 to 12 represent individuals from the Grahamstown population. Lanes 14 to 24 represent individuals from Oudtshoorn, Namibia and Oudtshoorn/Namibian crosses. A molecular weight marker (Lambda (λ) DNA digested with *Hind*III) is included in lanes 13 and 25.

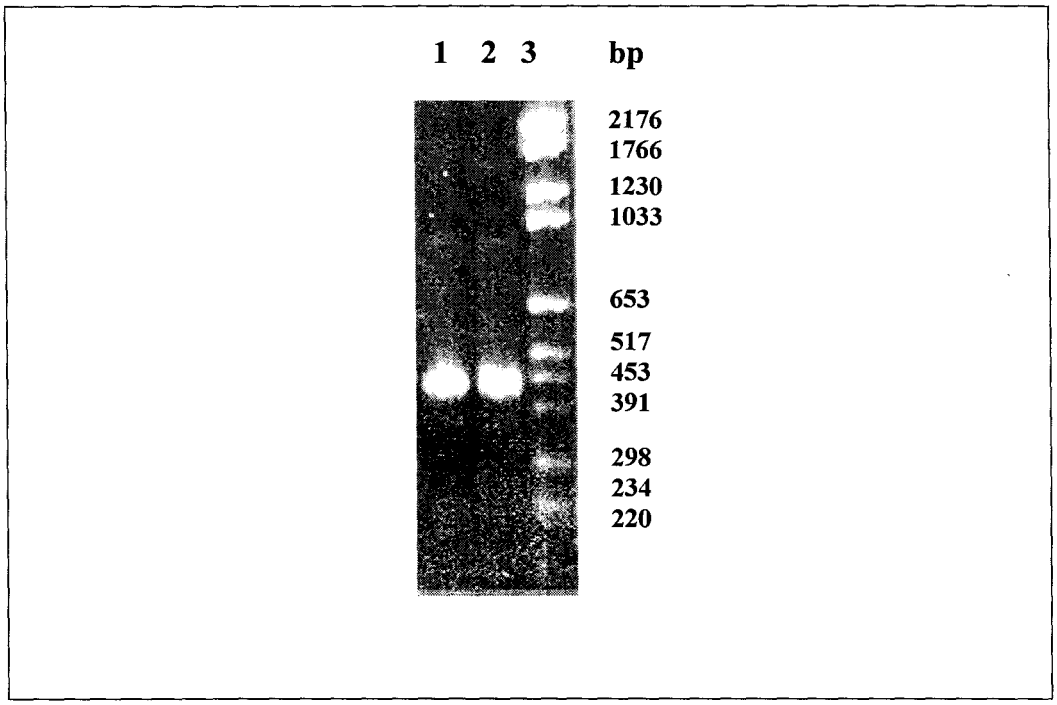


Figure 2.3: An ethidium bromide stained agarose gel (1 % w/v) showing PCR amplified 12S rRNA gene fragments. Lanes 1 and 2 depicts two examples of amplification products of ostrich mitochondrial 12S rRNA gene fragments. The size of the 12S rRNA gene fragment (450 bp) can be estimated, using the DNA molecular weight marker (DNA Molecular Weight Marker VI, Boehringer Mannheim, Germany) in Lane 3.

2.2.3.2 SEQUENCE ANALYSIS OF 12S rRNA GENE FRAGMENTS

The sequences of the 12S rRNA fragments from the different individuals of *S.c. australis* are shown in Table 2.4. A detailed sequence printout, as supplied by the DNA Sequence Laboratory of the ICBR, is contained in Appendix C. Sequences for the light chain fragments are displayed first. The sequences were visually aligned to the sequences of *S.c. australis* that were published by Cooper *et al.* (1992) and no sequence variation was observed within this 400 bp 12S rRNA gene fragment. The individuals in this study represented populations from three possible different lineages within the southern African domesticated ostrich populations. The sequences of all the individuals were identical to the published sequences of Cooper *et al.* (1992).

Table 2.4: Sequence data of 12S rRNA gene fragments. The sequence data of the Oudtshoorn, Oudtshoorn/Namibian crosses and the ostriches were compared to the ostrich 12S rRNA sequence data that were published by Cooper *et al.* (1992). The sequences were aligned from nucleotide 14 of the published sequence. The light chain sequences are 5'-3' and the heavy chain 3'-5'.

L-Strand Amplified by Primer L1091

Published 5'-TCATGATACTTACCCCACCTAAGTATCCGCCCGAGAACTACGAGCACAAACGCTTAAAACTCTAAGGACTTGGCGGTGCCCTAAACCCACCTAGAGGAGCCTGT
ODT/NAM 5'-TCATGATACTTACCCCACCTAAGTATCCGCCCGAGAACTACGAGCACAAACGCTTAAAACTCTAAGGACTTGGCGGTGCCCTAAACCCACCTAGAGGAGCCTGT
ODT 5'-TCATGATACTTACCCCACCTAAGTATCCGCCCGAGAACTACGAGCACAAACGCTTAAAACTCTAAGGACTTGGCGGTGCCCTAAACCCACCTAGAGGAGCCTGT
SWA 5'-TCATGATACTTACCCCACCTAAGTATCCGCCCGAGAACTACGAGCACAAACGCTTAAAACTCTAAGGACTTGGCGGTGCCCTAAACCCACCTAGAGGAGCCTGT
14→

Published TCTATAATCGATAATCCACGATTCACCCAACCACCCCTTGCCATGCAGCCTACATACCGCCGTCGCCAGCCCGCCTCATGAGAGAACAATAGCGAGCACAATAG
ODT/NAM TCTATAATCGATAATCCACGATTCACCCAACCACCCCTTGCCATGCAGCCTACATACCGCCGTCGCCAGCCCGCCTCATGAGAGAACAATAGCGAGCACAATAG
ODT TCTATAATCGATAATCCACGATTCACCCAACCACCCCTTGCCATGCAGCCTACATACCGCCGTCGCCAGCCCGCCTCATGAGAGAACAATAGCGAGCACAATAG
SWA TCTATAATCGATAATCCACGATTCACCCAACCACCCCTTGCCATGCAGCCTACATACCGCCGTCGCCAGCCCGCCTCATGAGAGAACAATAGCGAGCACAATAG

Published CCCACCCGCTAACAAGACAGGTCAAGGTATAGCATATGGAGTGGAAGAAATGGGCTACATTTTCTAACATAGAATACACACGAAAGAGGATATGAAATCNTCCT
ODT/NAM CCCACCCGCTAACAAGACAGGTCAAGGTATAGCATATGGAGTGGAAGAAATGGGCTACATTTTCTAACATAGAATACACACGAAAGAGGATATGAAATCNTCCT
ODT CCCACCCGCTAACAAGACAGGTCAAGGTATAGCATATGGAGTGGAAGAAATGGGCTACATTTTCTAACATAGAATACACACGAAAGAGGATATGAAATCATCCT
SWA CCCACCCGCTAACAAGACAGGTCAAGGTATAGCATATGGAGTGGAAGAAATGGGCTACATTTTCTNACATAGAATACACACGAAAGAGGATATGAAATCATCCT

Published CAGAAGGCGGATTTAGCAGTAAAATAGAATAAGAGAGTCTATTTTAAGTCGGCTCTAGGGC[381]→3'
ODT/NAM CAGAAGGCGGATTTAGCAGTAAAATAGAATAAGAGAGTCTATTTTAAGTCGGCTCTAGGGCACGTACACACCGCCCGTCACCCTCTGC→3'
ODT CAGAAGGCGGATTTAGCAGTAAAATAGAATAAGAGAGTCTATTTTAAGTCGGCTCTAGGGCACGTACACACCGCCCGTCACCCTCT→3'
SWA CAGNAGGCGGATTTAGCAGTAAAATAGAATAAGAGAGTCTATTTTAAGTCGGCTCTAGGGCACGTACACACCGCCCGTCACCCTCTG→3'

H-Strand Amplified by Primer H1478

ODT/NAM 3'-AGTACTATGAATGGGGTGGATTCATAGGCGGGCTCTTGATGCTCGTGTTTGCGAATTTTGAGATTCCTGAACCGCCACGGGATTTGGGTGGATCTCCTCGGAC
ODT 3'-AGTACTATGAATGGGGTGGATTCATAGGCGGGCTCTTGATGCTCGTGTTTGCGAATTTTGAGATTCCTGAACCGCCACGGGATTTGGGTGGATCTCCTCGGAC
SWA 3'-AGTACTATGAATGGGGTGGATTCATAGGCGGGCTCTTGATGCTCGTGTTTGCGAATTTTGAGATTCCTGAACCGCCACGGGATTTGGGTGGATCTCCTCGGAC
365

ODT/NAM AAGATATTAGCTATTAGGTGCTAAGTGGGTTGGTGGGGAACGGTACGTCGGATGTATGGCGGCAGCGGTCGGGCGGAGTACTCTCTTGTTATCGCTCGTGTTAT
ODT AAGATATTAGCTATTAGGTGCTAAGTGGGTTGGTGGGGAACGGTACGTCGGATGTATGGCGGCAGCGGTCGGGCGGAGTACTCTCTTGTTATCGCTCGTGTTAT
SWA AAGATATTAGCTATTAGGTGCTAAGTGGGTTGGTGGGGAACGGTACGTCGGATGTATGGCGGCAGCGGTCGGGCGGAGTACTCTCTTGTTATCGCTCGTGTTAT

ODT/NAM CGGGTGGGCGAATGTTCTGTCCAGTTCCATATCGTATACCTCACCTTCTCTACCCGATGTAAAAGANTGTATCTTATGTGTGCTTTCTCCTATACTTTAGTAGGA
ODT CGGGTGGGCGAATGTTCTGTCCAGTTCCATATCGTATACCTCACCTTCTCTACCCGATGTAAAAGANTGTATCTTATGTGTGCTTTCTCCTATACTTTAGTAGGA
SWA CGGGTGGGCGAATGTTCTGTCCAGTTCCATATCGTATACCTCACCTTCTCTACCCGATGTAAAAGANTGTATCTTATGTGTGCTTTCTCCTATACTTTAGTAGGA

ODT/NAM TCCTCCGCCTAAATCGTCANTTTATCTTATTCTCTCANATAAAA-5'
ODT TCCTCCGCCTAAATCGTCANTTTATCTTATTCTCTCANATAAAA→5'
SWA TCCTCCGCCTAAATCGTCANTTTATCTTATTCTCTCANATAAAA→5'
→14

2.2.4 SUMMARY

The results of the sequencing experiment did not yield any differences between the representatives from different populations. The sequences were identical to those published by Cooper *et al.* (1992). This indicates that the ostrich samples that Cooper *et al.* (1992) used in their experiments were probably from the same subspecies.

The results may also be indicative that the southern African ostrich populations may be closely related and/or that 12S rRNA gene fragments are too conserved to indicate any the population genetic differences that may exist between the different ostrich populations. Sequencing of DNA fragments is a high resolution method for the detection of genetic differences. It is however time and cost ineffective when the sample sizes are large.

2.3 PCR-RFLP ANALYSIS OF A 450 bp REGION OF THE 12S rRNA GENE

2.3.1 INTRODUCTION

An overview of the usefulness of 12S rRNA gene to resolve genetic differences is provided in Section 2.1.1.5 and the PCR-RFLP method is explained in Section 2.1.1.7. The 12S rRNA gene is a mitochondrial gene and thus maternally inherited. Differences observed in mtDNA sequences such as the 12S rRNA gene may reflect different maternal origins or divergence due to mutational events.

The rate of mutation, based on the entire mitochondrial genome, was estimated at 0.5 to 2 % nucleotide substitution per lineage per million years (Kreitman, 1991). Also using the entire mitochondrial genome, Freitag and Robinson (1993) estimated 7.16 % sequence divergence between *S.c. australis* and *S.c. molybdophanes* and 1.21 % divergence between *S.c. australis* and *S.c. camelus* (Table 1.1, Chapter 1). These observed differences between the *S.c. australis* and *S.c. molybdophanes* mitochondrial genomes may thus be sufficient to be reflected in RFLP analysis of the conserved coding genes such as the 12S rRNA gene. The divergence between *S.c. australis* and *S.c. camelus* may not be sufficient to reflect 12S rRNA gene differences between these two subspecies.

In this study the PCR-RFLP of a short (450 bp) 12S rRNA gene fragment was targeted as a potential source of genetic variability in ostriches (*Struthio camelus*), that could possibly resolve taxonomic relationships and population diversity levels in ostriches (*Struthio camelus*).

2.3.2 MATERIALS AND METHODS

2.3.2.1 SAMPLES, SAMPLE COLLECTION AND DNA ISOLATION

This study included representatives of ostriches (*S.c. australis*) from Swaziland (3 individuals), the Republic of South Africa (18 individuals) and Namibia (2 individuals). The South African populations were represented by ostriches from the Little Karoo (Oudtshoorn) and the Eastern Cape (Grahamstown). Representatives of crosses between individuals from Namibia and Oudtshoorn (4 individuals) were also included. One representative of *S.c. molybdophanes* was also included in this study. See Appendix B for details of the various individuals. Sections 2.2.2.1 and 2.2.2.2 have details of the collection and DNA isolation procedures.

2.3.2.2 PCR AMPLIFICATION OF A 12S rRNA GENE FRAGMENT FOR RFLP ANALYSIS

See Section 2.2.2.3.

2.3.2.3 RESTRICTION ENZYME SCREENING AND DIGESTION

The nucleotide sequence (381 base pairs) of the mitochondrial 12S rRNA gene of ostriches was obtained using the Entrez data base. The sequence information was then changed into the ASCII format usable by the computer program, GenePro and analysed for restriction sites, using GenePro software. The restriction enzymes that have restriction sites within this fragment are listed in Table 2.5. Five restriction enzymes from this list were selected to analyse the 12S rRNA gene fragment for RFLP's. The enzymes include three six-base pair cutters (*Dde*I, *Cla*I and *Nde*I), one five-base pair cutter (*Hinf*I) and a four-base pair cutter (*Taq*I). The restriction site recognition sequence of *Taq*I is the same as that of *Cla*I. The restriction site of the latter enzyme, however, has six nucleotides (an additional two nucleotides, one on each side of the restriction site of *Taq*I), thus sampling a larger proportion

of the DNA fragment. Twenty eight individuals were analysed using these 5 restriction enzymes.

Three µl of 12S rRNA PCR product was added directly to the appropriate restriction enzyme buffer (Boehringer Mannheim, Germany). Four units of restriction enzyme (Boehringer Mannheim, Germany) were used per 10 µl restriction reaction. The reactions were incubated at the appropriate temperature overnight. After incubation, the reaction was stopped by mixing with 3 µl loading buffer (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF and 50 % (v/v) glycerol in Milli-Q water). The samples were then run on gels as set out in Section 2.3.2.4.

Table 2.5: The restriction enzymes that have restriction sites within the 381 base pair 12S rRNA gene sequence. Recognition sequences are for 5' → 3' strand DNA. The restriction site location was determined using GenePro software. The arrow (↓) indicates the cleavage site of restriction enzymes. The restriction enzymes in **bold** were used in the analysis.

RESTRICTION ENZYME	RECOGNITION SEQUENCE	RESTRICTION SITE LOCATION			
*Ava*I	C↓T/CCGA/GG	43			
*Ban*I	G↓GT/CA/GCC	88			
*Bbv*I	G↓CAGC	162			
*Bsp*1286I	GA/G/TGCA/T↓C	55	89	208	
*Bsp*HI	T↓CATGA	14	198		
*Cel*II	GC↓TNAGC	1			
***Cla*I**	**AT↓CGAT**	**124**			
***Dde*I**	**C↓TNAG**	**2**	**32**	**75**	**319**
*Fnu*4HI	GC↓NGC	162			
*Hgi*AI	GA/TGCA/T↓C	55	208		
***Hinf*I**	**G↓ANTC**	**137**	**355**		
*Hph*I	GGTGAN	140			
*Mae*I	C↓TAG	104	375		
*Mbo*II	GAAGAN	262			
*Mnl*I	CCTCN	107	191	303	318
*Mse*I	T↓TAA	9	67	364	
***Nde*I**	**C↓ATATG**	**251**			
*Nla*III	CATG↓	15	159	194	
*Nla*IV	GGN↓NCC	88	109		
*Ple*I	CCTCN	355			
***Taq*I**	**T↓CGA**	**125**			

2.3.2.4 ELECTROPHORESIS AND STAINING

The two methods that were used to resolve DNA fragments: agarose gel electrophoresis as described in 2.2.2.4 and polyacrylamide gel electrophoresis, described below.

Discontinuous SDS-Polyacrylamide gel electrophoresis

After several unsuccessful attempts to use agarose electrophoresis (0.7 % - 3 % (w/v) gels) for the resolution of restriction fragments, it was decided to investigate the use of discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE: Laemmli, 1970). The gels were silver stained due to the high sensitivity of this staining method. This latter approach was successful and could be used for routine applications. Although PAGE and silver staining was more time consuming, it yielded reproducible results, and lower concentrations of DNA could be used for analysing restriction digests. Fragments of 100 base pairs in length were routinely resolved using gels that contained 10 % (w/v) acrylamide.

For these gels the resolving gel (10 % (w/v) acrylamide) was prepared in gel buffer containing 1.5 M Tris-base (pH 8.8, 25°C) and 0.8 % (w/v) SDS. The resolving gels were prepared and polymerised first, with 500 µl Milli-Q water layered on top of the gel to produce a smooth gel surface and prevent oxygen contamination as this would interfere with polymerization. Once polymerization had occurred, excess water was drained off. The stacking gel (4% (w/v) acrylamide) was then prepared in gel buffer which contained 0.5 M Tris-HCl (pH 6.8, 25°C) and 0.8 % (w/v) SDS. The stacking gel was poured on top of the resolving gel. The concentrations of the constituents of gel buffers (Tris and SDS) indicated above, were for stock solutions. The gels were prepared using protocols for preparing protein gels (Ausubel *et al.*, 1988; Maniatis *et al.*, 1989).

The entire restriction reaction (10 µl) was mixed with 3 µl loading buffer [0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF and 50 % (v/v) glycerol in Milli-Q water] and loaded into the gel wells. A molecular weight marker (Boehringer Mannheim, Germany, DNA molecular weight marker VI) was included on each gel as a reference marker. All gels were subjected to electrophoresis at 120 V for 3 hr, in 1 X TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA), using Hoefer (SE 280) vertical slab gel electrophoresis systems. Electrophoresis was carried out at room temperature.

Visualization of the resolved DNA fragments was by a silver staining method adapted from the Qiagen^{Tm} silver staining protocol for thermal gradient gel electrophoresis (TGGE). Using this protocol, gels were soaked in fixing solution [ethanol (10 %, v/v) and acetic acid (0.1 %, v/v) in Milli-Q water] for 3 minutes. The fixing solution was decanted and replaced with fresh solution and the procedure repeated for a further 3 minutes. This fixing step was followed by impregnation in silver nitrate solution (0.1 % (w/v) $AgNO_3$ w/v in Milli-Q water) for 10 minutes. The gel was then developed by soaking in a solution of sodium hydroxide (1.5 %, w/v), sodium borohydride (0.01 %, w/v) and formaldehyde (0.15 %, v/v) for 20 minutes. Development of the gels was stopped by washing in fixing solution [ethanol (10 %, v/v) and acetic acid (0.1 %, v/v) in Milli-Q water] for 30 seconds.

2.3.2.5 ANALYSIS OF RESTRICTION ENZYME PATTERNS OF THE 450 bp 12S rRNA GENE FRAGMENT

The wet gels were photographed and analysed using a UVP analysis system (UVP Gel Documentation System, GDS 2000) that was linked to a 80386SX computer running computer software UVP version 2.0 for DOS. The computer software compared the distance the selected restriction fragments migrated to the migration pattern of the molecular weight marker, and calculated band size in base pairs. Gels were analysed for polymorphisms in the

restriction fragment patterns. For 12S rRNA gene fragments, differences in RFLP patterns were scored as gains (+) or losses (-) in restriction sites when compared to the known *S. camelus* sequence (Cooper *et al.*, 1992).

Equation 21, $F = \frac{2N_{XY}}{(N_X + N_Y)}$, from Nei and Li (1979) was used to calculate the proportion of fragments shared (F) between the domesticated ostriches and the wild *S.c. molybdophanes*. In this equation, N_{XY} is the number of fragments shared between populations; N_X is the number of fragments in population X; and N_Y is the number of fragments in population Y. This value of F was used to estimate nucleotide sequence divergence (*p*)(Upholt, 1977) where $p = 1 - (\frac{-F+\sqrt{F^2+8F}}{2})]^{\frac{1}{n}}$ and n is the number of base pairs recognized by each restriction enzyme.

2.3.3 RESULTS AND DISCUSSION

Figures 2.4, 2.5 and 2.6 depict silver stained polyacrylamide (10 % w/v) gels showing restriction patterns when PCR amplified 12S rRNA gene fragments were digested with *Cla*I and *Taq*I, *Dde*I and *Hinf*I and *Nde*I, respectively. All the enzymes yielded two fragments for the *S.c. australis* that were uniform in size (for the particular enzyme) for all the individuals examined.

Figure 2.7 is a restriction map of the 12S rRNA amplified gene fragment of (A) the uniform genotype of the *S.c. australis* and (B) the genotype for *S.c. molybdophanes*. The restriction site is indicated by the first letter of the restriction endonuclease. These restriction maps were deduced from the restriction patterns as depicted in Figures 2.4 to 2.6 and published sequence data (Cooper *et al.*, 1992; Table 2.5). No new restriction sites were observed for any of the endonucleases used in the assay of ostrich 12S rRNA gene fragments.

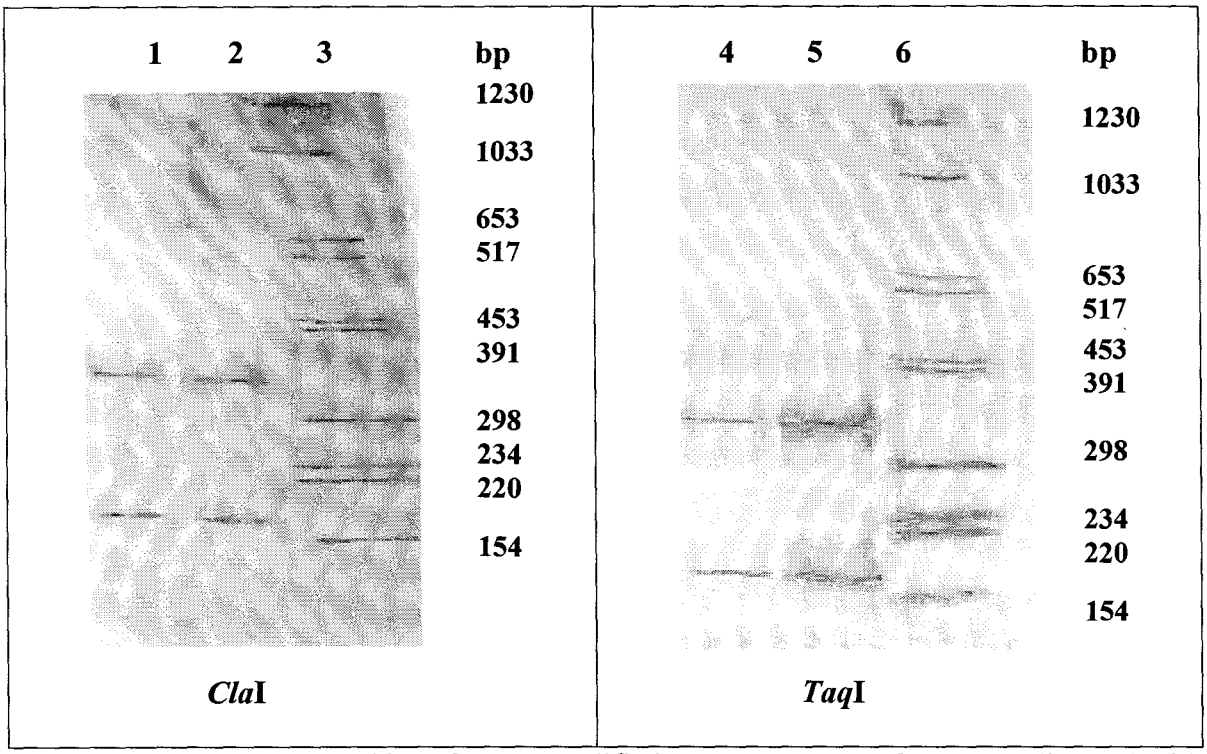


Figure 2.4: Restriction profiles of PCR amplified 12S rRNA gene fragments digested with *Cla*I and *Taq*I, independently. Lanes 1 and 2 show the *Cla*I profiles and lanes 4 and 5 show the *Taq*I profiles. These profiles were obtained with all individuals tested. The DNA molecular weight marker (DNA Molecular Weight Marker VI, Boehringer Mannheim, Germany) is in lanes 3 and 6. The restriction fragments were resolved by SDS-PAGE and visualized by silver staining.

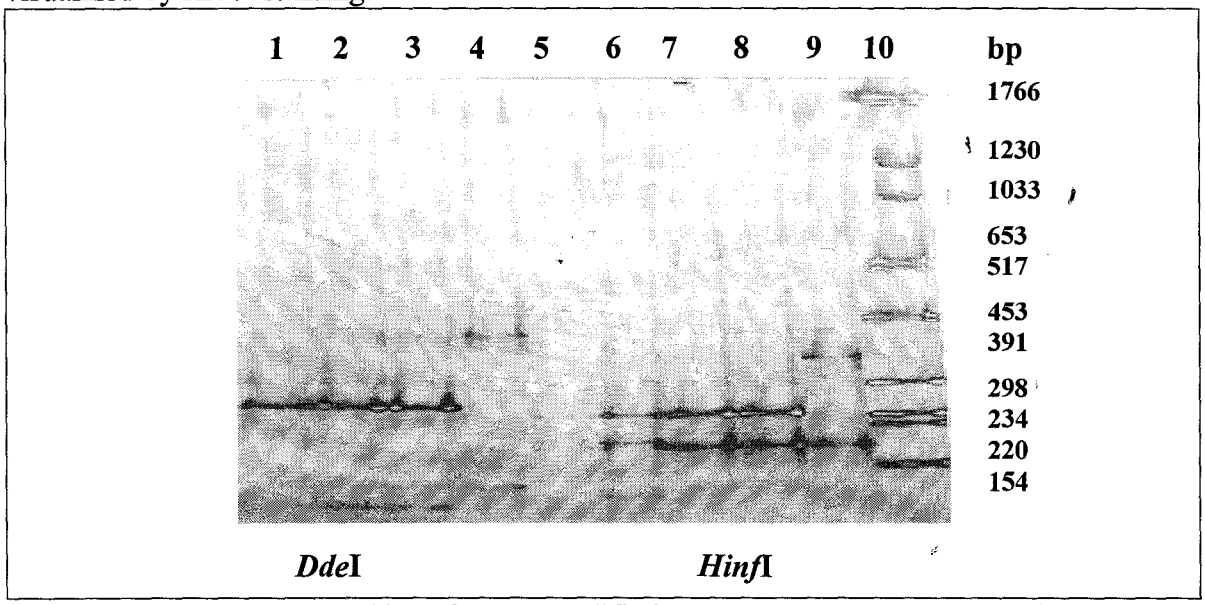


Figure 2.5: Restriction profiles of PCR amplified 12S rRNA gene fragments digested with *Dde*I and *Hinf*I, independently. Lanes 1 to 4 show the *Dde*I profiles and lanes 5 to 9 show the *Hinf*I profiles. The *Dde*I profiles in lanes 1 to 3 were observed for all the *S.c. australis* individuals tested. The *Dde*I profile of *S.c. molybdophanes* is depicted in lane 4. The *Hinf*I profiles in lanes 5 to 8 were observed for all the *S.c. australis* individuals tested. The *Hinf*I profile of *S.c. molybdophanes* is depicted in lane 9. The DNA molecular weight marker (DNA Molecular Weight Marker VI, Boehringer Mannheim, Germany) is in lane 10. The restriction fragments were resolved by SDS-PAGE and visualized by silver staining.

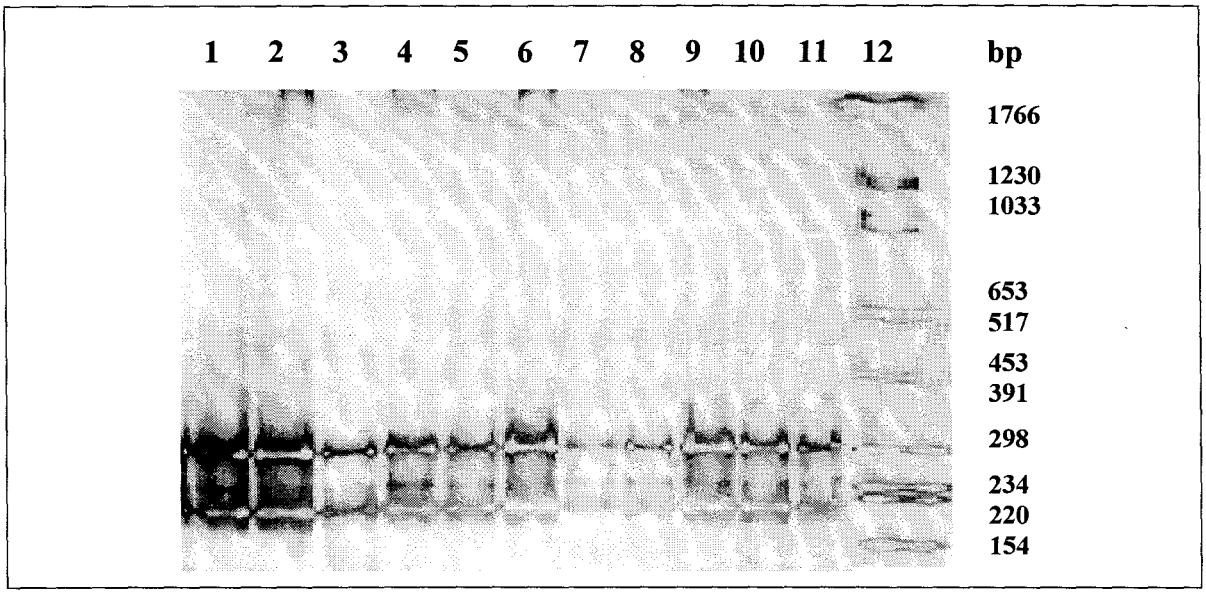


Figure 2.6: Restriction profiles of PCR amplified 12S rRNA gene fragments digested with *Nde*I. Lanes 1 to 11 show the *Nde*I profiles that were obtained with all individuals tested. The DNA molecular weight marker (DNA Molecular Weight Marker VI, Boehringer Mannheim, Germany) is in lane 12. The restriction fragments were resolved by SDS-PAGE and visualized by silver staining.

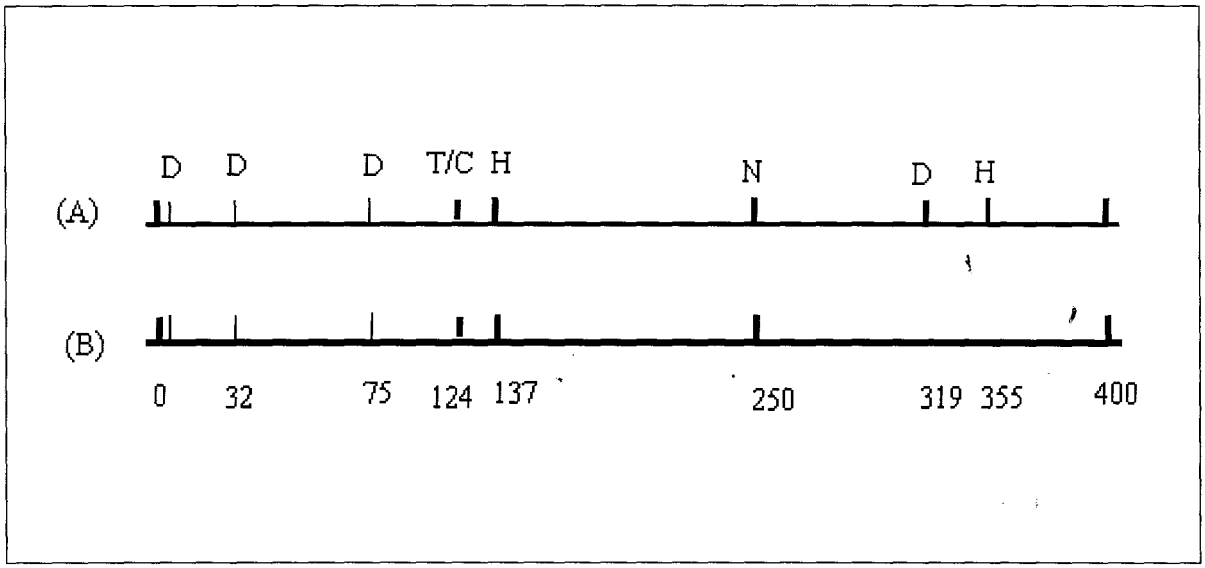


Figure 2.7: A restriction map of the 12S rRNA fragments that were assayed. The letters represent the first letter of the name of the restriction enzyme that is responsible for the restriction site (C = *Cla*I, D = *Dde*I, H = *Hinf*I, N = *Nde*I and T= *Taq*I). The numbers indicate the restriction site and the polarity of the fragment is 5' – 3' as indicated in Cooper *et al.* (1992). (A) represents the haplotype observed for *S.c. australis* and (B) the observed haplotype of *S.c. molybdophanes*.

Twenty eight individuals were analysed using 5 restriction enzymes. A total of 140 restriction sites were thus assayed. The only restriction fragment length polymorphisms observed were those between the subspecies *S.c. molybdophanes* and *S.c. australis*. Of the five restriction

enzymes employed, restriction sites for two of these enzymes (*Dde*I and *Hinf*I) were polymorphic at this level. These data are summarised in Table 2.6. The individuals from all the different domesticated populations were grouped and the RFLP information was compared to that of the *S.c. molybdophanes* individual.

Table 2.6: The results of the restriction analysis of a 450 bp12S rRNA gene fragment of ostrich (*Struthio camelus*). N_{XY} is the number of fragments shared between populations; N_X is the number of fragments in population X; and N_Y is the number of fragments in population Y. The table is split into the results for restriction enzymes that yielded restriction polymorphims (bottom section) and those that did not show any polymorphisms (top section).

RESTRICTION ENZYME	RECOGNITION SITE	NUMBER OF INDIVIDUALS ASSAYED	SCORED FRAGMENTS		
			Nx	N_Y	Nxy
*Cla*I	AT↓CGAT	28	2	2	2
*Nde*I	C↓ATATG	28	2	2	2
*Taq*I	T↓CGA	28	2	2	2
*Dde*I	C↓TNAG	28	2	1	0
*Hinf*I	G↓ANTC	28	2	2	1
		TOTAL	4	3	1

Only data from the two restriction enzymes that showed differences in the restriction patterns of the subspecies, *S.c. australis* and *S.c. molybdophanes*, were used to determine F (proportion of shared fragments). The value of F was calculated at 0.286. This latter value was then used to estimate sequence divergence (*p*)(Section 2.3.2.5). The value of *p* was estimated at 0.089. The time of divergence between the two subspecies was estimated at 4.5 million years ago (assuming a 2 % per million year rate of sequence evolution; Bown *et al.*, 1979; Kessler and Avise, 1987).

2.3.4 SUMMARY

Results presented in this study indicate that the subspecies *S.c. australis* and *S.c. molybdophanes* can be distinguished by PCR-RFLP of a 450 bp 12S rRNA gene fragments. Percentage sequence divergence based on this method was estimated at 8.9 %. A previous study (Freitag and Robinson, 1993) showed that the sequence divergence, based on RFLP analysis of the entire mtDNA genome, was 7.16 % between *S.c. australis* and *S.c. molybdophanes* (Table 1.1). They (Freitag and Robinson, 1993) also estimated that the divergence time of *S.c. molybdophanes* from all the other subspecies was between 3 and 4 million years ago. Data from PCR-RFLP analysis of a 450 bp 12S rRNA sequence (this study) indicate that divergence between *S.c. australis* and *S.c. molybdophanes* occurred 4.4 million years ago. Although the data (percentage sequence divergence and divergence times) presented here in this study were higher than those presented by Freitag and Robinson (1993), they are comparable to their data.

The PCR-RFLP method applied to 12S rRNA thus has applicability at the subspecies level of ostrich genetic studies, and may find uses in ostrich genetic studies that investigate intersubspecific hybridization. The cost and time effectiveness of the method would enable the rapid analysis of large sample sizes.

2.4 PCR-RFLP ANALYSIS OF A 550 bp REGION OF THE D-LOOP

2.4.1 INTRODUCTION

As discussed in Section 2.1.5.1, the greater intraspecific variation that is normally expected for the D-loop fragment could be explained by the duplication of sequences (Desjardins and Morais, 1990; Mindell *et al.*, 1998). This duplication phenomenon was observed in lizards (Brown and Wright, 1979; Moritz and Brown, 1986), humans (Anderson *et al.*, 1982; Cann and Wilson, 1983) and eels (Avise *et al.*, 1986). Intraspecific size variation within the D-loop of avian species has not yet been reported. However, tRNA-like sequences similar to those that cause duplications in the D-loop of lizards and humans have been observed in the D-loop of birds (Desjardins and Morais, 1990). A duplication of sequences was also observed in a non-coding region additional to the D-loop of *Smithornis sharpei* (Mindell *et al.*, 1998). This additional non-coding region and duplication of sequences have not been reported for ostriches (*Struthio camelus*).

Although the D-loop sequence has been used with success in population studies of vertebrates (Martin *et al.*, 1992; Cronin *et al.*, 1993; Meyer, 1994), its usefulness in population studies of ratite species has not been assessed. Some of the characteristics discussed in Section 2.1.5.1 may render this region (D-loop) of the mitochondrial genome suitable for the population studies of ostriches.

Most avian population studies of mtDNA used evidence from RFLP methods where the entire mitochondrial genome was analysed (Kessler and Avise, 1985; Shields and Helm-Bychowski, 1988; Freitag and Robinson, 1993). In this study the RFLP method was applied to a PCR amplified D-loop region.

The aim of this part of the study was to assess the suitability of PCR-RFLP of a 550 bp D-loop region in determining population genetic variability of ostriches (*S.c. australis*).

2.4.2 MATERIALS AND METHODS

2.4.2.1 SAMPLES, SAMPLE COLLECTION AND DNA ISOLATION

The same samples that were used for the PCR-RFLP analysis of the 12S rRNA gene fragment were used for this study. *S.c. molybdophanes* was not included in this study since the aim was only to assess for population genetic variability and not intraspecific variability. Twenty seven individuals of southern African ostriches (*S.c. australis*) were sampled. Details of sample collection and DNA isolation are described in Section 2.2.2.1 and 2.2.2.2.

2.4.2.2 PCR AMPLIFICATION OF THE 550 bp D-LOOP REGION FOR RFLP ANALYSIS

See Section 2.2.2.3.

The D-loop fragment was amplified using primers:

THR (5'-AGCTCAGCGCCAGAGCGCCGGTCTTGTAAA-3') (Kocher *et al.*, 1993) and

TDKD (5'-CCTGAAGTAAGGAACCAGATG-3') (Kocher *et al.*, 1993; Quinn and Wilson, 1993).

A Hybaid air-cooled thermal cycler (Omnigene) was used for PCR amplifications. The reaction profile was 94°C for 5 minutes initial denaturation, followed by 35 cycles of 94°C for 30 seconds, annealing 55°C for 30 seconds, and chain extension at 72°C for 60 seconds. After the final cycle the samples were held at 72°C for 5 minutes.

2.4.2.3 RESTRICTION ENZYME SCREENING AND DIGESTION

Twenty-three enzymes were screened for their ability to digest the PCR amplified D-Loop fragment. As sequence information was not available for this fragment at the time when the experiments were performed, several different restriction enzymes had to be screened. Subsequently, Härlid *et al.* (1997) published the sequence of the entire mitochondrion of ostriches. This provides the opportunity to search the sequence for restriction sites of enzymes.

The restriction enzymes that were included in the screening process in this study are listed in Table 2.7. Four of these enzymes were found to digest the D-loop fragment and were selected for subsequent analysis. These enzymes were, *Hinf*I, *Eco*RI, *Sca*I and *Rsa*I.

Table 2.7: Restriction enzymes that were screened for their ability to digest the PCR amplified D-loop fragment. Recognition sequences are for 5' → 3' strand DNA. The arrow (↓) indicates the cleavage site of restriction enzymes. The restriction enzymes in **bold** were used in the analysis.

	RESTRICTION SITE	RECOGNITION SEQUENCE
1.	*Aat*II	GACGT↓C
2.	*Alw*441	G↓TGCAC
3.	*Apa*I	GGGCC↓C
4.	*Bgl*I	GCCN↓NGGC
5.	*Bgl*II	A↓GATCT
6.	*Cla*I	AT↓CGAT
7.	*Dde*I	C↓TNAG
8.	*Dra*I	TTT↓AAA
9.	***Eco*RI**	**GGTAC↓C**
10.	*Hae*III	GG↓CC
11.	*Hind*III	A↓AGCTT
12.	***Hinf*I**	**G↓ANTC**
13.	*Kpn*I	GGTAC↓C
14.	*Mbo*I	GA↓TC
15.	*Nde*I	CA↓TATG
16.	*Pst*I	CTGCAG
17.	***Rsa*I**	**GT↓AC**
18.	*Sau*3AI	↓GATC
19.	***Sca*I**	**AGT↓ACT**
20.	*Sma*I	CCC↓GGG
21.	*Taq*I	T↓CGA
22.	*Xba*I	T↓CTGA
23.	*Xho*I	C↓TCGAG

Three µl of the PCR amplified D-loop fragment was added directly to the appropriate restriction enzyme buffer (Boehringer Mannheim, Germany). Four units of restriction enzyme (Boehringer Mannheim, Germany) were used per 10 µl restriction reaction. The reactions were incubated at the appropriate temperature overnight. After incubation, the reaction was stopped by mixing with 3 µl loading buffer (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF and 50 % (v/v) glycerol in Milli-Q water).

A double digest comprising of the enzymes *Eco*RI and *Sca*I was performed under the conditions mentioned above. This assisted in determining the orientation of the restriction sites and thus mapping the 550 bp D-loop fragment.

2.4.2.4 ELECTROPHORESIS AND STAINING

See Section 2.2.2.4 and Section 2.3.2.4

2.4.2.5 ANALYSIS OF RESTRICTION ENZYME PATTERNS OF THE 550 bp D-LOOP REGION

Differences in the restriction fragment patterns between individuals were noted, and scored as present or absent.

2.4.3 RESULTS AND DISCUSSION

2.4.3.1 ANALYSIS OF PCR AMPLIFIED D-LOOP DNA

Agarose electrophoresis was used to check whether the PCRs had worked and to determine the sizes of the fragments. Figure 2.8 depicts 1 % (w/v) agarose gels stained with ethidium bromide showing resolved PCR amplified D-loop fragments. The PCR amplified DNA fragments were consistently of uniform size and the size was estimated at 550 bp (Figure 2.8), using the UVP analysis system (Section 2.2.6). The molecular weight standard was the DNA molecular weight marker VI from Boehringer Mannheim (Germany).

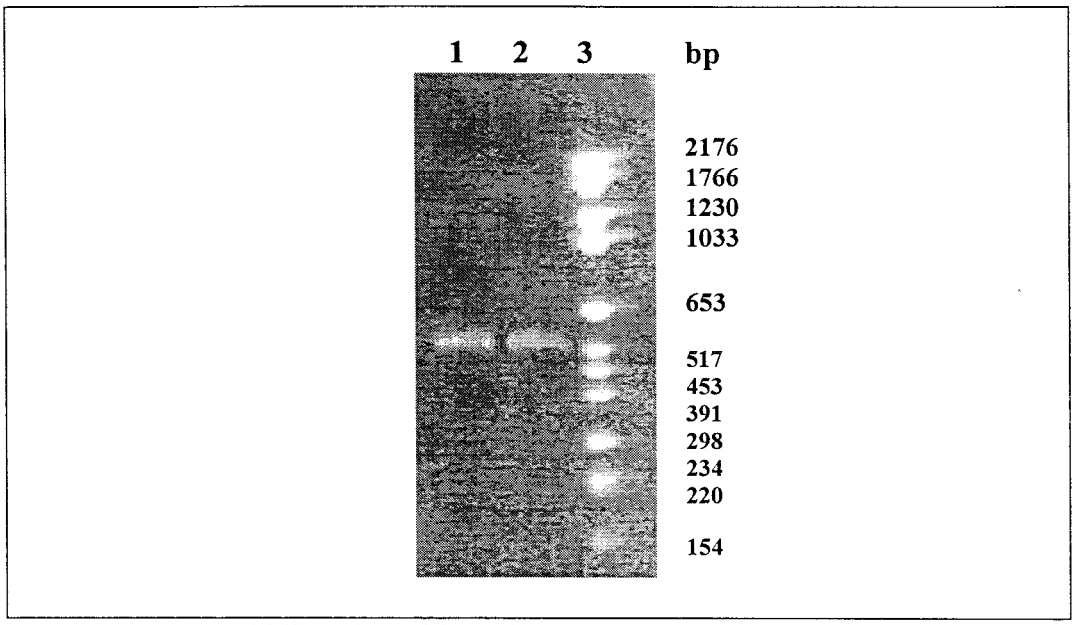


Figure 2.8: An ethidium bromide stained agarose gel (1 % w/v) showing PCR amplified D-loop fragments. Lanes 1 and 2 depicts two examples of amplification products of ostrich mitochondrial D-loop fragments. The size of the D-loop fragment (550 bp) can be estimated, using the DNA molecular weight marker (DNA Molecular Weight Marker VI, Boehringer Mannheim, Germany) in lane 3.

2.4.3.2 RESTRICTION ENZYME PATTERNS OF THE 550 bp D-LOOP REGION

Figure 2.9 is a diagrammatic representation of typical restriction profiles produced when the four enzymes were used to digest the PCR amplified 550 bp D-loop fragment. Only one of the digests was a double digest (Figure 2.9, Lane 2; *Eco*RI-*Sca*I). Lanes 1, 3, 4, and 5 represent profiles produced by the restriction enzymes *Hinf*I, *Eco*RI, *Sca*I and *Rsa*I, respectively. The actual restriction digest profiles are shown in Figures 2.10, 2.11 and 2.12.

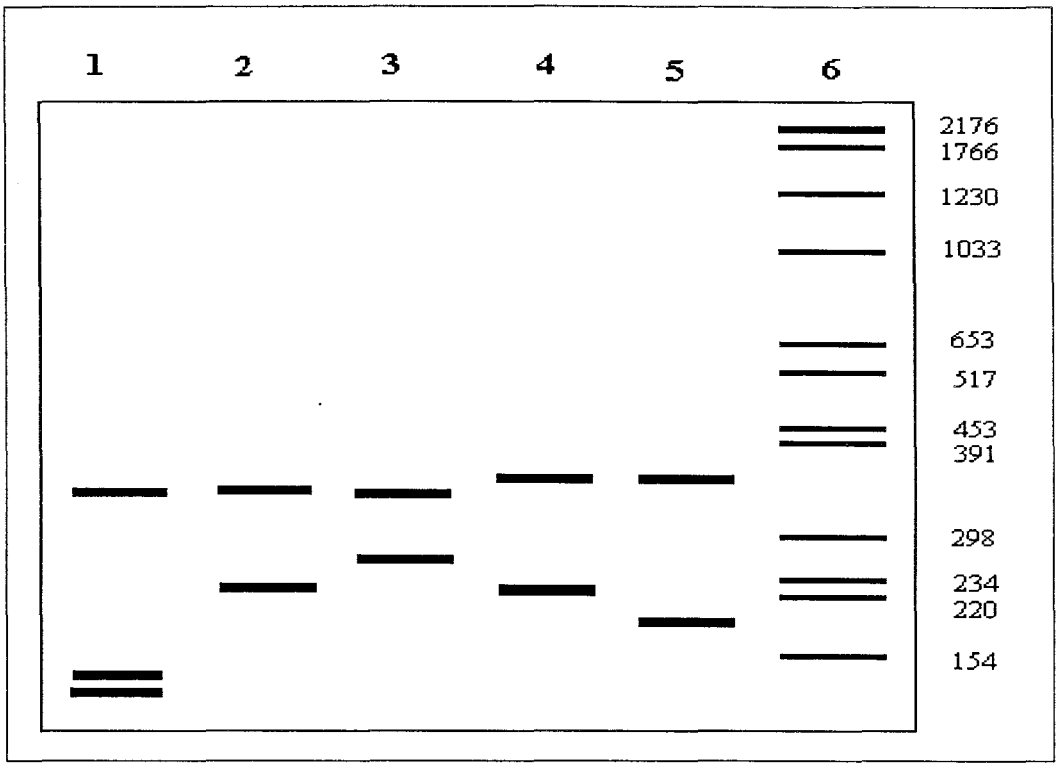


Figure 2.9: A diagrammatic representation of profiles of a PCR amplified D-loop fragment digested with different restriction endonucleases and resolved by SDS-PAGE. Lanes 1, 3, 4 and 5 represent the profiles generated by the enzymes *Hinf*I, *Eco*RI, *Sca*I and *Rsa*I respectively. Lane 2 represents the profile of a double digest (*Eco*RI-*Sca*I). Lane 6 represents the DNA molecular weight marker (DNA molecular marker VI, Boehringer Mannheim, Germany).

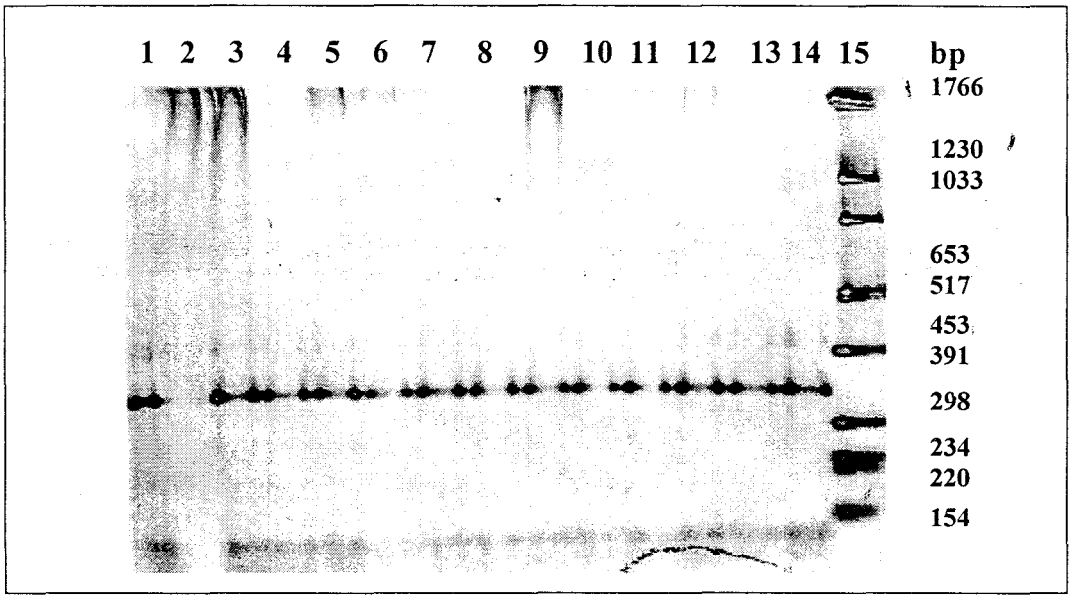


Figure 2.10: Restriction profiles of PCR amplified D-loop fragments digested with *Hinf*I. Lanes 1 to 14 show the *Hinf*I profiles that were obtained with all individuals tested. The DNA molecular weight marker (DNA Molecular Weight Marker VI; Boehringer Mannheim, Germany) is in lane 15. The restriction fragments were resolved by SDS-PAGE and visualized by silver staining.

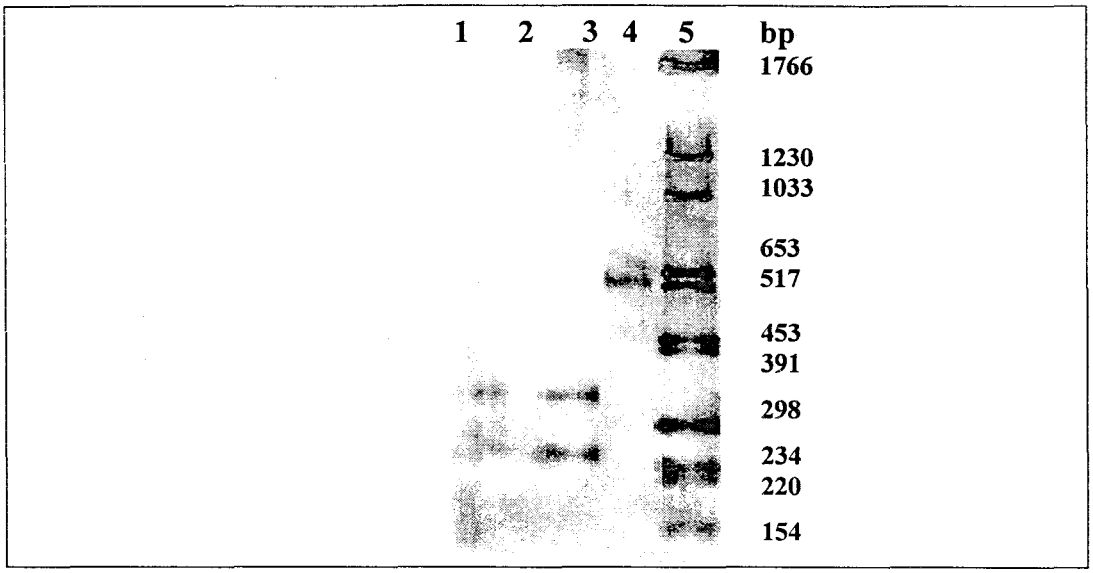


Figure 2.11: Restriction profiles of PCR amplified D-loop fragments digested with *Eco*RI. Lanes 1 to 3 show the *Eco*RI profiles that were obtained with all individuals tested. An undigested D-loop fragment is depicted in lane 4. The DNA molecular weight marker (DNA Molecular Weight Marker VI; Boehringer Mannheim, Germany) is in lane 5. The restriction fragments were resolved by SDS-PAGE and visualized by silver staining.

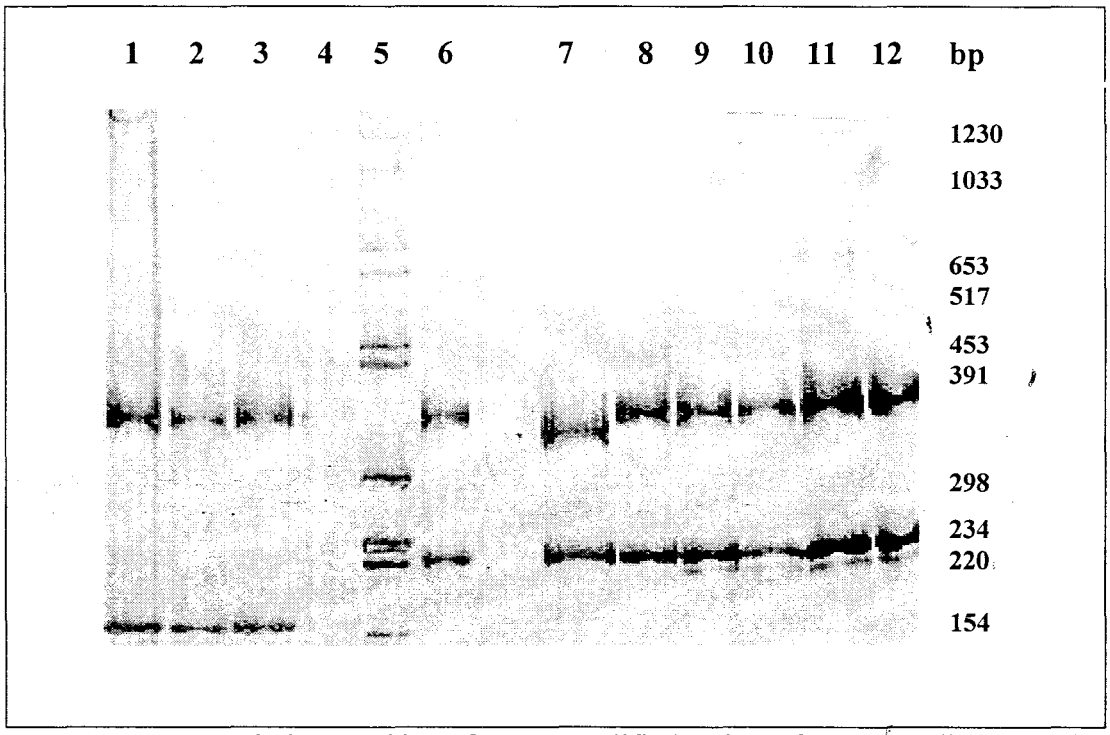


Figure 2.12: Restriction profiles of PCR amplified D-loop fragments digested with *Rsa*I, *Sca*I and the *Eco*RI-*Sca*I double digest. Lanes 1 to 4 show the *Rsa*I profiles and lanes 6 and 8 to 12 the *Sca*I profiles that were obtained with all individuals tested. Lane 7 shows the *Eco*RI-*Sca*I restriction pattern. The DNA molecular weight marker (DNA Molecular Weight Marker VI; Boehringer Mannheim, Germany) is in lane 5. The restriction fragments were resolved by SDS-PAGE and visualized by silver staining.

Multiplying the total number of restriction sites by the number of individuals (27) gives a total of 112 restriction sites that were assayed within the population of southern African ostriches (*S.c. australis*). A restriction map (Figure 2.13) could, however, be deduced for *S.c. australis* from the restriction pattern observed in the various gels (Figures 2.10 to 2.12) and the D-loop sequence data of Härlid *et al.* (1997).

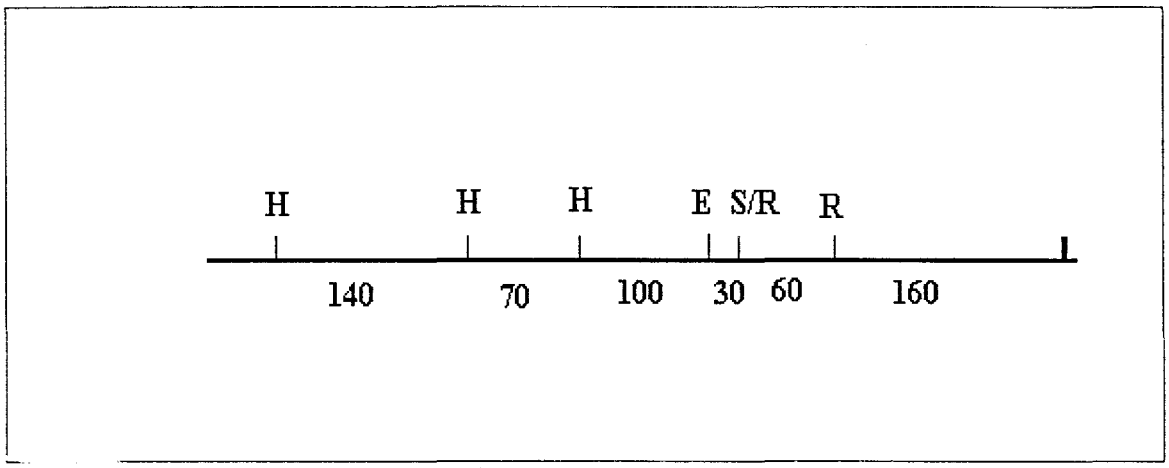


Figure 2.13: A restriction map of the D-loop fragments of *S.c. australis* that were assayed. The letters represent the first letter of the name of the restriction enzyme that is responsible for the restriction site (H = *Hinf*I, E = *Eco*RI, S = *Sca*I and R = *Rsa*I). The numbers indicate the approximate restriction fragment size as observed in the gels Figures 2.10 to 12 and the diagrammatic representation in Figure 2.9.

The fragment that was analysed in this study was 550 bp, representing about 50 percent of the 1 028 bp D-loop sequence of the ostrich (*Struthio camelus*: Härlid *et al.*, 1997). The PCR-RFLP analysis did not yield any polymorphisms between the different populations. This result was not expected although it is noted that the D-loop contains conserved sequences such as the consensus sequence that was found in the CSB-1 and in repeated sequences (Desjardins and Morais, 1990, Quinn and Wilson, 1993; Liu *et al.*, 1996; Härlid *et al.*, 1997, 1998). It should also be noted that whole blood was used for the extraction of DNA and genomic DNA was used as templates. The primers used in this study were universal primers and were extensively used for the amplification of vertebrate D-loop regions (Kocher *et al.*, 1993) and the result observed may thus suggest amplification of nuclear sequences homologous to the

D-loop as described by Sorenson and Fleischer (1996). Procedures that may prevent the amplification of possible nuclear sequences were not included in the protocol. However, the presence of such homologous nuclear D-loop sequences has not been shown in ratites, also the PCR amplification conditions were stringent and the restriction enzyme analysis results were comparable to sequence data of the ostrich D-loop that were published by Härlid *et al.* (1997). The fragments were thus assumed to be from mitochondrial origin.

The D-loop is heralded as the fragment of choice for population studies due to lower functional and structural constraints, showing greater mutational rates than fragments such as the cytochrome *b* or 12S rRNA gene (Cann *et al.*, 1987; Martin *et al.*, 1992; Meyer, 1994; Sorenson and Fleischer, 1996). In vertebrates and specifically in birds it was found that the nucleotide variation in the D-loop is greater than in the 12S rRNA gene (Desjardins and Morais, 1990; Quinn and Wilson, 1993; Härlid *et al.*, 1997, 1998).

2.4.4 SUMMARY

The D-loop fragments were not analysed by sequencing, but the results obtained here confirmed the results obtained by the analysis of the 12S rRNA. Both fragments (12S rRNA and D-loop fragments) were analysed by restriction enzymes and showed uniform haplotypes for all the individuals of *S.c. australis* from different geographic areas, representing presumably different lineages. Quinn and Wilson (1993) showed that the rate of sequence divergence in the D-loop of birds is much higher than the rate in mitochondrial rRNA genes. It was thus expected that the D-loop would show some differences, if any existed and that it would be more sensitive than the 12S rRNA gene. If D-loop differences were not observed, the opportunity of observing 12S rRNA differences would be expected to be even less.

The unexpected uniformity of haplotypes in the D-loop of the species presented here does not imply that the D-loop is unsuitable for population studies of ostriches. This result may be indicating that a low level of genetic variability exists between the populations in this study. It may, however, also be indicating that a much wider sample size should be included for such a study.

Martin *et al.* (1992) used the D-loop (2000 bp, entire fragment plus adjacent flanking regions) and a 12S rRNA to 16S rRNAfragment (1425 bp) in their study of the population structure of fish and found that the PCR-RFLP data generated from these fragments were more informative than the sequencing of a smaller portion of cytochrome oxidase I gene. They also found that the 12S rRNA fragment was not sufficiently variable to distinguish selected fish populations. They did, however, find several other advantages of the PCR-RFLP method that made it attractive for use in preliminary studies of nucleotide diversity (Martin *et al.*, 1992). The method is rapid, does not involve the purification of the mtDNA molecule and radioactivity for visualization of DNA and enables analysis of large numbers of individuals for a fraction of the cost of sequencing analysis (Martin *et al.*, 1992).

The PCR-RFLP method should be used to survey the mitochondrion for possible different haplotypes within the study groups. PCR amplified fragments from the various different haplotypes or different groups that may not show haplotype differences should be sequenced to determine the extent of the existing differences. The procedures should optimized to prevent the amplification of nuclear sequences that may be similar to the mtDNA sequences that are being studied.

The large volume of data that is available for avian genetic studies used analysis of sequences of the mitochondrial genomes as overviewed in Section 2.1. Sequence data are thus essential

for genetic studies of ostriches. The applicability of linking PCR-RFLP analysis methods to sequence analysis methods of the fragments that are being studied is demonstrated in this study. The results of the sequence analysis data and the PCR-RFLP data of the 450 bp 12S rRNA (Section 2.2. and Section 2.3) were compared to test the suitability of small conserved mitochondrial DNA fragments for resolving genetic diversity within southern Africa ostriches (*S.c. australis*). This fragment was unable to demonstrate any sequence differences between the populations of southern Africa included in this study.

The 450 bp 12S rRNA gene fragment of *S.c. molybdophanes* was also not sequenced. Sequence data could thus not be used for the intersubspecific analysis of ostriches. Future studies should attempt to sequence the entire 12S rRNA gene of *S.c. molybdophanes* and the other extant subspecies of ostriches.

Unfortunately financial constraints prevented determining the sequence of the PCR amplified 550 bp D-loop region of ostriches used in this study. The analysis approach that was conducted for the 12S rRNA gene fragment (comparing the PCR-RFLP analysis data to sequence data) could not be done for the D-loop fragment of the populations used in this study.

2.5 SYNTHESIS AND CONCLUSIONS

2.5.1 GENETIC STRUCTURING OF OSTRICHES

Results obtained here indicated that the populations of domesticated southern African ostriches, *S.c. australis,* that were sampled were indistinguishable by PCR-RFLP applied to the 450 bp 12S rRNA and 550 bp D-loop sequences of mtDNA. This observed lack of variation could be viewed as reflecting possible bottleneck events during domestication and subsequent selection processes of the southern African ostrich (*S.c. australis*) population.

However, care should be taken not to over-emphasise the importance of these observations since only a small section of the southern African population was sampled. Certain geographical populations that were part of a previous study (Freitag and Robinson, 1993) were not included in this study. The gene fragments that were used were also small and a limited number of restriction enzymes were used. The results are however in agreement with the study of Freitag and Robinson (1993) who also found low levels of genetic diversity.

The results indicate that approximately 1000 bp (about 6.02 %) of the mitochondrial genome of the southern African ostriches population (*S.c. australis*) were found to be invariant when analysed using 9 restriction enzymes. In contrast, Freitag and Robinson (1993) used 15 restriction enzymes and surveyed the entire ostrich mitochondrial genome (16 591 bp: Härlid *et al.*, 1997). The area that Freitag and Robinson (1993) sampled for their study covered the most of the geographic areas of southern Africa and they could only detect 6 maternal lineages in 78 individuals. They also found that lineages from Botswana were the most divergent when compared to those from the rest of southern Africa. The lowest genetic diversity was observed in populations from the Klein-Karoo (Oudtshoorn), Western Cape Province, Transvaal and Namibia (Freitag and Robinson, 1993). The lack of nucleotide variation in the small PCR-amplified mtDNA fragments (450 – 550 bp) within the sampled areas of the present study may thus not be extraordinary.

The low levels of intraspecific mtDNA sequence divergence observed for the ostrich population of southern Africa is comparable to that found in other avian species, such as red-winged Blackbirds (*Agelaius phoeniceus*; Avise *et al.*, 1988), Canada Goose (*Branta canadensis*; Shields and Helm-Bychowski, 1988) and Blue Tit (*Parus caeruleus*; Taberlet *et al.*, 1992) which are known to have suffered population bottle-necks in their recent evolutionary past. Low levels of mtDNA diversity were also observed in other vertebrates

such as fish (Avise *et al.*, 1986, 1988; Martin *et al.*, 1992; Moran and Kornfield, 1995) and mammals (koalas: Houlden *et al.*, 1996; African buffalo: Wenink *et al.*, 1998) that are known to have suffered population bottlenecks.

Factors that could have influenced the genetic variability of the present ostrich populations are the result of the recent evolutionary and agricultural history of ostriches. These factors include hunting, the effects of small founder populations, selection processes of the early ostrich farmers and abandoning of ostrich farms during economic depressions (Duerden 1920; Wagner 1986). Historical ostrich breeding practices are probably responsible for the observed lack of variation. The industry has been concerned with fixing traits favourable to feather production by using hybridization and inbreeding methods. These practices reduce genetic diversity resulting in a bottleneck and thus explain to some degree the low (zero) genetic diversity observed in *S. c. australis* populations.

Low levels of genetic diversity may also be a result of stochastic lineage-sorting and not bottleneck events alone (Avise *et al.*, 1994b). Events such as fluctuations in female numbers, random extinctions of populations (elimination of mtDNA haplotypes) or the selection of a particular mtDNA haplotype may also be the cause of limited genetic variability (Avise *et al.*, 1986; Bensch *et al.*, 1994). The low levels of genetic diversity observed within the domesticated southern African ostrich (*S. c. australis*) populations could thus be due to population bottleneck events and/or stochastic lineage-sorting processes (Freitag and Robinson, 1993).

The low levels of genetic diversity within the present populations do not necessarily imply that high variability existed in the past (Avise *et al.*, 1986; Moran and Kornfield, 1995; Houlden *et al.*, 1996). It is difficult to measure the effects of reduction in population size and

other disturbances on populations since the levels of genetic diversity that existed prior to these events are not known (Moran and Kornfield, 1995; Houlden *et al.*, 1996).

The impact of the rise in ostrich numbers after the domestication process was initiated, or the sudden decline in ostrich numbers after 1914 on the present limited genetic diversity is undetermined. It is however documented that small numbers of wild chicks were used to start the first domesticated ostrich populations during the mid 1800's (Wagner, 1986). These breeding populations were supplemented with new wild chicks until the industry was formalized towards the end of the previous century and inbreeding strategies were introduced (Duerden, 1920). The present breeding populations of the Little Karoo area (Oudtshoorn), which enjoyed special legal privileges since the 1940s, were restocked from populations that existed in the rest of southern Africa. This explains why Freitag and Robinson (1993) observed all the mitochondrial haplotypes present in the southern African ostrich population within the Oudtshoorn population. Although isolated populations from elsewhere in southern Africa were thought to be different lineages, evidence presented here shows that populations that were closed for 90 years (Swaziland and Grahamstown populations) exhibit haplotypes in small mitochondrial fragments that are indistinguishable from the rest the southern African ostrich population.

2.5.2 PROSPECTS OF THIS STUDY

2.5.2.1 APPLICATION OF PCR-RFLP ANALYSIS OF 12S rRNA SEQUENCES FOR INTERSUBSPECIFIC STUDIES OF OSTRICHES (*S.c. australis*)

The PCR-RFLP analysis of 12S rRNA sequence of *S.c. australis* and *S.c. molybdophanes* exhibited intersubspecific polymorphisms. This finding suggests the 12S rRNA gene could be highly informative across the extant subspecies. An extensive study that includes larger numbers of representatives from the different subspecies would be necessary to yield

conclusive results. PCR-RFLP analysis will afford the simultaneous analysis of individuals on single gels. The polymorphic DNA fragments can then be sequenced to determine the exact nucleotide substitution positions as well as base changes that do not affect restriction site changes (Martin *et al.*, 1992).

Freitag and Robinson (1993) could not detect any of the very divergent *S.c. camelus* haplotypes in the southern African domesticated populations and postulated that the sequence was diluted out of the population. Events that could have led to such a "dilution" would be deliberate extinction and back-crossing of the hybrids with *S.c. australis*.

It may be controversial to suggest the reopening of the search for *S.c. camelus* haplotypes but these haplotypes may still be present within the southern African population, even if in limited numbers. The breeding experiments are known to have yielded progenies with some of the desired feather characteristics, so it is feasible that some of the farmers would still have bred these birds, even if it was only in small numbers.

The introduced breeding stock of *S.c. camelus* consisted of both males and females. Duerden (1919) presented a detailed description of the breeding patters and origins of the parents. The study targeted direct crosses as well as back-crosses of both subspecies. Females and males of the two subspecies and hybrids were used in the experiments thus suggesting that no deliberate dilution of a particular mitochondrial genotype occurred by selective breeding.

An extensive study around the Middleburg-Cape area (where the breeding experiments were conducted), could detect the existence of remnants of the *S.c. camelus* genome. Since populations from Middleburg-Cape and surrounding areas were not included in the present or a previous study (Freitag and Robinson, 1993), the effects of the hybridization of *S.c. camelus*

and *S.c. australis* are still undetermined. As this area was the focal point of the 1912 hybridization experiments, a study specifically aimed at comparing the genetic diversity of this area to the rest of southern Africa would be interesting and should be conducted. In captivity, ostriches live for 30 to 40 years while some individuals reached higher ages (Wagner, 1986). It is thus likely that some of the mitochondrial genomes from individuals that were introduced 80 years prior to the survey may still be in the population. Although populations from the Middelburg area were not included in this study, the samples that were analysed were from populations with different breeding histories. It is also likely that the Oudtshoorn population may have had some of Middelburg hybrids as ancestors.

The 12S rRNA gene will not provide an opportunity to study the effects of hybridization but may present the opportunity to identify populations that contain *S.c. camelus* 12S rRNA haplotypes inherited from a *S.c camelus* maternal ancestor. However, the nuclear rRNA genes could be more readily targeted in such a study as they would show biparental inheritance. These genes were successfully used to indicate patterns of introgression in grasshoppers (Arnold *et al.*, 1987) and grass shrimps (Garcia and Davis, 1994), so the precedent for such a study has been set.

2.5.2.2 APPLICATION OF PCR-RFLP ANALYSIS OF mtDNA SEQUENCES FOR POPULATION STUDIES OF OSTRICHES (*S.c. australis*)

From the literature it is evident that large mtDNA fragments and nuclear fragments may be more informative when used for PCR-RFLP analysis than the smaller fragments used in this study. In previous studies larger fragments yielded more conclusive results than shorter fragments of the same gene (Martin *et al.*, 1992; Cronin *et al.*, 1993; Lee *et al.*, 1997; Evans *et al.*, 1998; van Tuinen *et al.*, 1998). Martin *et al.* (1992) used the entire D-loop fragment plus adjoining sequences for fish populations. This was found to be more informative than

sequence data sets from small coding genes. Cronin *et al.* (1993) made similar observations as Martin *et al.* (1992). Evans *et al.*, (1998) found that a 1300 bp fragment (that included the D-loop) digested by only three restriction enzymes provided genetic markers for inter- and intraspecies of asteriod larvae (*Asterias amurensis*) identification.

Studies by Mindell *et al.* (1998) on avian species demonstrated that the gene order in the vicinity of the D-loop (upstream and downstream) may be subjected to rearrangement. Their results suggest that these rearrangements may be diagnostic for the oscine and suboscine birds and that gene rearrangement characters may have phylogenetic utility. The discovery of an additional non-coding region that may or may not share similarities with the D-loop and that may or may not contain repeat sequences also has potential for phylogenetic analysis (Mindell *et al.*, 1998)

The examples that were referred to within this study clearly illustrate that the region of the mitochondrion where large quantities of genetic evolutionary information is contained stretches from the cytochrome *b* gene to the 16S rRNA region. This region of the ostrich mitochondrion is 5 580 bp (Härlid *et al.*, 1997) and, unlike other vertebrate mitochondria, contains the ND 6 gene. The latter gene showed significant nucleotide variations between avian taxa (Härlid *et al.*, 1998) and may have applications for use in ostrich genetic studies. Cytochrome *b* gene sequences were very informative for kiwi populations, providing genetic markers for some of the populations (Baker *et al.*, 1995). Cytochrome *b* is also able to resolve genetic relationships of taxa at various hierarchical levels of the avian evolutionary tree (Taberlet *et al.*, 1992; Meyer, 1994; Baker *et al.*, 1995; Härlid *et al.*, 1997, 1998; Lee *et al.*, 1997; van Tuinen *et al.*, 1998; Härlid and Arnason, 1999). Further investigation into the variability of this region is thus recommended for genetic studies of ostriches. The region from the cytochrome *b* gene to the D-loop (inclusive) is 2 900 bp in length and the region

from the D-loop to the 16S rRNA gene is 2 700 bp (excluding the D-loop). Primers can be designed that would amplify the entire region and allow subsequent RFLP analysis. Only through such additional research would answers be provided as to whether the mtDNA data are unable to provide resolution of population level differences in ostriches.

2.5.2.3 APPLICATION OF SEQUENCE ANALYSIS OF mtDNA FOR INTERSUBSPECIFIC AND POPULATION STUDIES OF OSTRICHES (*Struhio camelus*)

Although the 450 bp 12S rRNA sequence data presented in this study did not show any population differences, sequence data of this and other fragments may be informative in so far as intersubspecies relationships are concerned. The PCR-RFLP data of the 450 bp 12S rRNA revealed subspecies differences. These differences and possible additional differences may be revealed by the sequencing this fragment of the different subspecies. Future research on subspecies relationships of the ostrich (*S. camelus*) should pursue sequencing of various mitochondrial fragments (such as the D-loop and cytochrome *b* fragments) of the different subspecies and determining the nucleotide divergence between these fragments. Data from such studies should be compared to that of other avian data sets of the same fragments.

The variation patterns of these fragments within and between domesticated and wild populations of the various extant subspecies of ostrich should also be investigated. This aspect may be important for systematic as well population genetic purposes. Data obtained by sequencing methods may also (or not) reflect genetic bottle-neck events within other subspecies of ostriches.

Although sequencing is more costly than methods such as PCR-RFLP, it is an important high resolution method for determining nucleotide diversity and should be used for genetic studies

of ostriches. The PCR-RFLP is a cost and time efficient method that could be used for preliminary studies. Strategies that apply PCR-RFLP to specific genetic questions of ostriches are discussed in Sections 2.5.2.1 and 2.5.2.2. The results from these strategies should be verified by sequence data.

Chapter 3

Randomly Amplified Polymorphic DNA (RAPD) Analysis of Genetic Diversity of Domesticated Ostriches from Southern Africa

3.1 GENERAL INTRODUCTION

DNA fingerprinting methods based on Jeffreys and other probes (Jeffreys *et al.*, 1985b; Gazit and Gazit, 1990) or PCR-based fingerprinting such as RAPDs (randomly amplified polymorphic DNA sequences: Welsh and McClelland, 1990; Williams *et al.*, 1990) are very informative at the intrapopulational level. Both DNA fingerprinting methods can analyse more loci per individual than direct sequencing (Dowling *et al.*, 1990; Dodgson *et al.*, 1997), and several individuals can be analysed on a single gel (Jeffreys *et al.*, 1985b; Williams *et al.*, 1990; Hedrick, 1992; Hadrys *et al.*, 1992). These methods are thus suitable for analysis of large population sizes and more cost effective than direct sequencing (Chambers, 1994; Dodgson *et al.*, 1997).

The RAPDs analysis method evolved from the Jeffreys approach and has several advantages over its predecessor (Welsh and McClelland, 1990; Williams *et al.*, 1990). No sequence information about the genome to be studied is required. The isolation and cloning of probes is eliminated since detection could be done through labelling the primer (Carrano *et al.*, 1989; Kessler, 1994; Preus and Russel, 1994), by staining procedures (Goldman and Merril, 1982) or fluorescence (Lins *et al.*, 1996). Minute quantities of DNA are required as template for PCR (Jeffreys *et al.*, 1988; Li *et al.*, 1988; Higuchi *et al.*, 1992), and non-destructive sampling procedures could be followed, even for very small organisms (Grimberg *et al.*, 1989; Kirby, 1990; Williams *et al.*, 1990). A universal set of primers could be used to analyse the genome of a variety of taxa and species (Williams *et al.*, 1990). Procedures (from DNA isolation to the analysis of RAPD fingerprints) can be standardized for collaborative work. An added advantage is that this relatively uncomplicated, time and cost effective procedure does not require handling of radioactive material for detection of amplified fragments (Goldman and Merril, 1982; Williams *et al.*,1990; Kazan *et al.*,1993a; Neilan, 1995). The possibility of automating the technique is one of the major advantages (Williams *et al.*, 1990). This would

facilitate and streamline large-scale applications such as in epidemiological and agricultural breeding studies. Another advantage is tailoring the RAPD profiles to produce simple patterns for genetic mapping and more complex patterns for genotyping. (Caetano-Anollès *et al.*, 1991).

3.1.2 DNA FINGERPRINTING WITH RAPD MARKERS

In the RAPD technique, a single (sometimes two) oligonucleotide primer of random sequence is used to amplify polymorphisms by means of the polymerase chain reaction. The set of amplified DNA fragments is resolved on agarose (Williams *et al.*, 1990) or polyacrylamide gels (Welsh and McClelland, 1990) and can be visualized using ethidium bromide in agarose gels (Williams *et al.*, 1990) or by silver staining in polyacrylamide gels (Welsh and McClelland, 1990). These staining methods could, however, be used in either of the electrophoresis matrices. Although silver staining is more time consuming and more expensive, it is also more sensitive than ethidium bromide (Maniatis *et al.*, 1989; Welsh and McClelland, 1990; Caetano-Anollès *et al.*, 1991). Polymorphisms are scored as absence or presence of bands (Welsh and McClelland, 1990; Williams *et al.*, 1990).

3.1.2.1 INHERITANCE OF RAPD MARKERS

Using inbred lines of soya bean strains, Williams *et al.* (1990) was the first study to show Mendelian inheritance of RAPD markers. Test crosses in conifers (Carlson *et al.*, 1991) and experiments with honey bees (Hunt and Page, 1992) confirmed the findings of Williams *et al.* (1990). Carlson *et al.* (1991) showed how RAPD data from the test crosses of conifers could be applied to construct segregation maps for the different genotypes. Hunt and Page (1992) used honey-bees (these are haplo-diploid) and showed how several types of RAPD polymorphic markers were inherited in these organisms. They could conclusively show that the RAPD method would be useful for population studies of honey bees. The Mendelian

inheritance of RAPD markers was shown by Lu *et al.* (1995) studying *Pinus sylvestris*. RAPD technology could be be applied to natural populations, where no pedigree information was available (Matioli and de Brito, 1995; Pérez *et al.*, 1998).

The implication from the Mendelian inheritance patterns of RAPD markers is that they are usually nuclear. Aagaard *et al.*, (1998) however, showed that RAPDs could also originate from mitochondrial DNA. The RAPD markers of mitochondrial origin showed lower diversity than those of nuclear origin.

3.1.2.2 SOME PITFALLS AND PRECAUTIONS IN RAPDs APPLICATIONS

The major concerns associated with this technique lie in understanding the basis of the variation that is targeted, the repeatability of experiments and problems concerning the dominance of the markers (Lamboy, 1994; Lynch and Milligan, 1994; Schweder *et al.*, 1995; Pérez *et al.*, 1998). Although RAPD analysis is a technically uncomplicated procedure, it requires a fair amount of skill and practical experience to yield reproducible results. These results should be statistically analysed and interpreted without unnecessary bias introduced by technical inconsistencies (Lamboy, 1994). It is a multivariate analysis system that requires optimization for a particular application (Schweder *et al.*, 1995; Virk *et al.*, 1995; Boleda *et al.*, 1996). A slight change in one of the parameters will have a great effect on the final result (Boleda *et al.*, 1996). Researchers should thus include regulatory steps in the procedure to prevent any distortions of the results (Apostol *et al.*,1993; Lynch and Milligan, 1994; Virk *et al.*, 1995).

Methods of DNA extraction should be standardized. Unclean DNA may prevent PCR amplification of certain fragments and promote the amplification of others, and the results of duplicate experiments may vary considerably (Schweder *et al.*, 1995; Virk *et al.*, 1995). A

method should be selected that provides clean, high quality DNA of considerable yield from minimum amounts of tissue. For a single set of investigations the same thermal cycler should be used under a standard set of conditions (Schweder *et al.,* 1995). PCR mixtures should be optimised and remain constant throughout the experiments (Innes and Gelfand, 1990).

Differential staining results in varying intensities of bands. For applications where densitometers or automated scanners are used, this practice is highly unsuitable. Identical bands in a gel or in different gels will be perceived as different and included in the analysis as such, influencing the result negatively. Standard staining methods and protocols should thus be used (Bassam *et al.,*1991).

Duplicate runs are also essential and standard conditions should be used for all experiments that will be included in a particular analysis (Schweder *et al.,* 1995). Besides the potential technical problems that may be encountered, PCR carry over (contamination of PCR mixes by extraneous DNA or other PCR products) must be prevented (Heinrich, 1991).

3.1.2.3 APPLICATIONS AND USEFULNESS OF RAPDs

Initial evaluations of RAPDs were conducted using the method without directly comparing the data to other established molecular methods (Carlson *et al.* 1991; Welsh *et al.,* 1991; Hunt and Page, 1992; Tulsieram *et al.*, 1992; van Heusden and Bachmann, 1992). Due to the concerns about this method several laboratories started using these highly informative markers in tandem with other genetic markers (Kaemmer *et al.*, 1992; Barua *et al.*, 1993; Cruzan and Arnold, 1993, 1994; Kazan *et al.*, 1993a; Thorpe *et al.*, 1994; Williams *et al.*, 1994; Neilan, 1995; Cohen *et al.*, 1997).

In one of the first evaluations of the technique a whole range of applications was investigated, from genetic diversity in bacteria, plants and mammals to zygosity in human twins (Caetano-Anollès *et al.*, 1991). This study demonstrated inheritance patterns of RAPD bands, the ability of the method to distinguish self-pollinating plants and that twins produced identical RAPD profiles. RAPD analysis was also successfully employed in applications of plant and animal genetic studies. Several of these applications are listed in Table 3.1.

The effectiveness of RAPDs had been also been shown in applications such as forensic medicine, human genetics and inheritance of human diseases, animal husbandry, determining zygosity in twins (Welsh and McClelland, 1990; Williams *et al.*, 1990; Caetano-Anollès *et al.*, 1991; Hadrys *et al.*, 1992; Cushwa and Medrano, 1996; Rao *et al.*, 1996; Smith *et al.*, 1996; Sharma *et al.*, 1998), biogeography and systematics (Chalmers *et al.*, 1992; Chapco *et al.*, 1992b; van Heusden and Bachmann, 1992; Thorpe *et al.*, 1994; Dawson *et al.*, 1995; Kappe *et al.*, 1995; Stammers *et al.*, 1995; Tollefsrud *et al.*, 1998), mating patterns (Hunt and Page, 1992; Apostol *et al.*, 1993), identification of cross-contamination of cell lines with cell cultures (Schlegel *et al.*, 1996), sexing of birds (Lessells and Mateman, 1998) and, especially pertinent to this study, sexing of ostriches ostriches (Bello and Sánchez, 1999).

Table 3.1: Some applications of RAPDs at various taxonomic hierarchic levels of animals and plants showing (where releveant) methods that the result of the RAPD study was compared to. In the table the following abbreviations were used: B = Birds, M = Mammals, R = Reptiles, I = Insects, P = Plants and NR = not relevant, MLLE = multilocus enzyme electrophoresis.

	TAXON	COMMON NAME	APPLICATION	TAXONOMIC HIERARCHIC LEVEL	METHOD COMPARED TO	REFERENCE
B	*Struthio camelus*	Ostriches	Sexing	Intraspecific	NR	Bello and Sánchez, 1999
B	*Parus major* *Haematopis ostralegus*	Great tits Oyster catcher	Sexing	Intraspecific	Sequencing	Lessels and Mateman, 1998
B	*Numida meleagris*	Guinea fowl	Breeding	Intraspecific	NR	Sharma *et al.*, 1998
B	*Stercorarius pomarinus*	Skua	Systematic	Inter- and Intraspecific	Allozymes, mtDNA RFLP	Cohen *et al.*, 1997
B	*Gallus gallus.*	Chickens	Breeding	Intraspecific/ Population	NR	Smith *et al.*, 1996
	Meleagris gallopavo	Turkeys	Breeding	Intraspecific/ Population	NR	Smith *et al.*, 1996
B	*Picoides borealis*	Woodpeckers	Conservation	Intraspecific/ Population	Allozymes, Classical	Haig *et al.*, 1994
M	*Cervus elaphus* *Sus scrofa*	Red deer Wild boar	Conservation	Population	NR	Pérez *et al.*, 1998
M	*Bos sp*	Cattle	Systematic/ Breeding	Interspecific/ Intraspecific	NR	Cushwa and Medrano, 1996 Rao *et al.*, 1996
M	*Ovis aries*	Sheep	Systematic /Breeding	Interspecific	NR	Cushwa and Medrano, 1996 Rao *et al.*, 1996
M	*Capra hircus*	Goat	Breeding/ Systematic	Interspecific	NR	Cushwa and Medrano, 1996 Rao *et al.*, 1996
M	*Sus scrofa*	Pig	Breeding	Intraspecific	NR	Cushwa and Medrano, 1996
M	*Phoca vitulina* *Halichoerus grypus*	Seals	Conservation	Inter- and Intraspecific/ Population	DNA fingerprinting	Kappe *et al.*, 1995
R	*Galotia sp.*	Lizards	Conservation / Biogeography	Inter- and Intraspecific	RFLP of mtDNA, Sequence	Thorp *et al.*, 1994
I	*Quadraspidiotus sp.*	Insects	Pheromone tests	Interspecific	Chemical	Frey and Frey, 1998
I	*Aphis gossypii*	Aphids	Biocontrol- host based differentiation	Intraspecific	Classical	Vanlerberghe-Musutti and Chavigny, 1998
I	*Ceratitis capitata*	Medfly	Population study	Intraspecific	MLEE	Baruffi *et al.*, 1995
I	*Solenopsis*	Fire ants	Hybrid zone	Intraspecific	Allozymes	Schoemaker *et al.*, 1994

	TAXON	COMMON NAME	APPLICATION	TAXONOMIC HIERARCHIC LEVEL	METHOD COMPARED TO	REFERENCE
I	*Aedes aegypti*	Mosquito	Pedigree	Intraspecific/Population	NR	Apostol *et al.*, 1993
I	*Anax parthnope*	Dragonflies	Paternity in polygamous mating systems	Intraspecific/Population	Field studies	Hadrys *et al.*, 1993
I	*Apis mellifera*	Bees	Pedigree	Intraspecific	NR	Hunt and Page,1992
I	*Melanoplus sp.*	Locust	Conservation	Intraspecific Population	NR	Chapco *et al.*, 1992
P	*Pinus Strobus*	White Pine	Paternity	Inter- and intraspecific	Isozymes	Isabel *et al.*, 1999
P	*Tragopogon*	Asteraceae	Tracing plant origins	Intraspecific	NR	Cook *et al.*, 1998
P	*Pseudotsuga menziesii*	Douglas fir	Conservation	Intraspecific	mtDNA + cpDNA	Aagaard *et al.*, 1998
P	*Haloragedendron lucasii*	Haloragaceae	Clone identification/ Conservation	Population	Allozymes	Sydes and Peakall, 1998
P	*Saxifraga sp.*		Conservation	Interspecific	NR	Tollefsrud *et al.*, 1998
				Population	NR	Bauret *et al.*, 1998
P	*Oryza glumeapatula*	Rice	Breeding	Population	Isozymes	Busso et al., 1998
P	*Scizachyrium scoparium*	Grass	Environment restoration	Population	NR	Huff *et al.*, 1998
P	*Acorus gramineus*	Araceae	Conservation	Inter- and Intraspecific	NR	Liao and Hsiao, 1998
P	*Aloe sp.*	Aloe	Hybrid zone	Interspecific	Morphological	Barker *et al.*, 1996
P	*Argyroxiphium sp.*	Mauna Kea silversword	Population/genetic bottle-neck/ Conservation.	Inter- and intraspecific	NR	Friar *et al.*, 1996
P	*Pinus pinaster*	Pine	Breeding	Population	Protein	Plomion *et al.*, 1995
	Pinus sylvestris		Pedigree analylsis		NR	Lu *et al.*, 1995
P	*Picea sitchensis*	Spruce	Clone identification /Breeding	Population	NR	Van De Ven and McNicol, 1995
P	*Picea abies*	Spruce	Breeding	Population	NR	Bucci and Menozzi, 1995
			Geneflow/Breeding	Population	PCR-RFLP cpDNA	Dawson *et al.*, 1995
P	*Lolium/Festuca*	Grasses	Systematic	Inter- and Intraspecific	Classical	Stammers *et al.*, 1995
P	*Iris sp.*	Iris	Conservation – Hybridzones	Inter- and Intraspecific	cpDNA, mtDNA	Cruzan and Arnold, 1993, 1994
P	*Stylosanthes sp.*	Legumes	Systematic/ Breeding	Inter- and Intraspecific	Morphology	Kazan *et al.*, 1993a,b

The results of these carefully constructed experiments and simulations by the investigators cited above and in Table 3.1, makes allowance for the conclusion that RAPD markers are suitable for:

(1) genetic linkage mapping,

(2) genetic diversity studies,

(3) generation of hybridization probes from total DNA for RFLP analysis,

(4) generation of single copy RAPD markers from cDNA,

(5) determining the degree of heterozygosity within individual parents by amplifying DNA from single gametes,

(6) determining zygosity in twins,

(7) epidemiological studies,

(8) paternity testing,

(9) forensics: medicine and illegal trade in wild life,

(10) detecting disease resistant strains of plants and animals,

(11) developing breeding programmes

(12) sex determination in birds.

The primary objective of this part of the research was to use the RAPD method to collect information on genetic variation patterns in ostriches. The RAPD technique was evaluated for its ability to:

1. distinguish different ostrich subspecies;
2. distinguish different geographic populations and to determine genetic variability within and between these populations;
3. provide information that may be useful for breeding and management decisions;
4. determine zygosity of twin ostriches.

These four objectives compromise four studies, each discussed as a separate entity below.

3.2 APPLICATION OF RAPDs TO OSTRICH TAXONOMY

3.2.1 INTRODUCTION

The aim of this study was to evaluate RAPDs for its ability to produce markers that could be used for taxonomic purposes. Previous genetic studies of ostriches at subspecies level either targeted cytoplasmic DNA (mtDNA: Freitag and Robinson, 1993; Chapter 2 of this thesis) or nuclear DNA (microsatellites: Kumari and Kemp, 1998). Limitations of these cited methods lie in the time that may be consumed when large sample sizes need to be analysed and the level of expertise required to generate results. The RAPD method, on the other hand, is cost and time effective, technically reasonably simple and could be advantageous to ostrich genetic studies at the subspecies level.

The taxonomic status of ostriches and implications of the hybridization experiments of the early 1900s were discussed in Chapter 1. Commercially, the different subspecies are cross-bred to improve breeding and production qualities of domesticated ostriches. To maintain the diversity that is still prevalent amongst ostrich subspecies, without compromising agricultural progress, it would be important to focus on the taxonomy of the subspecies. The characters that are to be used for analysing taxonomic relationships have important ramifications for population and subspecies management. Some of these ramifications include arguments about the value of the type of data, and philosophical approaches, that are used to analyse the data (Hillis and Moritz, 1990). It is thus essential that these characters reflect systematic differences that are of evolutionary significance, without compromising the effectiveness of the method (Ballou and Cooper, 1992). Many researchers have thus employed multi-marker approaches in which characters from two or more different sources, such as cytoplasmic DNA, nuclear DNA and allozymes (eg. Cruzan and Arnold, 1993, 1994; Shoemaker *et al.*, 1994; Baker *et al.*, 1995; Cohen *et al.*, 1997; Buso *et al.*, 1998; Aagaard *et al.*, 1998; Sydes and Peakall, 1998), are used.

Examples of this multi-marker approach in avian studies include taxonomic studies of skuas (Cohen *et al.*, 1997). In this study ectoparasite, allozyme, RAPD, sequence and mtDNA RFLP data were analysed and used to resolve phylogenetic relationships between skuas and gulls. Baker *et al.* (1995) used a similar multi-marker approach to study kiwi populations in New Zealand. Their results showed extreme population structuring of the kiwi populations. The multi-marker approach was also used to resolve issues in the phylogeny of the ratite group (Lee *et al.*, 1997). All the available data sets (DNA-DNA hybridization, several large mtDNA sequences and morphology data) for ratites were pooled to provide a solution to the conflict that existed for decades between morphological and molecular data sets. The pooled data supported the relationships based on the morphological data (Lee *et al.*, 1997).

Genetic studies of ostriches in which mtDNA data sets (Freitag and Robinson, 1993) and microsatellite information (Kumari and Kemp, 1998) were independently generated showed relationships between ostrich subspecies (Figure 1.2 in Chapter 1). The relationships that were indicated by these data sets were similar to the classification that was based on morphological data such as those presented by Brown *et al.* (1982). The divergence times between subspecies (Table 1.1) within the species *Struthio camelus* is, however, only based on RFLP analysis data of mtDNA. The deep division between *S.c. molybdophanes* and the other three subspecies were confirmed by microsatellite analysis (Kimwele *et al.*, 1998). It may thus be necessary to confirm the relationships of the different ostrich subspecies and divergence times by data for other methods that target a range of molecular markers. RAPDs could be one of these methods.

Uncontrolled crossing of ostrich subspecies by ostrich breeders may also present the ostrich industry with "new" hybrid (cross-breed) ostriches. In practice ostriches are traded on the basis of their phenotypic characteristics and their 'genetic' lineage. However, limited genetic

methods are available that could be utilised for diagnostic tests of ostriches. The diagnostic tests available can be summarised as:

(i) distinguishing ostrich subspecies by RFLP analysis of mtDNA (eg. Freitag and Robinson, 1993) or by microsatellite analysis (eg. Kumari and Kemp, 1998)

(ii) parentage determination in *S.c. massaicus* by microsatellite analysis (eg. Kimwele *et al.*, 1998)

(iii) determining the sex of infant ostriches by a RAPD based analysis method (eg. Bello and Sánchez, 1999).

The microsatellite tests are applicable to two of the four extant subspecies (*S.c. massaicus* and *S.c. molybdophanes*), and have not yet been tested on *S.c. australis* and *S.c. camelus* and hybrids of these two subspecies. Taxonomic data from nuclear markers for some of the ostrich subspecies are thus still lacking. Studies of ostrich taxonomy that utilises nuclear markers and include all the subspecies are still required.

In the light of these limited tests and data that are available some commercial breeders may be unsure about the origin of their ostrich lineages. This was evident in a claim by an American ostrich breeding concern, which claimed to have used Israeli ostriches to produce a unique ostrich lineage. However, true Israeli ostriches were from the extinct subspecies *S.c. syriacus* (Grizmek, 1970, Brown *et al.*, 1982; Freitag and Robinson 1993). The ostriches that were used by these breeders were probably from another subspecies although they might have been purchased from Israeli farms. Only genetic information could provide conclusive evidence about the origin of these "Israeli" ostriches.

Bello and Sánchez (1999), the research team that developed a RAPD method to determine the sex of juvenile ostriches, was recently presented with a problem of identifying the subspecies of the ostriches that they worked with. Their attempts using microsatellite analysis

were unsuccessful (Bello, personal communication). This example reflects unfavourably on the methods presently available to resolve taxonomic questions of ostriches at the subspecies level

It is thus necessary to focus on taxonomic studies of ostriches and to generate more data from a range of mtDNA and nuclear DNA markers. Data from such studies would be essential in the future management of these birds. In the process methods that are more time and cost effective than the presently available methods and with a capacity to analyse large data sets (such as RAPD analysis) could be developed. The intention of this limited study was to determine if significant differences could be shown between selected subspecies, using the RAPD method.

3.2.2 MATERIALS AND METHODS

3.2.2.1 SAMPLES

The DNA samples that were used in this part of the study were supplied by the Mammal Research Institute of the University of Pretoria and are the same as those used in the analysis of Freitag and Robinson (1993). The origin of the DNA samples is provided in Appendix B. The DNA was isolated by the method of Wetton *et al.* (1987). The DNA was stored at –20°C.

Three subspecies (*S.c. massaicus*- one representative sample, *S.c. molybdophanes*- one representative sample and *S.c. australis*- two representative samples) were used in this study.

3.2.2.2 RAPD-PCR

The 50µl PCR mixture contained 100 ng genomic DNA, 400 mM dNTPs (Boehringer Mannheim, Germany), 100 picomoles of primer, 1 unit *Taq* polymerase (Biolabs, New England), 1 X *Taq* reaction buffer [50 mM Tris·HCl (pH 9.0), 500 mM KCl, 15 mM MgCl,

1% triton X-100) (Promega, USA)]. Additional $MgCl_2$ (Promega, USA) was added to a final concentration of 3.5 mM. One percent (w/v) acetamide (Sigma, USA) was included to enhance the reproducibility of the PCR reactions. These PCR conditions were found to be most suitable for generating reproducible RAPD profiles. Precautions were taken to avoid PCR carry-over and contamination of PCR reagents. The precautions included autoclaving reagents and equipment (where reagents were or equipment was autoclavable); using fresh, sterile DNA free reagents; separating work areas into DNA isolation, PCR preparation and PCR analysis areas; capping microfuge tubes immediately after transfer of reagents when PCRs were prepared; cleaning the work areas with 70 % ethanol immediately before and after use. Pipette tips were only used to transfer one type of reagent and only once for transfer of primer and DNA. New latex gloves were worn when PCRs where prepared.

A James Duncan (model 8012) water-cooled thermal cycler was used for PCR amplifications. The reaction profile was 94°C for 3 minutes initial denaturation followed by 40 cycles of: 94°C for 30 seconds, 37°C for 30 seconds and 72°C for 60 seconds. After the final cycle the samples were held at 72°C for 5 minutes.

3.2.2.3 PRIMER SELECTION

Synthetic oligonuleotides of random sequence (G-C content of 50, 70 and 80 %) were used as primers. Details of these primers are indicated in Table 3.2. The primers were screened for their ability to produce individual-specific RAPD profiles of ostrich genomes. Eight primers and a primer combination [multiplex RAPDs] (primer RUM-P3 and primer RUM-P4 at equal concentrations) were screened.

3.2.2.4 ELECTROPHORESIS

Discontinuous SDS-Polyacrylamide gels (Laemmli, 1970) were used to separate the PCR amplification products (See Section 2.2.6 for details). All gels were subjected to electrophoresis at 150 V for 5 hr in 1 X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), using a Hoefer (SE 400) vertical electrophoresis system. The gel dimensions were: 18 cm wide, 16 cm long and 0.15 cm thick.

Ten percent (w/v) acrylamide gels were used for resolving the PCR amplified fragments. These gels resolved fragments of sizes between 200 bp and 1600 bp. This size range contained a large number of DNA fragments. Very few bands were detected that were smaller than 150 bp, eliminating the use of resolving gels of higher acrylamide concentrations. Bands greater than 1600 bp were not very well resolved, even when 5 % (w/v) acrylamide resolving gels were used. As the gel system was very sensitive to pH changes, the resolving gel buffer and stacking gel buffer had to be maintained at pH 8.8 and 6.8 respectively. The running buffer was 1 x TBE (pH 8.3). A molecular weight marker, pBR322 (Boehringer Mannheim), digested with *Hinf*I (Boehringer Mannheim) was included on each gel as a reference.

3.2.2.5 VISUALIZATION OF RESOLVED DNA FRAGMENTS BY SILVER STAINING

Visualization of the resolved DNA fragments was by a silver staining method (Bassam *et al.*,1991; Caetano-Anollès *et al.*, 1991). The procedure is the same as described in Section 2.3.2.4.

The silver staining protocols were strictly adhered to and fresh reagents were used for development of the silver/DNA complexes in the gel. The silver nitrate solution, however, was used more than once. After a maximum of 5 gels it was replaced by fresh solution. The

silver staining procedure was highly sensitive and detected a large number of bands in each profile.

3.2.2.6 ANALYSIS OF DATA SETS

Two methods were used to analyse the RAPD data from the gels. Both are computer based but utilise RAPD data differently. The RAPDistance version 1.03 is a semi-automatic and the GelCompar version 4.0 a completly automatic method. GelCompar software allows for manual manipulation after the gel image is loaded in the database. Similarity matrices can then be calculated by various algorithms. The matrices can be used to generate trees such as dendrograms.

The RAPDistance version 1.03 software uses matrices scored for present/absent of bands in the RAPD profiles (Armstrong *et al.*, 1994). The matrices are manually scored and can be subjective. However it, has a major advantage in that the matrices of several primers can be pooled. Several algorithms are available for determining similarity matrices. The algorithms used in this study are shown in Table 3.4. Similarity data can be graphically depicted in the form of Neighbour Joining Trees and UPGMA (Unweighted Pair Group Method using Arithmetic averages) trees.

(a) Semi-automated Analysis by RAPDistance version 1.03

Photographs were used to determine the positions of clearly distinguishable bands. Only these distinct bands were included in the data set. Presence/absence (1/0) matrices were compiled for each of the primers RUM-P2, RUM-P8 and RUM-P12. Band positions in each individual RAPD profile were recorded against a molecular weight marker (pBR322 digested with *Hinf*I) and assigned a '1' for presence and a '0' for absence of that band.

The RAPD data sets for the different primers were pooled and the pairwise distances were calculated using algorithms from Excoffier *et al.* (1992), Upholt (1977), Dice (1945), Kulczynski (1927)(Table 3.4), supplied by RAPDistance software. Neighbour Joining Tree (NJTREE) version 2.0 software was used to construct Neighbour Joining Trees for each of the algorithms and these are presented in Section 3.2.3.2.

(b) Automated Analysis by GelCompar version 4.0

GelCompar version 4.0 (GelCompar) was used to analyse the RAPD profiles generated by primers RUM-P2, RUM-P8 and RUM-P12. The Pearson product-moment correlation coefficient clustering, using Ward's algorithm, provided consistent results and was selected for analysis. Ward's algorithm is similar to the UPGMA. The resultant dendrograms are depicted in Section 3.2.3.2

Besides band position, the program also takes band intensity into account for analysis of profiles.

3.2.3 RESULTS AND DISCUSSION

3.2.3.1 PCR, ELECTROPHORESIS AND VISUALIZATION

A prerequisite for the use of RAPD data in estimation of genetic diversity or identification, is proven reproducibility (Virk *et al.*, 1995; Pérez *et al.*, 1998). Figure 3.1 indicates a gel in which different PCR mixtures were tested and illustrates the suitability of the selected PCR mix. The middle set of profiles (Figure 3.1, B), lanes 1 to 4, indicates conditions that were used for further analysis. Well-resolved and distinct bands can be observed in each of the profiles. This type of RAPD profile may be suitable for analysis of the genetic relationships between individuals.

The RAPD profiles for the different primers were characteristic of the primer/genome combination. Figure 3.2 depicts silver stained polyacrylamide gels that show the RAPD profiles that were used in this part of the study. The number of bands ranged from 4 to 17 (Table 3.2) and the sizes from 200 to 1600 bp. Only the bands that were of high intensity and present in duplicate PCRs were used for the analysis by RAPDistance and GelCompar.

Table 3.2: The list of RAPD primers that were used in the various sections of this study. The GC-content, sequence, size range of bands, sections where the primer were used and figures depicting their profiles are presented. The RUM primers were designed by the Rhodes University Biochemistry and Microbiology and synthesized by the University of Cape Town, Biochemistry Department. The OPA primer was obtained from Operon Technologies, USA.

PRIMER NAME	GC (%)	PRIMER SEQUENCE	SIZE RANGE OF USEFUL BANDS	NUMBER OF BANDS PER PRIMER
RUM-P2	50	5'-GCAAGTAGCT-3'	280 –1600	8 – 10
RUM-P3	80	5'-CGGCCCCTGT-3'	220 – 1000	7 – 10
RUM-P4	50	5'-CACATGCTTC-3'	200 – 1000	7 – 11
RUM-P3 & P4 (Multiplex PCR)	80 + 50	5'-CGGCCCCTGT-3' 5'-CACATGCTTC-3'	220 – 1600	6 – 9
RUM-P7	50	5'-TCACGATGCA-3'	220 – 1000	9 –12
RUM-P8	50	5'-TCTCGATGCA-3'	220 – 1000	7 – 17
RUM-P12	50	5'-ATTGCGTCGA-3'	450 – 1000	7 – 10
OPA-10	70	5'-TCGGGGCATC-3'	220 – 600	4 – 6

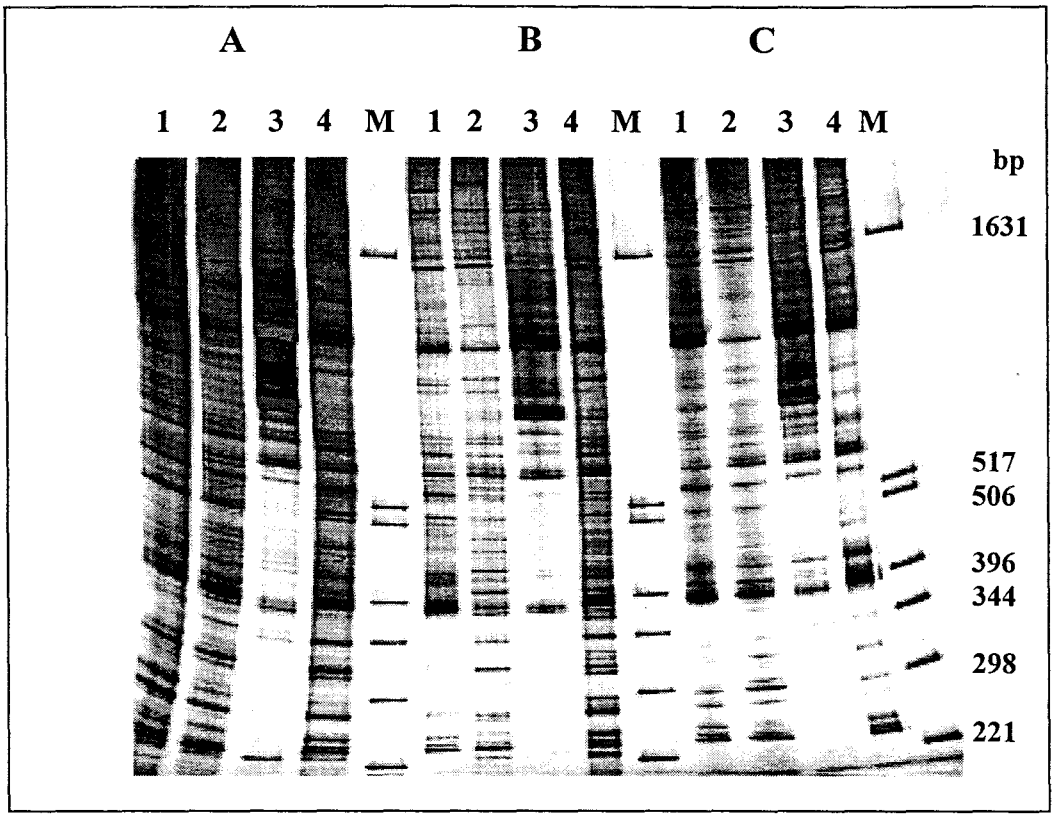


Figure 3.1: A silver stained polyacrylamide gel showing the RAPD fingerprints that were generated when genomic ostrich DNA was amplified by PCR under different conditions. The Hoefer (SE 400) electrophoresis system was used. The PCR mixtures contained:

A: 2 units of *Taq*, 400 µmole dNTPs and 1 % (w/v) acetamide
B: 1 unit of *Taq*, 400 µmole dNTPs and 1 % (w/v) acetamide
C: 2 units of *Taq*, 100 µmole dNTPs and NO acetamide

The DNA samples that were used were as follows: In gels A, B and C:
Lanes 1 and 2: *S.c. australis*,
Lane 3: *S.c. molybdophanes*
Lane 4: *S.c. massaicus*.
Lane M: molecular marker (pBR322 digested with *Hinf*I)

3.2.3.2 RAPD PROFILES OF SELECTED OSTRICH SUBSPECIES

Eight primers were assessed for their ability to produce RAPD profiles that would be able to differentiate between individuals from different subspecies. The profiles that were generated by the primers RUM-P3, RUM-P4, combination of primers RUM- P3 and RUM-P 4, RUM-P7 and OPA-10 were inconsistent or yielded profiles of the different subspecies that did not reveal any differences and were thus excluded from the analysis. Primers RUM-P2, RUM-P8 and RUM-P12 generated results that could be analysed by semi-automated and automated

means and are depicted in Figure 3.2, gels A, B and C respectively. In all three gels the loading order was the same, i.e. Lanes 1 and 2 contained samples *S.c. australis*, Lane 3 *S.c. molybdophanes* and Lane 4 *S.c. massaicus*. The RAPD profiles that were generated by these three primers were very well resolved on 10 % (w/v) acrylamide gels.

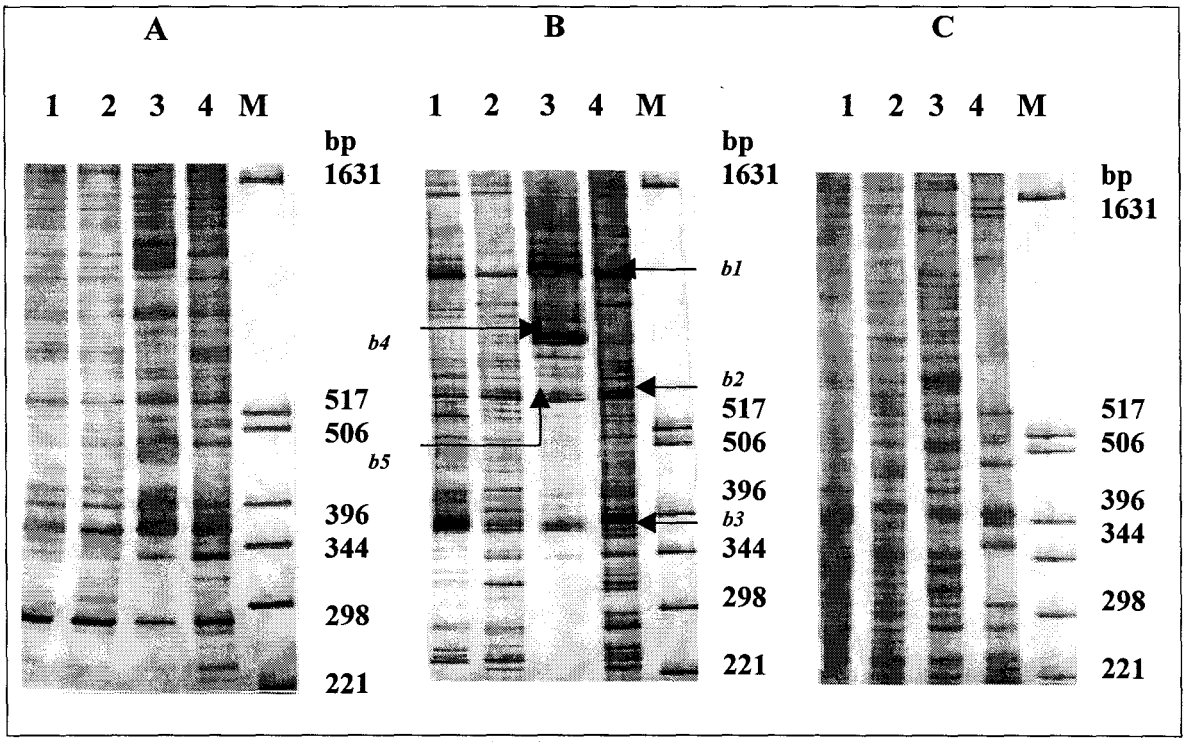


Figure 3.2: Silver stained polyacrylamide gels showing the ostrich RAPD fingerprints generated by primers RUM-P2 (A), RUM-P8 (B) and RUM-P12 (C). The Hoefer (SE 400) electrophoresis system was used to resolve RAPD fingerprints
The DNA samples that were used were as follows: In gels A, B and C:

Lanes 1 and 2:	*S.c. australis*,
Lane 3:	*S.c. molybdophanes*
Lane 4:	*S.c. massaicus*
Lane M:	molecular marker (pBR322 digested with *HinfI*)

The profiles of the individual representatives of the different subspecies presented various degrees of similarity and variation. A summary of the observed banding patterns and band-sharing is provided in Table 3.3. In the profiles of primer RUM-P2 a large percentage of the bands (71.1 %) was shared between all individuals. On the other hand, in the profile of primer RUM-P8 only 25 % of the bands was shared on average between all individuals. Average band-sharing per representative subspecies could not be conducted because only individual

(with the exception of *S.c. australis* that had two) representatives of the subspecies were included.

Table 3.3: A summary of the total number of bands and the proportion of bands shared in the RAPD profiles in Figure 3.2. The proportion of bands (contributing to the data base, the proportion of bands common to all samples and the proportion of common bands per individual primer) is expressed as a percentages.

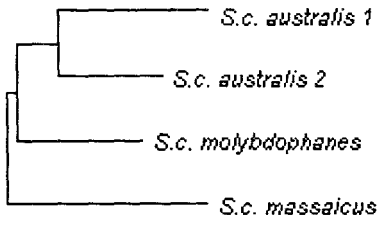
Description	Total	RUM-P2	RUM-P8	RUM-P12
Number of bands	148	45	48	55
Proportion (%) of bands from each primer	-	30.4	32.4	37.4
Number of bands common to all samples	68	32	12	24
Proportion (%) of common bands in the profiles of each primer	45.9	71.1	25.0	43.6

The results, however, showed differences between the RAPD profiles of *S.c. australis* representatives (Figure 3.2: gels A, B & C, Lanes 1 & 2) and those from the representatives of *S.c. molybdophanes* and *S.c. massaicus* (Figure 3.2: gels A, B & C, Lanes 3 & 4, respectively). The profiles of *S.c. molybdophanes* (Figure 3.2: gels A, B & C, Lanes 3), showed the most differences when compared to the profiles of the other two subspecies representatives. For example, in gel B, Lane 3 (Figure 3.2), fragments smaller than 600 bp (*b5*) did not amplify efficiently, except the possible species marker bands at 390 bp (*b3*). A high intensity marker band was present at 800 bp (Figure 3.2 B, Lane 3, *b4*). Several bands were observed in the gels that were present in all subspecies and could be species specific markers such as those marked with the straight arrows in Figure 3.2, gel B [1020 bp (*b1*), 600 bp (*b2*), 390 bp (*b4*)]. This could, however, not be conclusively established because one of the extant subspecies, *S.c. camelus*, was not represented.

(a) Results of Semi-automated Analysis by RAPDistance version 1.03

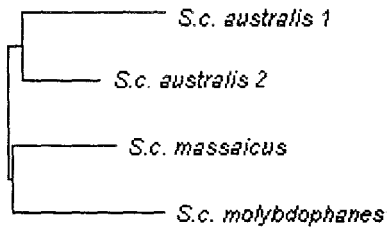
Presence/absence matrices were constructed for primers RUM-P2, RUM-P8 and RUM-P12. A total of 148 bands was included in the pooled data set. Each of the primers contributed

substantially towards the data base (Table 3.3: Primer RUM-P2 contributed 30.4 %, RUM-P8 contributed 32.4 % and RUM-12 contributed 37.4 %). The average percentage of bands that were common to all samples was 45.9 %. This is within the range observed in other animal species (eg. Chapco *et al.*, 1992b).



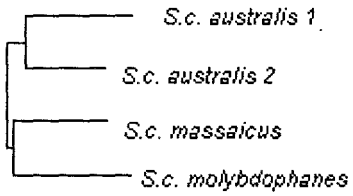
A

Neighbour Joining Tree generated by the algorithm of Excoffier *et al.* (1992).



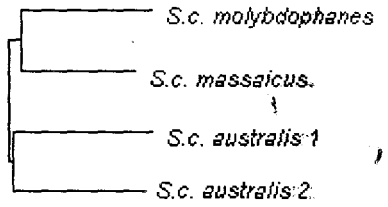
B

Neighbour Joining Tree generated by the algorithm of Upholt (1977).



C

Neighbour Joining Tree generated by the algorithm of Kulczynski (1927).



D

Neighbour Joining Tree generated by the algorithm of Dice (1945).

Figure 3.3: Neighbour Joining Trees that were constructed from data obtained using primers RUM-P2, RUM-P8 and RUM-P12. Data was combined and analysed using RAPDistance software. The different trees (A to D) were generated by algorithms of Excoffier *et al.* (1992), Upholt (1977), Kulczynski (1927) and Dice (1945) respectively. Trees B, C and D show the same topology. Details of these algorithms are shown in Table 3.4.

The matrices of individual primers were pooled using RAPDistance version 1.03 software and analysed by algorithms of Excoffier *et al.* (1992), Upholt (1977), Dice (1945) and Kulczynski

(1927) (Table 3.4) All the resultant Neighbour Joining Trees are represented in Figure 3.3 (A to D). Three Neighbour Joining Trees in Figure 3.3, B to D (B: Upholt, 1977; C: Kulczynski, 1927; D: Dice, 1945) showed similar relationships amongst the ostrich subspecies. These trees indicate that *S.c. australis* representatives are different from *S.c. massaicus* and *S.c. molybdophanes*. The two *S.c. australis* representatives and *S.c. massaicus* and *S.c. molybdophanes* formed separate clusters. The results corroborate Freitag and Robinson (1993) who, using RFLP analysis of entire genomes, showed that *S.c. australis* and S.c. *molybophanes* were very distant.

Table 3.4: The algorithms that were used to analyse the RAPD profile data using RAPDistance software.

$S = N_T(1 - \frac{N_{AB}}{N_T})$ Excoffier *et al.*, 1992	$S = 0.5(\sqrt{F.F + 8F} - F)(\frac{1}{N_T}) where F = \frac{2N_{AB}}{(N_A + N_B)}$ Upholt, 1977
$S = \frac{2N_{AB}}{[(2N_{AB}) + (N_{AoB} + N_{ABo})]}$ Dice, 1945	$S = 0.5(\frac{N_{AB}}{N_{AB} - N_{AoB}}) + \frac{N_{AB}}{N_{AB} + N_{ABo}}$ Kulczynski, 1927

Where:

S	Similarity between profiles
NT	Total number of band positions in the profile
NA	Total number of bands present in the profile of A
NB	Total number of bands present in the profile of B
NAB	Total number of band positions where A = 1 and B = 1
NAoBo	Total number of band positions where A = 0 and B = 0
NAoB	Total number of band positions where A = 0 and B = 1
NABo	Total number of band positions where A = 1 and B = 0

The Neighbour Joining Tree that was generated by analysis of the data by the Excoffier *et al.* (1992) algorithm (Figure 3.3, A) shows that the two *S.c. australis* representatives are more closely related to *S.c. molybdophanes* than to *S.c. massaicus*. This is in conflict with the relationships that were suggested by Freitag and Robinson (1993).

The differences observed in the Neighbour Joining Trees may be the result of the different algorithms that were used to determine the relationships. All the algorithms (Table 3.4) that were used, take into account the presence of bands, band-sharing and band positions between taxa to determine relatedness. The algorithms by Upholt, (1977), Dice (1945) and Kulczynski (1927) bear greater similarity to each other than to the algorithm of Excoffier *et al.* (1992). The former three equations generated Neighbour Joining Trees that showed similar relationships between the representative ostrich subspecies. The Excoffier *et al.* (1992) algorithm uses band-sharing and total number of band positions to determine similarity between the taxa. The tree that showed *S.c australis* as more closely related to *S.c molybdophanes* than to *S.c. massaicus* was based on the calculations by the Excoffier *et al.* (1992) algorithm. Although this algorithm takes shared presence and shared absence into account when determining relatedness between taxa, greater emphasis is on bands present.

(b) Results of Automated Analysis by GelCompar version 4.0

Figure 3.4 depicts the dendrograms that were produced when the RAPD profiles of different ostrich subspecies were analysed using GelCompar software. The RAPD profiles were generated by the primers RUM-P2, RUM-P8 and RUM-P12.

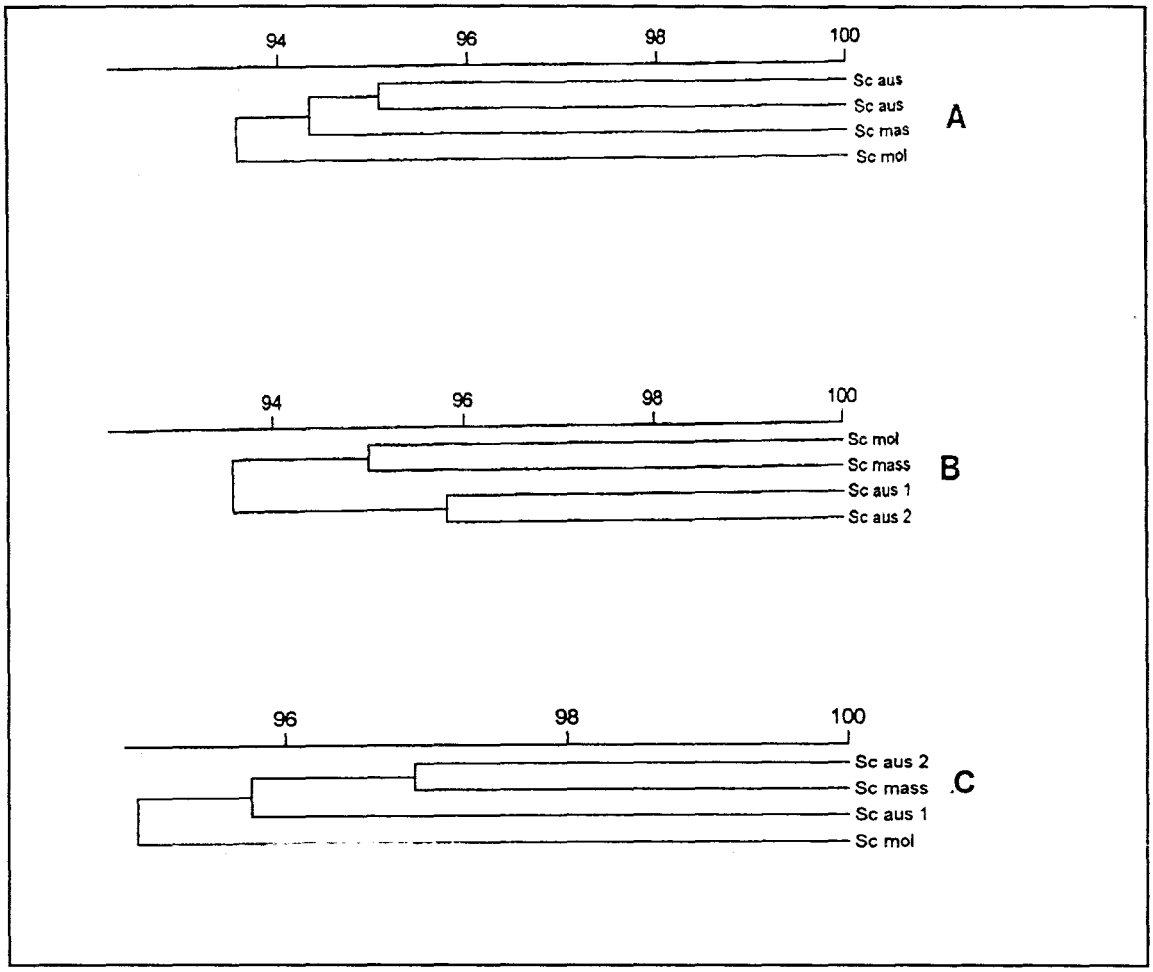


Figure 3.4: Dendrograms that were produced when RAPD profiles were analysed using GelCompar software. The dedrograms were generated using RAPD profiles of the different subspecies, produced by primers RUM-P8 (A), RUM-P12 (B) and RUM-P2 (C). Sc aus indicates representatives of subspecies *S.c. australis*; Sc mass is the individual representative of *S.c. massaicus*; Sc mol is the individual representative of the subspecies *S.c. molybdophanes*.

The dendrogram that was generated by analysis of RUM-P8 RAPD profiles (Figure 3.4, A) shows the relationships obtained in previous studies (Freitag and Robinson, 1993; Kumari and Kemp, 1998). The dendrogram that was produced by primer RUM-P12 (Figure 3.4, B) showed a relationship between the ostrich subspecies similar to that observed in three of the four Neighbour Joining Trees (Figure 3.3, B to D). Dendrogram C was produced by primer RUM-P2 (Figure 3.4) and showed that the relationship between *S.c. australis* representative number 2 and *S.c. massaicus* was closer than the relationship between the two *S.c. australis* representatives. This relationship was in contrast to that shown by two other RAPD primers

using the same analysis software (GelCompar) and semi-automated analysis of pooled RAPD data.

Freitag and Robinson (1993) also observed that a common *S.c. massaicus* and *S.c. australis* haplotype existed when the entire mtDNA genomes of these two subspecies were analysed using RFLP analyis. The common (*S.c. massaicus*/*S.c. australis*) haplotypes exhibited by the representatives of the *S.c. australis* were from locations in the Kruger National Park, Zimbabwe and the Kalahari of South Africa (Freitag and Robinson, 1993). The *S.c. australis* representative number 2 originated from the Kruger National Park.

The result of close relationship between *S.c. massaicus* and *S.c. australis*, based on the RAPD profile from a single primer (RUM-P2) and analysed by GelCompar, agrees with the RFLP data observed by Freitag and Robinson (1993). This common haplotype result between the two subspecies (*S.c. massaicus* and *S.c. australis*) was, however, in contrast to the pooled data of the three primers (RUM-P12, P8 and P2: Figure 3.3) as well as the data from the individual primers RUM-P12 and RUM-P8.

The observed relationships between ostrich subspecies as demonstrated by the RAPD profiles from three different primers that were analysed by GelCompar version 4 were different. This observation could be attributed to the weakness of GelCompar version 4 that, besides band position also takes into account band intensity. The results presented here indicate the unsuitability of GelCompar version 4 software for analysis of RAPD profiles of ostrich subspecies, when the above analysis conditions are used.

Further studies on using RAPDs for ostrich taxonomy should include more samples representing the different extant subspecies. A larger number of individuals from different

areas within the range of the various extant subspecies as well as more primers should also be included. This should generate results that would provide greater insights into the relationships of ostrich subspecies, based on RAPDs.

3.2.4 SUMMARY

These results showed that RAPDs revealed characters that could be useful in taxonomic studies. Visual and computer analysis of RAPD profiles of the representatives from three of the four extant subspecies of ostriches showed relationships that were also obtained in previous studies (Freitag and Robinson, 1993, Kumari and Kemp, 1998). The results showed that *S.c. australis* was more closely related to *S.c. massaicus* than to *S.c. molybdophanes*. This limited study thus showed that RAPDs have the potential to distinguish ostriches of different subspecies.

3.3 APPLICATION OF RAPDs IN DETERMINATION OF INTER- AND INTRAPOPULATION GENETIC DIVERSITY OF *S.c. australis* OF SOUTHERN AFRICA

3.3.1 INTRODUCTION

For effectively managing the present populations of ostriches (domesticated and wild), it would be essential to have comprehensive genetic information based on the entire genome. This study attempts to determine the genetic diversity within and between selected populations of *S.c. australis* in southern Africa, and to show any geographic genetic structure that might exist. The results from this study should complement the results from the study by Freitag and Robinson (1993) who found limited genetic structuring of mtDNA in the ostrich subspecies *S.c australis*, They could not, however, detect genetic diversity within populations of this subspecies (see Chapter 1 for detailed discussion). These results could be explained in terms of events such as possible genetic bottle-necks, or considerable gene flow between the different populations. Freitag and Robinson (1993) admit that one of the limitations of their results was that they did not detect nuclear introgressions and their study was thus uninformative in so far as nuclear diversity was concerned. Since RAPD markers were shown to be usually nuclear in origin, analysis by this method would give an indication of genetic diversity at the nuclear level (Lu *et al.*, 1995). RAPD data sets may thus contribute extensively towards our knowledge of genetic diversity of southern African ostriches (*S.c. australis*).

RAPDs has a record of showing greater resolution for inter- and intrapopulation genetic differentiation studies, than mtDNA (Chapco *et al.*, 1992a,b) and allozymes (Puterka *et al.*, 1993; Haig *et al.*, 1994; Buso *et al.*, 1998; Sydes and Peakall, 1998). RAPDs was also used in the

genetic diversity study of commercial strains of chickens, turkeys (Smith *et al.*, 1996) and guinea fowl (Sharma *et al.*, 1998).

Chapco *et al.* (1992b) used RAPD technology to show that higher levels of genetic diversity existed between grasshopper (*Melanopus sanguinipes*) populations than were indicated by a previous study (Chapco *et al.*, 1992a), in which mtDNA markers were used. These grasshoppers also have high dispersal rates and the observation that low mtDNA diversity existed, was explained in terms of clonal inheritance of mtDNA and disperal rates of females. The results obtained with RAPDs clearly demonstrated usefulness in detecting genetic variability in migratory locust species. It may thus be useful to evaluate the usefulness of this technology for other species where migrations and/or translocations could have caused low levels of mtDNA diversity between populations, such as ostriches.

In comparing RAPD phenotypes to allozyme data, Haig *et al.* (1994) found that both techniques gave similar inter- and intrapopulation differences for red-cockaded woodpeckers. RAPDs, however, showed greater differences than allozymes. This result was expected since allozyme differentiation was shown to be less profound among birds than amongst other vertebrates (Evans, 1987). In the recent studies of plant species, greater inter- and intrapopulation differentiation could be observed when RAPDs data was compared to allozyme data (Buso *et al.*, 1998; Sydes and Peakall, 1998; Isabel *et al.*, 1999).

There were, however, recent cases where RAPDs could not detect variation between populations. One such case was that of harbour seals (*Phoca vitulina*: Kappe *et al.*, 1995). Multi-locus DNA fingerpinting could, however, distinguish the seal populations. The authors admitted that the

RAPD protocols were not optimised for this application, and found only limited numbers of polymorphic bands (Kappe *et al.*, 1995).

In a population study of highly mobile squid species, Shaw *et al.* (1999) compared microsatellite data to mtDNA data and obtained similar values for inter- and intrapopulation differentiation. The information provided by microsatellites is not directly comparable to RAPDs but was significant since both are nuclear markers showing similar trends in population analysis.

RAPD markers generated data that were consistent with, or more informative than, data from other genetic markers such as multi-locus DNA fingerprinting and allozymes. It could indicate the evolutionary histories of the populations, such as inbreeding levels, hybridization levels, genetic drift and clonal development, (van Heusden and Bachmann, 1992; Cruzan and Arnold, 1993, 1994; Baruffi *et al.*, 1995; Buso *et al.*, 1998; Sydes and Peakall, 1998). In the case of a global analysis within *Diuraphis noxia* (Russian wheat aphid) populations, allozyme data were uninformative about the genetic diversity and RAPDs highly informative (eg. Puterka *et al.*, 1993).

This part of the study was an evaluation of the RAPD technique for the ability to generate markers that could be used to determine the genetic diversity within and between domesticated *S. c. australis* populations of southern Africa.

3.3.2 MATERIALS AND METHODS

3.3.2.1 SAMPLING STRATEGY, COLLECTION AND DNA ISOLATION

See Appendix B for the origins of the samples. The samples were from a Grahamstown farm and from an Oudtshoorn farm. Although this sampling approach represented only two populations they also represented two different breeding lineages. The Grahamstown population was closed for a prolonged time (more than 90 years). The Oudtshoorn population on the other hand was exposed to active breeding practices with regular introduction of fresh genetic stock from other geographic regions. This is illustrated by the Oudtshoorn/Namibian crosses that were included in this study. These crossed individuals were the progeny of individuals from the Oudtshoorn farm and individuals from a Namibian farm. This sampling strategy thus included a genetically closed population, an open population and crosses between geographically different populations.

Blood samples were collected by venupuncture. Blood samples were placed in vacu-tainers which contained 50 mM EDTA as anti-coagulant. The EDTA-blood was then transported to the laboratory at 4°C and then stored at –20°C. Section 2.2.2 contains the details of the DNA isolation procedures

3.3.2.2 RAPD-PCR

The standardised protocol as described in Section 3.2.2.2 was followed for all PCRs.

A Hybaid (Omnigene) air-cooled thermal cycler was used for PCR amplifications. The reaction profile was 94°C for 3 minutes initial denaturation followed by 40 cycles of: 94°C for 30 seconds, 37°C for 30 seconds and 72°C for 60 seconds. After the final cycle the samples were held at 72°C for 5 minutes.

3.3.2.3 PRIMER SELECTION

Details of the 10 bp RAPD primers that were used are indicated in Table 3.2. Five primers and a primer combination [multiplex RAPDs] (primer RUM-P3 and primer RUM-P4 at equal concentrations) were screened.

3.3.2.4 ELECTROPHORESIS

Discontinuous SDS-Polyacrylamide gels (Laemmli, 1970) were used to resolve aliquotes (10 µl) of the amplification products (See Section 2.3.2.4 and 3.2.2.4 for details). All gels were subjected to electrophoresis at 150 V for 6.5 hr in 1 X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), using an Owl (Omeg P2) vertical electrophoresis. The gel dimensions were: 20 cm wide, 15 cm long and 0.15 cm thick.

See Sections 2.3.2.4 and 3.2.2.4 for further details.

Although the Hoefer vertical slab unit (SE 400) produced the best resolution, the smiling effect seen when using the two lanes on the edge of the gel, (Figure 3.1) reduced the number of individuals that could be included on one gel. The vertical slab gel unit from Owl Scientific Plastics, South Africa (Omeg P2), was tested and found to be useful for resolution of large numbers of PCR samples. Although this system affected the resolving power of the gels, it was considered a useful alternative system as it was time efficient. The Omeg P2 vertical slab units were thus used for the resolution of the PCR samples in this section.

3.3.2.5 VISUALIZATION OF RESOLVED DNA FRAGMENTS BY SILVER STAINING

The method described in Sections 2.3.2.4 and 3.2.2.5 was strictly adhered to.

3.3.2.6 ANALYSIS OF DATA SETS

(a) Semi-automated Analysis by RAPDistance version 1.03

See Section 3.2.2.6 for details. Presence/absence (1/0) matrices were compiled for each of the primers RUM-P3, RUM-P8, RUM-P12, OPA-10 and the primer combination RUM-P3+P4. Band positions in each individual RAPD profile were recorded against a molecular weight marker (pBR322 digested with *Hinf*I) and assigned a '1' for presence and a '0' for absence of that band.

The RAPD data sets for the different primers were pooled and the pairwise distances were calculated using the following algorithms, supplied by RAPDistance software: Excoffier *et al.* (1992), Upholt (1977), Kulczynski (1927)(from Table 3.4) and Jaccard (1901) shown below. In this equation (Jaccard, 1901)

$$S = \frac{2N_{AB}}{(N_T - N_{AoBo})}$$

where S is the similarity between profiles, N_T is the total number of band positions in the profile, N_{AB} is the total number of band positions where A = 1 and B = 1 and N_{AoBo} is the total number of band positions where A = 0 and B = 0. Neighbour Joining Tree (NJTREE) version 2.0 software was used to construct Neighbour Joining Trees for each of the algorithms and are presented in Section 3.3.3.2 (Figure 3.10).

(b) Automated Analysis by GelCompar version 4.0

GelCompar version 4.0 (GelCompar) was used to analyse the RAPD profiles generated by primers RUM-P3, RUM-P3+4, RUM-P8, RUM-P12 and OPA-10. The Pearson product-moment correlation coefficient clustering, using Ward's algorithm, provided consistent results and was selected for analysis. The resultant dendrograms are depicted in Section 3.3.3.2 (Figure 3.11 to 3.15)

3.3.3 RESULTS AND DISCUSSION

Figures 3.5 to 3.9 depict silver stained polyacrylamide gels that show typical RAPD profiles that were used in this part of the study. Only the bands that were of high intensity and present in duplicate PCRs were used for the analysis. The number of bands ranged from 4 to 17 (Table 3.2) and the sizes from 200 to 1600 bp. Table 3.5 is a summary of the band data that were scored. A total of 887 bands were included in the data matrix of which 39.7 % were shared by all individuals. These could be species or subspecies markers. Band-sharing is significant in RAPD studies as it is indicative of relationships (Chapco *et al.*, 1992b). A high band-sharing rate indicates a very close relationship. In populations that consist of individuals that are very closely related it is expected that the number of bands shared within this population will be very high.

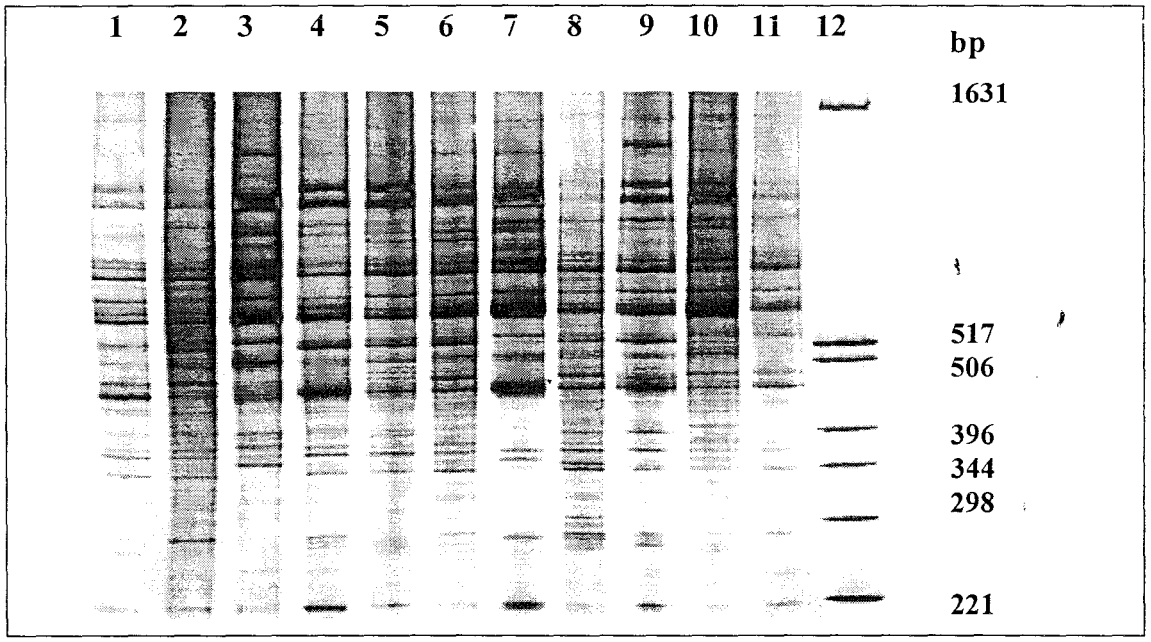


Figure 3.5: A silver stained polyacrylamide gel showing the ostrich RAPD fingerprints generated by primer RUM-P12. The different lanes (Lane 1 to 12) contained the following samples in order: GHT-F2, GHT-F3, GHT-F4, Colleen, Cecil, GHT-F5, GHT-M1, GHT-M2, GHT-M3, GHT-M4, GHT-U and molecular weight marker pBR322 digested with *Hinf*I.

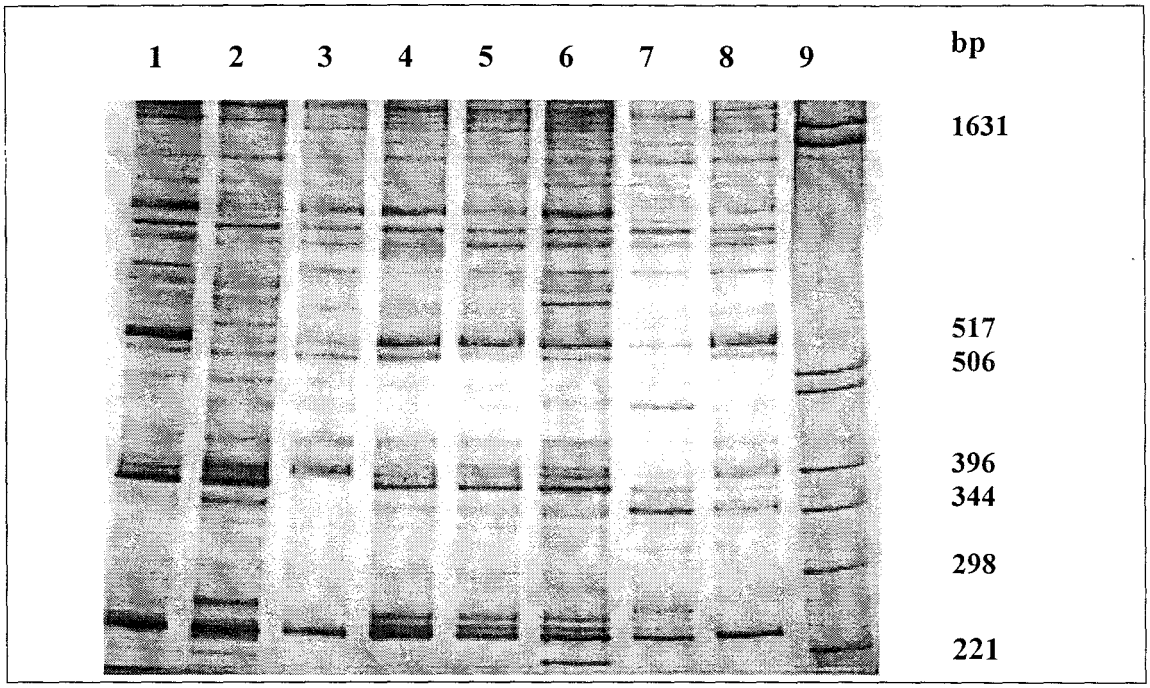


Figure 3.6: A silver stained polyacrylamide gel showing the ostrich RAPD fingerprints generated by primer RUM-P8. The different lanes (Lane 1 to 9) contained the following samples in order: Cecil, Colleen, GHT-M3, GHT-F1, GHT-M4, GHT-F2, GHT-M4, GHT-F4 and molecular weight marker pBR322 digested with *Hinf*I.

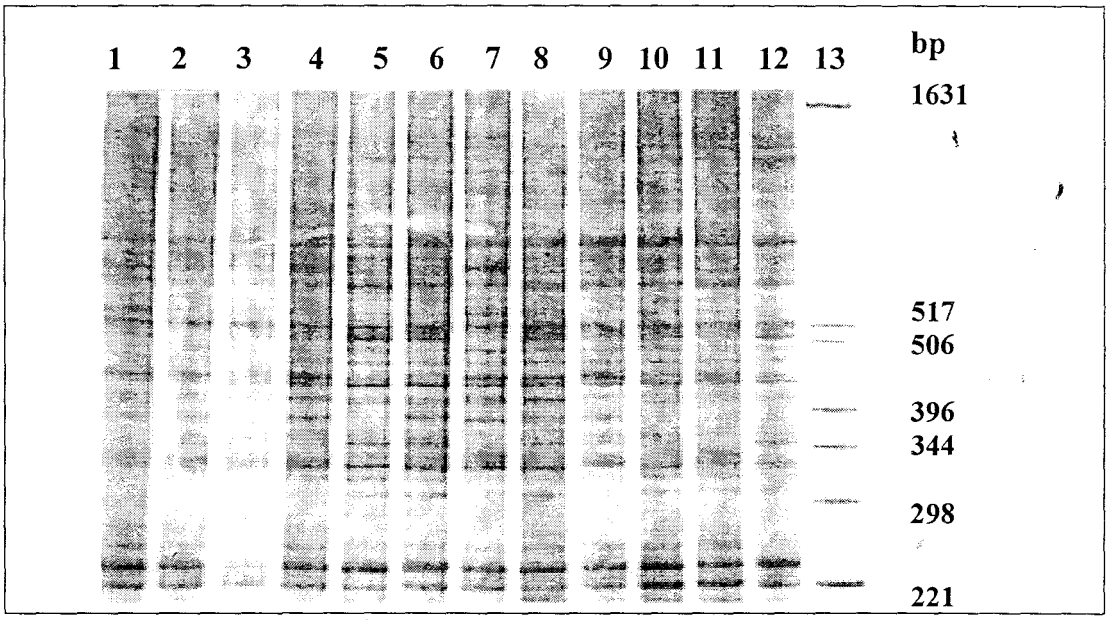


Figure 3.7: A silver stained polyacrylamide gel showing the ostrich RAPD fingerprints generated by primer combination RUM-P3 and RUM-P4. The different lanes (Lane 1 to 13) contained the following samples in order: GHT-F1, GHT-F2, GHT-F3, GHT-F4, Colleen, Cecil, GHT-F5, GHT-M1, GHT-M2, GHT-M3, GHT-M4, GHT-U and molecular weight marker pBR322 digested with *Hinf*I.

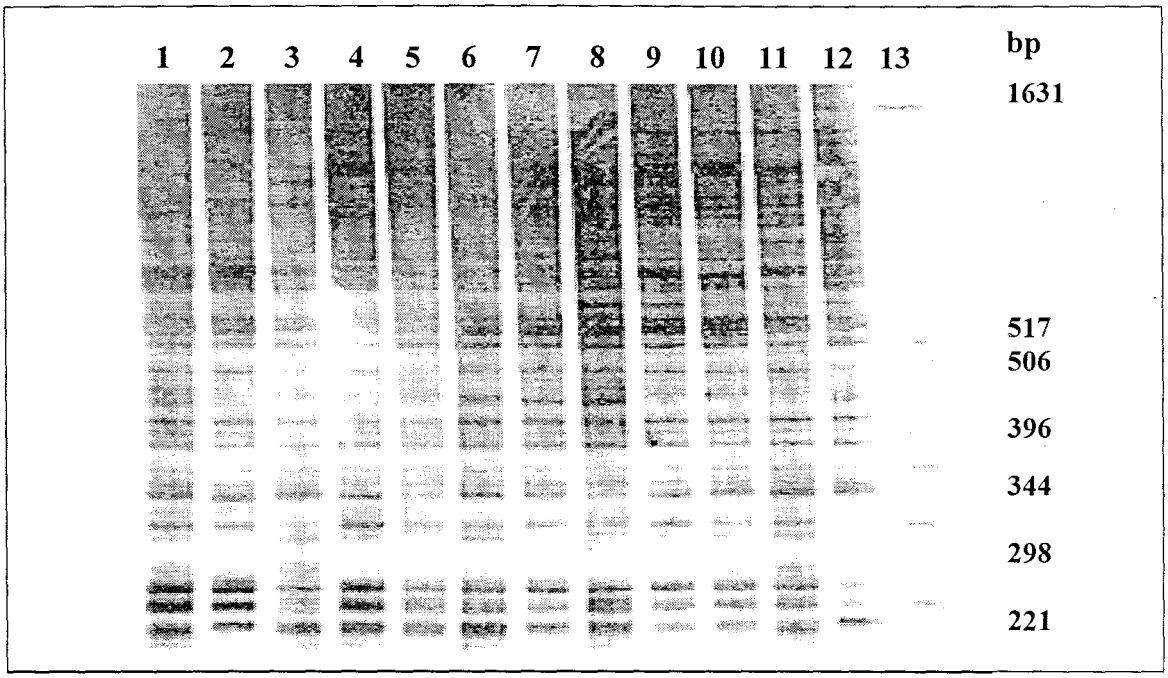


Figure 3.8: A silver stained polyacrylamide gel showing the ostrich RAPD fingerprints generated by primer RUM-P3. The different lanes (Lane 1 to 13) contained the following samples in order: GHT-F1, GHT-F2, GHT-F3, GHT-F4, Colleen, Cecil, GHT-F5, GHT-M1, GHT-M2, GHT-M3, GHT-M4, GHT-U and molecular weight marker pBR322 digested with *Hinf*I.

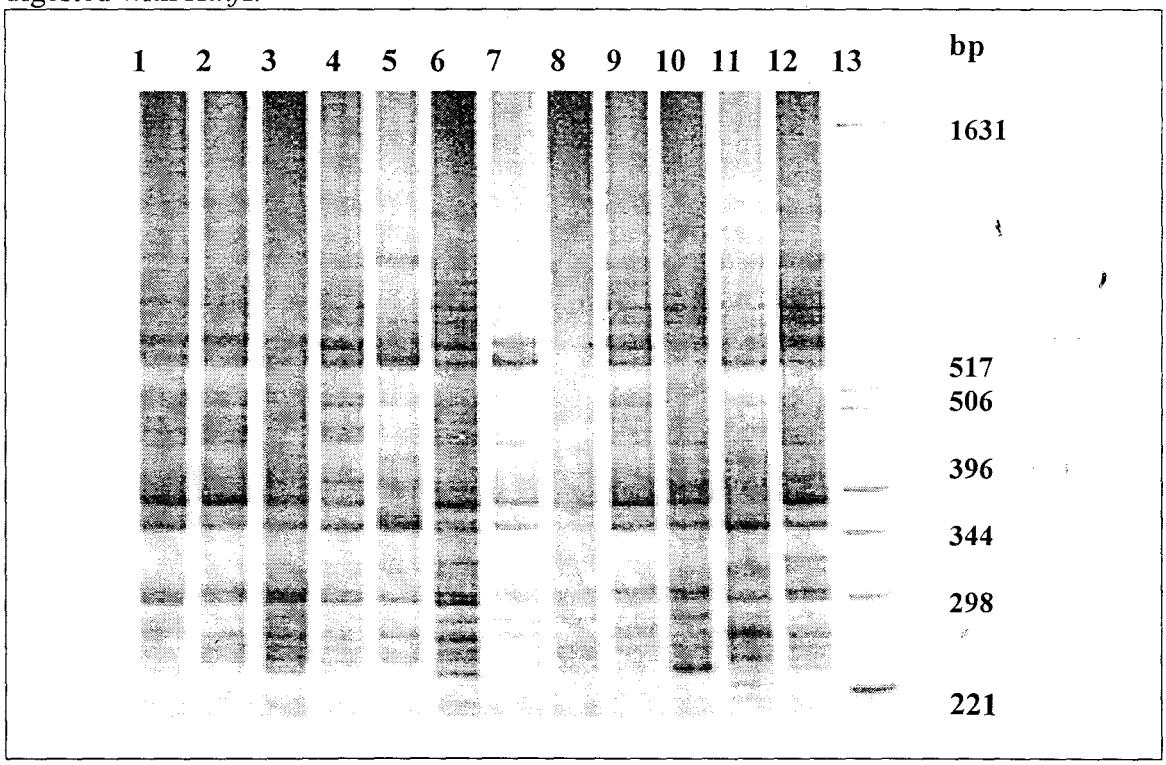


Figure 3.9: A silver stained polyacrylamide gel showing the ostrich RAPD fingerprints generated by primer OPA-10. The different lanes (Lane 1 to 13) contained the following samples in order: ODT-3, ODT-4, ODT-5, ODT-6, ODT-7, ODT-8, O/N-1, O/N-2, O/N-3, O/N-4, NAM-1, NAM-2 and molecular weight marker pBR322 digested with *Hinf*I.

The average percentage common bands within the Grahamstown and Oudtshoorn populations was fairly similar (29.8 % and 27.1 % respectively, Table 3.5). Two of the RAPD profile sets (generated by RUM-P12 and the primer combination RUM-P3+P4) however, showed greater percentage common bands in the profiles of Oudtshoorn population than in the Grahamstown population. Three out of five primers showed that the percentage common bands were greater in the Grahamstown population than in the Oudtshoorn population. Considering the different breeding histories of these two populations and the finding that the pattern of proportion of common bands in the profiles of the populations were not very different could be indicative of high levels of nuclear similarity. The presence/absence matrix was analysed by RAPDistance and the gels by GelCompar as described in Sections 3.2.2.6 and 3.3.2.6.

Table 3.5: A summary of the total number of bands and the number of shared bands observed in the RAPD profiles in Figures 3.5 to 3.9.

Description	Total	OPA-10	RUM-P3	RUM-P3 + P4	RUM-P8	RUM-P12
Number of bands	887	111	195	154	239	188
Proportion (%) of bands contributed by each primer	-	12.5	22.0	17.4	26.9	21.2
Number of bands common to all samples	352	66	88	66	44	88
Proportion (%) of bands common to all samples	39.7	59.5	45.1	42.9	18.4	46.8
Proportion (%) of common bands in the Grahamstown samples	-	77.8	72.7	64.0	35.6	49.5
Proportion (%) of common bands in the Grahamstown samples expressed as a % of the total number of bands	29.8	43.2	36.9	31.2	20.1	25.5
Proportion (%) of common bands in the Oudsthoorn / Namibian samples	-	81.6	72.9	63.3	19.2	65.9
Proportion of common bands in the Oudsthoorn/Namibian samples expressed as a % of the total number of bands	27.1	36.0	35.9	32.5	8.4	31.9

3.3.3.1 NUMERICAL ANALYSIS OF RAPD PROFILES

(a) Results of Semi-automated Analysis by RAPDistance version 1.03

The pooled RAPD data sets that were generated by the different primers (RUM-P3, RUM-P3+P4, RUM-P8, RUM-P12 and OPA-10) were used to calculate pair wise distances using algorithms by Excoffier *et al.* (1992), Upholt (1977), Kulczynski (1927) indicated in Table 3.4 and the algorithm by Jaccard (1901) (Section 3.3.2.6.a).

Neighbour Joining Tree (NJTREE) version 2.0 software used these distances to construct Neighbour Joining Trees for each of the metrics and are depicted in Figure 3.10 (A to D). All the Trees (Figure 3.10; A to D) showed trends that generally grouped the Grahamstown and the Oudtshoorn individuals into area-related clusters. The branching patterns and the combination of individuals found within the clusters varied among the different trees. These differences between the trees could be an algorithm related phenomenon as explained in Section 3.2.3.2.

In Figure 3.10 (A to D) cluster 1 was formed by individuals from the Grahamstown population, cluster 2 by individuals predominantly from Oudtshoorn and cluster 3 by individuals from Oudtshoorn, Namibia, Oudtshoorn/Namibian crosses and in two Trees (B and D) by the same two individuals from Grahamstown (Colleen and GHT-M1). Cluster 2 consistently contained the same individuals. Four out of the six individuals that formed this cluster were from the Oudtshoorn population. One of the individuals (O/N 4) within cluster 2 was a Oudtshoorn/Namibian hybrid. Due to the close genetic relationship it was not extra-ordinary for this individual to cluster within the Oudtshoorn population. The other individual (GHT-U), however, was thought to be a representative of the Grahamstown population. The GHT-U was an

unlabelled sample and its origin was unknown. From this result it appears as if this individual, GHT-U, was probably from the Oudtshoorn population.

Although these Neighbour Joining Trees (Figure 3.10) were not identical, they shared certain common characteristics such as clustering the Grahamstown and Oudtshoorn/Namibian populations into separate groups and sometimes forming a distinctive Oudtshoorn group. The fact that the Oudtshoorn population could not be effectively separated from the Oudtshoorn/Namibian individuals shows the close genetic relationship between these individuals.

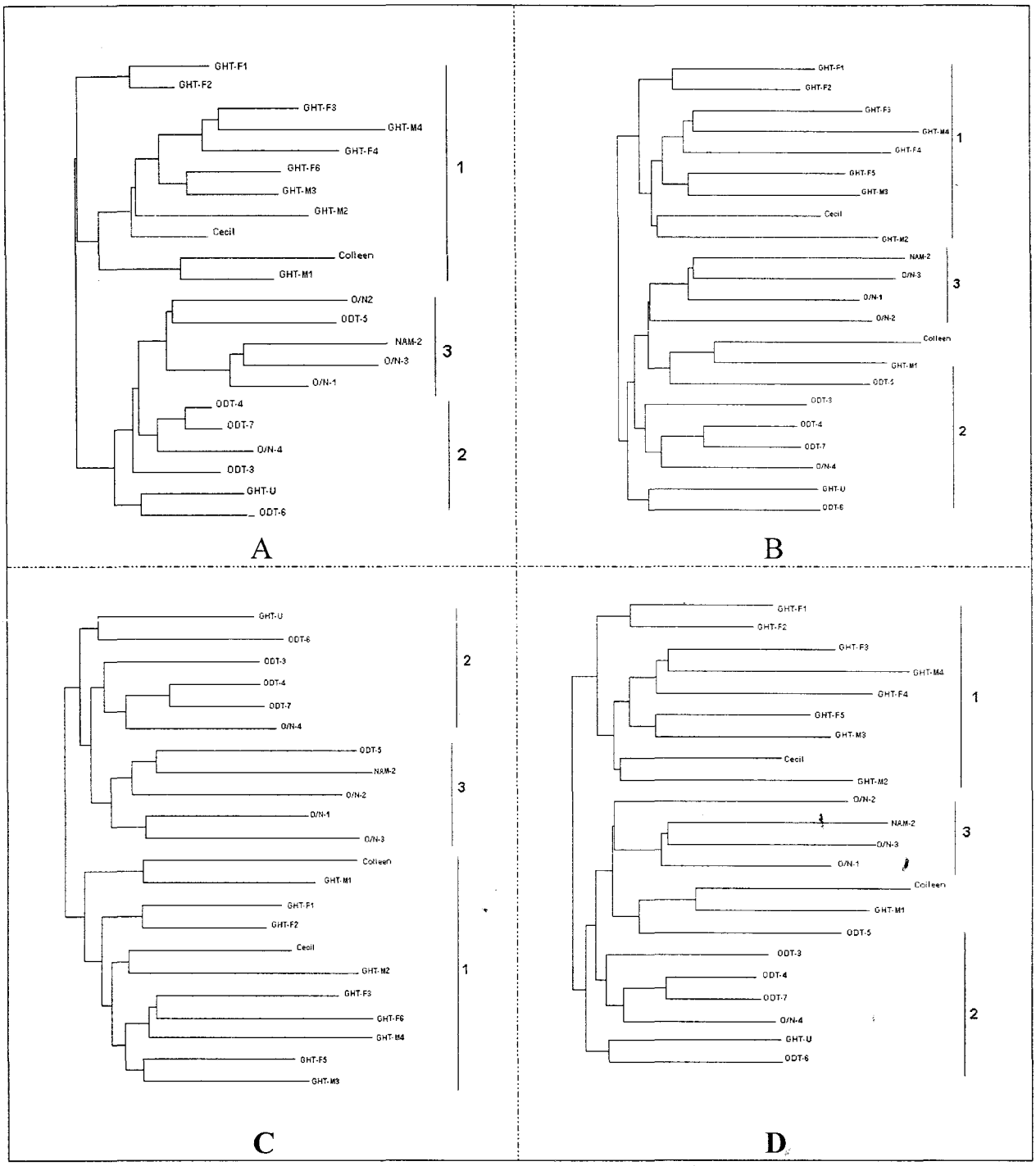


Figure 3.10: Neighbour Joining Trees that were produced when ostrich RAPD profile data generated by primers RUM-P12, RUM-P8, RUM-P3+P4, RUM-P3 and OPA-10 were combined in a single matrix and analysed using RAPDistance software. The numbers **1**, **2** and **3** in the Neighbour Joining Trees (A to D) indicate the Grahamstown, Oudtshoorn and Oudtshoorn/Namibian populations, respectively. The following algorithms were used to calculate similarity:

Fig. 3.10 A = Excoffier *et al.* (1992);
Fig. 3.10 B = Upholt (1977);
Fig. 3.10 C = Kulczynski (1927);
Fig. 3.10 D = Jaccard (1901).

From the above trees it is evident that RAPD marker data analysed by RAPDistance software could resolve a certain degree of genetic structure between closely related ostrich individuals from different populations.

(b) Results of Automated Analysis by GelCompar version 4.0

The computer program GelCompar version 4.0 (GelCompar) was also used to analyze the RAPD profiles that were generated by the different primers as well as the primer combination (Figures 3.5 to 3.9). The resulting dendrograms are depicted in Figures 3.11 to 3.15. All the dendrograms showed two major clusters, one which contained most of the Grahamstown individuals and the other which contained predominantly the Oudtshoorn and Nambian representatives. In the dendrograms of primers RUM-12 (Figure 3.11), RUM-P3 (Figure 3.14) and OPA-10 (Figure 3.15) the latter cluster also showed two separate smaller clusters. One cluster consisting predominantly of individuals from Oudtshoorn and the were other constituted of individuals from Oudtshoorn, Namibia as well as Oudtshoorn/Nambian crosses. Overall clustering patterns observed in the dendrograms for these primers were thus showed trends of separating the individuals and thus populations into geographical groups.

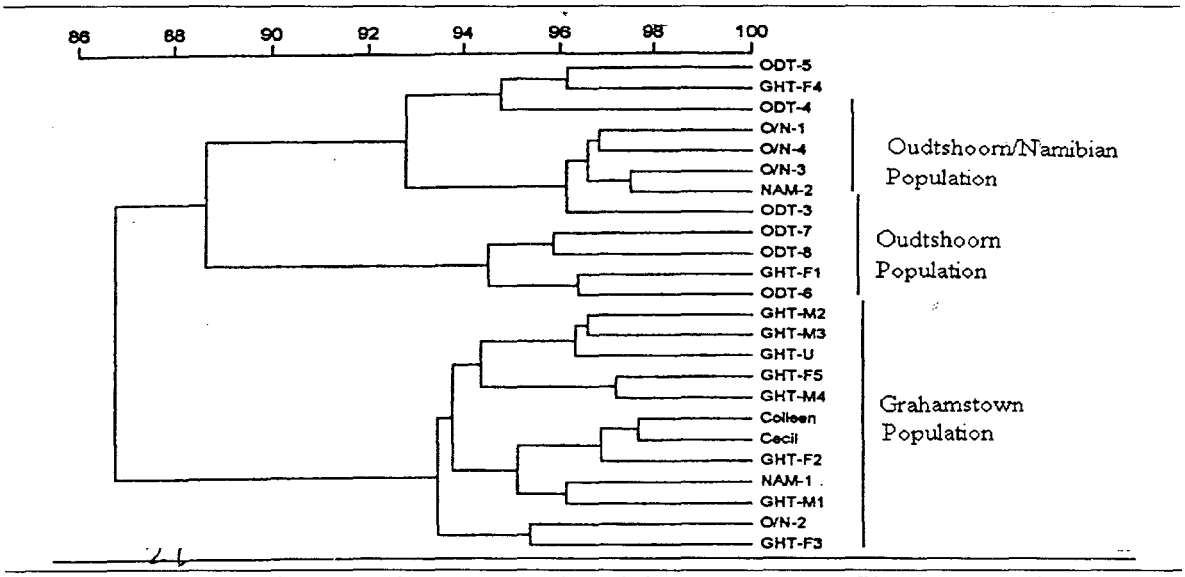


Figure 3.11: A dendrogram that was produced from RAPD profiles generated by primer RUM-P12 and analysed by GelCompar software.

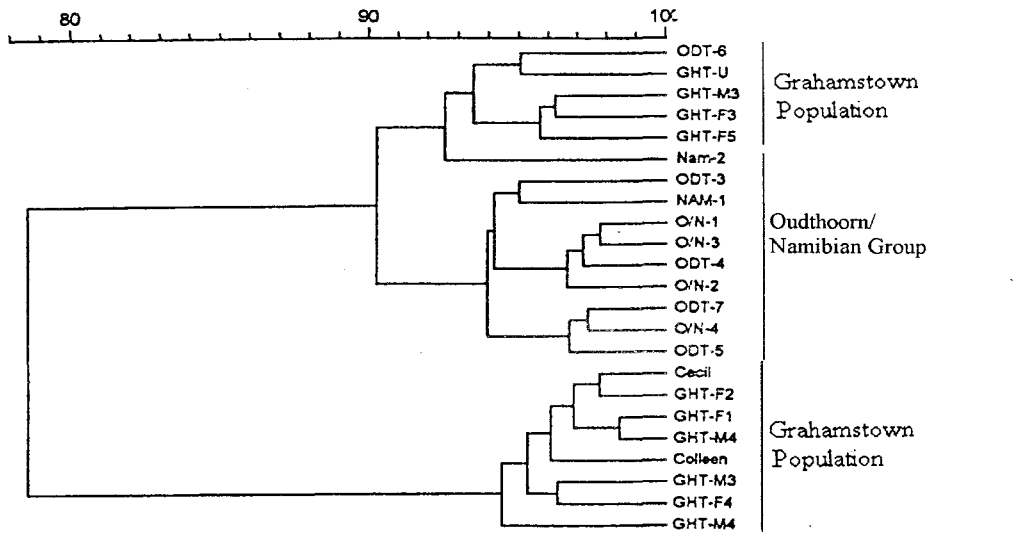


Figure 3.12: A dendrogram that was produced from RAPD profiles generated by primer RUM-P8 and analysed by GelCompar software.

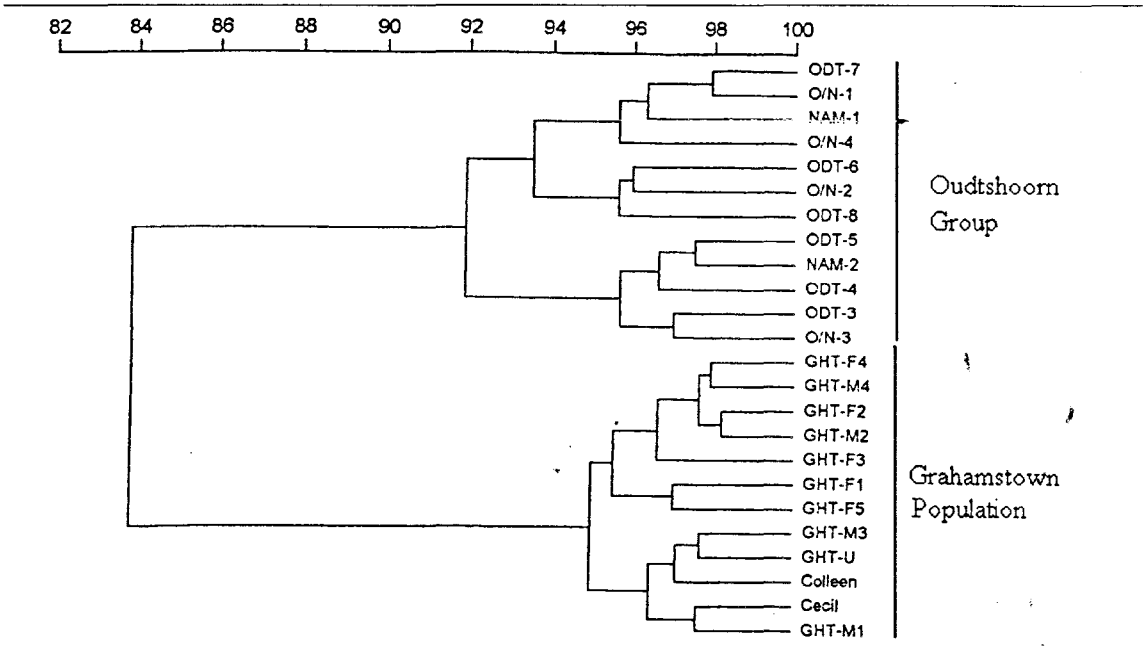


Figure 3.13: A dendrogram that was produced from RAPD profiles generated by the combination of primers RUM-P3 and P4 and analysed by GelCompar software.

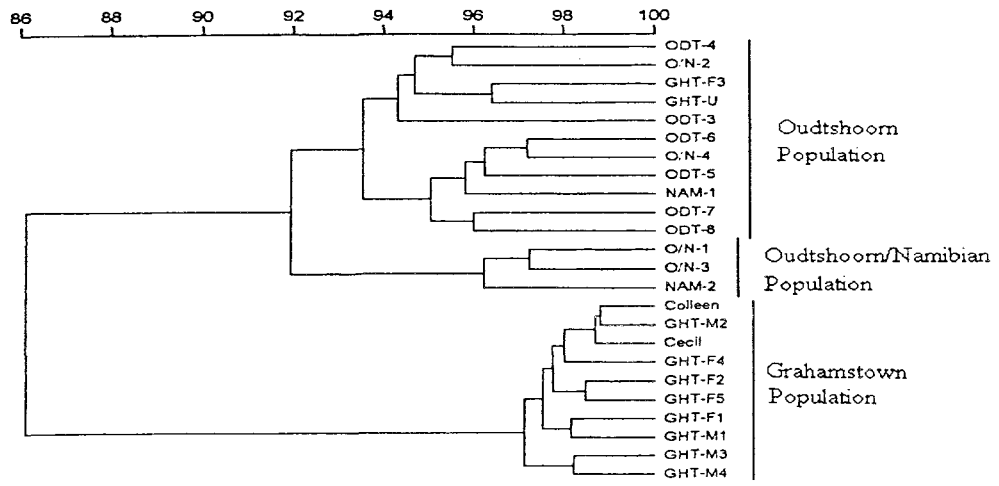


Figure 3.14: A dendrogram that was produced from RAPD profiles generated by primer RUM-P3 and analysed by GelCompar software.

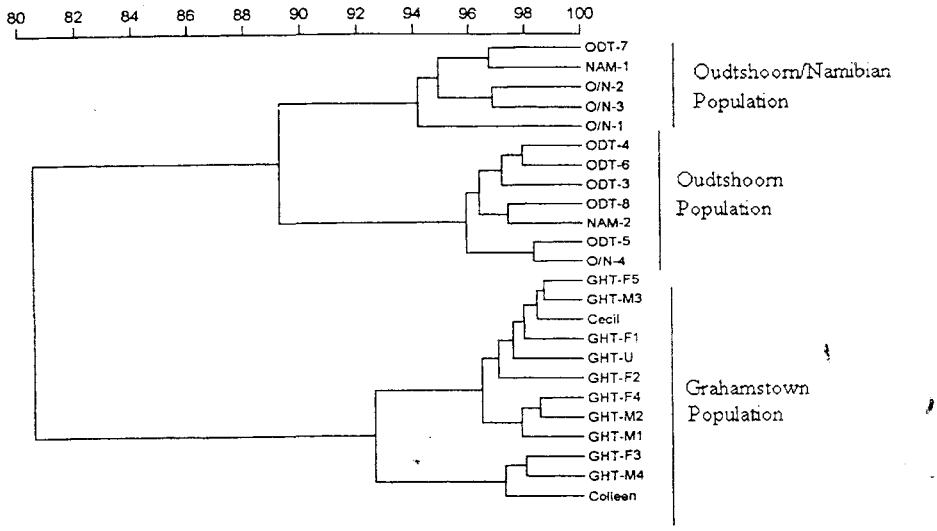


Figure 3.15: A dendrogram that was produced from RAPD profiles generated by primer OPA-10 and analysed by GelCompar software.

In all the dendrograms (Figures 3.11 – 3. 15), the cluster of the Grahamstown population consists mainly of individuals from that particular geographical area. However, individuals from the Oudtshoorn group clustered with the Grahamstown individuals. These individuals are O/N-2, NAM1 (Figure 3.11: dendrogram by primer RUM-P12), ODT-6 and NAM-2 (Figure 3.12: dendrogram by primer RUM-P8). This phenomenon can be observed in the dendrograms

generated by primer RUM-P12 (Figure 3.11) and RUM-P8 (Figure 3.12). Individuals from the Grahamstown population, on the other hand, also clustered within the Oudthoorn group. These individuals are GHT-F1, GHT-F4 (Figure 3.11: dendrogram by primer RUM-P12), GHT-F3 and GHT-U (Figure 3.14: dendrogram by primer RUM-P3).

Three of the dendrograms (Figure 3.11, 3.14 and 3.15) show that the cluster representing the Oudtshoorn group can be divided into an Oudtshoorn population and an Oudtshoorn/Namibian population, based on the geographical/genetic origin of the individuals predominantly found within the cluster. Primer RUM-P8 (Figure 3.12) and the primer combination (RUM-P3 and RUM-P4; Figure 3.13), however, could not clearly distinguish the Oudtshoorn and an Oudtshoorn/Namibian populations. This phenomenon was also not unexpected due to the close genetic relationship between the Oudtshoorn and the Oudtshoorn/Namibian individuals. The dendrograms produced by these primers could, however, distinguish the Oudtshoorn group from the Grahamstown population.

Although the Oudtshoorn population cluster contained individuals predominantly from Oudtshoorn, some individuals from Namibia and the Oudthoorn/Namibian hybrids were also observed within the clusters of this population. The same applied to the Oudtshoorn/Namibain population in which individuals from the Oudtshoorn populations were also observed. The dendrograms that were produced by the primer combination of RUM-P3 and RUM-P4 (Figure 3.13), primers RUM-P3 (Figure 3.14) and OPA-10 (Figure 3.15) show this occurrence. The dendrogram that was produced by primer OPA-10 (Figure 3.15) showed a clustering pattern that grouped the majority of individuals with those from the same geographic (genetic) origin.

The percentage similarities within the Grahamstown population and the Oudtshoorn group was estimated from the dendrograms and are shown in Table 3.6. Percentage similarities were read from the dendrograms and were taken from the point where the cluster of a particular group is connected to that of another group.

Table 3.6: Summary of similarities estimated from the dendrograms that were produced when the RAPD profiles of the different primer-DNA combinations were analysed by GelCompar.

Description	PERCENTAGE SIMILARITY					
	OPA-10	RUM-P3	RUM-P3+P4	RUM-P8	RUM-P12	AVE.
Similarity within the entire sample	85.0	86.0	84.0	78.5	86.5	84.0
Similarity within the Grahamstown population	92.5	96.5	95.0	94.5	93.5	94.4
Similarity within the Oudtshoorn group	89.5	92.0	92.0	90.5	88.5	90.5

This table also indicates that the average percentage similarity within the Grahamstown population (94.4 %: Table 3.6) is greater than the average percentage similarity within the Oudtshoorn group (90.5: Table 3.6). This difference in similarity between the two groups is displayed by dendrograms of all the individual primers in the analysis. The difference in similarity between the two groups is meaningful when compared to the average similarity (84.0 %: Table 3.6) in the data base. The data presented above could not be compared to similarity data from Neighbour Joining Trees due to the differences in the tree building methods. If the connecting points between the various groups within the clusters for the different populations in Figure 3.10 are considered, then it appears that the similarity trends observed with the GelCompar analysis may also be prevalent in the Neighbour Joining Trees.

Analysis of RAPD markers by RAPDistance and GelCompar demonstrated the sensitivity of RAPDs to detect genetic variation among ostrich populations. The analysis of pooled RAPD data

by RAPDistance showed comparable trends as RAPD data of single primers analysed by GelCompar, forming clusters consisting predominantly of individuals from the same geographic origin. When all the trees and dendrograms are considered then it is clear that it is only amongst the Neighbour Joining Trees, that one 'possible' Grahamstown individual, GHT-U consistently clusters within the Oudtshoorn group. Analysis of the RAPD profiles of primer RUM-P3 analysed by GelCompar showed a dendrogram that also clustered GHT-U with the Oudtshoorn individuals. Within the various trees and dendrograms different Grahamstown individuals also clustered within the Oudtshoorn group. The clustering of these individuals within the Oudtshoorn group was not consistent. Except for dendrograms produced by primer RUM-P12 (Figure 3.11) and primer RUM-P8 (Figure 3.12) the other dendrograms and all Neighbour Joining Trees show clusters of the Grahamstown group consisting only of individuals from that particular geographic origin.

The inconsistencies between GelCompar and RAPDistance analysis data could be due to disadvantages of automated analysis software such as GelCompar version 4 that takes band intensity into account in the analysis. Another reason could be that the entire profile was used for analysis and this may have incorporated inconsistently generated bands of very large or very small molecular size. On the other hand, the phenomenon such as the inconsistent clustering of GHT-U could indicate genetic relationship between this individual and the Oudtshoorn and Grahamstown populations. It may indicate that this individual is a hybrid of the two populations, indicating recent introduction of some Grahamstown individuals into the Oudtshoorn population.

3.3.4 SUMMARY

The dendrograms generated by GelCompar and the Neighbour Joining Trees generated by RAPDistance showed similar trends in so far as distinguishing the Grahamstown population from the Oudtshoorn population and the hybrids between Oudtshoorn and Namibia. Based on RAPD markers and deduced from the similarity matrices it is evident that the genetic variability within the Grahamstown population is lower than the genetic variability within the Oudtshoorn population (Tables 3.4 and 3.5). This is in agreement with the known history of the populations.

In the genetic diversity study of commercial strains of chickens (*Gallus gallus*: Smith *et al.*, 1996), turkeys (*Meleagris gallopavo*: Smith *et al.*, 1996) and guinea fowl (*Numida meleagris*: Sharma *et al.*, 1998), high levels of band-sharing was also observed. This phenomenon was attributed to low levels of genetic diversity. Multi-locus DNA fingerprinting of inbred chicken strains previously demonstrated that high levels of band-sharing was due inbreeding (Kuhnlein *et al.*, 1989, 1990). The varying levels of band-sharing observed within the different ostrich populations may thus a real phenomenon that is affected by the genetic factors.

It can thus be concluded that analysis of RAPD profiles that were generated by five different primers and two analytical software programmes showed that nuclear genetic structuring of southern African ostriches exists. The results also indicated that different levels of similarity may exist within populations of ostriches from different geographical regions.

The two major regions (Grahamstown and Oudtshoorn) that were included in this analysis represented different ostrich breeding histories. Oudtshoorn was until recently, for economic, climatic, legal and other strategic reasons, the hub of ostrich breeding activity. Fresh genetic

material from other parts of southern Africa, such as Namibia and Botswana, was constantly added to the breeding population to avoid inbreeding. This thus represented an open population. Other ostrich populations were very small and were left for prolonged periods (more than 90 years) without any fresh genetic stock entering the population. These different populations were represented in the sample, and these genetic differences could be detected by RAPD analysis.

These results also demonstrate that RAPDs could be useful in breeding management of ostriches. Genetic information such as band-sharing between individuals can be employed in breeding management decisions. The RAPDs method may also be developed to identify markers that may be specific for certain geographic areas or populations. Ostrich breeders normally claim their stake by presenting a lineage which usually includes the "finest genetics" from Oudtshoorn ostriches. By demonstrating the genetic origin of breeding pairs/trios/groups, the value of the breeding individuals may also be increased.

3.4 APPLICATION OF RAPDs TO BREEDING MANAGEMENT OF OSTRICHES (*S.c. australis*) OF SOUTHERN AFRICA

3.4.1 INTRODUCTION

The aim of this aspect of the study was to evaluate the RAPD method for providing markers that could be useful in breeding programs. In particular it was hoped that RAPD markers could be used to:

(a) group individuals based on egg production capabilities.

(b) determine if similarity of the RAPD patterns could be linked to egg production.

Egg production is a single trait that can be linked to reproductive fitness of oviparous animals if the rate of producing eggs under natural and artificial conditions is known (Oldfield, 1989). Egg production rates in wild and captive (domesticated) ostrich populations have been well studied and reported (Leuthold, 1977; Bertram, 1978, 1992; Brown *et al.*, 1982; van Schalkwyk *et al.*, 1996).

Reproductive performance of birds was shown to be affected by high levels of inbreeding (Greenwood, 1987). Breeding patterns and behaviour of ostriches under natural conditions ensure that inbreeding is avoided (Brown *et al.*, 1982; Bertram, 1992). However, conditions in captivity and breeding practices over the past 150 years favoured the inbreeding of these birds (Duerden, 1908, 1920; Brown *et al.*, 1982). Although the genetic fitness and reproductive performance of ostriches have not been formally linked, the symptoms of inbreeding were identified in the southern African population of *S.c. australis*. Symptoms such as high infant mortality, high susceptibility to disease, low breeding fitness such as low egg production ability and low hatchability have been reported in the ostrich population of southern Africa from as early as the 1900's (Duerden, 1912, 1917; 1919; 1920), and are still evident. These phenomena are symptoms of inbreeding but could also be attributed to factors such as

nutritional state, flock size, etc. (Billet, 1984, 1991). Genetic data sets of *S.c. australis* suggest high levels of gene flow between individual populations from geographically isolated areas of southern Africa (Freitag and Robinson, 1992; this study Chapter 2). These studies also suggest high levels of inbreeding within the populations. The studies were based on mtDNA and they do not account for nuclear introgression or nuclear diversity. It would thus be important to determine levels of genetic diversity between and within the ostrich populations and to establish if inbreeding is affecting the genetic fitness of the present population.

Multi-locus analysis methods such as DNA fingerprinting with Jeffreys probes were successfully applied to breeding programmes of avian species such as ducks and turkeys (Hillel *et al.*, 1989) and aided in the identification of inbred chicken lineages (Kuhnlein *et al.*, 1989, 1990). Since the RAPD technique has several advantages over conventional DNA fingerprinting (Welsh and McClelland, 1990; Williams *et al.*, 1990; Dodgson *et al.*, 1997; Perés *et al.*, 1998), it could be a useful method for application in breeding programmes of ostriches. A RAPD method was recently developed to determine the sex of juvenile ostriches (Bello and Sánchez, 1999). This application of RAPDs will have a major impact on the ostrich industry. Until this method, unreliable methods were used to sex chicks or farmers had to wait until the male plumes appeared before the sex of juveniles could be determined. This development allows for the early detection of sex which in turn means that breeding pairs could be sold at a very young age. It was also demonstrated that RAPD technology would be useful in resolving breeding questions of several livestock species such as cattle (*Bos sp.*), sheep (*Ovis aries*) goat (*Capra hircus*) (Cushwa and Medrano, 1996; Rao *et al.*, 1996) and pig (*Sus scofa*) (Cushwa and Medrano, 1996).

A single primer (RUM-P8) was evaluated for its ability to distinguish domesticated ostriches based on a single qualitative trait: that of egg production. This primer was selected because it

produced distinct bands in the range of 2000 bp to 150 bp and only a small percentage of bands were shared (18.4 %: Table 3.5) amongst the different geographical populations. It was anticipated that by using a primer that provided such high levels of polymorphisms, markers that could be linked to egg production would be more easily identified.

3.4.2 MATERIALS AND METHODS

3.4.2.1 SAMPLING STRATEGY, COLLECTION AND DNA ISOLATION

The individual used in this study were from the Kalani Investments ostrich farm. Blood samples were supplied by the company and were delivered to the laboratory. The blood samples were accompanied by egg production data per camp (Appendix B; Tables 3.7 and 3.8).

These farms purchased the ostriches from a variety of farms from across the southern African. The breeding ostriches may thus have a broad genetic base. On these farms, breeding camps consisted of 10 individuals (6 females and 4 males) instead of breeding pairs. This simulated a breeding structure as observed in the wild (Leuthold, 1977; Bertram, 1978, 1992). Farm workers regularly collected the eggs from the nest and placed them in incubators.

Blood samples were collected by venupuncture and were placed into a vacu-tainer which contained 50 mM EDTA as anti-coagulant. The EDTA-blood was then transported to the laboratory at 4°C and then stored at –20°C. Section 2.2.2 contains the details of the DNA isolation procedures.

3.4.2.2 RAPD-PCR

The standardised protocol as described in Section 3.2.2.2 was followed for all PCRs.

A Hybaid (Omnigene) air-cooled thermal cycler was used for PCR amplifications. The reaction profile was 94°C for 3 minutes initial denaturation followed by 40 cycles of: 94°C for 30 seconds, 37°C for 30 seconds and 72°C for 60 seconds. After the final cycle the samples were held at 72°C for 5 minutes.

3.4.2.3 PRIMER SELECTION

Details of the 10 bp RAPD primer (RUM-P8) that was used is indicated in Table 3.2

3.4.2.4 ELECTROPHORESIS

Discontinuous SDS-Polyacrylamide gels (Laemmli, 1970) were used to resolve aliquotes (10 µl) of the amplification products (See Section 2.2.6 for details). All gels were subjected to electrophoresis at 150 V for 6.5 hr in 1 X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), using an Owl (Omeg P2) vertical electrophoresis. See Sections 2.3.2.4 and 3.2.2.4 for details.

3.4.2.5 VISUALIZATION OF RESOLVED DNA FRAGMENTS BY SILVER STAINING

The method described in Sections 2.3.2.4 and 3.2.2.5 was strictly adhered to.

3.4.2.6 ANALYSIS OF DATA SETS

(a) Semi-automated Analysis by RAPDistance version 1.03

See Section 3.2.2.6 for details. A presence/absence (1/0) matrix was compiled for RAPD profiles of the individuals from the different camps. Band positions in each individual RAPD profile were recorded against a molecular weight marker (pBR322 digested with *Hinf*I) and assigned a '1' for presence and a '0' for absence of that band.

Pairwise distances were calculated using the algorithm of Excoffier *et al.* (1992) (Table 3.4), supplied by RAPDistance software. Neighbour Joining Tree (NJTREE) version 2.0 software was used to construct the Neighbour Joining Tree that is presented in Section 3.4.3.1.

(b) Automated Analysis by GelCompar version 4.0

GelCompar version 4.0 (GelCompar) was used to analyse the RAPD profiles generated by primer RUM-P8. The Pearson product-moment correlation coefficient clustering, using Ward's algorithm was employed to determine similarities between the ostriches. The resultant dendrogram is depicted in Section 3.4.3.1.

3.4.3 RESULTS AND DISCUSSION

The general RAPD patterns observed in all the profiles (Figures 3.16 and 3.17) were similar to those previously observed (Sections 3.2.3, 3.3.3). Figure 3.16 depict RAPDs profiles generated by primer RUM-P8 and DNA from the individuals associated with good egg production. Figure 3.17 depict RAPD profiles generated by the primer RUM-P8 and DNA from individuals associated with poor egg production.

Table 3.7 gives a summary of the banding patterns observed in the RAPD profiles. A total of 325 bands was scored and amount to 53.2 % of the total available band positions. The RAPD profiles of the individuals associated with good egg production contributed 41.8 % towards the total number of bands in the database. Of these bands, 19.4 % was shared among all individuals. The RAPD profiles of the individuals associated with poor egg production contributed 55.3 % towards the total number of bands in the database. Twenty four percent of these bands were bands that were shared among all individuals.

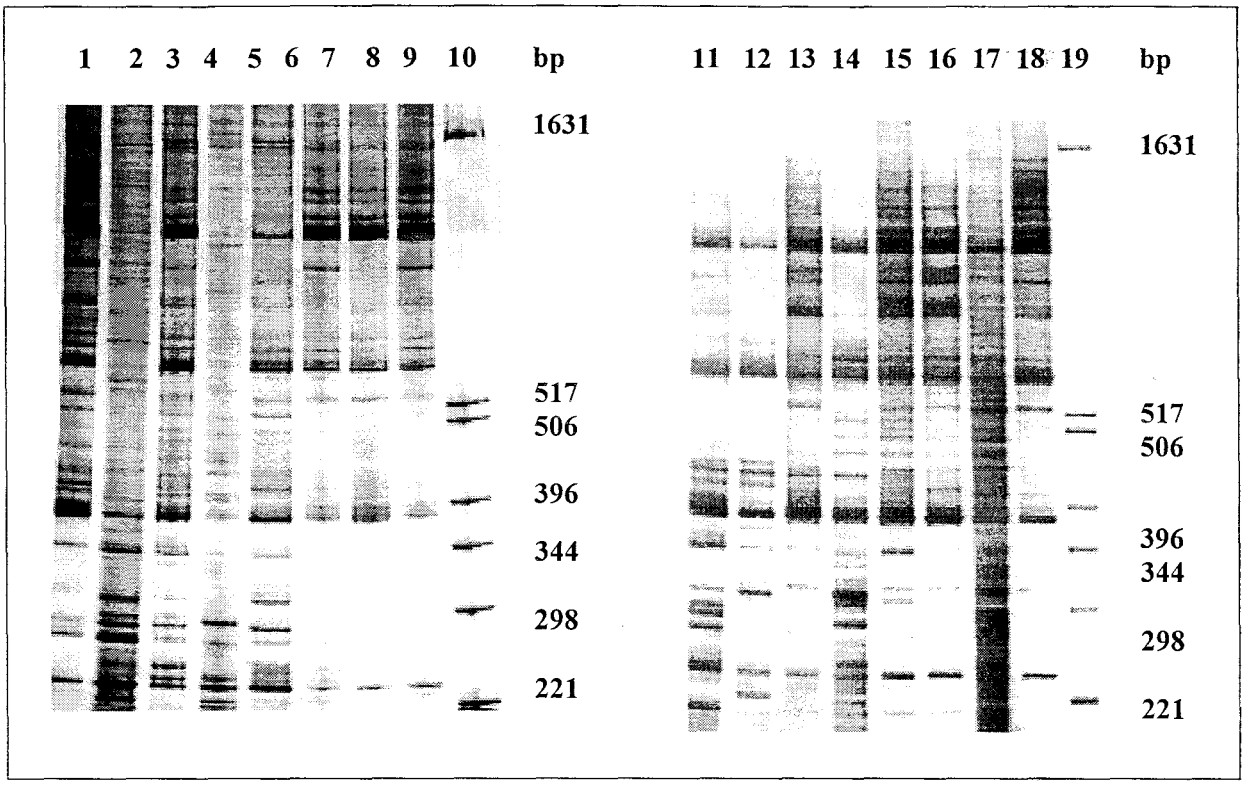


Figure 3.16: Two silver stained polyacrylamide gels, showing the ostrich RAPD fingerprints generated by primer RUM-P8. Lanes 1 to 9 contained the samples from the Kalani farm camp B3 and lanes 11 to 18 the samples from the Kalani farm camp E3. Lanes 10 and 19 contained the molecular weight marker pBR322 digested with *Hinf*I.

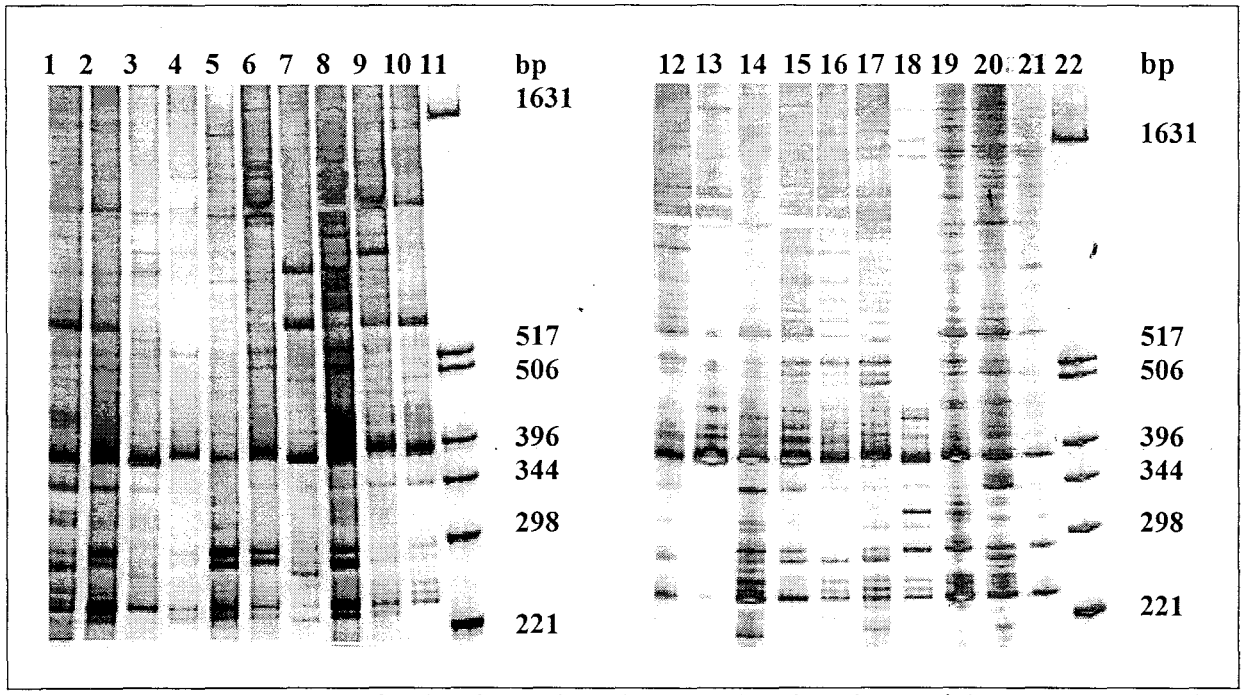


Figure 3.17: Two silver stained polyacrylamide gels, showing the ostrich RAPD fingerprints generated by primer RUM-P8. Lanes 1 to 10 contained the samples from the Kalani farm camp A2 and Lanes 12 to 21 the samples from the Kalani farm camp B2. Lanes 11 and 22 contained the molecular weight marker pBR322 digested with *Hinf*I.

Table 3.7: A summary of the total number of bands, contributions by the good and poor egg producers and the number and proportion of common bands observed in the RAPD profiles in Figures 3.16 and 3.17.

DESCRIPTION	TOTAL	GOOD EGG PROD.	POOR EGG PROD.
Number of bands	325	136	189
Proportion (%) of bands contributed by the two groups	-	41.8	58.2
Proportion (%) of bands common to all the samples	-	19.4	24.0
Proportion (%) of bands common to the profiles within each group	-	46.3	41.3

Band-sharing (common bands) within the profiles of the group of individuals associated with good egg production was 46.3 %. The profiles of the group associated with poor egg production showed that 41.3 % of the bands within this group were common bands. When common bands across all samples were compared (and not only within groups) then a lower percentage of the bands (19 %) were from the group of predominantly good egg producers were common, compared to the 24 % of the predominantly poor egg producer group. This may illustrate a relationship between egg production, poor egg producers having a higher proportion of shared bands. However, as only one primer was used, it is possible that this result is coincidental.

Nonetheless, this possible relationship between the egg production phenotype and band-sharing may be important since band-sharing is regarded as an indication of relatedness and egg production may be influenced by the degree of genetic relatedness (Greenwood, 1987). The group associated with poor egg production had a higher overall proportion of common bands (24 % common to the whole sample) than the good egg producer group (19 %). Thus when compared across the total sample range, individuals from the camps associated with good egg production are genetically more diverse than the ones from the poor egg producing camps. This may be due to independent breeding histories of the two groups but it may also indicate a linkage of the egg production phenotype to band-sharing.

When the band-sharing statistics are compared on within-group basis, there is a trend of higher within-group band-sharing (46.3 %) amongst the ostriches associated with good egg production and lower band-sharing (41.3 %) within the group associated with poor egg production. This trend is in reverse to that noted above. However, these figures are rather close, and it is possible that the difference in band-sharing between these groups is insignificant.

Further investigations are needed to test the explanations given for the above observations. These investigations should include testing more primers and monitoring the egg production behaviour of the test birds over more than one season. A larger number of primers that show similar tends would eliminate the possibility that the trends shown here were obtained by chance. If the camps would consistently show similar egg production trends as indicated above then this phenotype could be linked to the observed band-sharing and genetic similarity. Information about the origin (breeding histories) of the birds would also be valuable to support or refute reasons that the observed associations of ostriches based on egg production capabilities were due to breeding histories alone.

3.4.3.1 NUMERICAL ANALYSIS OF RAPD PROFILES

(a) Results of Semi-automated Analysis by RAPDistance version 1.03

The presence/absence matrix was analysed using RAPDistance software. Figure 3.18 depicts a Neighbour Joining Tree generated by RAPDistance using the Excoffier *et al.* (1992) algorithm.

The tree in Figure 3.18 shows three clusters. Two of these clusters include individuals from good egg production camps, and comprised 60.0 % of these individuals (Table 3.8). The other cluster contained the individuals associated with poor egg production camps and

compromised 57.0 % those individuals (Table 3.9). The percentage of individuals that were clustered with individuals of contrasting egg production capabilities was approximately 40 % (Tables 3.7 and 3.8). This tree (Figure 3.18), however, showed trends of separating individuals on the basis of egg production capabilities.

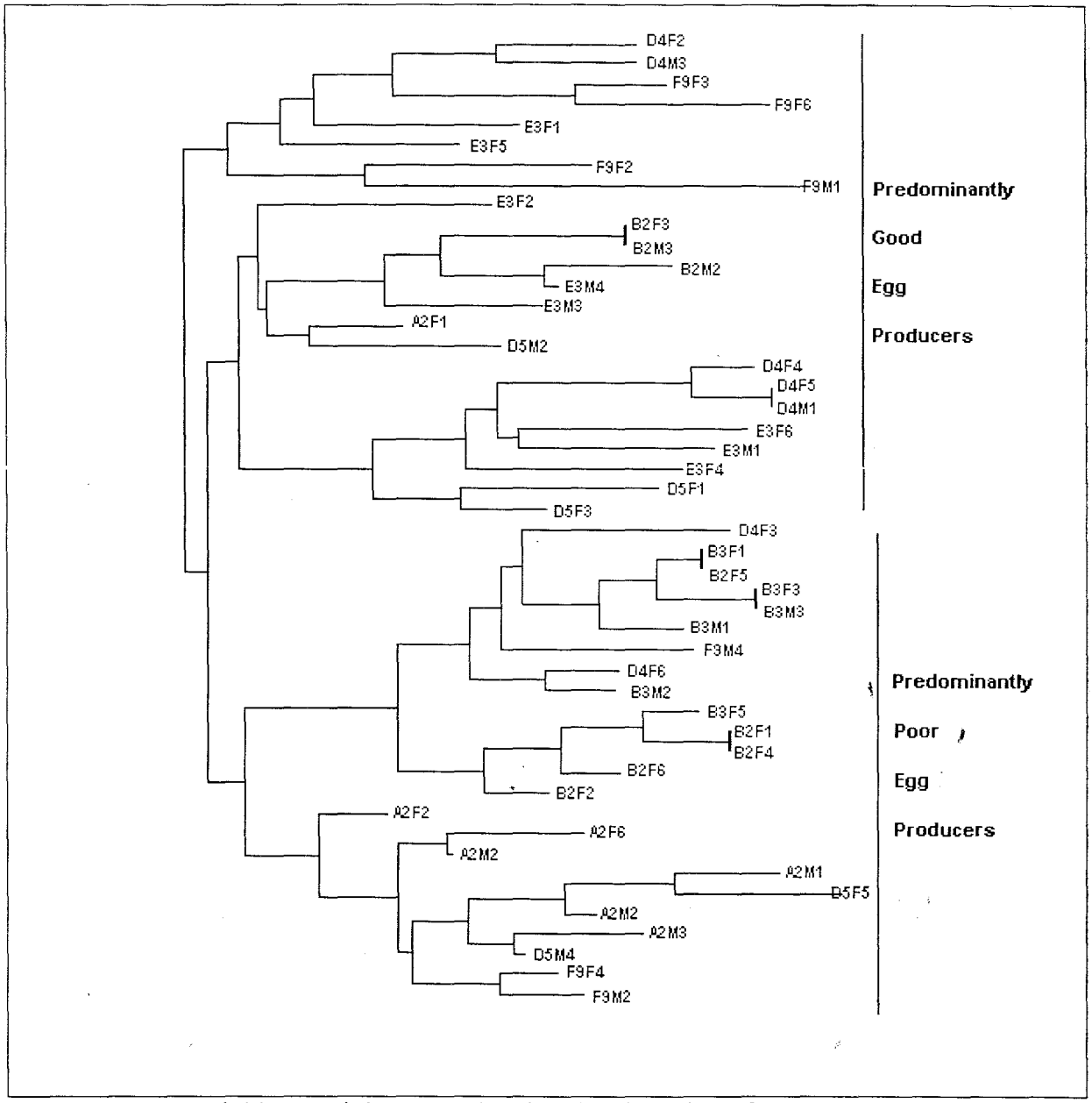


Figure 3.18: A Neighbour Joining Tree showing the clustering of domesticated ostriches based on RAPD profile data produced by primer RUM-P8. This was produced using RAPDistance software and the algorithms of Excoffier *et al.* (1992). Details of egg production capabilities are added to the tree, showing a cluster of predominantly good egg producers and a cluster of predominantly poor egg producers.

Table 3.8: A summary of the distribution of ostriches of good egg production capabilities within clusters of a Neighbour Joining Tree and a dendrogram. The Neighbour Joining Tree (Figure 3.18) was generated when RAPD profiles of ostriches were analysed by the algorithm of Excoffier *et al.* (1992). The dendrogram (Figure 3.19) was generated when RAPD profiles of ostriches were analysed by GelCompar. The egg production data per camp was for the 1992-1993 breeding season.

Camp Name	Number of eggs per camp	Neighbour Joining Tree by RAPDistance			Dendrogram by GelCompar		
		Good Egg Production Cluster	Poor Egg Production Cluster	Total	Good Egg Production Cluster	Poor Egg Production Cluster	Total
B3	350	0	6	6	4	1	5
E3	391	4	2	6	7	0	7
F9	423	8	0	8	7	1	8
Total		12	8	20	18	2	20
% of Predominantly Good Egg Producers per Cluster		**60.0**	**40.0**		**90.0**	**10**	

Table 3.9: A summary of the distribution of ostriches of poor egg production capabilities within clusters of a Neighbour Joining Tree and a dendrogram. The Neighbour Joining Tree (Figure 3.18) was generated when RAPD profiles of ostriches were analysed by the algorithm of Excoffier *et al.* (1992). The dendrogram (Figure 3.19) was generated when RAPD profiles of ostriches were analysed by GelCompar. The egg production data per camp was for the 1992-1993 breeding season.

Camp Name	Number of eggs per camp	Neighbour Joining Tree by RAPDistance			Dendrogram by GelCompar		
		Good Egg Production Cluster	Poor Egg Production Cluster	Total	Good Egg Production Cluster	Poor Egg Production Cluster	Total
A2	87	1	6	7	0	7	7
B3	87	3	5	8	2	6	8
D4	80	5	2	7	1	6	7
D5	47	2	2	4	1	3	4
Total		11	15	26	4	22	26
% of Predominantly Poor Egg Producers per Cluster		**42.0**	**57.0**		**15.4**	**84.6**	

(b) Results of Automated Analysis by GelCompar version 4.0

Cluster analysis was also performed using GelCompar. The clustering pattern was expressed as a dendrogram that is shown in Figure 3.19. This dendrogram is similar to that observed in the Neighbour Joining Tree (Figure 3.18) but shows that 90 % (Table 3.8) of ostriches

predominantly associated with good egg production camps formed a cluster. A cluster of predominantly poor egg producers contained 84.6 % of individuals associated with this egg production capability. Between 10 and 15 % of individuals were clustered with individuals of predominantly contrasting egg production capabilities. This percentage was still very high but might be relevant as egg production is camp based and not individual based.

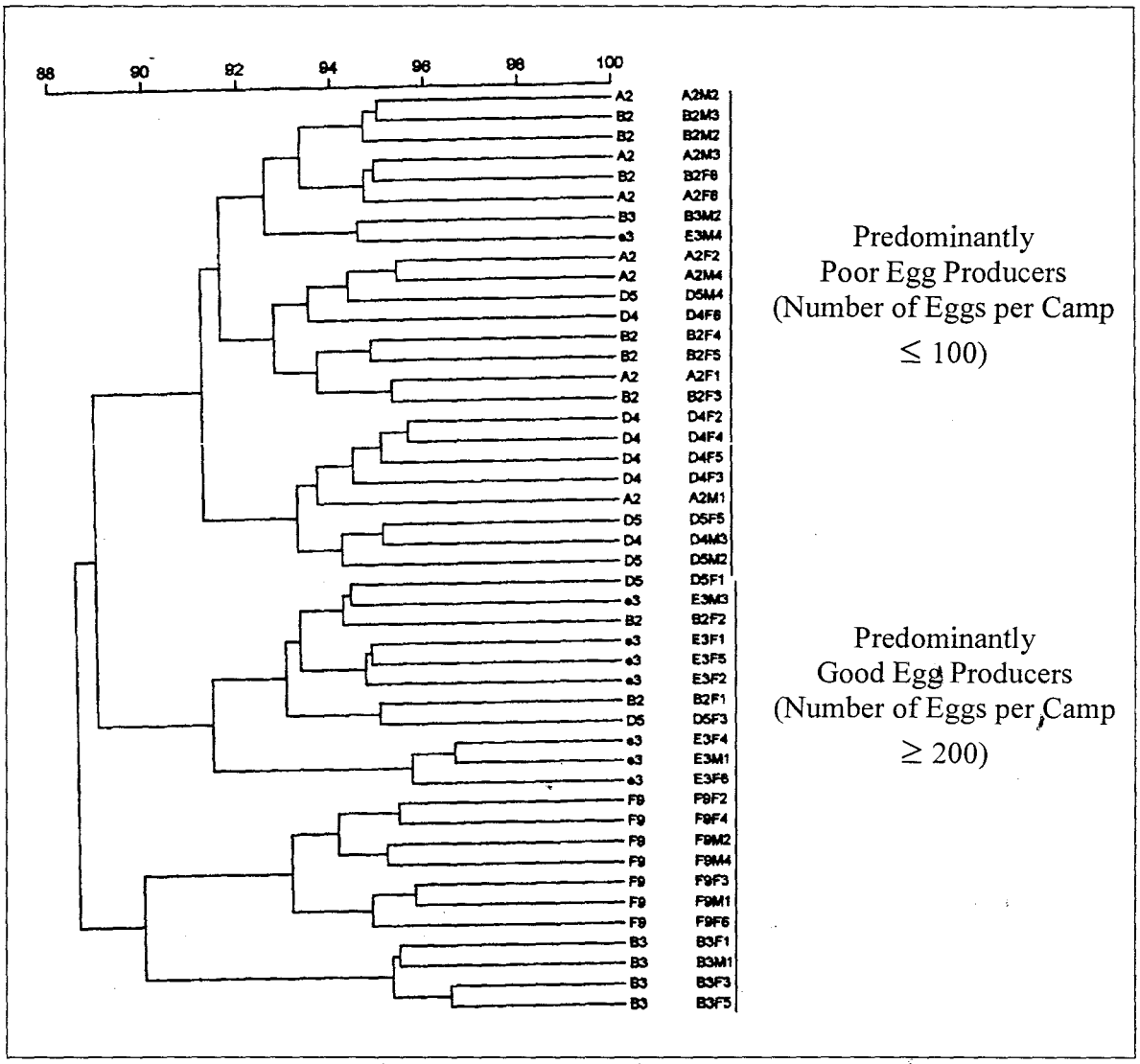


Figure 3.19: A dendrogram that was generated when ostrich RAPD profiles produced by RUM-P8 were analysed by GelCompar. All the individuals were from the Kalani breeding farm.

Percentage similarities were read from the dendrograms and were taken from the point where the cluster of a particular group is connected to that of another group. The overall average similarity within the groups was then calculated. The overall average percentage similarity

estimate within the two clusters (the one associated with good egg production and the one associated with poor egg production) was exactly the same (93.9 %). This observation supports the possibility that band-sharing differences between the two groups may be insignificant. If the genetic relationship between these two groups were determined based on the point at which the two cluster groups join then a similarity value of 89,0 % is obtained. Since the predominantly good egg producer cluster has two points at where it joins the poor egg producer cluster (89,0 % and 88,5 %), the diversity between the clusters were determined at between 2.9 % and 3.4 %. Although these values are close they indicate that genetic diversity and egg production may be linked.

3.4.4 SUMMARY

In this section of the research RAPDs was evaluated for its ability to distinguish ostriches according to good and poor egg production capabilities. The results from a single-primer RAPD approach showed separation of ostriches based on egg production trends. These data sets were not conclusive but demonstrated that RAPD technology may be useful for selecting ostriches according to breeding traits.

It should, however, be borne in mind that in this approach a single RAPD primer was used. More work needs to be done in this regard, which should include a larger database of individuals and more primers. Ideally, one or more RAPD markers associated with a specific qualitative trend should be identified and this could then be developed into a diagnostic test. It would also be interesting to determine the amount of band-sharing and levels of relatedness of the individuals that do not contribute towards egg production within camps.

3.5 APPLICATION OF RAPDs TO DETERMINING ZYGOSITY OF TWIN OSTRICHES

3.5.1 INTRODUCTION

This aspect of the research attempted to evaluate RAPDs for its ability to produce markers that could distinguish between heterozygous and homozygous twin ostriches.

DNA fingerpinting has been used in determining the zygosity of polyandrous ape species (Dixson *et al.*, 1988) and humans (Hill and Jeffreys, 1985). No literature in which this method (DNA fingerprinting) was used to determine zygosity of twin birds could be found.

Eggs with double yolks are common in poultry but the effect of this phenomenon on the efficiency of the breeding process is undetermined. It is speculated that spatial and nutritive constraints may negatively affect the development of the foetuses and could lead to decreased hatching or increased nestling mortality (Duerden, 1911b). The incidence of twinning in ostriches and the information on the zygosity could be important to the industry. Determining the zygosity of the twin birds may reflect on the genetic aspects of the twinning phenomenon.

Caetano-Anollès *et al.* (1991) showed that RAPDs could be used for twin studies. However, there are more data available in which multi-locus DNA fingerprinting was used for twin studies. Hill and Jeffreys (1985) used this DNA fingerprinting method to determine the zygosity of human twins at birth. They also showed that the DNA fingerprints of monozygotic twins were identical. Faber *et al.* (1989) used DNA fingerprinting to demonstrate spontaneous XX/XY chimerism in a human female individual that had a twin brother. The DNA fingerprinting method eliminated the need for preparatory steps that are required with cytogenetic and immunochemical methods. It also proved to be a faster method and reliable for determining chimerism.

The results of multi-locus DNA fingerprinting of other closely related individuals such as clones compared favourably to RAPD data (Kaemmer *et al.*, 1992; Baruffi *et al.*, 1995), once again illustrating the suitability of the RAPD method for studying closely related individuals. RAPD technology has a proven record in distinguishing individuals that are very closely related such as siblings (Carlson *et al.*, 1991; Hunt and Page, 1992; Apostol *et al.*, 1993; Lu *et al.*, 1995; Matioli and de Brito, 1995; Schlegel *et al.*, 1996) and clones (Van De Ven and McNicol, 1995; Sydes and Peakall, 1998). It was thus the intention to use the RAPD method to determine if it could distinguish zygosity of ostrich twins.

3.5.2 MATERIALS AND METHODS

3.5.2.1 SAMPLE COLLECTION AND DNA ISOLATION

The twin fetus samples were obtained from Dr. C. Brown Zoology Department, Rhodes University, Grahamstown. The brains of the two sets of twins were carefully excised using sterile scalpel blades and stored at –20°C. The brain material of the other representatives was obtained from the University of Cape Town Medical School. Section 2.2.2 contains the details of the DNA isolation procedures (Also see Appendix B).

3.5.2.2 RAPD-PCR

The standardised protocol as described in Section 3.2.2.2 was followed for all PCRs.

A James Duncan (model 8012) water-cooled thermal cycler was used for PCR amplifications. The reaction profile was 94°C for 3 minutes initial denaturation followed by 40 cycles of: 94°C for 30 seconds, 37°C for 30 seconds and 72°C for 60 seconds. After the final cycle the samples were held at 72°C for 5 minutes.

3.5.2.3 PRIMER SELECTION

Details of the 10 bp RAPD primers (RUM-P8 and RUM-P12) used are indicated in Table 3.2

3.5.2.4 ELECTROPHORESIS

Discontinuous SDS-Polyacrylamide gels (Laemmli, 1970) were used to resolve aliquotes (10 µl) of the amplification products (See Section 2.3.2.4 for details). All gels were subjected to electrophoresis at 150 V for 5 hr in 1 X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), using a Hoefer (SE 400) vertical electrophoresis system. See Sections 2.3.2.4 and 3.2.2.4 for details.

3.5.2.5 VISUALIZATION OF RESOLVED DNA FRAGMENTS BY SILVER STAINING

The method described in Sections 2.3.2.4 and 3.2.2.5 was strictly adhered to.

3.5.2.6 ANALYSIS OF DATA SETS

GelCompar version 4.0 was used to analyse the RAPD profiles generated by primer RUM-P8. The Pearson product-moment correlation coefficient clustering, using Ward's algorithm was employed to determine similarities between the ostriches. The resultant dendrograms are depicted in Section 3.5.3.2

3.5.3 RESULTS AND DISCUSSION

Two primers RUM-P8 and RUM-P12 were selected to determine their usefulness in establishing zygosity of two different sets of twins. Figures 3.20 and 3.21 indicate two silver stained polyacrylamide gels (10 %, w/v) that depict the profiles produced by these two primers.

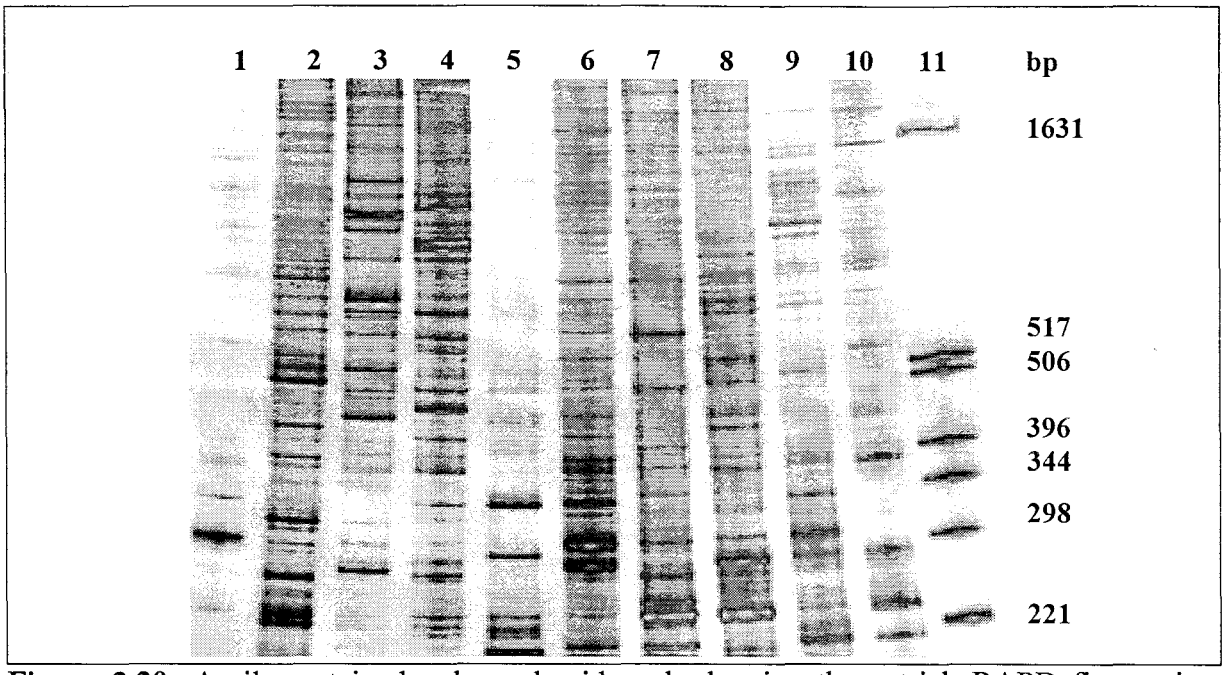


Figure 3.20: A silver stained polyacrylamide gel, showing the ostrich RAPD fingerprints generated by primer RUM-P8. Lanes 1 and 2 represented the twins ODT-A1 and ODT-B1, and Lanes 3 and 4 the twins ODT-A2 and ODT-B2. Lanes 5 to 10 contained RAPD profiles representing the following individuals GAB-1, GAB-2, GAB-3 (Gaberone representatives), ODT-1, ODT-2 (Oudtshoorn representatives) and KLEIN (Kleinzee representative). Lane 11 contained the molecular weight marker pBR322 digested with *Hinf*I.

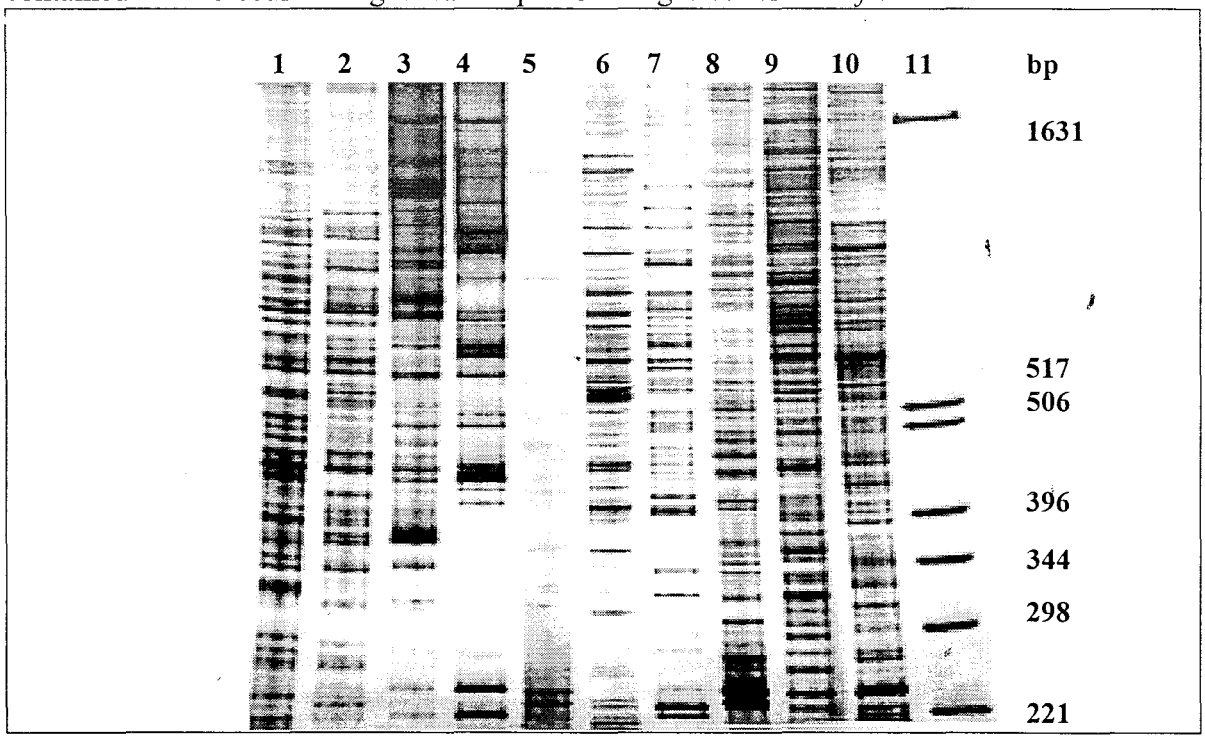


Figure 3.21: A silver stained polyacrylamide gel, showing the ostrich RAPD fingerprints generated by primer RUM-P12. Lanes 1 and 2 represented the twins ODT-A1 and ODT-B1, and Lanes 3 and 4 the twins ODT-A2 and ODT-B2. Lanes 5 to 10 contained RAPD profiles representing the following individuals GAB-1, GAB-2, GAB-3 (Gaberone representatives), ODT-1, ODT-2 (Oudtshoorn representatives) and KLEIN (Kleinzee representative). Lane 11 contained the molecular weight marker pBR322 digested with *Hinf*I

Individuals from domestic and "wild" populations are included in the database. In Figures 3.20 and 3.21, Lanes 1 and 2 indicate RAPDs profiles produced by DNA from Egg 1 (ODT-A1 and ODT-B1) and Lanes 3 and 4 show profiles generated by DNA from Egg 2 (ODT-A2 and ODT-B2). Lanes 6 to 10 depict RAPDs profiles generated by DNA from the following individuals: Gaborone 2 (GAB2), Gaborone 3 (GAB3), ODT-1, ODT-2 and Kleinzee (KLEIN).

The profiles shown here were different from those observed in the preceding sections due to the different conditions used to produce the RAPDs profiles. The information could not be used comparatively, but provided ample information about the zygosity of the twins.

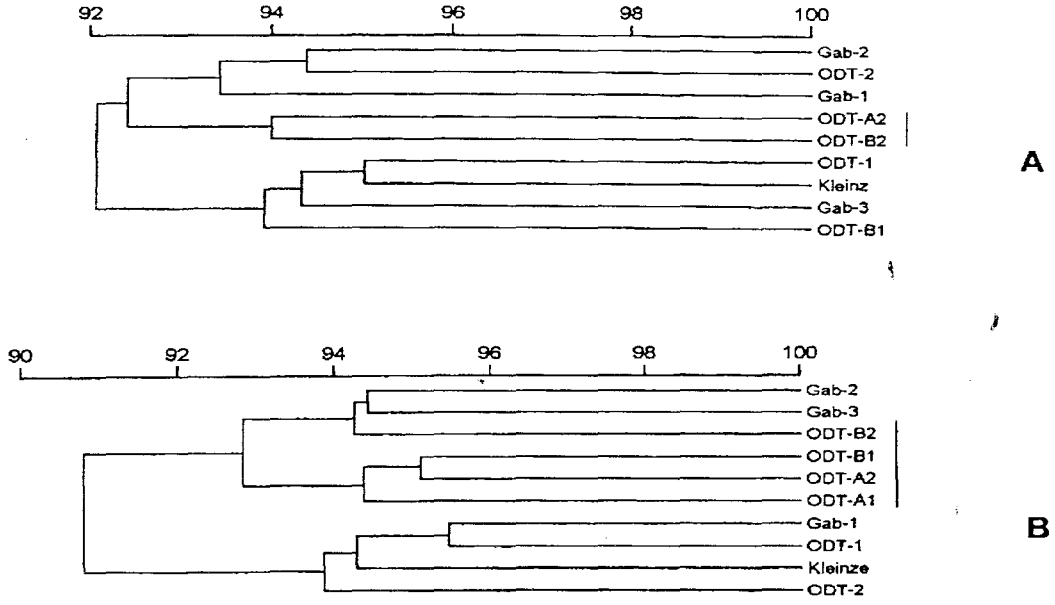


Figure 3.22: Two dendrograms that were produced when ostrich RAPD profiles were analysed using GelCompar. The dendrograms were generated using RAPD profiles from ostrich twin DNA and the primers RUM-P8 (A) and RUM-P12 (B). The clustering associations of the twins were indicated by both the dendrograms.

Visual inspection of the profiles from the two sets of twins showed that each individual had a different profile. It was also established from Figure 3.21 (Lanes 1 and 2), that the RAPDs profiles produced by primer RUM-P12 for ODT-A1 and ODT-B1 were similar but not

identical. The profiles for Egg 2 (ODT-A2 and ODT-B2: Figure 3.21, Lanes 3 and 4) were very different. This difference was particularly evident in the profiles produced by primer RUM-P8 (Figure 3.20, Lanes 3 and 4). Due to the continuous poor amplification of ODT-A1 DNA by this primer (RUM-P8), the profile of this individual was excluded from the analysis. Only GelCompar was used for the analysis of the RAPD profiles and the dendrograms are shown in Figure 3. 22 . The RAPD profiles from the gels and the dendrograms indicate that both sets of twins were not identical.

3.5.4 SUMMARY

The observation that these twins were non-identical indicates that the formation of these twins could have been due to environmental factors. The twinning phenomenon was probably due to the physical condition of the hens that produced these twins. Unfortunately information on the condition of the hen(s) that produced the sets of twins was not available. In twin studies of humans it was found that the incidence of non-identical twins increases with maternal age or is dependent on environmental factors (Duerden, 1911b; Edwards, 1968; Gaines and Elston, 1969; Emery, 1976). It is known that the breeding life of ostriches continues until they are close to thirty years old.

RAPD methods were also developed for pedigree analysis in polygamous mating systems (Hadrys *et al.*, 1993). This will have a direct application in ostrich breeding systems, whether in the wild or on certain farms that simulate conditions in the wild by keeping a sex ratio of 1:1,5 (Bertram, 1992). Microsatellites that were isolated for application in parentage determination of ostriches may also be useful for determining whether ostrich twins are identical or non-identical (Kimwele *et al.*, 1998). This method is very sensitive but RAPD technology may have advantages (Section 3.1.3) that would make it highly effective to apply to large sample sizes and routine testing of many individuals (Hadrys *et al.*, 1992).

3.6 SYNTHESIS AND CONCLUSIONS OF THE RAPD STUDY

The aim of the application of RAPD methodology was to identify markers that could be used for ostrich taxonomic purposes, the management and improvement of the genetic composition of ostrich breeding populations and determining the zygosity of twin ostriches. Considerations pertaining to the genetic improvement of organisms depend on the existence, nature and extent of genetic variability available for manipulation. The breeding strategy that is eventually adopted will be influenced by variability that exists between and within populations (Ballou and Cooper, 1992; Chalmers *et al.*, 1992). To date, assessment of ostrich genetic variability has indicated definite subspecific variability of mitochondrial genomes (Freitag and Robinson, 1993; Chapter 2, this study) and nuclear markers (Kumari and Kemp, 1998). In addition, limited phylogeographic structuring of mtDNA was observed in southern African ostrich populations (Freitag and Robinson, 1993).

The lack of genetic variability within populations as indicated by cytoplasmic DNA (mtDNA; Freitag and Robinson, 1993; Chapter 2, this study), necessitated the present study in which nuclear markers were evaluated for their ability to determine genetic variability at different taxonomic levels. It is thus essential that methods that generate nuclear markers (such as RAPDs) are evaluated and their usefulness carefully determined before decisions regarding breeding strategies for ostriches are implemented.

This study has shown that RAPD data sets possess a variety of applications. The results indicate that the RAPD technique could provide characters that are useful in the study of ostrich genetics at and below the species level.

3.6.1 APPLICATION OF RAPDs TO OSTRICH TAXONOMY

The results presented and discussed in Section 3.2.3 compare favourably with results of

previous studies (Freitag and Robinson, 1993; Kumari and Kemp, 1998; Chapter 2, this study). Although two of the subspecies (*S.c. massaicus* and *S.c. molybdophanes*) were represented by single individuals in this study, the analysis of the RAPD profiles of 8 primers (independently and collectively) showed the presence of RAPD markers unique to each ostrich subspecies. The results also showed that RAPD fingerprints for the subspecies *S.c. molybdophanes* are markedly different from those of the other two subspecies (*S.c. australis* and *S.c. massaicus*).

According to Neighbour Joining Tree analysis (Figure 3.3), *S.c. australis* is more closely related to *S.c. massaicus* than to *S.c. molybdophanes*. Geographical isolation due to barriers such as the Ethiopian Great Valley Drift separated *S.c. molybdophanes* from *S.c. massaicus* and *S.c. australis* for more than 3 million years. This provided sufficient time for disrupting gene flow between *S.c. molybdophanes* and the *S.c. massaicus* (Freitag and Robinson, 1993). The findings of this study provide support for the previous proposal that *S.c. molybdophanes* be treated as a distinct species based on mtDNA (Freitag and Robinson, 1993) and microsatellite data (Kumari and Kemp, 1998). The results of the RAPD study are thus in agreement with the other data. The RAPD technique should be further investigated for application to ostrich taxonomic issues.

Recently microsatellite markers were developed that can be used to study the relationships between ostrich subspecies (Kumari and Kemp, 1998). In the first application these markers generated data sets that agreed with those from a previous study by Freitag and Robinson (1993). Kimwele *et al.* (1998), on the other hand, isolated microsatellite markers that could be used for parental typing of the *S.c. massaicus* subspecies. These ostrich microsatellite markers will surely find many applications. Other nuclear markers such as RAPDs have also shown application in avian genetics, e.g. population studies (Haig *et al.*, 1994), sexing of birds in

general (Lessells and Mateman, 1998) and ostriches in particular (Bello and Sánchez, 1999) and taxonomy (Cohen *et al.*, 1997). The technique has shown that it provides markers that can reliably be used for species and subspecies identification of plant and animal taxa. The major advantage of the RAPD technique over other PCR based studies such as microsatellites is that no previous sequence data are required (Williams *et al.*, 1990). This study showed that RAPDs could be utilised for ostrich taxonomic purposes.

3.6.2 APPLICATION OF RAPDs IN DETERMINATION OF INTER- AND INTRAPOPULATION GENETIC DIVERSITY OF *S.c. australis* OF SOUTHERN AFRICA

The results presented in Section 3.3.3 show that geographic population structures exist within southern African ostriches (*S.c. australis*). It shows spatial distribution of genetic diversity and may have implications for future quantitative nuclear introgression studies. Such data are essential for ostrich management decisions and breeding strategies, translocation of ostriches between geographic regions and controls on import and export of ostrich products (especially live birds earmarked for breeding purposes elsewhere).

The report by Freitag and Robinson (1993) about the limited genetic variability within the gene pool of southern African ostriches did not account for nuclear introgression. They suggested that limited levels of gene flow observed between the populations could have been due to factors such as genetic bottle-necks, small founder populations and stochastic lineage sorting processes. RAPD markers, used in the present study, showed that the genetic variability between and within populations could be detected.

The RAPD results demonstrated that this technique is sensitive enough to detect genetic diversity between the populations from different southern African geographic regions, which

also represented different breeding lineages. The Grahamstown population represented a highly inbred population and the Oudtshoorn population an open population. Domesticated Namibian populations, on the other hand, might have had recent contact with presumably wild populations. Inclusion of crosses of the Oudtshoorn and Namibian populations and an individual from Namibia into the analysis presented the opportunity to observe the clustering pattern of these individuals.

The clustering pattern observed within the Neighbour Joining Trees showed groups that were predicted from the known geographical and lineage data. Although the present study was not specifically designed to determine genetic diversity within a large number of specific breeding populations, it was set up to investigate the genetic variation within regional populations which represented different lineages. The observed genetic diversity, based on this small sample size, did not reveal major differences between the Oudtshoorn population and the Grahamstown population.

Similar intrapopulation genetic diversity patterns, based on RAPD data, have been observed in other species (Chalmers *et al.*, 1992; Chapco *et al.*, 1992b; Baruffi *et al.*, 1995; Buso *et al.*, 1998; Huff *et al.*, 1998; Liao and Hsiao, 1998). Interpopulational genetic diversity (based on RAPD markers) was lower than intrapopulational in populations of certain plant (Kazan *et al.*, 1992a,b; Buso *et al.*, 1998; Huff *et al.*, 1998; Isabel *et al.*, 1999), insect (Chapco *et al.*, 1992b) and avian species (Haig *et al.*, 1994). Reasons for this phenomenon were ascribed to a variety of factors ranging from sampling techniques (Buso *et al.*, 1998; Isabel *et al.*, 1999) to actual genetic events such as population fragmentation and dispersal mechanisms (Haig *et al.*, 1994). Larger sample sizes, including more representative populations and more geographical areas, should provide greater significance to the RAPD result (Pérez *et al.*, 1998). The results obtained here can thus be treated as relevant, although the sample size was small.

Reduced intra- and interpopulation genetic diversity may be a real phenomenon in southern African ostriches (*S.c. australis*) that can be explained in terms of the recent history of the ostrich industry. High dispersal rates of ostriches prior to domestication and small founder populations (Douglass, 1881; Layard, 1884; Schreiner, 1898; Brown *et al.*, 1982; Bertram, 1992), major translocations and inbreeding strategies (Duerden, 1908, 1910, 1911a, 1919; Wagner, 1986; Swart, *et al.*, 1987) could all have resulted in the reduced genetic variability that was observed in this and previous studies (Freitag and Robinson, 1993).

It can thus be concluded that RAPD technology is useful for inter- and intrapopulation genetic diversity of geographically distinct populations of southern African ostriches (*S.c. australis*) and that can it generate clustering patterns indicative of the known lineages of these ostrich populations/groups.

3.6.3 APPLICATION OF RAPDs TO BREEDING MANAGEMENT OF OSTRICHES (*S.c. australis*) OF SOUTHERN AFRICA

This study explored the usefulness of RAPDs as a tool, identifying ostrich groups that conform to certain reproductive standards. The high prices that are being paid for breeding pairs/groups require that these pairs/groups are of the finest quality only. A single avian reproductive trait (egg production) that has been linked to levels of intrapopulation diversity and inbreeding (Greenwood, 1987; Oldfield, 1989).

This study was not intended to determine inbreeding levels of ostriches, but investigated the ability of RAPDs to group ostriches from a single population according to their reproductive capability, and to determine if there was any relationship between the genetic variability and the egg production capabilities. The results based on one primer showed that individuals with different egg production capabilities could be clustered. Results showed that ostriches from

predominantly good egg production camps tended to form one cluster while birds from predominantly poor egg production camps tended to form a second cluster. This phenomenon should be further investigated, using more primers, samples from different populations in the same region and samples from different populations within different regions. Other traits that can be linked to inbreeding depression should also be investigated and compared to these findings.

Inbreeding is one of the possible causes for the low level of mtDNA diversity that was observed in ostriches (*S.c. australis*) (Freitag and Robinson, 1993; Chapter 2, this study). Inbreeding as well as outbreeding strategies were followed in the breeding of ostriches of southern Africa for most of this century (Duerden, 1912, 1917, 1920; Brown *et al.*, 1982; Bertram, 1992). The effects of these practices may only become more evident in future breeding stocks. It is thus essential that sufficient genetic data are accumulated to devise programmes to counter the negative effects of inbreeding or outbreeding depression. Inbreeding depression could lead to problems such as low fertility and decreased individual fitness (Bensch *et al.*, 1994). Inbreeding could also cause a complete loss of genetic diversity in small populations (Huff *et al.*, 1998). The expression of deleterious genes within inbred populations is also of great concern to breeders. These genes may be concentrated during successive inbreeding stages and could also have severe financial implications. If the expression of these genes is infrequent it may go unreported or individuals maybe eliminated by culling or natural death (Dolf *et al.*, 1991). The emphasis should thus be towards optimal outbreeding, in which optimum genetic variability is carefully maintained (Greenwood, 1987).

The avian industry has proud records of creating inbred strains of poultry with desired characteristics such as the White Leghorns that are resistant to Marek's disease and are good

egg producers (Kuhnlein *et al.*, 1989, 1990). In this case inbreeding is the desired genetic effect and since eggs are the commercial product for human consumption, hatchability is non-essential and thus not accounted for. However, in ostrich husbandry, egg production should also be linked to hatchability (Coetsee, 1993; van Schalkwyk *et al.*, 1996). It is the reproduction rates of the parents and survival rates of their offspring that are essential factors for breeders. Individuals with low reproduction rates are more valuable as slaughter birds than as breeding stock.

In some applications where RAPDs were used in plant breeding programmes, it was utilised as a cost effective selection tool by producing: (i) markers for progeny selection (Conifers: Carlson *et al.*, 1991) (ii) population specific markers (Legumes: Chalmers *et al.*, 1992); (iii) disease resistance markers (Barley: Barua *et al.*, 1993); (iv) markers identifying potential parents in the genetic mapping of populations (Legumes: Kazan *et al.*, 1993a, b); (v) separate markers for inbreeders and for outbreeders (Grasses: Stammers *et al.*, 1995). These applications of RAPD methodology, in conjunction with appropriate methods of numerical analysis, showed that it was a fast, efficient and reliable technology for effective management of breeding programmes.

3.6.4 APPLICATION OF RAPDs TO DETERMINE ZYGOSITY OF TWIN OSTRICHES

The results presented here suggested that both sets of twins were non-identical. In twin studies of humans it was found that the incidence of non-identical twins increases with maternal age or is dependent on environmental factors (Duerden, 1911b; Edwards, 1968; Gaines and Elston, 1969; Emery, 1976).

The real value of these results can only be determined if pedigree data were available or if the putative parents could also be sampled as controls. This was however not possible and individuals from a variety of diverse origins were used as controls.

Chapter 4

Conclusions and Prospects

4.1 CONCLUSIONS

Animal agriculture is a multimillion rand global industry. The success of the industry is, however, dependent on the heritability of fecundity (i.e. breeding efficiencies). Breeding programmes have mainly relied on selection to increase efficiency. Modern techniques such as artificial insemination, cryopreservation techniques, *in vitro* fertilization and identification and manipulation of genes may not yet be suitable or cost effective for use in ostrich breeding programmes. Selection is thus the immediately accessible process available for improving breeding efficiency.

The aim of this research project was to evaluate and adapt existing molecular techniques for application to genetic studies on the ostrich, *Struthio camelus*. The methods were evaluated for their applicability at different taxonomic and population levels, the cost and time-effectiveness thereof and the technical difficulty in executing the methods. Methods were regarded as useful for general use if large numbers of individuals could be studied simultaneously, rendering their application cost effective. It was the intention to select non-destructive sampling methods and user - and environment friendly techniques. The molecular methods used in this study can be divided into methods that targeted the cytoplasmic DNA (mtDNA) and methods that targeted nuclear DNA (RAPDs).

Restriction endonuclease fragment length analysis of mtDNA segments that were amplified by the polymerase chain reaction (PCR-RFLP) and randomly amplified polymorphic DNA sequences (RAPDs) were the methods that were investigated in a variety of applications deemed to be important to the ostrich breeding industry.

4.2 MITOCHONDRIAL DNA ANALYSIS: BY PCR-RFLP

Mitochondrial DNA variation was investigated by employing restriction enzyme analysis of fragments amplified by the polymerase chain reaction (PCR-RFLP). The method was found to be a very cost and time effective technology that could analyse relatively large numbers of individuals. The method is not as sensitive as direct sequencing but would be useful in preliminary studies at the intraspecific level in ostriches.

A total of 28 individuals, representing 3 different geographical areas in southern Africa and possibly also different genetic pools, was included in this part of the study. The study dealt extensively with *S.c. australis*, but one representative of the subspecies *S.c. molybdophanes* was included in some of the analysis.

4.2.1 ANALYSIS OF GENETIC DIVERSITY OF OSTRICH POPULATIONS

4.2.1.1 SEQUENCING

The 450 bp 12S rRNA gene fragment was sequenced to establish integrity of the amplified fragments when compared with published data (Cooper *et al.*, 1992; Lee *et al.*, 1997). The sequences of individuals from the Oudtshoorn, Oudtshoorn/Namibian hybrids and the Swaziland population were identical to the data published by Cooper *et al.*, (1992). This 12S rRNA gene fragment did not show any nucleotide differences for the populations included in this study. It appeared that the fragment was unsuitable for detecting population differences between ostriches (*Struthio camelus*) from different breeding histories. This finding was inconclusive due to the limited number of individuals and populations tested.

4.2.1.2 PCR-RFLP ANALYSIS OF mtDNA FRAGMENTS

PCR-RFLP analysis of a 450 bp12S rRNA gene fragment and a 550 bp D-loop fragment was

conducted. Both fragments (12S rRNA and D-loop) did not reveal any RFLPs in the *S.c. australis* populations that were sampled. A total of nine restriction enzymes surveyed these two fragments of 27 individuals and represented 243 restriction sites.

The 12S rRNA gene is highly conserved (discussed in Section 2.1.5.1) in avian species and observing no restriction site polymorphisms in fragments of this gene may not be extraordinary for domesticated ostriches (*S.c. australis*). This result is also in agreement with sequencing data.

Some variability was expected within the D-loop, normally a highly polymorphic region (Quinn and Wilson, 1993; Liu *et al.*, 1996; Härlid *et al.*, 1998). This fragment seemed to be the fragment of choice for population studies (Meyer, 1994). However, no variation in the D-Loop was found indicating that these populations are probably very closely related.

The ostrich industry was witness to several events such as population genetic bottle-necks, stochastic lineage-sorting processes, selection of female haplotypes and random extinctions (Duerden 1920; Brown *et al.*, 1982; Wagner, 1986; Bertram, 1992; Freitag and Robinson, 1993), that may have eroded the genetic variability of ostriches in domestication (Avise *et al.*, 1988; Houlden *et al.*, 1996; Shaw *et al.*, 1999). The results from the present study are similar to that of a previous study in which limited genetic variability for ostriches from southern Africa (*S.c. australis*) was observed (Freitag and Robinson, 1993) and can thus be accepted as a corroboration of the genetic status between the populations that were investigated. It confirms the hypothesis that the founder events associated with the start of ostrich domestication, the subsequent breeding strategies and the soaring and sudden decline in ostrich numbers over the past 150 years have all contributed towards low levels of ostrich genetic diversity. This could mean that the ostrich population of southern Africa is highly inbred and that the symptoms of inbreeding depression, such as low breeding fitness, observed within some populations, may be real phenomena. This

RAPDs provided data that were used to show similarities and differences between different ostrich subspecies. The data showed that the subspecies *S.c. australis* is more closely related to *S.c. massaicus* than to *S.c. molybdophanes*. This is in agreement with data from mtDNA, morphological characteristics and the biogeography of these birds, confirming existing theories of evolutionary relationships of ostrich subspecies (Figure 1.2: Freitag and Robinson, 1993; Kumari and Kemp, 1998).

RAPD technology was also used to investigate genetic variability between representative populations of ostriches from different geographically isolated areas in southern Africa. The histories of these areas (Grahamstown, Oudtshoorn, Klein Karoo, and Namibia) are closely linked to the history of the ostrich industry, both internationally and locally. The breeding populations from these areas may each represent a different gene pool.

Varying degrees of genetic variability were observed between the populations from the different geographical areas reflecting their historical isolation. Examination of the similarity matrices showed that the similarity was greater in the known inbred population than in the outbreeding population.

This study also showed that RAPDs data might be linked to a qualitative trait, that of egg production by camps of 8 to 10 birds. For this part of the study, single primer (RUM-P8) was selected that generated RAPD profiles of ostrich DNA that were least similar. The numerical analysis of these profiles showed trends of clustering ostriches from the same camps based on the predominant egg production capability of the camp. The clustering pattern and band-sharing of the predominantly poor egg production cluster also indicates lower genetic variability between these individuals.

It is known that low genetic variability (high similarity) causes reduced egg production and hatchability in birds (Greenwood, 1987; Oldfield, 1989; Bensch *et al.*, 1994). The reproduction rate of present ostrich populations may be under "genetic stress" and could be corrected by selecting individuals that are genetically different (Greenwood, 1987; Bensch *et al.*, 1994).

In the wild, birds tend to avoid inbreeding by several methods such as dispersal (Greenwood, 1987), non-incestuous mating (Bensch *et al.*, 1994), brood parasitism (Gibbs *et al.*, 1998) and polygamous breeding systems (Bertram, 1978, 1992). In captivity, however, the breeding patterns of animals change, as is the case in ostriches. The birds become sexually mature at a younger age and the mating behaviour may also change (Bertram, 1978, 1992; Brown *et al.*, 1982). The captive breeding systems should thus simulate natural conditions. Provisions should be made for the natural promiscuous mating behaviour of ostriches by including several ostriches into a breeding camp that has sufficient space. The natural adult sex ratio of 1:1,5 (male:female) should also be followed (Jarvis *et al.*, 1985; Bertram, 1992). These minimal requirements are not always provided by breeding farmers (Swart *et al.*, 1987; Bertram, 1992). Hatching and pedigree data would also be essential for the management of the breeding behaviour of ostriches. This information would be needed so that a link between genetic structure and productivity can be established and further investigated. DNA fingerprinting methods such as RAPDs showed that this technology is useful for such studies in the absence of pedigree data (Bensch *et al.*, 1994).

In another application RAPDs revealed that two sets of twin ostriches were genetically different indicating that the sets of twins had different parents. The potential of twin studies in the genetic analysis of ostriches is undetermined because the phenomenon of twins is a rare occurrence. Instances of twins occurring within a population should be carefully monitored and the possible causes identified. RAPD methodology provides a means of identifying the parents of the twins and may be useful in identifying possible genetic factors leading to the formation of twins.

Alternative methods for the study of twins include multi-locus DNA fingerprinting, microsatellite and double-stringency RAPDs approaches.

4.4 PROSPECTS

A prospect of the PCR-RFLP result presented in this study is that this method may be a useful alternative to direct sequencing even though it may not be as sensitive. The time and cost effectiveness of the PCR-RFLP method and the ability to analyse large sample sizes makes it an attractive method for routine use in ostrich genetic studies at the subspecies level. Identified polymorphic regions could be sequenced to determine the extent of the polymorphism.

This study has also shown that RAPD technology has the potential to provide markers that could help in addressing genetic questions of ostriches ranging from subspecies differentiation, population genetic variability studies to determining whether twin ostriches were identical or not. The results of the RAPD analysis were complementary to data obtained from mtDNA analysis (sequencing and PCR-RFLP). The data sets from this study (RAPDs, sequencing and PCR-RFLP) also agreed with data from a previous study (Freitag and Robinson, 1993).

The application of RAPDs to distinguish subspecies of ostrich could be developed to address taxonomic issues in this species. Subspecific markers will be useful to study the effects of hybridization on the present ostrich population. RAPDs have been previously used in studies of hybrid zone genetics in organisms such as fire ants (Shoemaker *et al.*, 1994) and plant species such as roses (Crawford *et al.*, 1993), lilacs (Marsolais *et al.*, 1993), irises (Cruzan and Arnold, 1993, 1994) and aloe (Barker *et al*, 1996). These studies clearly indicated the parent subspecies markers in the RAPD profiles of the hybrids. The application of RAPD technology for studies of subspecies hybrids could thus also be investigated.

The RAPD method also showed that it could generate markers that distinguished ostriches from different breeding populations. This implies that the RAPD method can be developed to study the genetic variability within and between ostrich populations. Such an application would address the problems that are associated with breeding closely related (inbreeding depression) or too distantly related (outbreeding depression) individuals. It will thus have a direct and immediate impact on the present breeding strategies of ostriches.

Another evaluation of the RAPD techniques was to determine whether RAPDs could distinguish ostriches based on a qualitative breeding trend. Breeding fitness in birds is coupled to egg production and in this study it was shown that RAPD analysis could group ostriches from camps with predominantly similar egg production capabilities. The prospects of the RAPDs thus show that this technology could be used for generating markers in ostriches that could be linked to traits such as reproductive fitness, a fast growth rate, high quality meat, hides and feathers, rapid maturity rate and high fecundity levels. Instead of phenotypic data, genotypic data could be used to select ostriches for breeding purposes and establishing breeding groups in which optimal outbreeding occur (Greenwood, 1987; Bensch *et al.*, 1994). Farmers could use RAPD evidence to negotiate a fair price for their birds, selling them at a very young age thus reducing their own production cost. They would also be able to identify birds that are unsuitable for breeding and these can be sold to farmers specializing in raising and selling slaughter birds. RAPD technology would thus streamline productivity in the ostrich breeding industry.

With careful laboratory techniques, sampling strategies and further research RAPDs could become a standard tool, addressing most, if not all, genetic questions that exist in the ostrich industry.

REFERENCES

Aagaard, J.E., Krutovskii, K.V. and Straus, S.H. 1998. RAPD markers of mitochondrial origin exhibit lower population diversity and higher differentiation than RAPDs of nuclear origin in Douglas fir. *Molecular Ecology*, **7:** 801 – 812.

Agriculture Information Sheet. 1989. Agriculture in South Africa. Department of Agriculture, South Africa.

Akopyanz, N., Bukanov, N.O., Westblom, T.U. and Berg, D.E. 1992. PCR-based RFLP analysis of DNA sequence diversity in the gastric pathogen *Helicobacter pylori. Nucleic Acids Research*, **20:** 6221 - 6225.

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, F.H., Smith, A.J.H., Staden, R. and Young, I.G. 1981. Sequence and organisation of the human mitochondrial genome. *Nature*, **290:** 457 - 465.

Anderson, S., de Bruijn, M.H.L., Coulson, A.R., Eperon, I.C., Sanger, F. and Young, I.G. 1982. complete sequence of bovine mitochondrial DNA: Conserved features of the mammalian mitochondrial Genome. *Journal of Molecular Biology*, **156:** 683 - 717.

Apostol, B.L., Black (IV), W.C., Miller, B.R., Reiter, P. and Beaty, B.J. 1993. Estimation of the number of full sibling families at an oviposition site using RAPD-PCR markers: Application to the mosquito *Aedes aegypti. Theoretical and Applied Genetic*, **86:** 991 - 1000.

Armstrong, J.A., Gibbs, A., Peakall, R. and Weiller. 1994. The RAPDistance version 1.03 package. ftp://life.anu.edu.au (150.203.38.740) pub/molecular_biology/rapd-pack.exe.

Arnold, M.L., Shaw, D.D. and Contreras. 1987. Ribosomal RNA-ecoding DNA introgression across a narrow hybrid zone between subspecies of grasshopper. *Proceedings of the National Academy of Science, USA.*, **84:** 3946 – 3950.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Strahl, K. 1988. Current protocols in molecular biology. John Wiley and Sons, New York.

Avise, J.C. and Ball, R.M. 1992. Mitochondrial DNA and avian microevolution. *Acta XX Congress of International Ornithology*. **1:** 514 – 524.

Avise, J.C., Ball, R.M., Arnold, J. 1988. Current versus historical population sizes in vertebrate species with high gene flow: A comparison based on mitochondrial DNA lineages and inbreeding theory for neutral mutations. *Molecular Biology and Evolution*, **5 (4):** 331 – 344.

Avise, J.C., Helfman, G.S., Saunders, N.C. and Hales, L.S. 1986. Mitochondrial DNA differentiation in north atlantic eels: Population genetic consequences of an unusual life history pattern. *Proceedings of the National Academy of Science, USA.*, **83:** 4350 - 4354.

Avise, J.C., Nelson, W.S. and Sibley, C.G. 1994a. DNA sequence support for a close phylogenetic relationship between some stork and New World vultures. *Proceedings of the National Academy of Science, USA.*, **91:** 5173 – 5177.

Avise, J.C., Nelson, W.S. and Sugita, H. 1994b. A speciational history of "living fossils": Molecular evolutionary patterns in horseshoe crabs. *Evolution*, **48 (6):** 1986 - 2001.

Baker, A.J., Daugherty, C.H., Colbourne, R. and McLennan, J.L. 1995. Flightless brown kiwis of New Zealand possess extremely subdivided population structure and cryptic species like small mammals. *Proceedings of the National Academy of Science, USA.*, **92:** 8254 – 8258.

Ballou, J.D. and Cooper, K.A. 1992.Genetic management strategies for endangered captive populations: The role of genetic and reproductive technology. **In:** Biotechnology and conservation of genetic diversity. **pp 183 - 206.** (eds. Moore, H.D.M., Holt, W.V. and Mace, G.M.). Clarendon Press, Oxford.

Barker, N.P., Kruger, L.Y., Vlok, J.H. and Harley, E.H. 1996. RAPD data suggest the Gamkapoort Aloe to be of hybrid origin. **62 (2):** 292 – 295.

Barua, U.M., Chalmers, K.J., Hackett, C.A., Thomas, W.T.B., Powel, W. and Waugh, R. 1993. Identification of RAPD markers linked to a *Rhynchosporium secalis* resistance locus in barley using near-isogenic lines and bulked segregant analysis. *Heredity*, **77:** 177 - 184.

Baruffi, L., Damian, G., Guglielmino, C.R., Bandi, C., Malacrida, A.R. and Gasperi, G. 1995. Polymorphism within and between populations of *Ceratitis capitata*: comparison between RAPD and multilocus enzyme electrophoresis data. *Heredity*, **74:** 425 - 437.

Bassam, B.J., Caetano-Anollès, G. and Gresshoff, P.M. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Analytical Biochemistry*, **196:** 80 - 83.

Bauert, M.R., Kälin, M., Baltisberger, M. and Edwards, P.J. 1998. No genetic variation detected within isolated relict populations of *Saxifraga cernua* in the Alps using RAPD markers. *Molecular Ecology*, **7:** 1519 – 1527.

Becker, I.I. 1987. Biochemical and mitochondrial population studies of southern African hake *Merluccius capensis* and *Merluccius paradoxus*. PhD Thesis, University of Cape Town, South Africa.

Bell, A. 1991. Bird on the wing. *Cosmopolitan*, **May:** 96.

Bello, N. and Sánchez, A. 1999. The identification of a sex-specific DNA marker in the ostrich using a random amplified polymorphic DNA (RAPD) assay. *Molecular Ecology*, **8:** 667 – 669.

Bensch, S., Hasselquist, D. and von Schantz, T. 1994. Genetic similarity between parents predicts hatching failure: Non-incestuous inbreeding in the great reed warbler? *Evolution,* **48 (2):** 317 - 326.

Bertram, B.C.R. 1978. Breeding system and strategies of ostriches. *Proceedings of the17th International Ornithological Congress*, West Berlin.

Bertram, B.C.R. 1992. The ostrich. Communal nesting system. Princeton University Press, New Jersey.

Bibb, M.J., van Etten,R.A., Wright, C.T., Walberg, M.W. and Clayton, D.A. 1981. Sequence and gene organisation of mouse mitochondrial DNA. *Cell*, **26:** 167 - 180.

Billet, R. 1984. Ostrich feedlotting. *Farmers Weekly*, **July 13:** 6 - 9.

Billet, R. 1991. Ostriches can make money. *Farmers Weekly*, **July 19:** 66 - 69.

Blackstone, N.W. 1995. A unitis-of-evolution perspective on the endosymbiont theory of the origin of the mitochondrion. *Evolution* **49 (5):** 785 – 796.

Boleda, M.D., Briones, P., Farrès, J., Tyfield, L. and Pi, R. 1996. Experimental design: A useful tool for PCR optimization. *BioTechniques,* **21:** 134 -140.

Brown, L.H., Urban, E.K. and Newman, K. 1982. Birds of Africa. **Volume I**. Academic Press Inc., London.

Brown, W.M., and Vinograd, J. 1974. Restriction endonuclease cleavage maps of animal mitochondrial DNAs. *Proceedings of the National Academy of Science, USA.*, **71:** 4617 - 4621.

Brown, W.M. and Wright, J.W. 1979. Mitochondrial DNA analysis and the origin and relative age of parthenogenetic lizards (Genus *Cnemidophorus*). *Science*, **203:** 1247 - 1249.

Brown, W.M., George, M. and Wilson, A.C. 1979. Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Science, USA.*, **76:** 1967 - 1971.

Bucci, G. and Menozzi, P. 1995. Genetic variation of RAPD markers in a *Picea abies* Karst. population. *Heredity*, **75:** 188 - 197.

Burke, T. and Burford, M.W. 1987. DNA fingerprinting in birds. *Nature*, **327:** 149 - 152.

Buso, G.S.C., Rangel, P.H. and Ferreira, M.E. 1998. Analysis of genetic variability of South American populations (*Oryza glumaepatula*) with isozymes and RAPD markers. *Molecular Ecology*, **7:** 107 – 117.

Caetano-Anollès, G., Bassam, B.J. and Gresshoff, P.M. 1991. DNA amplification using very short arbitrary oligonucleotide primers. *Bio/technology*, **9:** 553 - 557.

Cann, R.L. and Wilson, A.C. 1983. Length mutations in human mitochondrial DNA. *Genetics,* **104:** 699 - 711.

Cann, R.L., Stoneking, M. and Wilson, A.C. 1987. Mitochondrial DNA and human evolution. *Nature,* **325:** 31 - 36.

Carlson, J.E., Tulsieram, L.K., Glaubitz, J.C., Luk, V.W.K., Kauffeldt, C. and Rutledge, R. 1991. Segregation of random amplified DNA markers in F1 progeny of conifers. *Theoretical and Applied Genetics,* **83:** 194 - 200.

Carrano, A.V., Lamerdin, J., Ashworth, L.K., Watkins, B., Branscomb, E., Slezak, T., Raff, M., De Jong, P.J., Kieth, D., McBride, L., Meister, S. And Kronick, M. 1989. A high-resolution, fluorescence-based, semi-automated method for DNA fingerprinting. *Genomics,* **4:** 129 - 136.

Chalmers, K.J., Waugh, R., Sprent, J.I., Simons, A.J. and Powell, W. 1992. Detection of genetic variation between and within populations of *Gliricidia sepium* and *G. maculata* using RAPD markers. *Heredity,* **69:** 455 - 472.

Chambers, G. K. 1994: DNA fingerprinting: A versatile technology. *Australasian Biotechnology,* **4:** 99 - 102.

Chapco, W., Kelln, R.A. and Mcfadyen, D.A. 1992a. Intraspecific mitochondrial DNA variation in the migratory grasshopper, *Melanoplus sanguinipes*. *Heredity*, **69:** 547 - 557.

Chapco,W., Ashton, N.W., Martel, R.K.B., Antonishyn, N. and Crosby, W.L. 1992b. A feasibility study of the use of random amplified polymorphic DNA in the population genetics and systematics of grasshoppers. *Genome*, **35:** 569 – 574.

Clary, D.O., and Wolstenholme, D.R. 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: Nucleotide sequence, gene organisation and genetic code. *Journal of Molecular Evolution*, **22:** 252 - 271.

Coetsee, J. 1993. Oos-Kaap wil Sterk Volstruisbedryf Vestig. *Landbouweekblad*, **Oktober 22:** 34 - 38.

Cohen, B.L. Baker, A.J., Blechschmidt, K., Dittmann, D.L., Furness, R.W., Gerwin, J.A., Helbig, A.J., de Korte, J., Marshall, H.D., Palma, R.L., Peter, H.-U., Ramli, R, Siebold, I., Willcox, M.S., Wilson, R.H. and Zink, R.M. 1997, Enigmatic phylogeny of skuas (Aves: Stercorariidae). *Proceedings of the Royal Society, London, Series B.*, **264:** 181 – 190.

Cook, L.M., Soltis, P.S., Brunsfeld, S.J. and Soltis, D.E. 1998. Multiple independent formations of *Tragopogon* tetraploids (Asteraceae): evidence from RAPD markers. *Molecular Ecology*, **7:** 1293 – 1302.

Cooper, A., Mourer-Chauvire, C., Chambers, G.K., von Haeseler, A., Wilson, A.C. and Pääbo, S. 1992. Independent origins of New Zealand moas and kiwis. *Proceedings of the National Academy of Science, USA.*, **89:** 8741 - 8744.

Correspondent. 1991. Karoo co-op 'Stifles Trade in Ostrich Skins'. *Business Day*, **May, 13.**

Correspondent. 1993. Ostriches spread their wings. *Farmer's Weekly*, **April, 2.**

Correspondent. 1993. Abattoir is costly "Albatross": Relief for ratepayers if ostrich slaughter starts. *Grocott's Mail,* **August, 31.**

Correspondent. 1993. Grahamstown ostrich industry set to boom. *Grocott's Mail,* **October, 29.**

Cracraft, J. and Mindell, D.P. 1989. The early history of modern birds: a comparison of molecular and morphological evidence. **In:** The Hierarchy of life. **Pp 389 – 403.** (eds. Fernholm, B., Bremer, K. and Jörnvall, H.). Elsevier Science Publishers, USA.

Crawford, D.J., Brauner, S., Cosner, M.B. and Stuessy, T. F 1993. Use of RAPD markers to document the origin of the intergeneric hybrid *xMargyracaena skottsbergii* (Rosaceae) on the Juan Fernandez Islands. *American Journal of Botany*, **80 (1):** 89 – 92.

Cronin, M.A., Spearman, W.J., Wilmot, R.L, Patton, J.C. and Bickham, J.W. 1993. Mitochondrial DNA variation in chinook *(Oncorhynchus tshawytscha)* and chum salmon (*O. keta*) detected by restriction enzyme analysis of polymerase chain reaction (PCR) products. *Canadian Journal of Fisheries and Aquatic Sciences,* **50:** 708 - 714.

Cruzan, M. B. and Arnold, M.L. 1993. Ecological and genetic associations in an *Iris* hybrid zone. *Evolution*, **47(5):** 1432 - 1445.

Cruzan, M. B. and Arnold, M.L. 1994. Assortative mating and natural selection in an *Iris* hybrid zone. *Evolution*, **48 (6):** 1946 - 1958.

Cushwa, W.T. and Medrano, J.F. 1996. Application of the random amplified polymorphic DNA (RAPD) assay for genetic analysis of livestock species. *Animal Biotechnology*, **7 (1):** 11 – 31.

Dawson, I.K., Simons, A.J., Waugh, R. and Powell, W. 1995. Diversity and genetic differentiation among subpopulations of *Gliricidia sepium* revealed by PCR-based assays. *Heredity*, **74:** 10 - 18.

De la Cruz, V.F., Neckelmann, N., and Simpson, L. 1984. Sequences of six genes and several open reading frames in the kinoplast maxicircle DNA of *Leishmania tarentolae*. *Journal of Biological Chemistry*, **259:** 15136 - 15147.

De Robertis, E.D.P. and De Robertis, E.M.F. 1980. Cell and molecular biology. Seventh edition. Saunders College/Holt, Rinehart and Winston, Philadelphia.

Desjardins, P., and Morais, R. 1990. Sequence and gene organisation of chicken mitochondrial genome. A novel gene order in higher vertebrate. *Journal of Molecular Biology*, **212:** 599 - 634.

Dice, L.R. 1945. Measures of the amount of ecological association between species. *Ecology*, **26:** 297 -302.

Dixson, A.F., Hastie, N., Patel, I. and Jeffreys, A.J. 1988. DNA "fingerprinting" of captive family groups of common marmosets (*Callithrix jacchus*). *Folia Primatologica*, **51:** 52 - 55.

Dodgson, J.B., Cheng, H.H. and Okimoto, R. 1997. DNA marker technology: a revolution in animal genetics. *Poulty Science*, **76 (8):** 1108 – 114.

Dolf, G., Glowatzki, M-L. and Gaillard. 1991. Searching for genetic markers for heredity diseases in cattle by means of DNA fingerprinting. *Electrophoresis*, **12:** 109 – 112.

Douglass, A. 1881. Ostrich farming in South Africa. Cassel, Petter, Galpin and Co., London.

Dowling, T.E., Moritz C. and Palmer, J.D. 1990. Nucleic acids II. Restriction site analysis. **In:** Molecular systematics. **pp 250 - 315.** (eds. D..M. Hillis and C. Moritz). Sinauer Associates, Inc., Massachusetts, USA.

Duerden, J.E. 1908. The domesticated ostrich in South Africa. African Book Company Limited, Grahamstown.

Duerden, J.E. 1909. Experiments with ostriches–XI. Department of Agriculture. Cape Times Limted, Cape Town.

Duerden, J.E. 1910. The application of science to the ostrich industry. Experiments with ostriches - XV.*Bulletin. South African Department of Agriculture*, **71:** 1 - 7.

Duerden, J.E. 1911a. The future of the ostrich industry. Experiments with ostriches-XVII. Department of Agriculture, Cape Times Limited, Cape Town.

Duerden, J.E. 1911b. Twin ostrich chicks from a single egg. Department of Agriculture, Cape Times Limited, Cape Town.

Duerden, J.E. 1912. Feather irregularities. Experiments with ostriches-XXI. *Bulletin. South African Department of Agriculture*, (August) 1 - 17.

Duerden, J.E. 1917. The plumes of parents and chicks. Breeding Experiments with North African and South African ostriches. *Bulletin. South African Department of Agriculture*, **13:** 1 - 11.

Duerden, J.E. 1919. Crossing the North and South African ostriches. Breeding experiments with North and South African ostriches. *Bulletin. South African Department of Agriculture*, **3:** 1 - 36.

Duerden, J.E. 1920. Mutation and evolution. South African Association for the Advancement of Science. Johannesburg, South Africa, 300 - 315.

Edwards, J.H. 1968. Multiple pregnancy. *Proceedings of the Royal Society of Medicine*, **61:** 227 - 229.

Ellegren, H. 1991. DNA typing of museum birds. *Nature*, **354:** 113.

Emery, A.E.H. 1976. Methodology in medical genetics. An introduction to statistical methods. Churchill Livingstone, Edinburgh.

Evans, P.G.H. 1987. Electrophoretic variability of gene products. **In:** Avian genetics a population and ecological approach. **pp105 - 161.** (eds. Cooke, F. and Buckley, P.A.). Academic Press Inc. limited, London, England.

Evans, B.S., White, R.W.G. and Ward, R.D. 1998. Genetic identification of asteroid larvae from Tasmania, Australia, by PCR-RFLP. *Molecular Ecology*, **7:** 1077 – 1082.

Excoffier, L., Smouse, P.E. and Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics*, **131:** 479 - 491.

Farber, C. M., Georges, M., De Bock, G., Verhest, A., Simon, P. Verschragen-Spae, M. and Vassart, G. 1989. Demonstration of spontaneous XX/XY chimerism by DNA fingerprinting. *Human Genetics,* **82:** 197 - 198

Ferris, S.D., Sage, R.D., Wilson, A.C. 1982. Evidence from mtDNA sequences that coom laboratory strains of inbred mice are descended from a single female. *Nature*, **295:** 163 – 165.

Ferris, S.D., Sage, R.D., Huang, C.-M., Nielsen, J.T., Ritte, U. and Wilson, A.C. 1983. Flow of mitochondrial DNA across a species boundary. *Proceedings of the National Academy of Science, USA.*, **80:** 2290 - 2294.

Freitag, S. 1992. Intra specific mitochondrial DNA phylogeography of the ostrich *Struthio camelus*. M.Sc. thesis, University of Pretoria, South Africa.

Freitag, S. and Robinson, T.J. 1993. Phylogeographic patterns in mitochodrial DNA of the ostrich (*Struthio camelus*). *The Auk*, **110 (3):** 614 – 622.

Frey, J.E. and Frey, B. 1995. Molecular identification of six species of scale insects (*Quadraspidiotus* sp.) by RAPD-PCR: assessing the field-specificity of pheromone traps. *Molecular Ecology*, **4:** 777 – 780.

Friar, E.A., Robichaux, R.H., and Mount, D.W. 1996. Molecular genetic variation following a population crash in the endangered Mauna Kea silversword, *Argyroxiphium sandwicense* ssp. *Sandwicense* (Asteraceae). *Molecular Ecology*, **5:** 687 – 691.

Gaines, R.E. and Elston, R.C. 1969. On the Probability that a twin pair is monozygotic. *American Journal of Human Genetics*, **21:** 457 – 465.

Garcia, D.K. and Davis, S.K. 1994. Evidence for a mosaic hybrid zone in the grass shrimp *Palaemonetes kadiakensis* (Palaemonidae) as revealed by multiple genetic markers. *Evolution*, **48:** 376 - 391.

Gazit, E and Gazit E. 1990. DNA fingerprinting. *Israel Journal of Medical Sciences*, **26:** 158 -162.

Georges, M., Lathorp, M., Hilbert, P., Marcotte, A., Schwers, A., Swillens, S., Vassart, G. And Hanset, R. 1990. On the use of DNA fingerprints for linkage studies in cattle. *Genomics*, **6:** 461 - 474.

Gibbs, H.L., De Sousa, L., Marchetti, K. and Nakamura, H. 1998. Isolation and chraterization of microsatellite DNA loci for an obligate brood parasitic bird, the common cuckoo (*Cuculus canorus*). *Molecular Ecology*, **7:** 1431 – 1439.

Giles, R.E., Blanc, H., Cann, H.M. and Wallace, D.C. 1980. Maternal inheritance of human mitochondrial DNA. *Proceedings of the National Academy of Science, USA,* **77:** 6715 - 6719.

Glaus, K.R., Zassenhaus, H.P., Fechheimer, N.S., and Perlman, P.S. 1980. Avian mtDNA: structure, organisation and evolution. **In:** The organisation and expression of the Mitochondrial Genome. **pp 131 - 135** (eds. A.M. Kroon and C. Saccone) Elsevier/North-Holland, Amsterdam.

Goldman, D. and Merril, C.R. 1982. Silver staining of DNA in polyacrylamide gels: linearity and effect of fragment size. *Electrophoresis,* **3:** 24 - 26

Greenwood, P.J. 1987. Inbreeding, philopatry and optimal outbreeding in birds. **In:** Avian genetics, a population and ecological approach. **pp 207 - 222.** (eds. Cooke, F. and Buckley, P.A.) Academic Press, London.

Grimberg, J., Nawoschik, S., Belluscio, L., McKee, R, Turck, A. And Eisenberg, A. 1989. A simple and efficient nonorganic procedure for the isolation of genomic DNA from blood. *Nucleic Acids Research,* **17:** 8390.

Grzimek, B. 1970. Grzimek's animal life encyclopedia. Van Nostrand ReinHold Co. New York. **Volume** 7, 91 - 101.

Gyllensten, U., Wharton, D., Josefsson, A. and Wilson, A.C. 1991. Paternal inheritance of mitochondrial DNA in mice. *Nature,* **352:** 255 – 257.

Hadrys, H., Balick, M. and Schierwater, B. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Molecular Ecology,* **1:** 55 – 63.

Hadrys, H., Schierwater, B., Dellaporta, S.L., Desalle, R. and Buss, L.W. 1993. Determination of paternity in dragonflies by random amplified polymorphic DNA fingerprinting. *Molecular Ecology,* **2:** 79 – 87.

Haig, S.M., Rhymer, J.M. and Heckel, D.G. 1994. Population differentiation in randomly amplified polymorphic DNA of red-cockaded woodpeckers *Picoides borealis*. *Molecular Ecology,* **3:** 581 – 95.

Haley, C.S. 1991. Use of DNA fingerprints for the detection of major genes for quantitative traits in domestic species. *Animal Genetics,* **22:** 259 - 277.

Hammans, S.R. 1994. Mitochondrial DNA and disease. **In:** Essays in Biochemistry. **(28): pp 99 - 112.** (ed. Tipton, K.F) Portland Press, Ltd. London.

Härlid, A. and Arnason, U. 1999. Analysis of mitochondrial DNA nest ratite birds within the Neonathae: Supporting a neotenous origin of ratite morphological characters. *Proceedings of the Royal Society of London, Series B,* **266:** 305 – 309.

Härlid, A., Janke, A. and Arnason, U. 1997. The mtDNA sequence of the ostrich and the divergence between paleognathous and neognathous birds. *Molecular Biology and Evolution,* **14 (7):** 754 – 761.

Härlid, A., Janke, A. and Arnason, U. 1998. The complete mitochondrial genome *of Rhea americana* and early avian divergence. *Journal of Molecular Evolution*, **46:** 669 – 679.

Harrison, J. 1990. Ostrich mentality. *Finance Week*, **May 3 - 9:** 29 - 30.

Harrison, J. 1991. Head in sand. *Finance Week,* **May 2 - 8:** 38.

Hedges, S.B., Parker, P.H., Sibley, C.G. and Kumar, S. 1996, Continental breakup and the ordinal diversification of birds and mammals. *Nature*, **381:** 226 – 229.

Hedges, S.B. and Sibley, C.G. 1994. Molecules vs morphology in avian evolution: the case of the "pelecaniform" birds. *Proceedings of the National Academy of Science, USA.*, **91:** 9861 - 9865.

Hedges, S.B., Simmons, M.D., van Dijk, M.A.M., Caspers, G-J., de Jong, W.W. and Sibley, C.G. 1995. Phylogenetic relationships of the hoatzin, an enigmatic South American bird. *Proceedings of the National Academy of Science, USA.*, **92:** 11662 - 11665.

Hedrick, P. 1992. Shooting the RAPDs. *Nature*, **350:** 679 - 680.

Heinrich, M. 1991. PCR carry-over. *Biotech Forum Europe*, **8:** 594 - 597.

Higuchi, R., Dollinger, G., Walsh, P.S. and Griffith, R. 1992. Simultaneous amplification and detection of specific DNA sequences. *Bio/technology*, **10:** 413 - 417.

Hill, A.V.S. and Jeffreys, A.J. 1985. Use of minisatellite DNA probes for determination of twin zygosity at birth. *Lancet*, 1394 - 1395.

Hillel, J., Plotzy, Y., Haberfeld, A., Lavi, U., Cahaner, A. and Jeffreys, A.J. 1989. DNA fingerprints of poultry. *Animal Genetics*, **20:** 145 - 155.

Hillel, J., Schaap, T., Haberfeld, A., Jeffreys, A.J., Plotzky, Y., Cahaner, A. and Lavi, U. 1990. DNA fingerprints applied to gene introgression in breeding programs. *Genetics*, **124:** 783 - 789.

Hillis, D.M., Larson A., Davis S.K. and Zimmer E. 1990. Nucleic Acids III: Sequencing. **In:** Molecular systematics. **pp 318 - 369.** (eds. Hillis, D.M. and Moritz, C.). Sinauer Associates, Inc., Massachusetts, USA.

Hillis, D.M. and Moritz, C. 1990. An overview of applications of molecular systematics. **In:** Molecular systematics. **pp 502 - 515.** (eds. Hillis, D.M and Moritz, C.). Sinauer Associates, Inc., Massachusetts, USA.

Hoelzel, A.R. 1998. Molecular genetic analysis of populations. Second edition. Oxford University Press. New York. USA.

Houlden, B.A., England, P.R., Taylor, A.C., Greville, W.D. and Sherwin, W.B. 1996. Low genetic variability of the koala *Phascolarctos cinereus* in south-eastern Australia following a severe population bottleneck. *Molecular Ecology*, **5:** 269 – 281.

Huff, D.R., Quinn, J.A., Higgins, B. and Palazzo, A.J. 1998. Random amplified polymorphic DNA (RAPD) variation among native little bluestem [*Schizachyrium scoparium* (Michx.) Nash] populations from sites of low fertility in the forest and grassland biomes. *Molecular Ecology*, **7:** 1591 – 1597.

Hunt, G.J. and Page, R.E. 1992. Patterns of inheritance with RAPD molecular markers reveal novel types of polymorphism in the honey bee. *Theoretical and Applied Genetics*, **85:** 15 - 20.

Innes, M.A. and Gelfand, D.H. 1990. Optimization of PCRs. PCR protocols. **In:** A guide to methods and applications. **pp 3 - 11.** (eds. Innes, M.A., Gelfand, D.H., Sninky, J.J. and White, T.J.) Academic Press, Inc., California.

Isabel, M., Beaulieu, J., Thériault, P. and Bousquet, J. 1999. Direct evidence for biased estimates from dominant random amplied polymorphic DNA (RAPD) fingerprints. *Molecular Ecology*, **8:** 477 – 483.

Jaccard, P. 1901. Etude comparative de la distribution florale dans une portion des alpes et des Jura. *Bulletin. Societe Vaudoise des Sciences Naturelles*, **37:** 547 - 579.

Jarvis, M. 1993. A new approach to ostrich feeding. *Farmers Weekly*, **October 22:** 20 - 22.

Jarvis, M.J.F., Jarvis, C. and Keffen, R.H. 1985. Breeding seasons and laying patterns of the southern African ostrich *Struthio camelus*. *Ibis*, **127:** 442 - 449.

Jeffreys, A.J., Hillel, J. Hartley, N., Bulfield, G., Morton, D., Wilson, V., Wong, Z. and Harris, S. 1987. The implications of hypervariable DNA-regions for animal identification. *Animal Genetics,* **18:** 141 - 142.

Jeffreys, A.J. and Morton, D.B. 1987. DNA fingerprints of dogs and cats. *Animal Genetics*, **18:** 1 - 15.

Jeffreys, A.J., Wilson, V., Neumann, R. and Keyte, J. 1988. Amplification of human minisatellite by the polymerase reation: Towards DNA fingerprinting of single cells. *Nucleic Acids Research*, **16:** 10953 - 10971.

Jeffreys, A.J., Wilson, V. and Thein, S.L. 1985a. Hypervariable "minisatellite" regions in human DNA. *Nature*, **314:** 67 - 73.

Jeffreys, A.J., Wilson, V. and Thein, S.L. 1985b. Individual - Specific "Fingerprints" of Human DNA. *Nature*, **316:** 76 - 79.

Kaemmer, D., Afza, R., Weising, K., Kahl, G. and Novak, F.J. 1992. Oligonucleotide and amplification fingerprinting of wild life species and cultivars of banana (*Musa spp.*). *Bio/technology,* **10:** 1030 - 1035.

Kappe, A.L., Van De Zande, L., Vedder, E.J., Bijlsma, R. and Van Delden, W. 1995. Genetic variation in *Phoca vitulina* (the harbour seal) revealed by DNA fingerprinting and RAPDs. *Heredity*, **74:** 647 - 653.

Kawasaki, E.S. 1990. Sample preparation from blood, cells and other fluids. **In:** PCR protocols. **pp 146 - 152.** (eds. Innes, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. Academic press, Inc. San Diego, California.

Kazan, K., Manners, J.M. and Cameron, D.F. 1993a. Genetic variation in agronomically important species of *Stylosanthes* determined using random amplified polymorphic DNA markers. *Theoretical and Applied Genetics,* **85:** 882 - 888.

Kazan, K., Manners, J.M. and Cameron, D.F. 1993b. Genetic relationships and variation in the *Stylosanthes guianensis* species complex assessed by random amplified polymorphic DNA. *Genome,* **36:** 43 - 49.

Kessler, C. 1994. Non-radioactive analysis of biomolecules. *Journal of Biotechnology*, **35:** 165 - 189.

Kessler, L.G., and Avise, J.C. 1985. A comparative description of mitochondrial DNA differentiation in selected avian and other vertebrate genera. *Molecular Biology and Evolution*, **2:** 109 - 125.

Kimwele, C.N., Greaves, J.A., Burke, T. and Hanotte, O. 1998. Development of microsatellite markers for parentage typing of chicks in ostrich *Struthio camelus*. *Molecular Ecology*, **7:** 249 –251.

Kirby, L.T. 1990. DNA fingerprinting: An introduction. Stockton Press. New York.

Klein, N. K. and Brown, W.M. 1994. Intraspecific molecular phylogeny in the yellow warbler (*Dendroica petechia*), and implications for avian biogeography in the West Indies. *Evolution*, **48:** 1914 - 1932.

Kocher, T.D., Conroy, J.A., McKay, K.R., and Stauffer, J.R. 1993. Similar morphologies of ciclid fish in Lakes Tanganyika and Malawi are due to convergence. *Molecular Phylogenetics and Evolution*, **2:** 158 - 165.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V. Pääbo, S., Villablanca, F.X. and Wilson A.C. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Science, USA.*, **86:** 6196 - 6200.

Kornegay, J., Kocher. T., Williams, L., and Wilson, A. 1993. Pathways of lysozyme evolution inferred from the sequences of cytochrome *b* in birds. *Journal of Molecular Evolution,* **37:** 367 - 379.

Kreitman, M. 1991. Variation at the DNA level: Something for every one. **In:** Molecular Techniques in Taxonomy.**pp 15 - 32.** (eds. Hewit, G.M., Johnston, A.W.B., and Young, J.P.W.). Springer-Verlag, Berlin, Germany.

Kuhnlein, U., Dawe, Y., Zadworny, D. and Gavora. 1989. DNA fingerprinting: a tool for determining genetic distances between strains of poultry. *Theoretical and Applied Genetics*, 77: 669 - 672.

Kuhnlein, U., Zadworny, D., Dawe, Y., Fairfull, R.W. and Gavora, J.S. 1990. Assessment of inbreeding by DNA fingerprinting: Development of a calibration curve using defined strains of chickens. *Genetics*, **125:** 161 - 165.

Kulczynski, S. 1927. Die Pflanzenassoziationen der Pieninen. *Bulletin International. Academie Polonaise des Science et des Lettres. Classe des Sciences Mathematiques et Naturelles.* **(Serie B-2. Zoologie)**, 57 - 203.

Kumari, P. and Kemp, S.J. 1998. Polymorphic microsatellite markers in the ostrich (*Struthio camelus*). *Molecular Ecology*, **7:** 133 – 140.

Küntzel, H. and Köchel, H.G. 1981. Evolution of rRNA and origin of mitochondria. *Nature*, **293:** 751 - 755.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227:** 680 - 685.

Lake, J.A. 1988. Origin of the eukaryotic nucleus determined by rate-invariant analysis of rRNA sequences. *Nature*, **331:** 184 - 186.

Lamboy, W.F. 1994. Computing genetic similarity coefficients from RAPD data: Correcting for the effects of PCR artifacts caused by variation in experimental conditions. *PCR Methods and Applications*, **4:** 38 – 43.

Layard, L.E. 1884. The birds of South Africa. Bernard Quaritch, London.

Lee, K., Feinstein, J. and Cracraft, J. 1997. The phylogeny of ratite birds: Resolving conflicts between molecular and morphological data sets. **In:** Avian molecular evolution and systematics. **pp 173 – 211.** (ed. Mindell, D.P.). Academic Press, New York, USA.

Lessells, C.M. and Mateman, A.C. 1998. Sexing birds using random amplified polymorphic DNA (RAPD) markers. *Molecular Ecology*, **7:** 187 – 195.

Leuthold, W. 1977. Notes on the breeding biology of the ostrich *Struthio camelus* in Tsavo East National Park, Kenya. *Ibis,* **119:** 541 - 544.

Li, H., Gyllensten, U.B., Cui, X., Saiki, R.K. Erlich, H.A. and Arnheim, N. 1988. Amplification and analysis of DNA sequences in single human sperm and diploid cells. *Nature*, **335:** 414 - 417.

Liao, L.C. and Hsiao, J.Y. 1998. Relationship between population genetic structure and riptarian habitat as revealed by RAPD analysis of the rheophyte *Acorus gramineus* Soland. (Araceae) in Taiwan. *Molecular Ecology*, **7:** 1275 – 1281.

Lins, A.M., Sprecher, C.J., Puers, C. and Schumn, J.W. 1996. Multiplex sets for the amplication of polymorphic short tandem repeat loci - silver stain and fluorescence detection. *BioTechniques*, **20:** 882 - 889.

Liu, H.T., Hu, Y.H., Wang, C.T. and Lin, L.Y. 1996. Sequences and comparisons of duck mitochondrial DNA control regions. *Comparative Biochemistry and Physiolology.* **115(2):** 209-214.

Lu, M-Z., Szmidt, A. E. and Wang, X-R. 1995. Inheritance of RAPD fragments in haploid and diploid tissues of *Pinus sylvestris* (L.). *Heredity*, **74:** 582 - 589.

Lynch, M. and Milligan, B.G. 1994. Analysis of population genetic structure with RAPD markers. *Molecular Ecology,* **3:** 91 - 99.

Lynn, S. 1991. Rich: Boas, boots and golden eggs. *Ultra*, **February, 69:** 70 and 125.

Maclean G.L. 1990. Ornithology for Africa. University of Natal Press, Pietermaritzburg, South Africa.

Maniatis, T., Fritsch, E.F. and Sambrook, J. 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbour laboratory, New York, USA.

Marsolais, J.V., Pringle, J.S. and White B.N. 1993. Assessment of random amplified polymorphic DNA (RAPD) as genetic markers for determining the origin of interspecific lilac hybrids. *Taxon*, **42:** 531 – 537.

Martin, A.P., Humphreys, R. and Palumbi, S.R. 1992. Population structure of the armorhead, *Pseudopentaceros wheeleri*, in the North Pacific Ocean: Application of the polymerase chain reaction to fisheries problems. *Canadian Journal of Fisheries and Aquatic Sciences*, **49:** 2386 - 2391.

Matioli, S. R. and de Brito, R. A. 1995. Obtaining genetic markers by using double-stringency PCR with microsatellites and arbitrary primers. *BioTechniques*, **19:** 752 - 758.

Meyer, A. 1994. Short commings of the cytochrome *b* gene as a molecular marker. *Trends in Ecology and Evolution*, **9:** 278 – 280.

Mindell, D.P., Dick, C.W. and Baker, R.J. 1991. Phylogenetic relationships among megabats, microbats and primates. *Proceedings of the National Academy of Science, USA.*, **88:** 10322 - 10326.

Mindel, D.P., Sorensen, M.D. and Dimcheff, D.E. 1998. Multiple independent origins of mitochondrial gene order in birds. *Proceedings of the National Academy of Science, USA.*, **95:** 10693 - 10697.

Mindell, D.P., Sorenson, M.D., Huddleston, C.J., Miranda, H.C., Knight, A., Swachuk, S.J. and Yuri, Tamaki. 1997. Phylogeny relationships among and within selected avian orders based on mitochondrial DNA. **In:** Avian molecular evolution and systematics. **pp 173 – 211.** (ed Mindell, D.P.). Academic Press, New York, USA.

Moore, H.D.M., Holt, W. V. and Mace, G.H. 1992. Biotechnology and conservation of genetic diversity. Clarendon Press, Oxford.

Moran, P. and Kornfield, I. 1995. Were population bottlenecks associated with the radiation of the mbuna species flock (Teleostei: Cichlidae) of Lake Malawi? *Molecular Biology and Evolution*, **12 (6):** 1085 – 1093.

Moritz, C. and Brown, W.M. 1986. Tandem duplication of D-Loop and ribosomal RNA sequences in lizard mitochondrial DNA. *Science,* **233:** 1425 - 1427.

Nei, M. and Li, W.H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Science, USA.*, **76:** 5269 - 5273.

Neilan, B.A. 1995. Identification of phylogenetic analysis of toxigenic cyanobacteria by multiplex randomly amplified polymorphic DNA PCR. *Applied and Environmental Microbiology*, **61:** 2286 - 2291.

Oldfield, M.L. 1989. The value of conserving genetic resources. Sinauer Associates, Inc. Massachuttes.

Onadim, Z. and Cowell, J.K. 1991. Application of PCR amplification of DNA from paraffin embedded tissue sections to linkage analysis in familial retinoblatoma. *Journal of Medical Genetics,* **28:** 312 - 316.

Parkin, D.T. and Wetton, J.H. 1991. DNA fingerprinting. **In:** Molecular techniques in taxonomy. **pp 145 - 157.** (eds Hewit, G.M., Johnston, A.W.B. and Young, J.P.W.). Springer-Verlag, Berlin, Germany.

Perelman B., Cognono, E., Katchko, L., Agur. Y., Kuttin, E. and Carmy, R. 1995. An unusual mechanobullous skin disorder in ostrich (*Struhio camelus*). *Journal of Avian Medicine and Surgery*, **9 (2):** 122 – 126.

Pérez, T., Albornoz, J. and Dominquez, A. 1998. An evaluation of RAPD fragment reproducibility and nature. *Molecular Ecology*, **7:** 1347 – 1357.

Plomion, C., Bahrman, N., Durel, C-E. And O'Malley, D.M. 1995. Genomic mapping in *Pinus pinaster* (Maritime Pine) and protein markers. *Heredity*, **74:** 661 - 668.

Plummer, D.T. 1978. An introduction to practical Biochemistry. McGraw-Hill Book Company (UK) Limited, London, England.

Preus, H.R. and Russel, D.T. 1994. Use of nonradioactive genetic probe identified, synthesized, and labelled in the polymerase chain reaction. *Scandinavian Journal of Dental Research*, **102:** 161 - 167.

Puterka, G.J., Black, W.C., Steiner, W.M. and Burton, R.L. 1993. Genetic variation and phylogenetic relationships among worldwide collections of the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), inferred from allozyme and RAPD-PCR markers. *Heredity*, **70:** 604 – 618.

Quinn, T.W. and Wilson, A.C. 1993. Sequence evolution in and around the mitochondrial control region in birds. *Journal of Molecular Evolution*, **37:** 417 – 425.

Rao, K.B., Bhat, K.V. and Totey S.M. 1996. Detection of species-specific genetic markers in farm animals though random amplified polymorphic DNA (RAPD). *Genetic Analysis.* **13 (5):** 135 – 138.

Reysenbach, A-L., Giver, L.J., Wickham G.S., and Pace, N.R. 1992. Differential amplification of rRNA genes by polymerase chain reaction. *Applied and Environmental Microbiology,* **58:** 3417 - 3418.

Rieseberg, L.H. 1995. The role of hybridization in evolution: Old wine in new skins. *American Journal of Botany,* **82 (7):** 944 – 953.

Roberts, J.W., Grula, J.W., Posakony, J.W., Hudspeth, R., Davidson, E.H., and Britten, R.J. 1983. Comparison of sea urchin and human mtDNA: Evolutionary rearrangements. *Proceedings of the National Academy of Science, USA,* **80:** 4616 - 4618.

Roe, B.A., Ma, D.-P., Wilson R.K., and Wong, J.F.H. 1985. The complete nucleotide sequence of *Xenopus laevis* mitochondrial genome. *Journal of Biological Chemistry,* **260:** 9759 - 9774.

Saperstein, D.A. and Nickerson, J.M. 1991. Restriction fragment length polymorphism analysis using PCR coupled to restriction digests. *BioTechniques,* **10:** 488 - 489.

Saville, B.J., Kohli, Y. and Anderson, J.B. 1998. Recombination in a natural population. *Proceedings of the National Academy of Science,* USA., **95:** 1331 – 1335..

Schlegel, J., Vogt, T., Münkel, K. and Rüschoff, J. 1996. DNA fingerprinting mammalian cell lines using nonradioactive arbitrarily primed PCR (AP-PCR). *BioTechniques,* **20:** 178 - 180.

Schreiner, S.C.C. 1898. The ostrich. **In:** The angora goat. **pp 259 - 296.** Longmans, Green and Co., London.

Schwarz, Zs. and Kössel, H. 1980. The primary structure of 16S rDNA from *Zea mays* chloroplast is homologous to *E. coli* 16S rRNA. *Nature,* **283:** 739 -742.

Schweder, M.E., Shatters, R.G., West, S.H. and Smith, R.L. 1995. Effect of transition interval between melting and annealing temperatures on RAPD analysis. *BioTechniques,* **19:** 38 - 42.

Seutin, G., Lang, B.F., Mindell, D.P. and Morais, R. 1994. Evolution of the WANCY region in amniote mitochondrial DNA. *Molecular Biology and Evolution,* **11 (3):** 329 – 340.

Sharma D., Rao, K.B., Singh, H.P. and Totey, S.M. 1998. Randomly amplified polymorphic DNA (RAPD) for evaluating genetic relationships among varieties of guinea fowl. *Genetic Analysis* **14 (4):** 125 – 128.

Shaw, P.W., Pierce, G.J. and Boyle, P.R. 1999. Subtle population structuring within a highly vagile marine invertebrate, the veined squid *Loligo forbesi,* demonstrated with microsatellite DNA markers. *Molecular Ecology,* **8:** 407 – 417.

Shields, G.F. and Helm-Bychowski, K.M. 1988. Mitochondrial DNA of birds. **In:** Current ornithology. **(5). pp 273 - 295.** (ed. Johnston, R.F). Plenum Press, New York.

Shoemaker, D.D., Ross, K.G. and Arnold, M.L. 1994. Development of RAPD markers in two introduced fire ants, *Solenopsis invicta* and *S. richteri*, and their application to the study of a hybrid zone. *Molecular Ecology*, **3:** 531 – 539.

Shrikant, A. and Subramanian, K.N. 1992. Isolation of low molecular weight DNA from bacteria and animal cells. *Methods in Enzymology*, **216:** 20 - 39.

Simon, C. 1991. Molecular systematics at the species boundary: Exploiting conserved and variable regions of the mitochondrial genome of animals via direct sequencing from amplified DNA. **In:** Molecular techniques in taxonomy. **pp 33 - 71** (eds. G.M Hewit, A.W.B Johnston and J.P.W Young). Springer-Verlag, Berlin, Germany.

Smith, C.A., Jordan, J.M. and Vinograd, J. 1971. *In vivo* effects of intercalating drugs on the superhelix density of mitochondrial DNA isolated from human and mouse cells in culture. *Journal of Molecular Biology*. **59:** 255 - 272.

Smith C., and Simpson, S.P. 1986. The use of polymorphisms in livestock improvement. *Journal of Animal Breeding and Genetics*, **103 :** 205 -217.

Smith, E.J., Jones, C.P., Bartlett, J and Nestor, K.E. 1996. Use of randomly amplified polymorphic DNA markers for the genetic analysis of relatedness and diversity in chickens and turkeys. *Poultry Science*. **75 (5):** 579 – 584.

Soller, M. and Beckmann, J.S. 1986. Restriction fragment length polymorphisms in poultry breeding. *Poultry Science*. **65 :** 1474 – 1488.

Sorenson, M.D. and Fleischer, R.C. 1996. Multiple independent transpositions of mitochondrial DNA control region sequences to the nucleus. *Proceedings of the National Academy of Science, USA.*, **93:** 15239 - 15243.

Spencer, D.F., Schnare, M.N. and Gray, M.W. 1984. Pronounced structural similarities between the small subunit ribosomal RNA genes of wheat mitochondria and *Escherichia coli. Proceedings of the National Academy of Science., USA*. **81:** 493 - 497.

Stammers, M., Harris, J., Evans, G.M., Hayward, M.D. and Foster, J.W. 1995. Use of random PCR (RAPD) technology to analyse phylogenetic relationships in the *Lolium/Festuca* complex. *Heredity*, **74:** 19 - 27.

Stuart, C. 1982. He opted for ostriches. *Farmers Weekly*, **June 23:** 17.

Swart, D. Mackie, R.I. and Hayes, J.P. 1987. For feathers and leathers. *Nuclear Active*, **36:** 2 - 9.

Sydes, M.A. and Peakall, R. 1998. Extensive clonality in the endangered shrub *Haloragodendron lucasii* (Haloragaceae) revealed by allozymes and RAPDs. *Molecular Ecology*, **7:** 87 – 93.

Taberlet, P., Meyer, A. and Bouvet, J. 1992. Unusual mitochondrial DNA polymorphism in two local populations of blue tit *Parus caeruleus*. *Molecular Ecology*, **1:** 27 – 36.

Thorpe, R.S., McGregor, D.P., Cumming, A.M. and Jordan, W.C. 1994. DNA evolution and colonization sequence of island lizards in relation to geological history: mtDNA RFLP, cytochrome *b*, cytochrome oxidase, 12S rRNA sequence and nuclear RAPD analysis. *Evolution*, **48(4):** 230 - 240.

Tollefsrud, M.M., Bachmann, K., Jacobsen, K.S. and Brochmann, C. 1998. Glacial survival does not matter – II: RAPD phylogeography of Nordic *Saxifraga cespitosa*. *Molecular Ecology*, **7:** 1217 – 1232.

Tulsieram, L.K., Glaubitz, J.C., Kiss, G. and Carlson, J.E. 1992. Single Tree Genetic Linkage Mapping in Conifers using Haploid DNA from Megametophytes. *Bio/technology,* **10:** 686 - 690.

Upholt, W.B. 1977. Estimation of DNA sequence divergence from comparison of restriction endonuclease digestion. *Nucleic Acids Research*, **4:** 1257 - 1265.

Van De Ven, W.T.G. and McNicol, R.J. 1995. The use of RAPD markers for the identification of sitka spruce (*Picea sitchensis*) clones. *Heredity*, **75:** 126 - 132.

van Heusden, A.W. and Bachmann, K. 1992. Genotype relationships in *Microseris elegans* (Asteraceae, Lactuceae) revealed by DNA amplification from arbitrary primers (RAPDs). *Plant Systematics and Evolution*, **179:** 221 - 233.

Vanlerberghe-Masutti, F. and Chavigny, P. 1998. Host-based genetic differentiation in the aphid *Aphis gosypii*, evidenced from RAPD fingerprints. *Molecular Ecology*, **7:** 905 – 914.

van Tuinen, M., Sibley, C.G. and Hedges, S.B. 1998 Phylogeny and biogeography of ratite birds inferred from DNA sequences of the mitochodrial ribosomal genes. *Molecular Biology and Evolution*, **15 (4):** 370 – 376.

van Rooyen, C. 1995. Belangrike vereistes vir welslae met volstruise. *Landbouweekblad*, **June 30:** 10 – 13.

van Schalkwyk, S.J., Cloete, S.W. and de Kock, J.A. 1996. Repeatability and phenotypic correlation for body weight and reproduction in commercial ostrich breeding pairs. *British Poultry Science*, **37 (5):** 953 – 962.

Viljoen, B. 1991. Head in sand. *Farmers Weekly*, **January 4:** 6 - 8.

Virk, P.S., Ford-Lloyd, B-V., Jackson, M.T. and Newbury, H.J. 1995. Use of RAPD for the study of diversity within plant germplasm collections. *Heredity,* **74:** 170 - 179.

Wagner, P. 1986. The ostrich story. Chameleon Press, Cape Town.

Watson, J.D., Hopkins, N.H., Roberts, J.W., Steitz, J.A and Weiner, A.M. 1987. Molecular biology of the gene. Fourth edition. The Benjamin/Cummings Publishing Company Inc., California.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research*, **18:** 7213 - 7218.

Welsh, J., Honeycutt, R.J., McClelland, M. and Sobral, B.W.S. 1991. Parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (AP-PCR). *Theoretical and Applied Genetics*, **82:** 473 - 476.

Wenink, P.W., Groen, A.F., Roelke-Parker, M.E. and Prins, H.T. 1998. African buffalo maintain high genetic diversity in the major histocompatibility complex in spite of historically known population bottlenecks. *Molecular Ecology*, **7:** 1315 – 1322.

Wetton, J.H., Carter, R.E., Parkin, D. and Walters, D. 1987. Demographic study of a wild house sparrow population by DNA fingerprinting. *Nature,* **327:** 147 - 149.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **22:** 6531 - 6535.

Williams, S.L., Goldson, S.L., Baird, D.B. and Bullock, D.W. 1994. Geographical origin of an introduced insect pest, *Listronotus bonariences* (Kuschel), determined by RAPD analysis. *Heredity*, **72:** 412 - 419.

Wolstenholme, D.R, MacFarlane, J.L., Okimoto, R., Clary, D.O., and Wahleithner, J.A. 1987. Bizzare tRNAs inferred from DNA sequences of mitochondrial genomes of nematode worms. *Proceedings of the National Academy of Science, USA.*, **80:** 1324 - 1328.

APPENDICES

APPENDIX A

CHEMICAL	SUPPLIER
Acetamide	BDH, England
Acrylamide	Sigma, USA
Agarose	SeaKem, USA, BDH, England
Ammonium Persulphate	Sigma, USA
Bromophenol Blue	Merck, Germany
dNTPs	Promega, USA
EDTA	Sigma, USA
100 % Ethanol	BDH, England
Ethidium Bromide	Sigma, USA
Glacial Acetic Acid	BDH, England
Glycerol	BDH, England
HCl	BDH, England
Isoamyl alcohol	BDH, England
Lambda DNA	Boehringer Mannheim, Germany
$MgCl_2$	Promega, USA
Molecular Weight Marker (DNA)	Boehringer Mannheim, Germany
NaCl	BDH, England
NN', Methylenebisacrylamide (Bis acrylamide)	Sigma, USA
pBR322	Boehringer Mannheim, Germany

Primers	Random Hill, USA Operon Technologies, USA University of Cape Town, South Africa
Proteinase K	Boehringer Mannheim, Germany
Restriction Enzymes	Boehringer Mannheim, Germany
SDS	Sigma, USA
Silver Nitrate	Sigma, USA
Sodium Borohydrate	Sigma, USA
Sterile Mineral Oil	Sigma, USA
Sterile Water	Sigma, USA
Sucrose	BDH, England
Taq Polymerase	BioLabs, New England Promega, USA
10X *Taq* Buffer	BioLabs, New England Promega, USA
TEMED	Sigma, USA
Tris Base	Sigma
Triton X-100	BDH, England
Xylene Cyanol FF	Merck, Germany

APPENDIX B

Ostrich samples used in the various sections of this study

1.1 The following DNA samples were obtained from the Mammal Research Institute University of Pretoria, courtesy of Ms S. Freitag and Prof. T. Robinson. The DNA was extracted by the method of Wetton *et al.*, 1987. One of the samples (*S.c molybdophanes*) was used in the study in Chapter 2, Section 2.2. All the samples were used in Chapter 3, Section 3.2

No	Subspecies	Locality
1.	*Struthio camelus australis*	Mabuasehube Game Reserve, Botswana
2.	*Struthio camelus australis*	Kruger National Park
3.	*Struthio camelus molybdophanes*	Lewa Downs Farm, Kenya
4.	*Struthio camelus massaicus*	Kajrado, Kenya

1.2 The following samples were used in the study in Chapter 2, Section 2.2 and 2.3. The samples were supplied by Domesticated Ostrich Products (PTY) LTD.

ORIGIN	CODE	SAMPLE COLLECTED	SUPPLIED BY
Swaziland	Swa 1	Blood	D.O.P.
Swaziland	Swa 2	Blood	
Swaziland	Swa 3	Blood	

1.3 The following samples were used for the study in Chapter 3, Section 3.5

ORIGIN	CODE	SAMPLE COLLECTED	SUPPLIED BY
Oudtshoorn (twin 1)	ODT-A1	Brain	Zoology Dept R.U.
Oudtshoorn (twin 1)	ODT-B1	Brain	Zoology Dept R.U.
Oudtshoorn (twin 2)	ODT-A2	Brain	Zoology Dept R.U.
Oudtshoorn (twin 2)	ODT-B2	Brain	Zoology Dept R.U.
Gaborone 1	GAB1	Brain	Medical School UCT
Gaborone 2	GAB2	Brain	Medical School UCT
Gaborone 3	GAB3	Brain	Medical School UCT
Oudtshoorn 1	ODT1	Brain	Medical School UCT
Oudtshoorn 2	ODT2	Brain	Medical School UCT
Kleinzee	KLEIN	Brain	Medical School UCT

1.4 The following samples were used in the study in Chapter 2, Sections 2.2 and 3 and Chapter 3, Section 3.3. Origin of Grahamstown sample marked GHT-U was unknown.

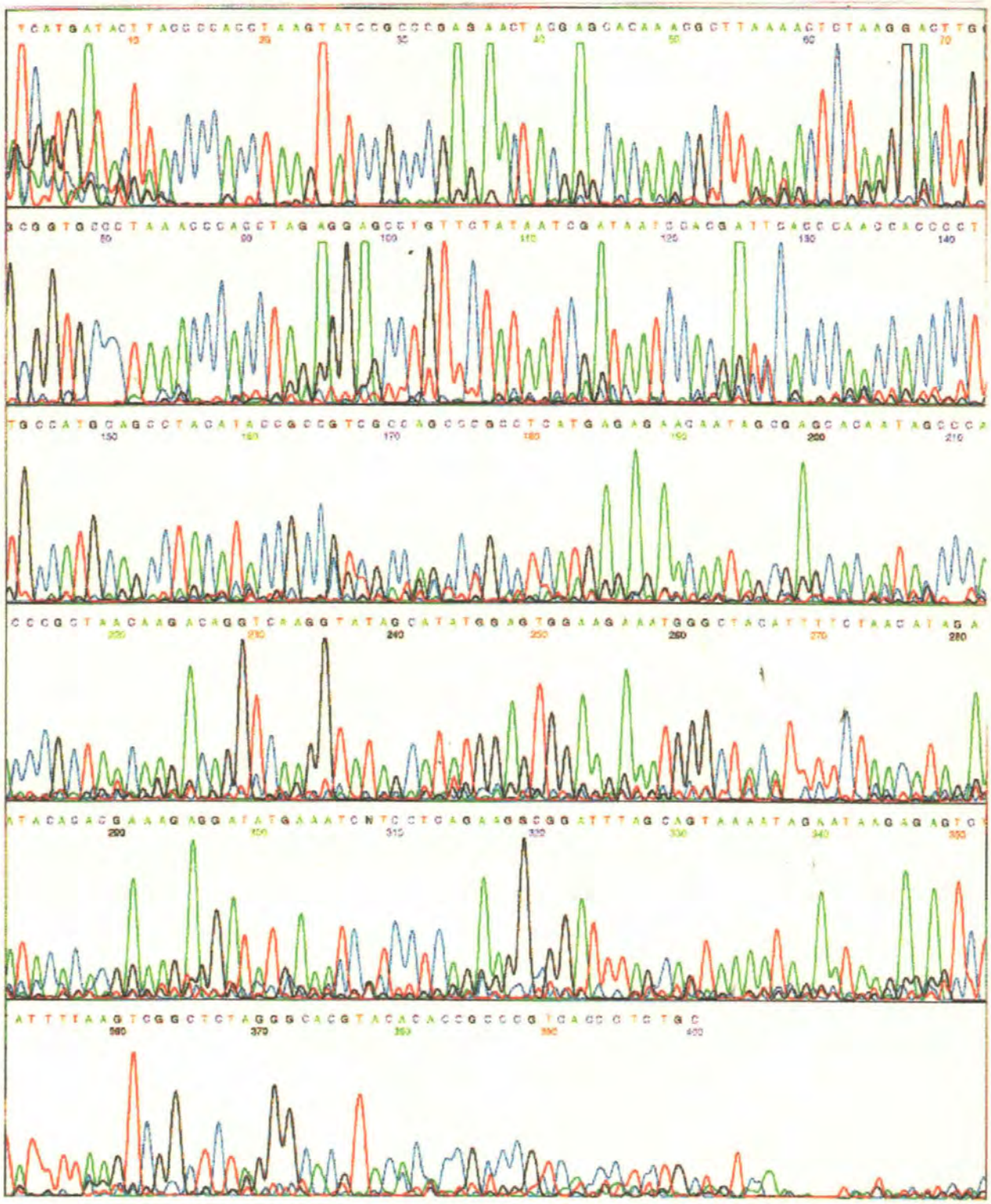
ORIGIN	CODE	SAMPLE COLLECTED	SUPPLIED BY
Grahamstown	Colleen	Blood	Zoology Dept R.U.
Grahamstown	Cecil	Blood	Zoology Dept R.U.
Grahamstown	GHT-F1	Blood	Zoology Dept R.U.
Grahamstown	GHT-F2	Blood	Zoology Dept R.U.
Grahamstown	GHT-F3	Blood	Zoology Dept R.U.
Grahamstown	GHT-F4	Blood	Zoology Dept R.U.
Grahamstown	GHT-F5	Blood	Zoology Dept R.U.
Grahamstown	GHT-M1	Blood	Zoology Dept R.U.
Grahamstown	GHT-M2	Blood	Zoology Dept R.U.
Grahamstown	GHT-M3	Blood	Zoology Dept R.U.
Grahamstown	GHT-M4	Blood	Zoology Dept R.U.
Grahamstown (?)	GHT-U	Blood	Zoology Dept R.U.
Oudtshoorn	ODT-3	Blood	Zoology Dept R.U.
Oudtshoorn	ODT-4	Blood	Zoology Dept R.U.
Oudtshoorn	ODT-5	Blood	Zoology Dept R.U.
Oudtshoorn	ODT-6	Blood	Zoology Dept R.U.
Oudtshoorn	ODT-7	Blood	Zoology Dept R.U.
Oudtshoorn	ODT-8	Blood	Zoology Dept R.U.
Oudtshoorn/Namibia	O/N-1	Blood	Zoology Dept R.U.
Oudtshoorn/Namibia	O/N-2	Blood	Zoology Dept R.U.
Oudtshoorn/Namibia	O/N-3	Blood	Zoology Dept R.U.
Oudtshoorn/Namibia	O/N-4	Blood	Zoology Dept R.U.
Namibia	NAM-1	Blood	Zoology Dept R.U.
Namibia	NAM-2	Blood	Zoology Dept R.U.

1.5 The following samples and breeding data were supplied by the Kalani Investments ostrich farms. These were all blood samples. These samples were used in Chapter 3 Section 3.4.

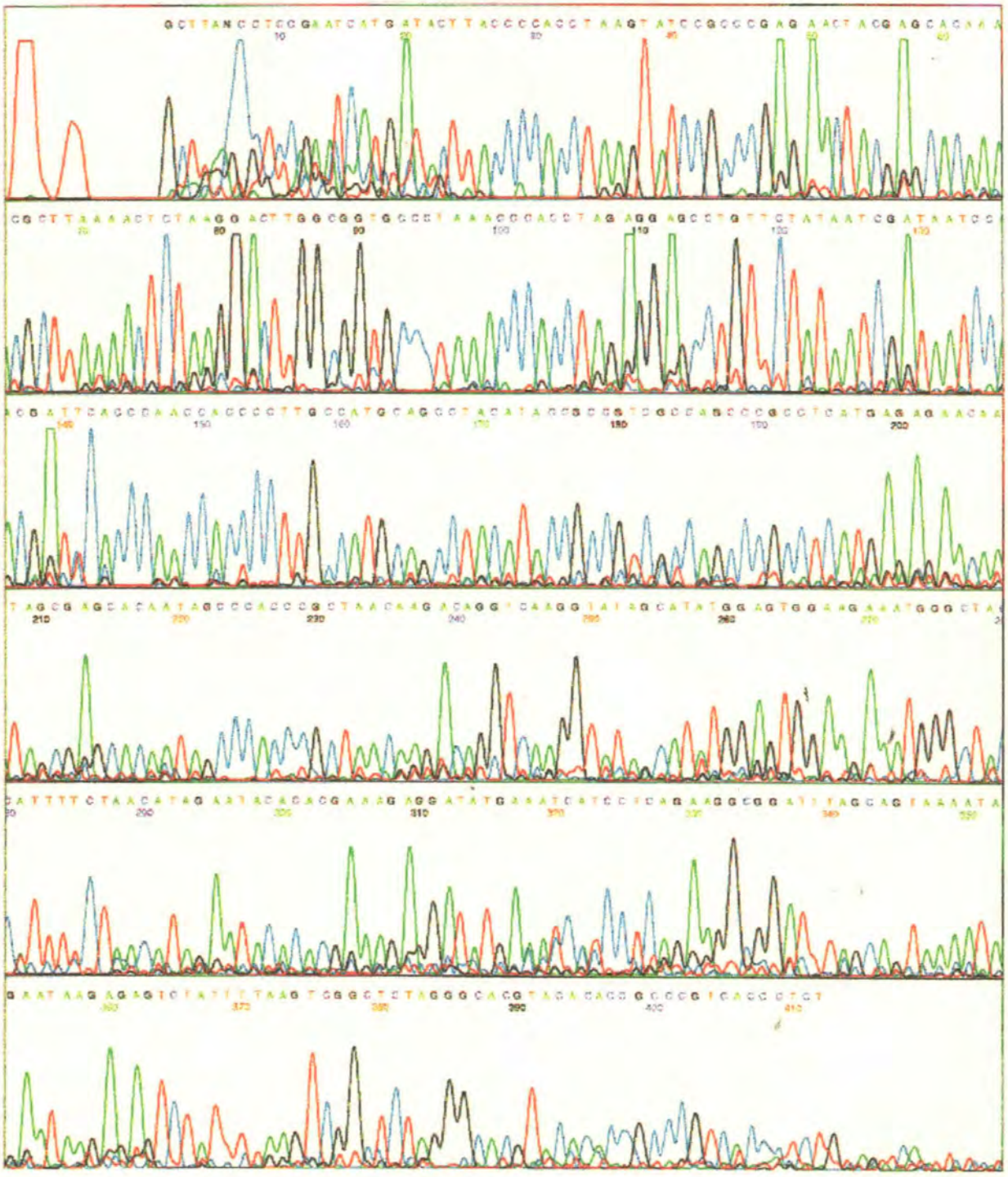
Index of Individuals	Camp Name	Number of eggs/camp in the breeding season 1992 –1993	Index of Individuals	Camp Number	Number of eggs/camp in the breeding season 1992 - 1993
A2F1 A2F2 A2F3 A2F4 A2F5 A2F6 A2M1 A2M2 A2M3 A2M4	A2	87	D5F1 D5F2 D5F3 D5F4 D5F5 D5F6 D5M1 D5M2 D5M3 D5M4	D5	47
B2F1 B2F2 B2F3 B2F4 B2F5 B2F6 B2M1 B2M2 B2M3 B2M4	B2	87	E3F1 E3F2 E3F3 E3F4 E3F5 E3F6 E3M1 E3M2 E3M3 E3M4	E3	391
B3F1 B3F2 B3F3 B3F4 B3F5 B3F6 B3M1 B3M2 B3M3 B3M4	B3	80	F9F1 F9F2 F9F3 F9F4 F9F5 F9F6 F9M1 F9M2 F9M3 F9M4	F9	423
D4F1 D4F2 D4F3 D4F4 D4F5 D4F6 D4M1 D4M2 D4M3	D4	350			

APPENDIX C

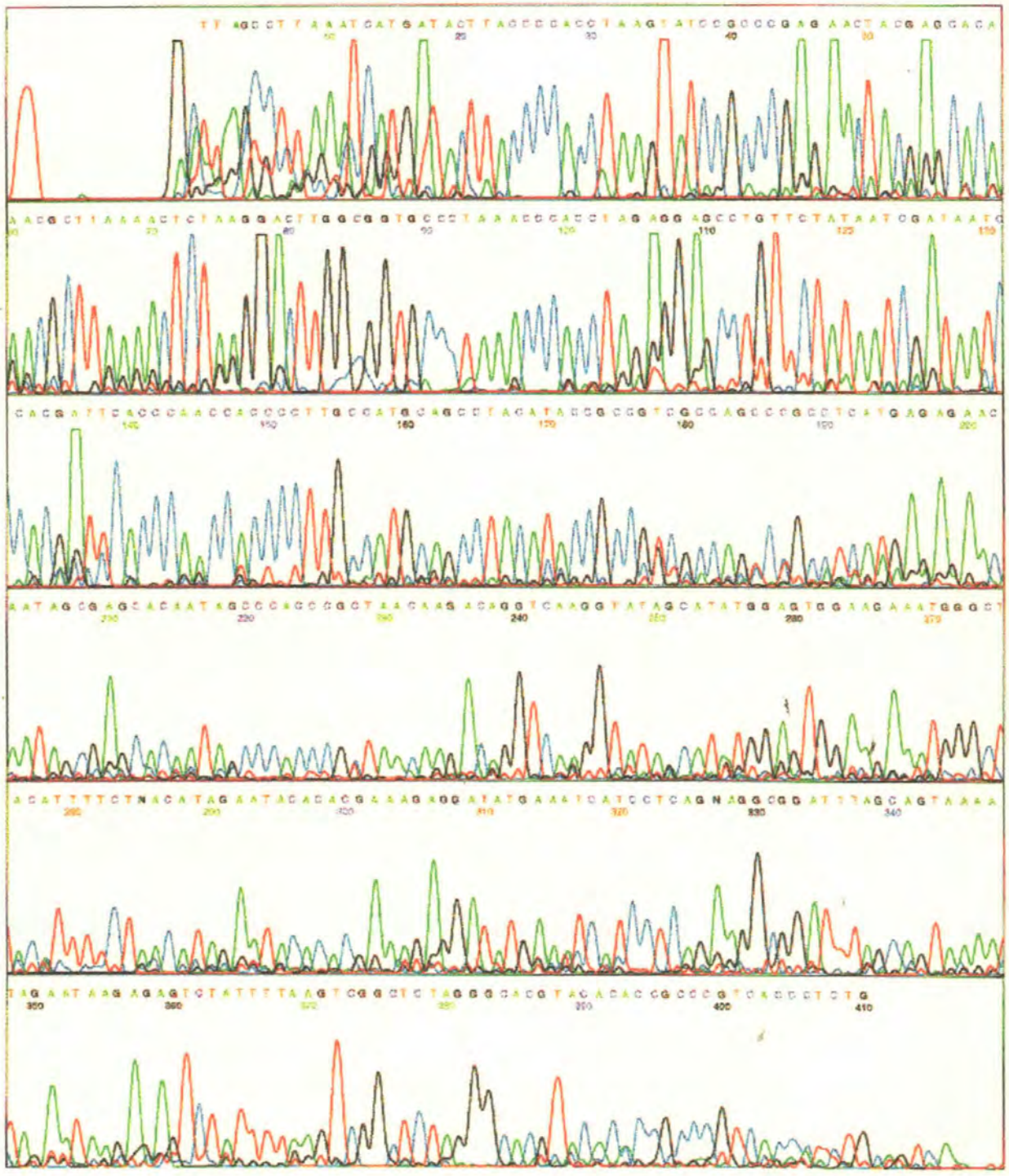
DNA sequence of the L-strand of a 12S rRNA gene fragment of ostriches. The DNA was from ostriches that represent crosses between the Oudtshoorn and Namibian populations. The primer L1091 was used for amplification and sequencing (Kocher *et al.*, 1989).



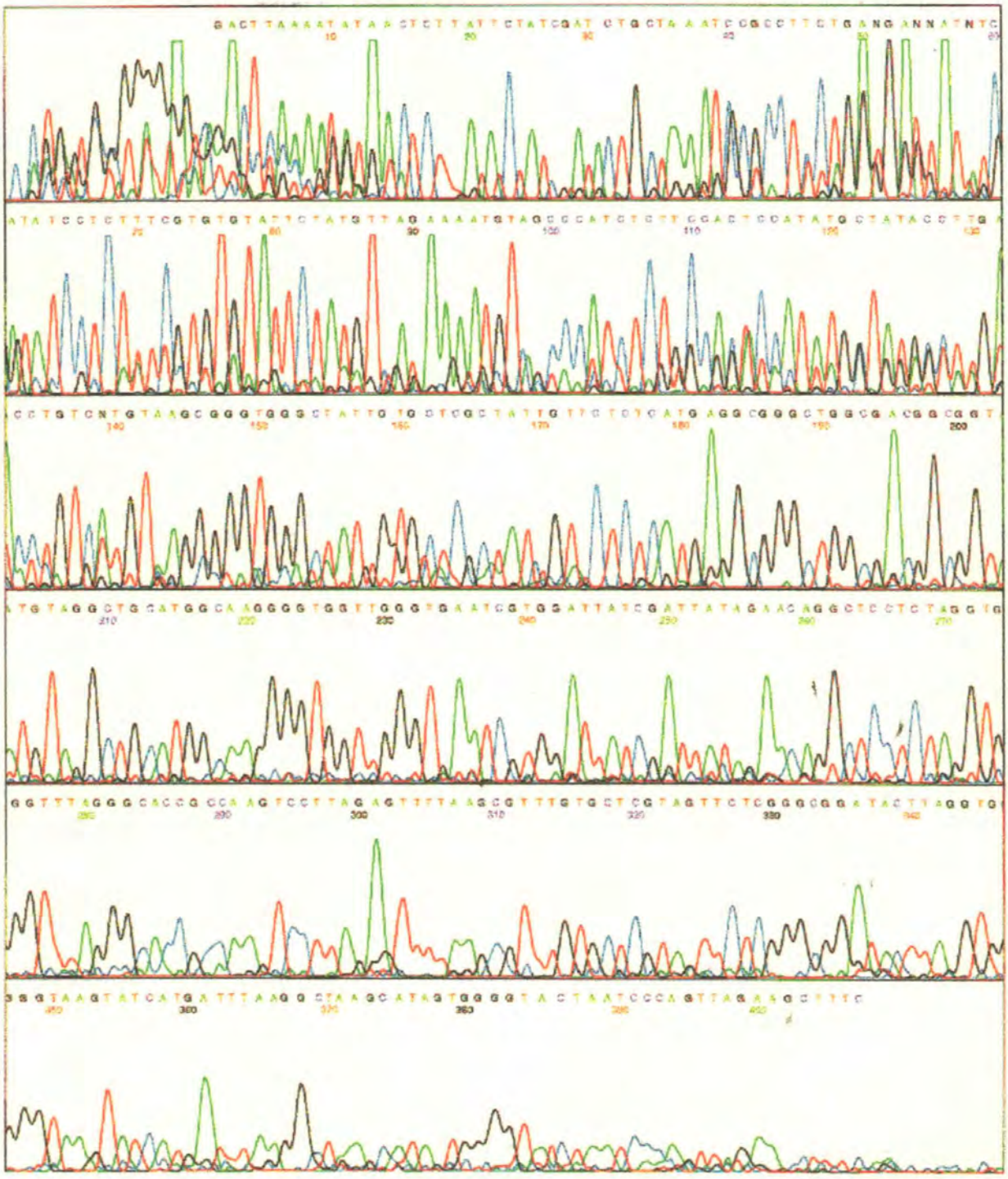
DNA sequence of the L-strand of a 12S rRNA gene fragment of ostriches. The DNA was from ostriches that represent the Oudtshoorn population. The primer L1091 was used for amplification and sequencing (Kocher *et al.*, 1989).



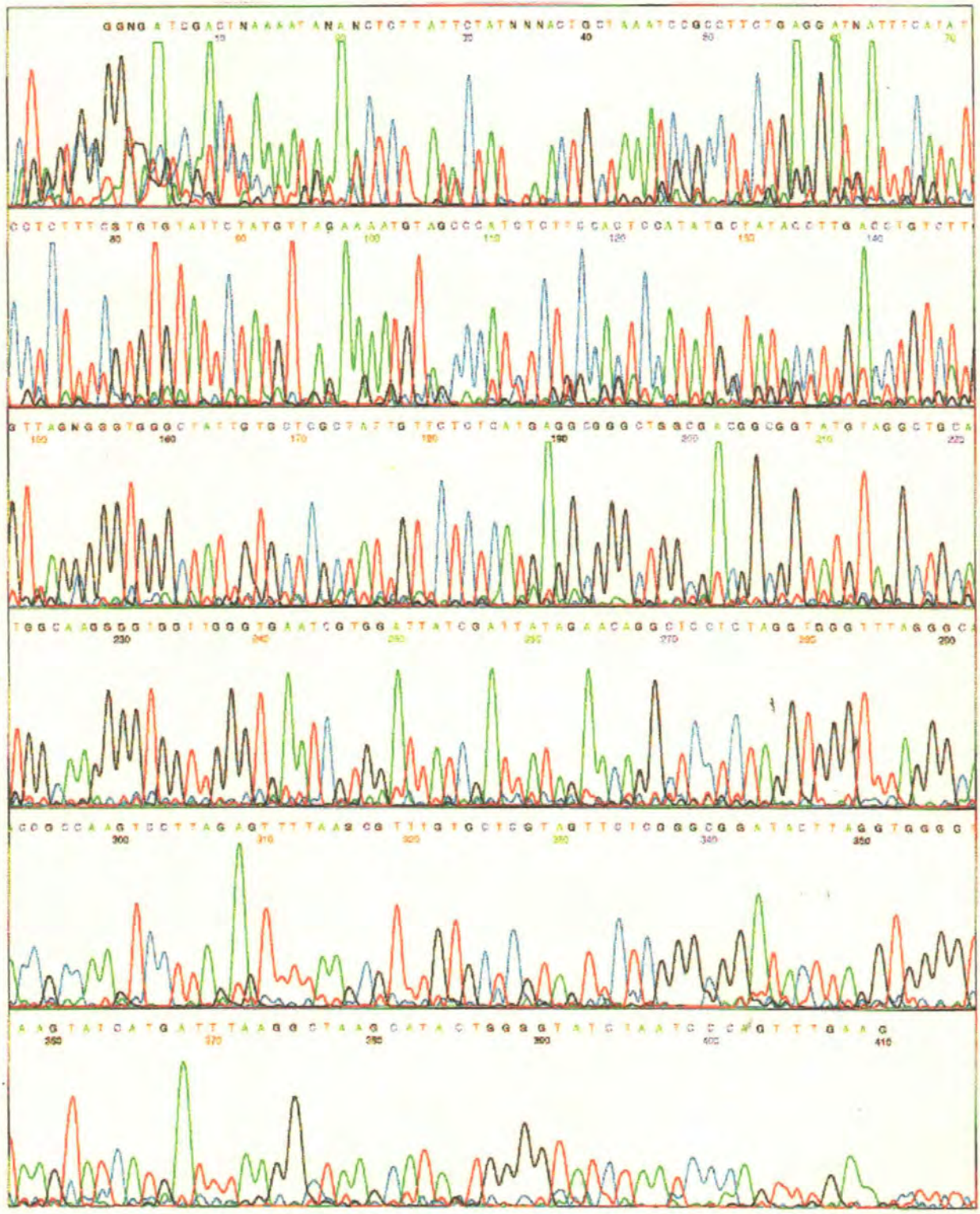
DNA sequence of the L-strand of a 12S rRNA gene fragment of ostriches. The DNA was from ostriches that represent the Swaziland population. The primer L1091 was used for amplification and sequencing (Kocher *et al.*, 1989).



DNA sequence of the H-strand of a 12S rRNA gene fragment of ostriches. The DNA was from ostriches that represent the crosses between the Oudtshoorn and Namibian populations. The primer H1478 was used for amplification and sequencing (Kocher *et al.*, 1989).



DNA sequence of the H-strand of a 12S rRNA gene fragment of ostriches. The DNA was from ostriches that represent the Oudtshoorn population. The primer H1478 was used for amplification and sequencing (Kocher *et al.*, 1989).



DNA sequence of the H-strand of a 12S rRNA gene fragment of ostriches. The DNA was from ostriches that represent the Swaziland population. The primer H1478 was used for amplification and sequencing (Kocher *et al.*, 1989).