GENETIC CHARACTERIZATION OF CONSPECIFIC POPULATIONS OF *TILAPIA SPARRMANII* (A. Smith 1840) IN THE DOLOMITIC SINKHOLES AND SPRINGS OF THE NORTH-WEST PROVINCE (SOUTH AFRICA), AND THEIR COMPARISON TO *TILAPIA GUINASANA* (Trewavas 1936).

THESIS

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by

CLIFFORD DAVID NXOMANI

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DECLARATION

I, CLIFFORD DAVID NXOMANI do hereby declare that the work reported on here is originally mine and all help and assistance obtained and utilised has been declared as such and that this work is not being submitted anywhere else for the purpose of examination.

SIGNED.....

PLACE: Grahamstown.....

DATE: 18/04/2002.....

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ABSTRACT

This study was undertaken to investigate the genetic relationships of allopatric populations of the cichlid fish, *Tilapia sparrmanii* (A. Smith 1840) inhabiting the sinkholes and springs of the North West Province, South Africa. It also examined the genetic relationships of *T. sparrmanii* to its polychromatic sister species, *Tilapia guinasana* (Trewavas 1936) which is endemic to the Guinas sinkhole in Namibia. Finally, the study investigated whether there is a genetic basis for *T. guinasana*'s colour polymorphism. The research was prompted by the concern of conservation authorities about the possible loss of unique fauna given the high demand for use of the subterranean waters for agricultural, domestic and industrial purposes. Such demands have the potential to drain these habitats. Further concerns related to habitat destruction and the introduction of alien species in the ecosystems inhabited by both fish species.

Three approaches were adopted in attempting to answer the above questions. First was the investigation of Sodium dodecylsulphate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) of total cellular proteins as a fast and relatively inexpensive indicator of genetic relatedness between the fish populations. Secondly, genetic differentiation between the *T. sparrmanii* populations and its relationship to *T. guinasana* were assayed using restriction endonuclease analysis of Polymerase Chain Reaction (PCR)-amplified regions of the cytochrome b gene and the d-loop of mitochondrial DNA, coupled with Temperature Gradient Gel Electrophoresis (TGGE) analysis of the same regions. The third approach involved the use of Random Amplified Polymorphic DNA (RAPD) fingerprinting of the populations of *T. sparrmanii* as an indicator of genetic differentiation between them. RAPD fingerprinting was further used to investigate the genetic relationships between *T. sparrmanii* and *T. guinasana* and to probe the genetic basis of the polychromatism of the latter.

SDS-PAGE did not reveal any genetic differentiation between the *T. sparrmanii* populations, nor could the analysis detect variation within them. It however clearly distinguished at a species level between *T. sparrmanii* and *T. guinasana* as well as between these and other fish species, thus indicating its possible utility as an indicator of genetic relatedness at a species level.

Mitochondrial studies employing the Restriction Fragment Length Polymorphism (RFLP) of Polymerase Chain Reaction (PCR)-amplified cytochrome b (1.1 kb) and d-loop regions (0.9 kb) with six and five restriction enzymes respectively, failed to reveal genetic differences within and between the allopatric populations. TGGE of 500 bp of the d-loop and 400 bp of the 12sRNA PCR-amplified fragments did not reveal any differences between the populations of *T. sparrmanii*, nor did the analysis reveal any differences between *T. sparrmanii* and *T. guinasana*. The lack of differentiation between the *T. sparrmanii* populations by these mitochondrial Dna analysis techniques, despite habitat fragmentation, indicated a recent origin of the populations from a common ancestral population. Failure to distinguish between *T. sparrmanii* and *T. guinasana* may be related to the sensitivity of the techniques utilized.

RAPD fingerprinting analysis indicated that the populations are genetically differentiated from each other. Using a measure of coefficient of variation, the population with the highest variation was the Wondergat population (13.99%), followed by the Klerkskraal population (8.29%), the Malmani and Marico Oog populations (each with 5.88%) and the least variation (4.95 and 4.83%) was with the Amalinda and Molopo Oog populations respectively. This high degree of intra population similarity points to the fact that this differentiation is still confined within the limits of conspecificity. The genetic distances between all of the *T. sparrmanii* populations across all primers ranged from 0.09 to 0.234 and averaged 0.146, a value that falls in the upper end of conspecific population differentiation. Such results indicate populational sub-division below the species level. RAPD fingerprinting therefore proved more sensitive than protein or mitochondrial studies. The differentiation it detected between the populations is a reflection of their adaptation to local conditions of the unique ecosystems they inhabit.

A comparison with a subset of primers between *T. guinasana* and *T. sparrmanii* confirmed the separate species status of the former from the latter. The mean genetic distance between the *T. sparrmanii* populations was 0.136, compared to that between *T. sparrmanii* and *T. guinasana* which was found to be 0.374. Statistical analysis of the difference between the mean genetic distances indicated significance with 95% confidence.

The polychromatism of *T. guinasana* was investigated to determine whether there were significant differences between its five colour morphs. RAPD fingerprinting indicated with 95% confidence that there were significant differences between the colour forms based on the genetic distances computed between them. These genetic differences appeared to correlate with the observed assortative mating between the colour forms of the species. The manifestation of the polychromatism at sexual maturity in *T. guinasana* probably indicates that colouration plays an important role in the breeding process.

The genetic uniqueness shown here between the populations of *T. sparrmanii* and the colour forms of *T. guinasana* indicate for protective measures to be put in place if the genetic resources of the isolated fish populations are to be preserved. These must be coupled with a thorough assessment of the temporal and spatial distribution of genetic variability of the populations as a guide to a long-term management strategy for the fish populations and the ecosystems they inhabit. This study therefore has shown that the allopatric populations of *T. sparrmanii* in the sinkholes and springs of the North-West Province are genetically unique, as well as show that the colour forms of *T. guinasana* are genetically distinct.

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CHAPTER 1.
GENERAL INTRODUCTION.

South Africa is characterized by high air temperatures, seasonal rainfall, a semi arid to arid climate with a scarcity of large permanent water bodies that can serve domestic, recreational, agricultural and industrial water requirements. The existence of numerous sinkholes and springs in the semi arid western part (in particular, the North West Province) of the country therefore offers the potential of a vast subterranean water supply (Pitman, 1973) that could be used to meet these various needs.

The sinkholes and springs are tiny water bodies which accommodate small, and in some cases diminishing populations of fish which are threatened by the growing need to tap the underground waters for agriculture, industry and domestic use in these relatively dry regions (Ribbink *et al.*, 1991). It has been suggested that sinkhole formation took place 50-65 million years ago as a result of aridification of the western part of the sub-continent, with subsequent disruptions of various drainage systems (Harger, 1922). Warmer, wetter conditions occurred around 11000 to 16000 years ago and colonization of these formations by fish is likely to have occurred during this period, with flash floods possibly aiding the distribution of fish. Aridification during the peak of the glacial period (around 18 000 years ago) which was characterized by drier conditions, may have resulted in fragmentation of the freshwater bodies thus formed, as well as the fish populations they now harboured.

Prior to fragmentation of these habitats and during wetter periods, it is likely that the fish populations were large and widespread. Upon fragmentation founder populations were created, probably undergoing several bottlenecks due to growing aridification of their environment. These populations may have adapted to their unique environments and in so doing diverged from one another such that today, the contemporary populations might represent unique components of South Africa's natural heritage. Each environment (i.e the sinkholes and springs) is unique in physical and biological characteristics (Ribbink *et al.*, 1994).

The populations are however faced with the genetic and demographic consequences of such isolation and fragmentation (Meffe & Vrijenhoek, 1988), namely

(a) local divergence via natural selection and/or genetic drift,

(b) little or no gene flow among isolated populations that otherwise might moderate losses of genetic variability after population crashes; and

(c) little or no recolonization of isolated habitats after local extinction.

To what extent are the biota (which include the fish) unique and therefore valuable as reservoirs of genetic resources? Do they warrant special protection as a result of their conservation status? The National Department of Environmental Affairs (South Africa) posed the questions above out of concern about the threats facing these ecosystems and the fish populations that inhabit them. A study was then set up to provide answers to these questions and to develop criteria for conservation and retention of the water bodies in the face of competing demands. The overall study was undertaken as a team effort involving ecologists, morphologists and entomologists as well as genetic characterization of the fish populations. This thesis focuses on the genetic characterization of populations in the sinkholes and springs. The genetic characterization of the fish was carried out using polyacrylamide gel electrophoresis (PAGE) of total soluble proteins, mitochondrial DNA (mtDNA) analysis of slow and fast evolving genes, and Random Amplified Polymorphic DNA (RAPD) analysis.

Previous conservation efforts have concentrated on individual species, but it is clear that whole communities and habitats may have to be protected (Lowe-McConnell, 1990). Lately there is agreement that important as conservation of unique and rare species may be, there is even greater value in the examination of the ecosystem as a whole Lowe-McConnell, 1990). This includes the processes that govern and maintain the ecosystem and its role in the broader environment (A Ribbink, personal communication) . The concept of ecosystem conservation is increasingly being recognized and applied in South Africa, albeit to river and catchment conservation in particular (O'keeffe *et al.*, 1989). Skelton (1990) notes that the conservation of threatened fishes has recently focused on both aspects, i.e ecosystems and species conservation.

This thesis probes only one component of this broader picture and set out to achieve the following specific objectives:

a) To determine if the *T. sparrmanii* populations from the sinkholes and springs of the North West Province of South Africa, are genetically distinct.

b) To determine if *T. guinasana* is genetically distinct from *T. sparrmanii* and investigate its species status

c) To investigate the extent of genetic differences between the colour polymorphs of *T. guinasana*

d) To suggest conservation measures for the fish populations in the sinkholes and springs.

The study found that the populations of *T. sparrmanii* and the ecosystems they inhabit are unique and represent part of South Africa's natural heritage and are worthy of conservation. *Tilapia guinasana* was confirmed as a separate species from *T. sparrmanii*. Further, the colour polymorphs of *T. guinasana* were found to be genetically unique. Based on these findings, recommendations for the conservation of both *T. sparrmanii* and *T. guinasana* were made.

CHAPTER 2.

MATERIALS AND METHODS.

2.1 Nature of sampling area/region

The area whose fish fauna was studied in this thesis was the south of the North West Province of South Africa (formerly western Transvaal). This is a dolomitic area covering approximately 6400 km^2, with over 5000 km^2 lying between Krugersdorp and Mafikeng. The area represents 200 km (in length) of dolomite that forms the basis of the watershed of the south North West Province (Harger, 1922). Figure 1 below is a map of southern Africa depicting some of the sampling sites for the fish.

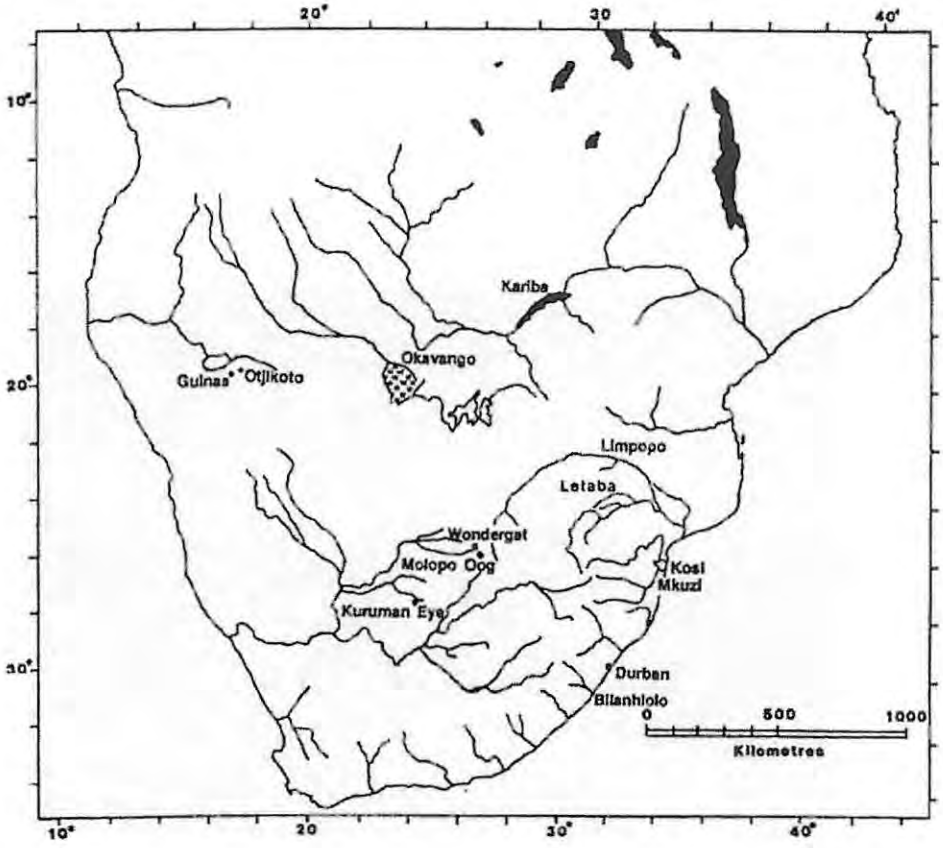


Figure 1. Map of southern Africa illustrating some of the collection sites of *T. sparrmanii* (Molopo Oog, Wondergat are visible), as well as that of *T. guinasana* (Guinas). Map after Ribbink *et al.*, (1994)

The action of rain and other water has had a profound effect in the transformation of rocky environments depending on the susceptibility of such systems to its percolating action, which has been determined by the chemical composition of such systems. The formation and transformation of the dolomitic rock systems the North West Province of South Africa, are a product of these processes.

Dolomite is by its nature porous owing to the high percentage of calcium and magnesium carbonate. Because of the soluble nature of these carbonates, they are leached out in horizontal bedding planes and vertical joints, resulting in apertures that may be sufficiently large to be called caverns. Being a magnesium limestone, dolomite is regarded as being amenable to the action of percolating waters. With an estimated 460 mm of rainwater falling annually in the North West Province, water has undermined the dolomite formations. The result of this has been the formation of caverns which eventually fall in and form numerous sinkholes (Harger, 1922). It has been suggested that sinkhole or cavern formation took place 65-75 million years ago, with their isolation occurring 50-65 million years ago (Harger, 1922) as a consequence of aridification of the western part of the sub-continent, with consequent disruptions of the drainage systems.

The subsequent colonization of the sinkholes by fish is however thought not likely to have taken place around the same time of formation of the sinkholes, since they probably would not have survived the glacial or dry periods. According to paleoclimatic and paleoecological evidence, warmer wetter conditions occurred around 11000 -16000 years ago (Twentyman-Jones, 1992). Colonisation of the sinkholes by fish may have taken place around this period, with flash floods possibly aiding the distribution of the fish. Fragmentation of the water bodies and consequently the fish populations inhabiting them, could have occurred during subsequent drier periods. A likely result of such fragmentation is the genetic isolation and possible differentiation of the fish populations in the absence of gene flow between the sink holes and other water bodies (Twentyman-Jones, 1992).

2.2 The fish species studied.

Two fish species were the main subject of this study, namely *T. sparrmanii* and *T. guinasana.* *Tilapia sparrmanii* is a widely distributed freshwater cichlid, occurring from the Orange river and KwaZulu/Natal south coast northwards to the upper reaches of southern Zaire/Congo tributaries, Lake Malawi and the Zambezi system. It has been extensively translocated south of the Orange in the Cape (Skelton, 1993). It has a wide habitat tolerance range; feeds on algae, soft plants, small invertebrates such as insects and even small fish. Its uses range from being mainly distributed as a forage fish for bass, as a component of subsistence fisheries, and is occasionally targeted as an angling fish (Skelton, 1993).

Tilapia guinasana differs from *T. sparrmani* in having fewer dorsal spines and highly variable colouration or polychromatism. It is naturally endemic to Lake Guinas in Namibia and has been translocated to Lake Otjikoto and several reserviors in Namibia (Skelton, 1993). With Guinas having a surface area of only 2800, *T. guinasana* is endemic in the smallest area for any known Tilapia (Ribbink *et al.*, 1991). It is listed in the IUCN Red Data book (Baillie & Groombridge, 1996) as critically endangered from depletion of groundwater resources and the potential impact of introduced species.

2.3 Description of sampling sites of *T. sparrmanii*

Six sites served as sources of material in this study, five of them situated in the south of North West province South Africa, ranging between longitude 25°47' - 26°17' and latitude 25°53' - 26°51' (see map in figure 2.1). The surface elevation ranges between 1497and 1440m asl. There are numerous springs to the north of the study sites, and these form the headwaters of the Groot Marico, Klein Marico and Malmani rivers (Limpopo system). Springs in the south and west form the headwaters of the Mooi, Schoonspruit & Harts river (Vaal system) and Molopo rivers respectively (Harger, 1922; Wellington, 1929). Of the six sites sourced in this study, four (Malemani, Klerksraal, Marico Oog and Molopo Oog) are classified as springs and one (Wondergat) as a sinkhole.

The names of the sites and the distances between the ones located in the North West Province are shown in Table 1. The sixth source of fish was a hatchery in Amalinda, East London in the Eastern Cape Province; used for the rearing endangered fishes as well as fish ear-marked for stocking water bodies for recreational fishing.

Table 1 Estimated distances between the sinkholes and springs in the North West Province of South Africa used as sources of fish samples.

	Locations	Distance
(A)	**(B)**	**(A☞B)**
Wondergat	Klerkskraal	133.00 km
	Molopo Oog	014.00 km
	Malemani	018.00 km
	Marico Oog	049.00 km
Molopo Oog	Klerksraal	120.00 km
	Malemani	008.00 km
Marico Oog	Klerkskraal	091.40 km
	Molopo Oog	036.00 km
	Malemani	030.70 km

The study area in the North West Province is situated within a summer rainfall region with hot, wet summers and cool dry winters (November to April and May to October respectively). The area incorporating the sites Molopo, Malmani, Wondergat & Marico has a yearly average rainfall of 560 mm (van Rensburg, 1922), while the Klerkskraal area averages 580.2 mm per year (Orpen, 1977).

Table 1 indicates the distances between the sites and illustrates the fact the fish populations are isolated from each other such that there are physical barriers to gene flow between them. Table 2 outlines the factors that pose threats to the continued existence of the *T. sparrmanii* populations in the sinkholes and springs. Figures 2 (a) through (f) provide a visual perspective of the different habitats in which the fishes are found, including the Guinas sinkhole, where *T. guinsana* is endemic. The legends to the figures describe the habitats within the sinkholes and springs respectively.

Figure 2(a). Wondergat sinkhole. Wondergat has a surface area of 3600m, with clear waters and visibility down to about 15-20m. Water levels fluctuate seasonally. The sinkhole has sheer vertical walls with very few crevices.

Figure 2 (b) Molopo Oog spring. Molopo Oog is about 1km long and 200m wide at its widest part and is dammed at the end of a narrow channel by a weir. It has a water depth of up to 3m and becomes shallower (0.3m) near the weir. The entire water body is delimited by reeds (*phragmytes sp.)*

Figure 2(c). Klerkskraal spring. This photograph shows one of two pools constituting the Klerkskraal spring. Livestock as shown in the photograph are responsible for some of the perturbation of the water body, in addition to recreational use supported by inadequate facilities for necessities such as ablution. Further threats are posed by potential fire hazards from un-demarcated camping sites destroying the surrounding vegetation.

Figure 2(d). One of two pools at Marico Oog spring showing a recreation platform. The spring is surrounded by dense reed beds, and is approximately 2-2.5m deep. The dense vegetation, also found in the water, may be a factor in limiting the impact of the introduced bass population, but also may act as a barrier to gene flow.

Figure 2(e). Malemani spring. People and cattle traverse some sections of the water body (not shown) and together with the existence of dwellings, pollution and alien introductions pose long term threats to the fauna of this water body. Features such as is shown in this photograph constitute barriers for fish movement and hence limit gene flow between populations.

Figure 2(f). Guinas sinkhole. Guinas is approximately 120m deep and has a surface area of 2800m, making it the smallest area of endemicity for any known tilapia. Water clarity is good to about 15-20m. The arid nature of the surrounding environment is evident in condition of the vertical walls of the sinkhole.

Table 2 Summary of information about the sampling sites from which *T. sparrmanii* were obtained (excluding Guinas). Further descriptions of these sites are given in the legends of Figures 2 (a) to (f) below.

Site	Site Type	Other fish species found	Threatening factors
Molopo Oog	Spring	*P. philander, Micropterus salmoides, Clarius gariepinus, Barbus cf. Brevipinnis*	• Extensive utilization for recreational activities • Introduction of alien fish species • Pollution and deterioration of water quality
Wondergat	Sinkhole	*P. philander* *C. gariepinus*	• Alien introductions • Degradation of surrounding environment • Extensive recreational use
Malemani Oog	Spring	*M. salmoides*	• Grazing pressure on surrounding environment • Presence of alien plant species (*Populus spp.*) • Alien introductions of fish species (*M. salmoides*)
Marico Oog	Spring	*M. salmoides, B. cf brevipinnis* *Amphilius uranuscopus* *Cheliglanis pretoriae*	• Utilization as a dive site • Proximity to a camp site and residences • Presence of alien fish species
Klerkskraal	Spring	*P. philander, C. gariepinus* *B. paludinosus, B. trimaculatus* *B. pallidus*	• Uncontrolled access
Amalinda	Hatchery	N/A, origin of fish not known.	N/A

It is evident from the table and the figures that each of these sites has different physical and biological characteristics that make them unique. The fish populations therein face different selection pressures ranging from water abstraction and pollution, problems of water quality linked to recreational activities that include uncontrolled fishing. Introductions of fish such as large mouth bass had already had deleterious effects on the numbers of *T. sparrmanii* fish in some of the systems (Ribbink, *et al.*, 1994, unpub report). These threats made it important that a study be undertaken to assess the level of genetic distinction and uniqueness of the *T. sparrmanii* populations in these systems as an indicator of the value that will be lost should the above interfering factors result in their extinction.

2.4 Techniques used for examining the genetic uniqueness of the fishes.

The molecular genetic techniques used were: Polyacrylamide gel electrophoresis (PAGE) of total soluble protein from muscle tissue, restriction fragment length polymorphism analysis of PCR-amplified mitochondrial DNA regions (PCR-RFLPs), Temperature gradient gel electrophoresis (TGGE) of PCR-amplified mitochondrial DNA regions and Random Amplified Polymorphic DNA (RAPD) analysis.

2.4.1 Polyacrylamide Gel electrophoresis (PAGE) of total protein.

The word electrophoresis is a combination of words rooted in Greek meaning electricity (electro) and movement (phoresis). The principle of electrophoresis is that molecules will migrate in an electric field according to their net charge, size and shape (Brock & Madigan, 1988).

In PAGE, the polyacrylamide acts as a gel matrix characterized by a cross-linked chemical structure that provides a porous network through which charged particles must move (Bohinski, 1987). A useful adaptation of the PAGE procedure in the analysis of proteins is the treatment of the protein with the anionic detergent Sodium dodecylsulphate (SDS) prior to application for eletrophoresis, and is the approach used in this study.

Sodium doecylsulphate is negatively charged and unravels the folded structure of a protein. The detergent is then adsorbed along the elongated surface of the polypeptide chain. This adsorption of many negatively charged SDS molecules on the polypeptide chain has the effect of conferring a net negative charge to the polypeptide or protein (Bohinski, 1987).

The PAGE technique therefore involves separation of proteins with uniform charge according to their molecular weights in a gel matrix that acts as a molecular sieve. The technique may be used to determine the molecular weights of proteins individually or in mixtures as well as for comparison of electrophoretic patterns from different organisms. The use of SDS-PAGE for electrophoretic pattern comparison of proteins from different organisms is the one that has found extensive application in organismal biology and population biology. The most extensive application has been mainly in the analysis of relationships within and between microorganismal populations. Polyacrylamide Gel Electrophoresis of proteins has been used extensively in the classification, grouping and identification of micro-organisms [Kersters & De Ley (1975); Owen & Jackman (1982); Vera Cruz *et al*, (1984); Jackman & Pelczynska (1986); Murphy *et al* (1990); Qhobela *et al* (1991); Qhobela & Claflin (1988, 1992)] but despite this and despite its potential value as an effective tool in systematics and conservation genetics of higher organisms, it has not been used to any significant extent in the characterisation of vertebrate and invertebrates. Part of the reason lies in the use of a general stain for the visualization and comparison of proteins and protein patterns.

Some uses of SDS-PAGE in fish studies have been in the forensic identification of processed fish products. Pineiro *et al.* (1999) used SDS-PAGE on fish samples to identify fish species in raw and cooked samples. The technique yielded reproducible and discriminant species specific protein patterns. In an international collaborative study among nine laboratories, Rehbein *et al.* (1999) used SDS-PAGE for species identification of cooked fish. They concluded that the technique used singly or in conjunction with isoelectric focusing, was suitable for checking the species declaration of fishery products.

In a study involving non-fish species, Etiene *et al.* (2000) used SDS-PAGE, isoelectric focusing and urea-isoelectric focusing for successful authentication and species identification of baby clams (*Meretrix lyrata*), grooved carpet shell (*Ruditapes decussatus*) and the common edible cockle (*Cerastoderma edule*).They had similar success with fish using the same techniques (Etiene *et al.*, 2001).

It is this relatively unexplored utility of the technique that was examined in this study, by employing the technique in the assaying of genetic relationships between conspecific populations of *T. sparrmanii* and *T. guinasana*. More importantly, the technique was used in this study to extend the work published in Nxomani *et al.* (1994). The utility of the technique in vertebrate species identification was also tested by the inclusion of two distantly related fish species in the study. The main reason for employing this relatively untested technique was to find a quick, simple, non-invasive and reasonably inexpensive molecular method that can be used by under-resourced conservation managers for at least the preliminary comparative analysis of unknown fish genetic systems. The species-level resolving power of the technique demonstrated in the authentication and identification of fishery products (Pineiro *et al.*, 1999; Rehbein *et al.*, 1999; Etiene *et al.*, 2000; Etiene *et al.*, 2001) means that it can be used in population and species level differentiation in fish species. A method like this could be used to lay the basis for preliminary understanding of relationships. This is critical in the light of the shortage of resources, especially in the southern African sub-region.

2.4.2 Mitochondrial DNA

Mitochondria are cytoplasmic organelles in eukaryotic cells which are the sites of respiration (Park & Moran, 1994) and are thought to have originated from free-living purple bacteria that became part of the eukaryotic organism by an occurrence called an "endosymbiotic" event (Meyer, 1993). They have their own DNA which encodes a number of genes that are important for cell respiration and other functions. Mitochondrial DNA is separate from the cells' nuclear DNA and such a physical separation accounts for the ease with which the molecule can be isolated (Park & Moran, 1994).

Mitochondria are cytoplasmically inherited and each mitochondrion contains about ten identical copies of the mitochondrial genome (Ovenden, 1990), but depending on the cell type up to several thousand copies of the genome may be found in the same cell (Meyer, 1993). This is a small circular double stranded molecule (16,500+- 500 base pairs in size, (Meyer, 1993)) that is homologous among widely divergent taxa (Bermingham, 1991). The homologous nature of the molecule allows the investigator to perform comparisons across independently evolving lineages.

The advantage of this is that such data would then make possible the study of the roles played by geologic history, demography and life-history strategies on the genetic structuring of populations (Bermingham, 1991). This was illustrated in a study of populational subdivision in freshwater fish species that are widely distributed in south-eastern United States. In a study of the bowfin (*Amia calva*) and spotted sunfish (*Lepomis punctatus*) Bermingham *et al.* (1986) found that the two species shared an East-West genetic break that was remarkable in its concordance across the two independently evolving lineages. This led them to suggest an historical biogeographic explanation for their observations. They also found a high level of concordance between Mitochondrial DNA and tagging studies in identifying the origin of Atlantic salmon, suggesting the usefulness of restriction analysis of Mitocohndrial DNA in the determination of continent of origin of Atlantic salmon (Bermingham *et al.*, 1986).

The appeal of mitochondrial DNA in genetic studies lies essentially in the ease with which it can be purified, relative to other forms of DNA (Berg & Ferris, 1987; Brown, 1983). The mitochondrial genome of animals (fish mitochondrial DNA has not been shown to be different), consists of 13 protein coding genes, two ribosomal RNA (rRNA, small 12S and large 16SrRNA) genes and a single major non-coding control region in vertebrates (Meyer, 1993). The molecule has several characteristics which make it a powerful tool for studying the genetic architecture of fish populations, namely:

- a rate of evolution 5-10 times that of single copy nuclear DNA, which provides for ease of detection of differences between recently separated populations (Bermingham, 1991; Meyer, 1993)
- an almost exclusive maternal inheritance, which is useful in detecting differential migration and spawning behaviour of the sexes (Bermingham, 1991; Park & Moran, 1994)
- lack of recombination, which makes sequence and phylogenetic analyses possible within a single lineage (Avise, 1994)
- homoplasmy, which obviates the need to examine more than one form of the same molecule (Bermingham, 1991)

2.4.2.1 Approaches to mitochondrial DNA analysis in fish populations.

Mitochondrial DNA analysis of populations has conventionally been performed using two approaches. The first involves the purification of the molecule from genomic DNA, restriction digestion using enzymes, separation of digestion products in a matrix such as agarose gels, visualization with ultraviolet stains or radio-active labelling, and finally scoring to determine the frequency of restriction sites or differences thereof between the units under study. This has been collectively termed Mitochondrial DNA Restriction Fragment Length Polymorphisms (RFLPs) (Ferris & Berg, 1987).

The second approach involves extraction of total genomic DNA, digestion with restriction enzymes, separation of products in a gel matrix, blotting onto nylon membranes and probing with digested and labelled mitochondrial DNA. RFLPs refer to changes in specific contiguous segments of DNA. Cleavage of genomic DNA results in an array of fragments of differing length. Detection of population/species differences is achieved with the use of a cloned DNA probe. Any changes or differences in sequence results in the alteration of the sites at which DNA is cleaved, thus individuals which contain different site changes, DNA deletions/insertions generate fragments that differ in length and number. These may be used as population specific markers (Kamaljit *et al*, 1991).

The regions of mitochondrial DNA that have been used so far in fish population analyses have been the cytochrome *b* (*cyt b*) gene (Imsiridou *et al.*, 1998; Triantafyllidis *et al.*, 1999; Nelson *et al.*, 2000), the cytochrome oxidase gene complex I, II and III (Carrera *et al.*, 1999; Chow *et al.*, 2000) the NADH Dehydrogenase (ND) genes (Turan *et al.*, 1998; Triantafillidis *et al.*, 1999; Shaw *et al.*, 2000) , 12S rRNA (Cespedes *et al.*, 2000) and/or the control region (Verheyen *et al.*, 1996; Innes *et al.*, 1998; Imsiridou *et al.*, 1998; Triantafallidis *et al.*, 1999; Tabata and Taniguchi, 2000; Wilson *et al.*, 2000; Shaw *et al.*, 2000; Terry *et al.*, 2000).

The selection of these particular sequences has been mainly due to the fact that they are flanked by highly conserved regions which have been described across many taxa (Waldman & Wirgin, 1994) and such regions can be used for the design of DNA primers to amplify intervening sequences for analysis by a wide variety of techniques.

With the development of the polymerase chain reaction (Saiki *et al.*, 1988), further approaches to the use of mitochondrial DNA in fish population analysis were made possible, including the ability to amplify specific genes or segments of mitochondrial DNA using universal primers (Kocher *et al.*, 1989). One such approach involves the amplification of the chosen region/sequence/gene(s), digestion of the amplification products with a battery of restriction enzymes, visualization by ethidium bromide and scoring of the resultant genotypes (Cronin *et al.*, 1993; Boulding *et al.*, 1993; Martin *et al.*, 1992; Chow *et al.*, 1993). Alternatively, the amplification products can be sequenced directly or cloned first into a vector of choice for subsequent sequencing (Carr & Marshal, 1991; Bartlett & Davidson, 1991; Beckenbach, 1991). Martin *et al.*, (1992) combined direct sequencing and RFLP analysis of PCR products.

The advantage of the PCR approach is that it makes non-invasive sampling possible and obviates the need for the sacrifice of whole animals, a few drops of blood or clippings of the fins suffice. The drawback of this approach has been that only short lengths of mitochondrial DNA could be amplified, which might not contain a large number of restriction sites, resulting in very few informative markers being detected. However, informative data have been derived using this approach, even in cases where only a few restriction enzymes have been used (Carrera *et al.*, 1999 in Atlantic salmon; Innes *et al.*, 1998 in marlin and other billfish and Cespedes *et al.*, 1998 in flatfish species).With rapid developments in PCR technology it is now possible to amplify large fragments of DNA (Boehringer Mannheim PCR manual, 1996) and to counteract the drawback mentioned above.

2.4.2.2 Examples of applications of mitochondrial DNA to analysis of fish population structure

The application of the different approaches of mitochondrial DNA population analysis have been wide and varied and could not all possibly be covered here. The following listings of mitochondrial DNA uses in fish population structure are limited to te illustration of specific strategies as contained in each, and are in addition to work mentioned above with respect to the use of specific mitochondrial genes.

- *Mitochondrial DNA RFLPs.*

Norway has both wild and farm populations of Salmon (*Salmo salar*), which have been shown to be different stocks (Knox & Verspoor, 1991). Scotland carries out extensive culture of Norwegian farm salmon, and there's been a great deal of concern about the effects and implications escapees of farm Atlantic salmon could have on the genetics on wild Scotish populations (Maitland, 1989). In a study carried out to assess the genetic implications of mixing of the two salmon populations, restriction endonuclease analysis identified an *Hae*III RFLP that was unique to the Norwegian salmon. This provided a genetic population marker for studies of genetic mixing of escaped salmon from Norway and the wild Scotish salmon (Knox & Verspoor, 1991), in that it could be used to identify and trace the two populations.

- *PCR/Sequencing.*

Four commercial species of tuna of the genus *Thunnus* are caught off the east coast of Canada. Of these, only the harvest of the bluefin tuna (*T. thynnus*) is regulated but not that of bigeye (*T. obesus*), yellowfin (*T. albacares*) or albacore (*T. alalunga*) . The diagnostic features used for the identification of the species have all been morphological. This has made the enforcement of regulations difficult since these features can be easily removed by poaching individuals or groups. Bartlett and Davidson (1991) compared the sequences of a 307 bp fragment of the cytochrome b gene from representatives of the four species. They not only found intra specific differences, but that the four species could be clearly differentiated from each other on the basis of these sequences. Such differences could be used as genetic markers to determine identity of an individual tuna species (Bartlett & Davidson, 1991).

- *PCR/Sequencing/RFLP.*

Amorhead (*Pseudopentaceros wheeleri*) is a species that occurs in the subarctic, epipelagic habitats of the northern Pacific Ocean and populations of the species are known to reproduce on seamounts in the central Pacific. Over exploitation of seamount populations has resulted in a dramatic decrease in the number of reproductive populations. In an effort to determine whether distinct stocks of the species exist in association with specific seamounts, Martin *et al* (1992) undertook a population genetics study thereof.

They carried out analysis of mitochondrial DNA variants for individuals collected from the open ocean and two of the seamounts, using sequencing and RFLP analysis of PCR amplified fragments of the D-loop and ribosomal genes. They found no geographical partitioning of mitochondrial DNA haplotypes, refuting the hypothesis that the different seamounts harbour genetically unique populations. The genetic similarity that they observed between the seamount and open ocean fish confirmed an earlier hypothesis that amorhead migrate between the central and northern Pacific ocean for reproduction and feeding, respectively (Martin *et al.*, 1992).

The particular relevance of mitochondrial DNA analysis to the present study is the fact that the systems studied consist of geographically isolated fish populations which were possibly founded by a relatively low number of individuals. Because of its largely maternal mode of inheritance, Mitochondrial DNA is particularly susceptible to founder effects (Meyer, 1993), and would be expected to yield relevant information about both the genetic relationships between the populations of *T. sparrmanii* studied and indications regarding their history.

2.4.3 Temperature Gradient Gel electrophoresis (TGGE).

Most analyses of mitochondrial DNA involve some sort or modification of the RFLP analytical technique. RFLPs however, result from base changes within restriction enzyme recognition sites or chromosomal rearrangements. Mutations in the DNA bases in the rest of the genome remain unassayed by conventional RFLP analysis of mitochondrial DNA.

This has resulted in the development of alternative strategies to assay genetic variation or mutations outside the restriction enzyme recognition sites namely:

- preparation of DNA:DNA, DNA:RNA, and RNA:RNA heteroduplexes of standard or wild type sequences and the mutated or comparative sequences and the cleavage of the heteroduplexes with a enzyme that is specific for single base pair mismatches in the heteroduplex
- chemical modification and cleavage of unpaired bases in the heteroduplex, which results in modification of the migration behaviour thereof in a gel system (Ganguly & Prockop, 1990)

- detection of single base mutations by differential melting properties which determine and modify the migration patterns of DNA homo/heteroduplexes under denaturing gradient gel conditions (Fischer & Lerman, 1980; Myers *et al.*, 1985b) or Denaturing Gradient Gel Electrophoresis (DGGE)

There are two types of denaturing gradient gels, (i) parallel and (ii) perpendicular gels. Parallel gels contain a linearly increasing gradient of DNA denaturants from top to bottom whereas perpendicular gels contain a linear gradient of denaturants from left to right across the gel. Both these gel systems can be used independently to assay for DNA polymorphisms in PCR -amplified genomic DNA. Parallel denaturing gradients allow the analysis of multiple samples on a single gel and are thus best suited for efficient identification of polymorphisms in multiple members of a large pedigree.

Perpendicular gels are best suited for identification of DNA polymorphisms in a single individual, thus allowing further directed characterization thereof (Sheffield *et al.*, 1989; Cox & Myers, 1990). Its utility in complementing PCR-RFLP for fish population structure analysis was examined in this study

2.4.4 Random Amplified Polymorphic DNA (RAPD) fingerprinting.

RAPD fingerprinting (Welsh & McLelland, 1990; Williams *et al.*, 1990) is based on the premise that nuclear DNA has multiple copies of short, inverted DNA sequences close to one another that can be used as primers to amplify intervening sequences. As opposed to conventional PCR, only a single short primer of random sequence is used and there's no need for *a priori* knowledge of the chromosomal location/identity of the amplified sequences (Waldman & Wirgin, 1994). Upon use of a short primer, the probability is high that the genome contains many priming sites that are close to each other and in opposite orientation. The technique provides a means of scanning the genome for these sequences and the resultant amplification of the intervening sequences (Hadrys *et al.*, 1992).

These intervening sequences are expected to differ in size between individuals for mainly two reasons: The first is that because the amplification process is sensitive to primer mismatch, a single change in the primer sequence will alter the number of amplification products. Secondly, differences in the lengths of the intervening sequences result in different molecular sizes of the amplified PCR products (Waldman & Wirgin, 1994).

In the estimation of relatedness between individuals and groups of samples, the amplification products are fractionated in an agarose or polyacrylamide gel, and the number and sizes of amplification products compared between individuals (Waldman & Wirgin, 1994). The polymorphisms detected by the technique serve as dominant genetic markers which are inherited in a Mendelian fashion (Williams *et al.*, 1990; Welsh & McClelland, 1991; Hadrys *et al.*, 1992; Bardacki & Skibinski, 1994).

The speed and simplicity of the procedure give it a particular appeal for a number of reasons:
(1) it is faster and less expensive than any other method for detecting sequence variation (Clark & Lanigan, 1993),
(2) there is no requirement for *a priori* knowledge of target sequences and a large number of primers (which are commercially available) can be tested (Waldman &Wirgin, 1994) and
(3) the technique can be easily automated for high throughput of samples (Wright, 1993).

Slight modifications of the technique have been referred to as (1) DNA Amplification Fingerprinting (DAF), which utilizes shorter random primers (5-8bp) and in which visualization is by polyacrylamide gel electrophoresis (PAGE) and silver staining; and Arbitrary Primed Polymerase Chain Reaction (AP-PCR), which involves the use of longer primers with radio-labelling of the amplification products and resolution by PAGE (Caetano-Anollés *et al.*, 1991; Welsh & McClelland, 1990; Welsh & McClelland 1991; Hardys *et al.*, 1992).

Random Amplified Polymorphic DNA analysis has found successful application in the detection of genetic variation (and other genetic analyses) in a large number of different taxa. These include *Gliricidia* (Chalmers *et al.*, 1992), cocoa (Russell *et al.*, 1993), conifers (Carlson *et al.*, 1991), isolation of molecular marker for tomato (Klein-Lankhorst *et al.*, 1991), genetic variation in related

species of black Aspergilli (Megnegneau *et al.*, 1993), location of disease resistance markers (Michelmore *et al*, 1991; Paran *et al.*, 1991). Hadrys *et al.* (1992) illustrates several potential applications of the technique in molecular ecology and explains how it could be used on determination of taxonomic identities, detection of intraspecies gene flow, assessment of kinship relationships, analysis of mixed genome samples and production of specific probes.

2.4.4.1 Applications of RAPD fingerprinting to studies of fish population structure

To what extent though, has RAPD analysis been utilised in fish biology? Bardakci & Skibinski (1994) applied RAPD analysis to three species of the *Tilapia* genus *Oreochromis* (O. *aureus*, O. *mossambicus* and O. *niloticus*) and four sub-species of *Oreochromis niloticus* (*O.n.vulcani*, *O.n. baringoensis*, *O.n. niloticus* and *O.n.(baobab*). They used the technique to successfully identify markers that distinguished the tilapia species as well as the sub-species of *O. niloticus*. Random Amplified Polymorphic DNA analysis detected intrapopulation differentiation (thus being more sensitive than mitochondrial DNA analysis which had failed to do so in the same species (Seyoum & Kornfield, 1992). It also proved to be at variance with studies which had suggested that *O. aureus* and *O. niloticus* are in a different sub-genus from *O. mossambicus*.

Dinesh *et al.* (1993) found that the technique could differentiate between twelve species of fishes (ten tropical and two temperate) that represented seven families. They concluded that RAPD analysis could be used to estimate the genetic variability, relatedness, species/strain verification, detection of economic traits and in other marker-based studies of fishes.

Other researchers used the technique to detect DNA polymorphisms in colour mutants of *Barbus tetrazona* and *Poecilia reticulata* (Dinesh *et al.*, 1992a & b). In the Japanese midaka fish, *Orizias latipes*, a variation of RAPD analysis, AP-PCR, was used to detect radiation induced DNA damage (Kubota *et al.*, 1992). Dinesh *et al.*. (1996) successfully used RAPD fingerprinting to estimate genetic variation and species differentiation in three species of tilapia (Nile, Mozambique and Aureus tilapia). They concluded that RAPD fingerprinting could be used to give estimates of genetic variation within and between species of Tilapia.

Gomes *et al.*, (1998) applied RAPD fingerprinting to discriminate between stocks of the four-wing flying fish, *Hirundichthys affinis*. Naish *et al.*, (1995) found that RAPD fingerprinting revealed similar genetic relationships between strains of *Oreochromis niloticus* as did multilocus DNA fingerprinting. Other uses have been in phylogenetic analysis of cichlid fishes (Sultman *et al.*., 1995; identification and characterization of Malaysian river catfish (Chong *et al.*, 2000); marker inheritance in Nile Tilapia (Appleyard & Mather, 2000); genetic variability in brown trout (Cagigas *et al.*, 1999); evaluating alterations of genetic diversity in sunfish populations (Nadig *et al.*, 1998), among the most recent.

These applications will certainly increase as more and more uses and applications for RAPD analysis are realized and the technique gains more acceptance in fish and fisheries biology. The reluctance for its wide application should be seen in the light of the general attitude to molecular genetics and its practical rewards for fisheries biologists and managers of cultured fishes. In this study RAPD fingerprinting was used to compare conspecific populations of *T. sparrmanii* to each other and to *T. guinasana*. It was also used to assess if the colour forms are genetically distinct from each other.

2.4.5 Techniques used on *T. sparrmanii* and *T. guinasana*

The PAGE (protein), mitochondrial DNA anaysis and RAPD fingerprinting studies carried out in this thesis compared not only the conspecific populations of *T. sparrmanii,* but them with *T. guinasana*. RAPD fingerprinting was also used to investigate the relationships between the colour forms of *T. guinasana*.

2.5 Collection and handling of fish samples.

Fish were collected from the study sites using scuba, hand-held nets and electro-fishing. Fish were transported from the study sites to the laboratory in aerated plastic bags and placed in prepared tanks at the J. L. B. Smith Institute of Ichthyology in Grahamstown, Eastern Cape. Fish considered to be under stress were immediately frozen in liquid nitrogen and kept at -20°C until processed.

2.6 Polyacrylamide Gel Electrophoresis (PAGE) of total cellular protein from muscle tissue.

2.6.1 Preparation of protein extracts.

After immersion in liquid nitrogen fish were stored at -20° C. Approximately one gram of muscle tissue was crushed with a pestle and mortar in 1ml of 0.1M phosphate buffer, pH 7.0. The tissue-buffer emulsion was then decanted into 1.5ml eppendorf tubes and centrifuged for 10 minutes at 13000 rpm. The supernatant was decanted into fresh tubes and stored at 4° C until used for electrophoresis. The amount of protein in each sample extract was determined using Bradford's method of protein assay (Hammond & Kruger, 1988).

2.6.2 Electrophoresis and analysis of samples.

Polyacrylamide slab electrophoresis in the presence of Sodium dodecylsulphate (SDS-PAGE) was performed using a Hoefer Scientific Instruments' SE-280 (CA, USA) apparatus in the buffer system of Laemmli (1970). Ten micrograms of the protein extract from each sample was loaded on the gel.

To facilitate direct comparison, samples from the different populations were electrophoresed in adjacent lanes, making it possible to perform visual comparison of electrophoretic patterns. Protein standards (Boehringer Mannheim, Germany) were included on all gels. Table 3 shows the names and the sizes of the protein standards.

Table 3. Protein markers and their sizes.

Marker	Size (kilodaltons)
α_2-macroglobulin	170
Phosphorylase b	97.4
Glutamate dehydrogenase	55.4
Lactate dehydrogenase	36.5
Trypsin inhibitor	20.1

The gels were run for 8 hours at 150V with water cooling or at 40V for 16 hours. For visualisation of proteins, the gels were stained in 45% Methanol, 44.8% distilled water, 10% Glacial acetic acid and 0.2% Coomasie Brilliant Blue G-250, (Fluka Chemica) for 12-16 hours. They were destained in 45% Methanol, 7% Glacial acetic acid and 48% distilled water for 4-6 hours. The gels were dried under vacuum for three hours and then photographed for permanent record.

2.7. Random Amplified Polymorphic DNA (RAPD) fingerprint analysis.

2.7.1. DNA extraction.

DNA was extracted from muscle tissue according to the method of Gold and Richardson (1991). Approximately 1g of muscle tissue was pulverized into a fine powder in liquid nitrogen using a pestle and mortar. The powder was resuspended in 0.5ml of STE buffer (0.1M NaCl, 50mM Tris, 1mM EDTA; pH 7.5) in an 1.5ml eppendorf tube and allowed to stand on ice until the same had been done for all the samples at any one time. Cell lysis was effected by the addition of 125µl of 20% SDS.

The mixture was vortexed vigorously and extracted twice with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol. This was followed by centrifugation in a microfuge for 5 minutes at 13000 rpm during each extraction, to separate the layers. The supernatant was then extracted twice with an equal volume of 24:1 chloroform:isoamyl alcohol and centrifuged as before. To the final supernatant, a 1/10 volume of 2M NaCl and two volumes of ice-cold 95% ethanol were added. Samples were mixed by gentle inversion and placed at -20°C overnight to precipitate the DNA. Precipitated DNA was recovered by centrifugation at 13000 rpm for 10 minutes at 4°C. The tubes were dried under vacuum for five minutes (care taken not to dry them excessively) and the pellets resuspended in 200µl of double distilled water, and stored at 4°C until further use.

2.7.2 Qualitative analysis of DNA extracts.

The DNA concentration in the extracts was determined by measurement of their absorbances in a Shimadzu UV-160 spectrophotometer, at 260nm. This was done by diluting the samples 1/100 in a total volume of 1ml and the concentration of DNA in each extract was estimated from the relationship **$1A_{260}$: 50µg/ml** (for double stranded DNA; Ausubel *et al.*., 1989). Ten microlitres of each sample was electrophoresed in a 1% agarose gel to check the quality of the DNA in the extracts.

2.7.3 DNA amplification.

DNA amplification was performed in an automated JDI (Cape Town, South Africa) water-cooled thermocycler. Into each reaction tube (0.5ml eppendorf tubes were used) was added: 100ng template DNA, 100pM of the respective primer (synthesized at the department of Biochemistry, University of Cape Town, South Africa), 2.5mM of each of the deoxynucleotides (Boehringer Mannheim, Germany), 1.6 mM magnesium chloride, 1X Taq polymerase buffer (Promega, USA) and 1 unit of Taq polymerase (Bio Labs, UK).The reactions were carried out in a total volume of 50µl (made up to volume with sterile distilled water). Each reaction mixture was overlayed with 50µl of sterile paraffin oil to prevent evaporation. The samples for enzymatic amplification were subjected to the following cycle: initial denaturation at 94°C for 270 seconds, and 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 36°C for 30 seconds, primer extension at 72°C for 60 seconds. This was followed by a final extension step at 72°C for 240 seconds. Following amplification, samples were either directly subjected to electrophoretic separation or stored at 4°C A total of nine decamer primers was used in this study and these are shown in Table 4..

Table 4. Primers, their numbers/codes (as in the primer collection of the department of Biochemistry and Microbiology, Rhodes University), and sequences used to genetic relatedness of *T. sparrmanii* conspecific populations. The primers were synthesized for the department for RAPD use and their sequences were completely random and bear no resemblance to commercially available stocks.

Primer code	sequence (5'-3')
1	TCACATGGCA
2	TCGATGAACG
3	TGTCCCCGGC
4	CTTCGTACAC
6	AACCGATGCT
7	ACGTAGCACT
10	AGTCGTTGTC
11	GTGATGAAGG
12	ACCTGCGTTA

2.7.4 Electrophoretic analysis/separation of amplification products.

Amplification products were separated electrophoretically in 5 and 10% gels using the buffer systems of Laemmli (1970). The stacking gel was prepared in 0.5M Tris-HCl, pH 6.8 and contained 0.4% sodium dodecylsulphate (SDS). The resolving or separation gel was prepared in 1.5M Tris-HCl, pH 8.8; and also contained 0.4% SDS. Gels were run on Hoefer Scientific Instruments'(CA, USA) "Tall Mighty Small" SE 280 electrophoresis units for an average of 2.5 hours. These "Tall Mighty Small" units were used to check for the success and extent of the amplification. Successfully amplified samples from different populations were electrophoresed adjacent to each other in a 50-well Owl electrophoresis unit to facilitate direct comparison. Electrophoresis in the Owl systems was performed overnight at 70-80V. The running/tank buffer was 1X Tris-Borate-EDTA (TBE) [0.089M Tris-base (Sigma, USA); 0.089M boric acid (BDH Ltd, England) and 0.02M EDTA (Sigma, USA)] Once electrophoresis was completed, gels were removed from the running chambers and stained with silver nitrate.

2.7.5 Silver staining of acrylamide gels.

Gels were placed in glass/plastic trays covered with buffer A [10% (v/v) ethanol; 0.5% (v/v) acetic acid] and incubated twice for 3 minutes. This was followed by incubation in (impregnation with) 0.1% (w/v) silver nitrate (BDH Ltd, England) solution for 10 minutes. Gels were then washed twice in double distilled water for 1 minute. Developing solution B [1.5% (w/v) sodium hydroxide (BDH Ltd, England); 0.01% (w/v) sodium borohyride (Sigma; USA); 0.15% (v/v) formaldehyde; in a total volume of 300ml] was added and the gels left indefinitely until the stain had developed. Amplification products were visualized as brown bands on a light yellowish-brown background. The gels were photographed for permanent record.

2.7.6 Numerical analysis of gel images (fingerprints).

Ten individuals from each population were analysed. The sex of the individuals was not known or determined. Comparison of RAPD patterns was done within and between populations. Amplification products were scored manually as "1" and "0" for presence and absence respectively and differences in band intensities were not considered. An index of similarity was determined between individuals within and between populations, using the formula:

$S_{xy} = 2n_{xy}/(n_x + n_y)$.....1

where n_{xy} is the number of fragments shared by individuals x and y and n_x and n_y are the number of fragments scored for each individual (Lynch, 1990). Similarity within a population (S) was determined as the average across all possible comparisons between individuals in a population. Between population similarity was corrected for within population similarity and determined using the formula:

$S_{ij} = 1 + S'_{ij} - 0.5 (S_i + S_j)$.....2

where Si and Sj are the values of S for populations i and j respectively, and S'_{ij} is the average similarity between randomly paired individuals from populations i and j (Lynch, 1990).

S'_{ij} was converted to a measure of genetic distance (Dij) using the formula:

$D_{ij} = -\ln[S'_{ij}/\sqrt{(S_i S_j)}]$.....3 (Lynch, 1990)

D_{ij} values obtained were used to construct dendrogram(s) using the unweighted pair-group method of analysis (UPGMA) in NEIGHBOR of the Phylip package (Felsenstein, 1993). This statistic was calculated for each of the primers and an average estimate determined, which was then used to construct dendrogam (s). For the combined data only four individuals from each population were used in the analysis.

2.8 Mitochondrial DNA Analysis.

2.8.1 Restriction endonuclease analysis of PCR-amplified fragments.

2.8.1.1 Mitochondrial DNA extraction.

Mitochondrial DNA extraction and purification was done using the method of Gold and Richardson (1991), which utilizes phenol:chloroform to separate the DNA from tissue material, followed by ethanol precipitation of the DNA.

2.8.1.2 PCR amplification.

PCR amplifications were performed in a JDI water-cooled programmable thermocycler (James Duncan Industries, Cape Town) in 50µl total reaction mixtures containing 1X PCR buffer, 2 mM $MgCl_2$ (Advanced Biotechnologies, UK), 50µM of each of the dATP, dCTP, dTTP and dGTP (Boeringer Mannheim, Germany), 10µM of each primer and 2 units of Taq polymerase (Advanced Biotechnologies, UK). The reaction mixtures were overlayed with sterile paraffin oil and subjected to the following conditions: initial denaturation at 94°C for 300 seconds, followed by 40 cycles of

94°C for 60 seconds, primer annealing at 50°C for 60 seconds and primer extension at 72°C for 120 seconds. A final extension step at 72°C for 300 seconds was also included to ensure complete extension of all the amplified products. Four mitochondrial DNA segments were amplified using PCR and four sets of universal primers and the same conditions were used for their amplification. The names/codes of the primers, the regions/genes they amplify, their sequences and the references in which they have been described are shown in Table 5. The sizes of the regions/fragments they amplified and their locations in the mitochondrial DNA genome are provided in the results section.

The terminology used for the primers is as was used in the original references. All primers, except for CB3R-L and TDKD, were synthesized at the Department of Biochemistry, University of Cape Town, South Africa. CB3R-L and TDKD were synthesized at Ransome Hill Bioscience Inc., USA. Primer names follow the Kocher *et al.* 's (1989) convention of naming the primer by the most 3' position of the primer in the human mitochondrial DNA sequence. L and H are the light and heavy strands respectively. The primer sequences are given 5' to 3' and the amplification strategy employed in the use of the above primers is shown below in Figure 3.

Table 5 Primers used to amplify regions of mitochondrial DNA from conspecific populations of *T. sparrmanii*.

Gene and primer name	Sequence	Reference
cytochrome b		
L14724	CGAAGCTTGATATGAAAAACCATCGTTG	(Kocher *et al.*, 1989)
H15915	AACTGCATGCATCTCCGGTTTACAAGA	(Irwin *et al.*, 1993)
H15149	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	(Kocher *et al.*, 1989)
control region		
THR	AGCTCAGCGCCAGAGCGCCGGTCTTGTAAA	(Kocher *et al.* 1993)
TDKD	CCTGAAGTAGGAACCAGATG	(Kocher *et al.*,1993)
cytochrome b-/d-loop		
CB3R-L	CATATTAAACCCGAATGATATTT	(Martin *et al.*, 1992)
TDKD	CCTGAAGTAGGAACCAGATG	(Kocher *et al.*, 1993)
12S RNA		
L1091	AAAAAGCTTCAAACTGGGATT	(Kocher *et al.*, 1989)
H1478	TGACTGCAGAGGGTGACGGGCGGTTGTGT	(Kocher *et al.*, 1989)

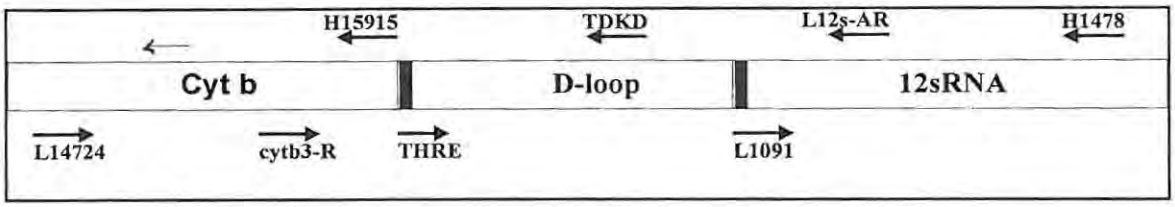


Figure 3. Strategy employed in the amplification of mitochondrial DNA genes and gene segments. The arrows indicate positions of priming and extension with accompanying text indicating the primer codes. The DNA sequences amplified were the cytochrome b (cyt b), D-loop region (D-loop), and the 12sRNA gene. Conditions for amplification were as detailed in paragraph 2.8.1.2.

2.8.1.3 Electrophoretic analysis of amplified products.

Ten microlitres of the amplification products were checked by electrophoresis through a 2% agarose (Promega, USA) gel in 1 X Tris-Borate-EDTA buffer containing 1μg/ml ethidium bromide. Gels were electrophoresed for 1 hour, visualised under ultraviolet light, photographed and their sizes estimated using the UVP Gel Documentation System (UVP, UK). The size standard used was generated from digestion of the plasmid pBR322 with the enzyme HinfI.

2.8.1.4 Endonuclease digestion of amplified DNA fragments.

The PCR-amplified cytochrome b gene was digested with the following enzymes: ApaI, HinfI, DraI, RsaI, DdeI and HaeIII. whereas the cyt-b/d-loop fragment was digested with DraI, RsaI, DdeI HaeIII and AatII. Restriction digests were performed in 10μl reactions containing 1μl of each of the amplified DNA (carefully removed from the PCR reaction mixture), appropriate enzyme and restriction buffer, according to manufacturer's (Boehringer Mannheim, Germany) instructions. The rest of the volume was made up with sterile distilled water. The reactions were incubated overnight at 37°C in a water bath. Digestion products were electrophoresed through a 5% stacking and 10% resolving acrylamide gel using the discontinuous system of Laemmli (1970), either in a Tall Mighty Small^{R} (Sigma, USA) or an Owl (S.A. Scientific) gel systems at 100V till the dye reached the end of the gel or overnight respectively. Digestion products were visualized by staining of the acrylamide gels in Silver Nitrate ($AgNO_3$) as in section 2.7.5.

2.8.1.5 Data analysis.

Size estimation of the restriction products was done by the inclusion of pBR322/HinfI digest as marker in each gel. Gels were analysed visually for absence or presence of bands.

2.9 Temperature Gradient Gel Electrophoresis (TGGE).

2.9.1 Casting the gel.

Gels were cast and run using the DIAGEN TGGE gel system (Qiagen, USA). The gel casting plates were cleaned using alcohol first followed by distilled water until they were sufficiently free of grease and traces of alcohol. The plain glass plate was moistened with distilled water to create hydrophobicity and the hydrophobic side of a gel support film (GelBond) was placed on top of it. The latter was cut to fit the size of the glass plate. The gel support film was fixed in position by rubbing it against the glass plate with the aid of a straight edge. The protective paper was then removed leaving the film in position. The spacers were greased and placed on the well-forming plate, two spacers down the sides of the plate and one across the bottom. Corners were sealed with silicon grease to prevent leaks. The two plates were clamped together, the gel cassette placed at an angle and the gel solution poured until it filled the cassette to the top. An 8% acrylamide gel was used for resolution of PCR-amplified fragments. The gel was allowed to polymerize for 60 minutes.

2.9.2 Temperature Gradients.

The temperature gradient ranges used to resolve the PCR-amplified DNA fragments were: 25-60°C, 40-80°C , 35-75°C, 40-80°C, 45-85°C and 55 to 90°C using two water baths on either side of the gel electrophoresis apparatus.

2.9.3 Electrophoresis of samples.

The gel cassette was dismantled while electrophoresis wicks were being boiled in distilled water for 5 minutes. Electrophoresis tanks were filled with 1litre of electrophoresis buffer (0.1 X TBE, 0.0089M Tris base, 0.0089M Boric acid and 0.002M EDTA) . The gel was placed in position and the wicks positioned such that they were at least 1cm from the wells at the top and the 1-2cm at the bottom of the gel, and pre-electrophoresis effected at 300V for 20 minutes.

Samples were then loaded on the gel and electrophoresed (70mA) to about 2cm below the wells, the spacers replaced, wells covered with the anode wick and the gel covered first with a plastic sheet and then a glass plate, to prevent evaporation and drying out of the gel. The temperature gradient was allowed to equilibrate for 30 minutes before running the samples at 50mA until the dye reached the bottom wick.

2.9.4 Staining of gels.

Staining of gels was as described in section 2.7.5.

CHAPTER 3.

THE USE OF POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF CELLULAR PROTEINS AS AN INDICATOR OF GENETIC RELATEDNESS BETWEEN ISOLATED, CONSPECIFIC POPULATIONS OF *TILAPIA SPARRMANII* (AND OTHER FISH SPECIES).

3.1 INTRODUCTION.

In a preliminary population analysis of *T. sparrmanii* (Nxomani *et al.*, 1994; attached as appendix B), SDS-PAGE was used to estimate the degree of genetic relatedness between four conspecific populations of the species. The outgroups in the analysis were *T. guinasana*, a closely related sub-stratum spawning polychromatic cichlid endemic in the Guinas sinkhole in Namibia (Ribbink *et al.* 1991); and *P. philander*, a distantly related mouth-brooder of the haplochromine lineage (Twentyman-Jones, 1992).

The findings of the study were:

(1) that the four conspecific populations of *T. sparrmanii* analysed (from Malemani spring, Amalinda Hatchery , Molopo Oog spring and the Wondergat sinkhole) differed from each other genetically when examined using SDS-PAGE of total soluble protein,

(2) that the Malemani spring and Amalinda hatchery populations were more closely related to each other than to those from the Wondergat sinkhole and the Molopo Oog spring; and

(3) that *T. guinasana* appeared to form part of the *T. sparrmanii* population complex

It was further concluded from the study that SDS-PAGE could distinguish between fish species and between conspecific populations, and that the isolated populations of the dolomitic ecosystems of the western Transvaal (the sources of the fish) are unique and should be conserved.

The fact that the results of Nxomani *et al.* (1994) indicated *T. guinasana* as being part of the *T. sparrmanii* population complex prompted the present study. The focus of the present study was to re-examine the genetic relationships between the *T. sparrmanii* populations using SDS-PAGE of protein by introducing a number of variations not included in Nxomani *et al.* (1994), to improve both interpopulational and interspecies resolution.

3.2 (VARIATIONS INTRODUCED TO) MATERIALS AND METHODS

Details of SDS-PAGE methodology used in this and the earlier study are described in Chapter 2. The present study introduced the following variations to the Nxomani *et al.* (1994) study:

1. Two additional populations of *T. sparrmanii* were added to the analysis, namely the Marico Oog and Klerkskraal (both springs) populations in the belief that more fish populations would improve the resolution of populational differences.
2. To investigate further the utility of the technique in detection of interspecies differences, two other unrelated fish species were included in the analysis. These were *Sandelia bainsii* and *Barbus andrewi.*
3. All the *T. sparrmanii* samples representing the different populations were electrophoresed and compared to each other on the same gel. This was done in order to reduce the effect of gel to gel variation in differentiation between the populations.

Four representative samples of each of *S. bainsii* (lanes a-e) and *B. andrewi* (lanes e-h) and *P. philander* (i-k) were also electrophoresed adjacent to each other on the same gel as those of *T. sparrmanii.* The purpose of this was to investigate internal differences in these species, inter species differences between these species on the one hand and between them and *T. sparrmanii* on the other.

A further direct species level comparison was performed between *T. sparrmani, T. guinasana, S. bainsii, B. andrewi* and *P. philander.* Two samples of each of these species (except for *P. philander* where only one was used) were compared to each other for differences in protein migration patterns. Results of this comparison are shown in Figure 5 where lanes 1and 2, 3 and 4, 5 and 6, 7 and 8 and 9 are *T. sparrmanii, T. guinasana, S. bainsii, B. andrewi* and *P. philander* respectively.

3.3 RESULTS.

To assess the genetic relatedness of the *T. sparrmanii* conspecific populations, representative protein patterns from each of the populations were electrophoresed adjacent to each other (Figure 4). In the figure one individual from each of the six conspecifics was run on the gel together with two from a single location/population. Lanes 1-6 in the figure show the protein patterns of fish from Wondergat, Malemani, Molopo, Klerkskraal, Marico and Amalinda; while in lanes 7 and 8, are protein patterns of fish from Amalinda. This was done in the former to investigate inter-population differences and in the latter case, intra-population differences.

Figure 4 illustrates results obtained in the direct comparison of SDS-PAGE protein patterns of *T. sparrmanii* conspecific populations and those of *S. bainsii*, *B. andrewi* and *P. philander*. Neither intra- (lanes 1-6) nor interpopulational (lanes 1-6, 7 and 8) differences could be detected between the *T. sparrmanii* populations. The SDS-PAGE patterns were identical for all the *T. sparrmanii* samples analysed. When the protein patterns of *B. andrewi*, *S. bainsii* and *P. philander* were compared to those *T. sparrmanii* (Figure 4: lanes a-d, e-h, i-k and 1-6 respectively), the differences were evident. The lack of intraspecies differences in the protein patterns of these species was also obvious, as was observed in *T. sparrmanii*.

The arrows in Figure 4 illustrate areas containing protein pattern differences between adjacent groups/lanes of samples. Arrow 1 indicates a pair of bands that was present in *T. sparrmanii* (Amalinda) and absent in *S. bainsii* (lane a), where the former species has a double band whereas the latter species has only one band that is slightly larger than the lowest of the former's. This band was also not found in *B. andrewi* and P. *philander*.

Arrow 2 shows a second band that was present in *T. sparrmanii* but absent in *S. bainsii* and in *P. philander*. Arrow 3 indicates the presence of a band in *S. bainsii* that was not present in *T. sparrmanii* and *B. andrewi* and which only appeared faintly in *P. philander*. In lane h, arrow 4 indicates a double band that was present in *B. andrewi* only and not in *T. sparrmanii*, *S. bainsii* or *P. philander*. The latter three species only had the lower one of these two bands.

Arrow 5 shows a double band unique to *B. andrewi*. Arrow 6 indicates a band unique to *P. philander*. These arrows show the most clearly identifiable differences in protein patterns between *T. sparrmanii* and the other fish species and illustrate genus level differences.

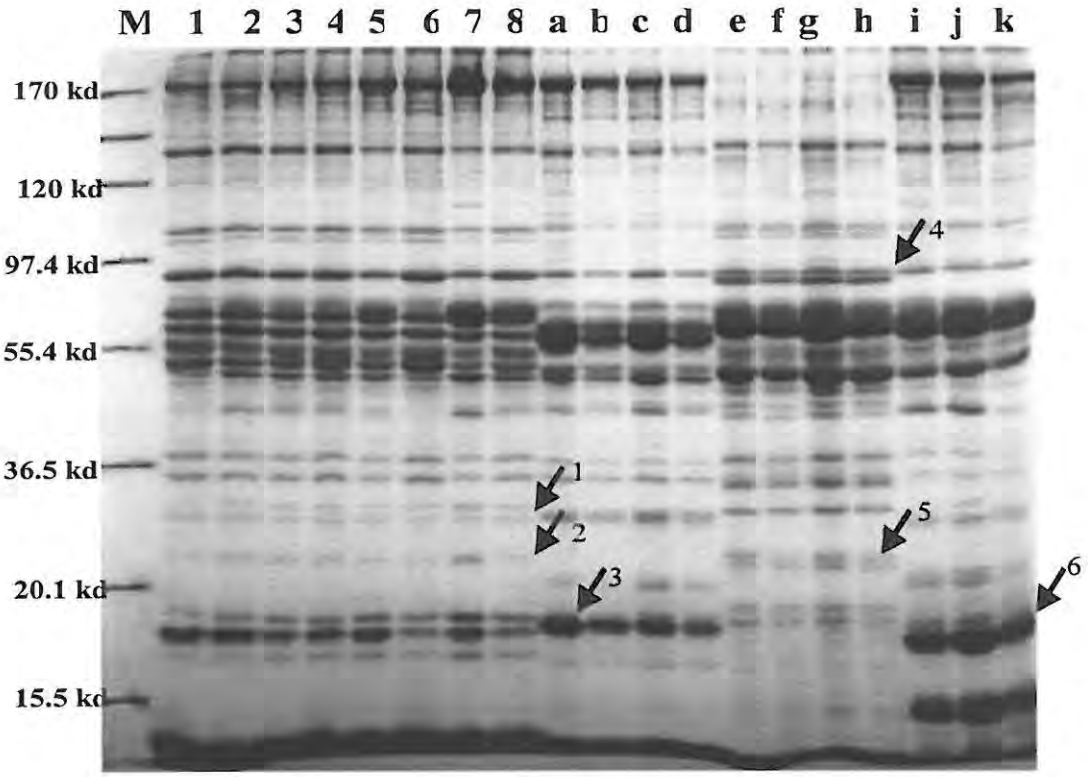


Figure 4. Comparison of PAGE protein patterns of conspecific *T. sparrmanii* and those of the other fish species. Lane M, marker proteins; lanes 1-6, protein patterns of individuals of *T. sparrmanii* from each of Wondergat, Malemani, Molopo, Klerkskraal, Marico and Amalinda respectively; lanes 7&8, protein patterns of fish from Amalinda; lanes a-d, e-h, and i-k are protein patterns of *S. bainsii*, *B. andrewi* and *P. philander* respectively. There were no apparent differences in the patterns of the *T. sparrmanii* from the different sinkholes and springs. The arrows indicate differences in the banding patterns between the *T. sparrmanii*, *S bainsii* and *B. andrewi* fish, showing differentiation by PAGE at the species level.

In Figure 5 is shown a direct comparison of the protein patterns of the different fish species in which *T. guinasana* was included. Differences between the protein patterns of *T. sparrmanii* (lanes1and 2) and those of *T. guinasana* (lanes 3 and 4) were located in the region indicated between the arrows 1 and 2 in Figure 5 . Both the *T. sparrmanii* and *T. guinasana* patterns had five major bands in the region between the arrows. The migration patterns however differed, with the *T. guinasana* bands migrating in a pattern of two well-separated bands and a group of three that migrated closely to each other. In the *T. sparrmanii* patterns, the major bands migrated equidistantly and together.

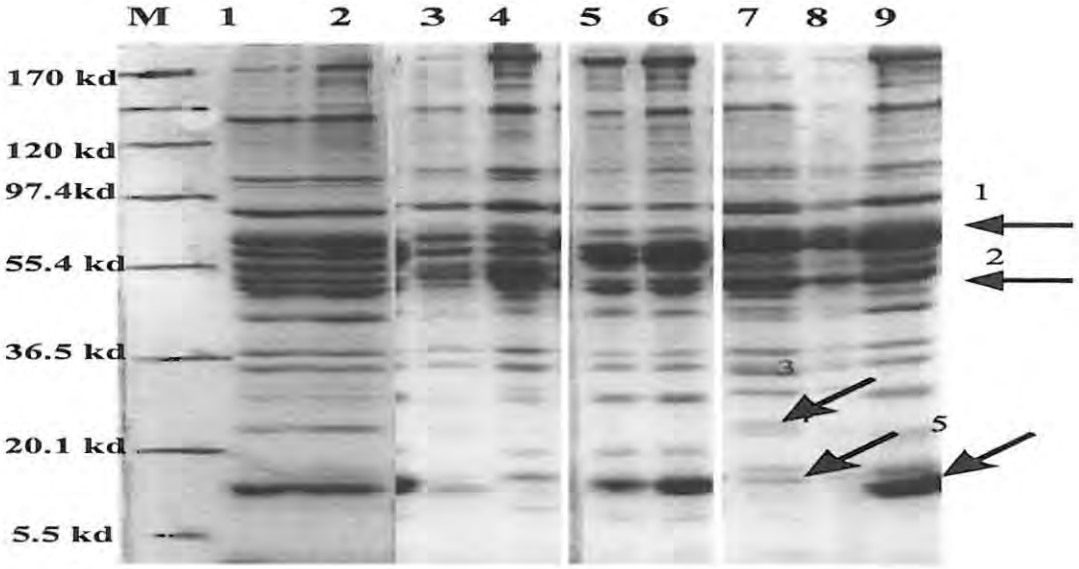


Figure 5. Direct comparison between the protein patterns of *T. sparrmanii* (lanes 1&2), *T. guinasana* (lanes 3&4), *S. bainsii* (lanes 5&6), *B. andrewi* (lanes 7&8) and *P. philander* (lane 9) respectively. Arrows 1 and 2 indicate the major banding area where banding pattern differences were clearly discernible, arrows 3-5 indicate differences in the minor bands. The indicated differences are at both a genus (as in the Tilapia compared with *B. andrewi* and *P. philander*) and a species level (between *T. sparrmanii* and *T. guinasana*), illustrating the resolving power of SDS-PAGE at these levels. The resolving power of SDS-PAGE at an intraspecific level could not be demonstrated.

3.3 DISCUSSION.

SDS-PAGE of total soluble protein from muscle tissue did not reveal any apparent intra- or interpopulational differences among the *T. sparrmanii* populations examined in this study (Figure 4). The lack of intraspecific variation observed in *T. sparrmanii* was also evident in the protein patterns of *S. bainsii*, *B. andrewi*, and *P. philander*. However, protein pattern differences were clearly discernible between *T. sparrmanii* and the other fish species (shown by arrows in Figure 4). In a direct comparison of the protein patterns between *T. sparrmanii* and *T. guinasana* (Figure 5), differences between the two species could be clearly seen, for example in the presence of a band which is third from the bottom of the gel not present in *T. guinasana*.

This finding indicates lack of below species level differentiation between the populations of *T. sparrmanii*. If the differences were at a species level, i.e the populations had differentiated into independent species, the technique would have been able to detect such differences. This is borne out by the fact that the technique, in this study, not only revealed interspecies differences between *T. sparrmanii* and the distantly related fish (*B. andrewi*, *S. bainsii* and *P. philander*), but also between *T. sparrmanii* and the only other *tilapia* used in this study, *T. guinasana*.

It is also possible that the lack of distinction between the *T. sparrmanii* populations as shown here by SDS-PAGE is indicative of the technique's limited capability for detecting intraspecific differentiation. The lack of intraspecific variation observed in *T. sparrmanii* was mirrored by that in *S. bainsii*, *B. andrewi* and *P. philander* (Figure 4). The same phenomenon was clearly discernible in the SDS-PAGE patterns published in Nxomani *et al.*, (1994). The technique's ability to discriminate between species however, was clearly demonstrated in this study.

Differences between Nxomani *et al.*. (1994) and the present study

Apart from indicating *T. guinasana* as being part of the *T. sparrmanii* population complex, the Nxomani *et al.* (1994) study indicated populational sub-division in *T. sparrmanii* in the springs and sinkholes studied. The current study detected only differences of an interspecific nature. The likely explanation of the differences between the two studies is that although GelManager as used in Nxomani *et al.* (1994) allowed for normalization (reduction of the effect of gel-to-gel variation where samples being compared are run on different gels) between electrophoretic

patterns contained on different gels, gel-to-gel variation enhanced the differences between the populations of *T. sparrmanii* and blurred the distinction between *T. sparrmanii* and *T. guinasana*. By electrophoresing samples under comparison on the same gel, the present study eliminated this effect.

In conclusion, on the basis of the SDS-PAGE results obtained in this study :

- the *T. sparrmanii* populations are uniform genetically
- *T. sparrmanii* and *T. guinasana* are distinct species
- SDS-PAGE of proteins can distinguish between fish genera but may have limited capability to detect intraspecific variation.

CHAPTER 4.

MITOCHONDRIAL DNA STUDIES OF CONSPECIFIC POPULATIONS OF *TILAPIA SPARRMANII*: PCR-RFLP AND TGGE ANALYSIS.

4.1 INTRODUCTION.

4.1.1 PCR/RFLP

Conventional mitochondrial DNA analysis has relied on physical purification of the molecule and subsequent cleavage thereof with a series of Type II restriction endonucleases. The resulting fragments are then analysed for restriction fragment length polymorphisms (RFLPs) (Carr & Marshall, 1991). The methodology used for these is, although well established, labour intensive compared to modern techniques and makes the analysis of large numbers of samples cumbersome. An alternative approach is that used by Johansen (1990) and Beckenbach (1991), in which they determined directly the sequences of interest and compared them between and among populations. Previously, direct sequence determination has required cloning, a method that was both technically demanding and labourious (Carr & Marshall, 1991), but now both methods have been improved and have become routine.

The polymerase chain reaction (PCR, Mullis *et al.*, 1986; Saiki *et al.*, 1988) permits the amplification of selected sequences from small amounts of tissue to provide quantities sufficient for many purposes, eg. direct sequencing (Beckenbach, 1991). The amplified sequences can then be subjected to sequence analysis or restriction enzyme analysis (Cronin *et al.*, 1993). Upon appropriate choice of gene segments, it has now become possible to study DNA sequence variation (and structure) among individuals, local populations or species (Carr & Marshall, 1991; Kocher *et al.*, 1993). Restriction enzyme analysis of PCR fragments has been used in other species (Kar *et al.*, 1992; Cronin *et al.*, 1993; Boulding *et al.*, 1993; Martin *et al.*, 1992).

This has resulted in much more efficient analysis of mitochondrial DNA variation in natural populations (Kocher *et al.*, 1989) and has revolutionized DNA sequence variation and phylogenetic analysis for fish populations (Turan *et al.*, 1998; Imsiridou *et al.*, 1998; Triantafyllidis *et al.*, 1999; Brykov, *et al.*, 2000; McLean *et al.*, 1999; Chow *et al.*, 2000),

evolutionary studies (Cronin *et al.*, 1993; Beckenbach, 1991), genetic variation and differentiation between fish species (Cespedes *et al.*, 2000; Carrera *et al.*, 1999; Cespedes *et al.*, 1999; Billington, 1998; Tabata & Taniguchi, 2000) and species identification (Innes *et al.*, 1998; Cespedes *et al.*, 1998).

PCR allows the selective amplification of specific sequences using oligonucleotide primers. The technique has a number of advantages over other conventional methods of DNA analysis:

- It is rapid. Once optimization of reaction conditions has been completed, analysis can be completed in a short space of time, compared to conventional RFLP analysis, cloning and sequencing .
- It requires small amounts of tissue/starting material. This obviates the need to sacrifice whole animals (especially endangered ones) or tissue.
- It is robust. PCR selectively amplifies specific sequences even if the starting material/tissue is in poor condition. The method can also cope with different methods of preservation (Beckenbach, 1991).

In this chapter, restriction endonuclease analysis of PCR amplified fragments was used to investigate the existence, or otherwise, of genetic differentiation between conspecific populations of *Tilapia sparrmanii*. In addition to this, selected fragments were subjected to Temperature Gradient Gel electrophoretic (TGGE) analysis.

4.1.2 TGGE: Approach adopted in this study.

Separation of DNA molecules in this (TGGE) system progresses according to conventional gel systems up to the point where the DNA fragment reaches a position where the melting begins. Upon destabilization of the domain with the lowest melting temperature, a transition occurs from an orderly helix to a partially denatured DNA molecule. This results in a strong retardation of its migration in the gel. If the sequence variation occurs in this domain it alters the melting temperature. This means that sequence differences can be identified by virtue of their altered migration patterns/behaviour in the TGGE gel (Schlotterer, 1995). Given the above, Fischer (1983) maintained that all substitutions that result from point mutations should be detectable by denaturing gradient gels if such substitutions occur in the lowest melting domain of the molecule being analyzed.

Two determinants of sensitivity then become the net stability change and the domain length over which the effect of the substitution is averaged. Destabilizations of the magnitude of those resulting from substitutions can be expected from events such as single base pair insertions and deletions as well as some base modifications (Fischer & Lerman, 1980).

Some new approaches to conventional DGGE have several advantages, namely:

- ability to screen large regions of DNA without the need for a labelled probe
- obviation of the need for preliminary DNA melting determinations,
- standardized denaturing concentrations and electrophoresis conditions, and
- increased sensitivity thus allowing use of small amounts of DNA.

A further advantage of the strategy followed in this study (see below) over conventional DGGE methods of assaying for polymorphisms, is that although such techniques preclude the use of labelled probes with repeat sequences (because of the hybridisation to multiple sites in the genome), this technique examines specifically amplified DNA with repeat sequences contained therein having no effect in the analysis. This study adopted the approach of Sheffield *et al.* (1990) to analyse PCR-amplified 12SRNA, d-loop and cytochrome b sequences from individuals representing conspecific populations of *T. sparrmanii* and the five colour forms of *T. guinasana*.

In Sheffield *et al.*'s (1990) approach, 1-5kb regions of DNA are amplified via PCR, digested with frequent-cutting enzyme(s) and run in two overlapping linear gradient ranges. Polymorphisms are scored as band shifts in samples with respect to others or appearance of additional bands in some samples not present in others. They estimated that by digesting PCR-amplified DNA with two restriction endonucleases and analysing the products in the above-mentioned system, they should be able to detect between 50 and 80% of polymorphisms. This approach is more simplified than previous ones in that

- PCR-amplified DNA can be visualized directly with ethidium bromide staining and does not require the use of labelled probes,
- the need to determine optimal gel conditions for each DNA fragment is eliminated as standardized gel conditions are used.

Ideally, when the amplified DNA is restricted with an enzyme, the most appropriate fragments for analysis are those in the 100-500bp region. In this study, since the fragments amplified were around the maximum ideal size, there was no need to cut them any further and they were run on the gel as they were, following amplification. Instead of using ethidium bromide to stain the gels, silver nitrate was used which is more sensitive for detection of DNA fragments. Two DNA denaturants were combined in a single gel, by the inclusion of urea in the gel and eletrophoresing the gel in a temperature gradient. The latter gradient was the one used to create the overlapping gradient ranges. The inclusion of the urea as a second denaturant was done with the intention of increasing the sensitivity of the system.

This chapter discusses the results obtained after amplification of selected regions of mitochondrial DNA via PCR, restriction analysis of these using a selection of restriction endonucleases, as well as TGGE analysis of said regions.

4.2 RESULTS.

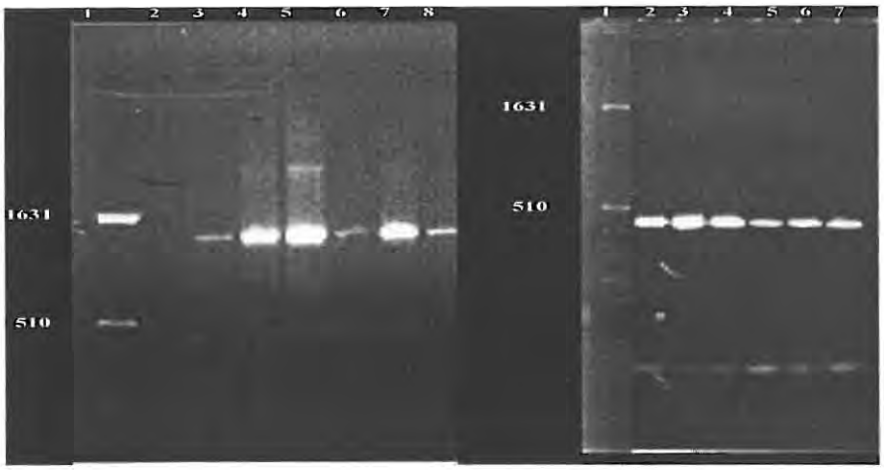
4.2.1 PCR amplification:

All the primer pairs employed in the amplification of mitochondrial genes and gene segments routinely amplified all the six conspecific populations of *T. sparrmanii*. Ten individuals from each population were used as sources of template DNA for amplification.

The 5' end of the cytochrome gene was amplified using the primer pair L14724/H15149 (Kocher *et al.* (1989), resulting in a fragment of approximately 500 bp (Figure 6B) and this fragment was subsequently used in TGGE analysis. The primer set L14724/H15915 (Kocher *et al.*, (1989); Irwin *et al.* (1993)) successfully amplified approximately 1.1 kb of the cytochrome b gene (Figure 6A).

Amplification of a portion (0.51kb, Figure 7B) of the control region was achieved using the primer pair THR/TDKD (Kocher *et al.* (1993)). The primer pair CB3R-L (Martin *et al.* (1992)) and TDKD (Kocher *et al.* (1993)) amplified a fragment of approximately 0.9 kb (Figure 7A), straddling the cytochrome b gene and the d-loop region. The primer TDKD was previously used by Kocher *et al.* (1993) as a reverse primer to amplify a segment of the d-loop (control) region of which they sequenced 350bp from twelve species of East African lacustrine cichlids.

A 400 bp fragment consisting of the initial portion of the small ribosomal RNA (12S RNA) was amplified using the primer pair L1091/H1478 (Kocher *et al.*, 1989). This fragment (Figure 8) together with cytochrome b and the d-loop fragments referred to above were subsequently used for TGGE analysis. These fragments were amplified routinely and with ease from the *T. sparrmanii* but amplification from *T. guinasana* DNA samples was difficult and infrequent, thus impeding its routine inclusion in further analyses carried out on the amplified fragments. There were no detectable differences in the sizes of the amplified products for all the genes and gene segments investigated among the populations.



A B

Figure 6 (A). Amplification of the entire cytochrome b (1.2 kb) gene using the universal primers of Irwin *et al.* (1993). In lane 1 is a pBR322/H*inf*I size marker used to determine the sizes of amplification products. Lanes 2-8 are amplification products from single representatives from each of Wondergat, Molopo Oog, Malemani, Amalinda, Klerkskraal and Marico Oog *T. sparrmanii.* (B) Amplification of the 0.5 kb fragment of cytochrome b using the universal primers of Irwin *et al.* (1993). Lane 1, pBR322/H*inf*I size marker. Lanes 2-7 are amplification products sourced and arranged as in B above.

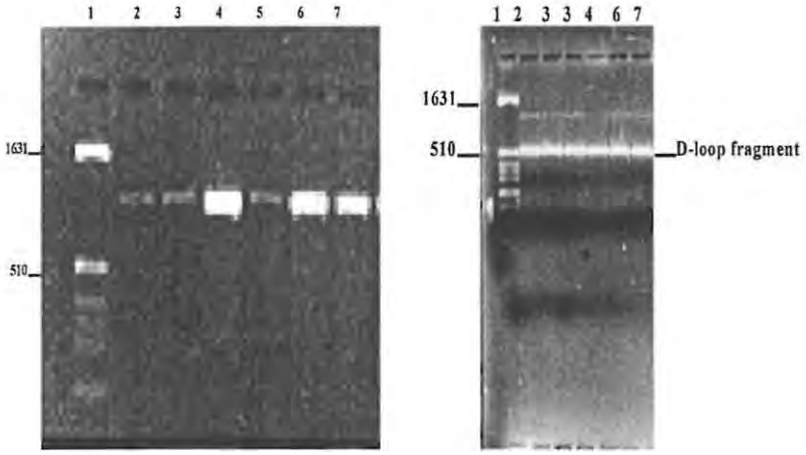


Figure 7 (A). Amplification of the cytochrome b/d-loop region (±0.9 kb) fragment from single representatives of each of the *T. sparrmanii* populations. Lane1, pBR322/H*inf*I size marker; lanes 2-7, amplification products from Wondergat, Molopo Oog, Malemani, Amalinda, Klerkskraal and Marico Oog individual fish. (B) Amplification of the initial portion (±0.51kb) of the d-loop region from single representatives of each of Wondergat, Molopo Oog, Malemani, Amalinda, Klerkskraal and Marico Oog *T. sparrmanii* fish (lanes 2-7). Lane 1, pBR322/H*inf*I size marker.

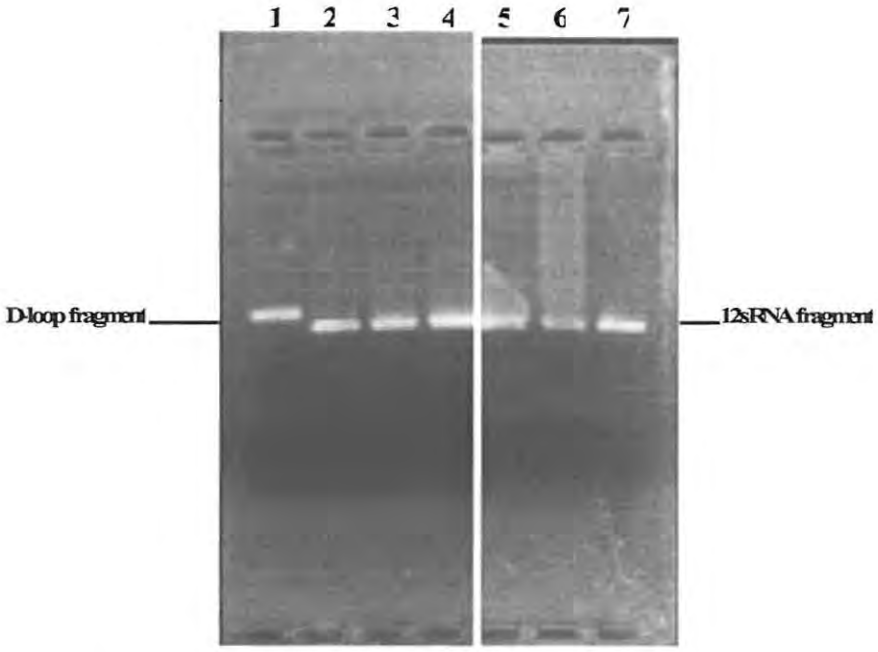


Figure 8. Amplification of the 5' end of the 12sRNA gene. Lane 1, amplification product of the d-loop region which was used as a size reference; lanes 2-7, amplification products of the 12sRNA gene fragment from individuals fish from Wondergat, Molopo Oog, Malemani, Amalinda, Klerkskraal and Marico Oog.

4.2.2 Restriction endonuclease analysis of amplified fragments.

The long amplification products described above were each subjected to restriction digestion with a number of restriction endonuclease enzymes. Six restriction endonucleases were used to digest the cytochrome b gene (1.2 kb) amplified from the *T. sparrmanii* conspecific populations. Of these, four had restriction sites, namely *Hinf*I, *Dra*I, *Rsa*I and *Aat*II. H*inf*I (Figure 9) produced four fragments in all samples representing the six conspecific populations The fragment patterns obtained with digestion with *Dra*I, *Rsa*I and *Aat*II are shown in Figure 10. However, these were monomorphic across all populations examined, both within and between populations. *Dra*I, RsaI and *Aat*II all produced two fragments which, although being of different sizes, were also monomorphic within and between populations.

For the cyt-b/d-loop fragment, the enzymes AatII, HaeIII, DraI, MspI each produced two fragments that were monomorphic within and between populations. The fragment patterns produced by the other enzymes mentioned above are shown in Figure 12 (with Figure 11 showing a photograph of the restriction digestion products generated by MspI). The fragment patterns were all different for the different enzymes yet monomorphic between the individuals and across the populations of fish examined.

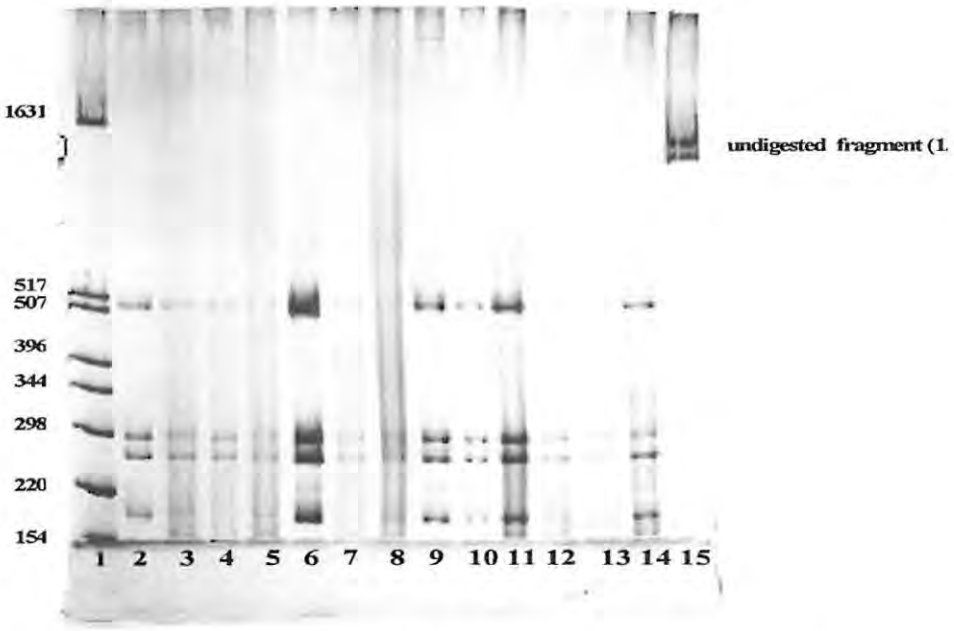


Figure 9. H*inf*I *restriction* fragment profiles of the PCR-amplified cytochrome b gene electrophoresed on a 10% acrylamide gel. Lane 1, pBR322/H*inf*I size marker; lanes 2-7, restriction fragment profiles of six individuals representing the six conspecific *T. sparrmanii* populations Wondergat, Molopo Oog, Malemani, Amalinda, Klerkskraal and Marico Oog; lanes 8-14, restriction fragment profiles from seven individuals of the Amalinda population; lane 15, undigested cytochrome b gene amplicon. The figure shows that there were neither intra (lanes 8-14) or inter population (lanes 2-7) differences detected by digestion of the cytochrome b gene fragment with H*inf*I.

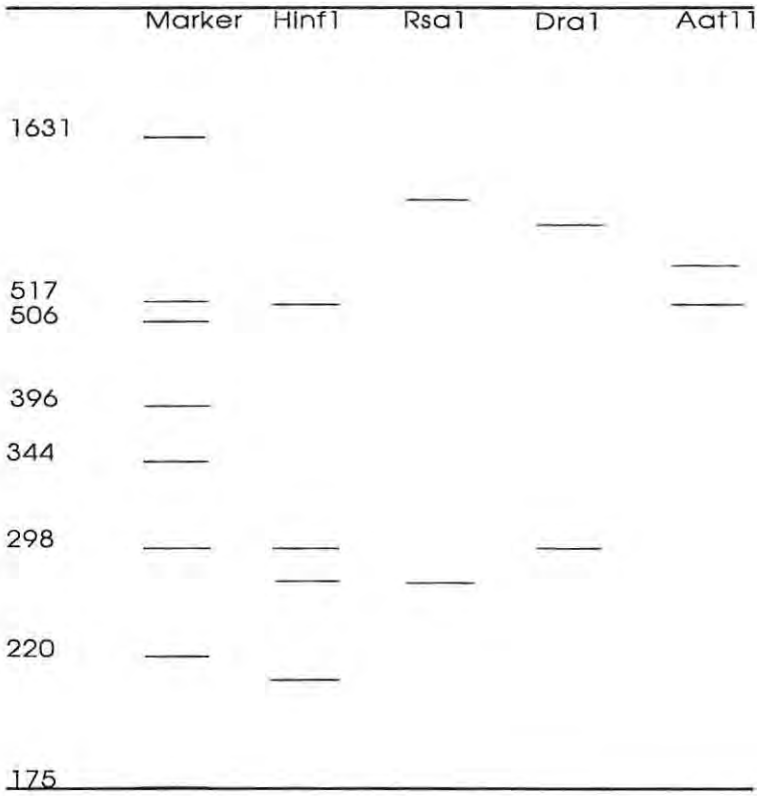


Figure 10. Diagrammatic representation of the restriction fragment profiles of the cytochrome b gene obtained for the six conspecific populations of *T. sparrmanii* examined. Lane 1, pBR322/H*inf*I size marker; lanes 2-5, representative cytochrome b gene restriction fragment profiles obtained from all the *T. sparrmanii* populations with each of the enzymes H*inf*I, RsaI, DraI and AatII respectively. These profiles were identical within and between the *T. sparrmanii* populations for each of the enzymes shown.

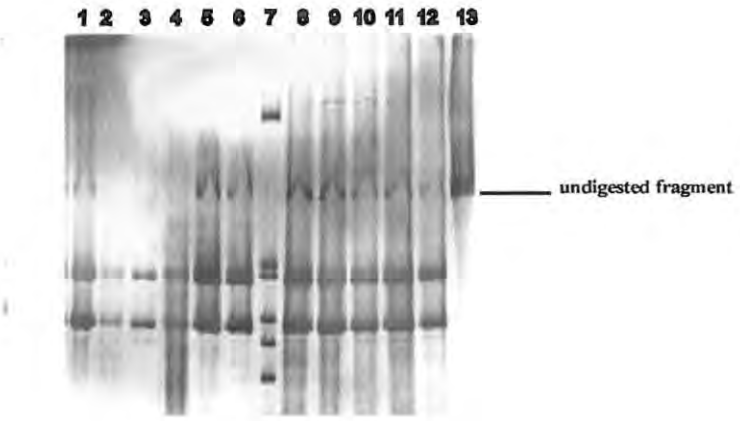


Figure 11. Msp I restriction fragment profiles of the cytochrome b/d-loop fragment obtained for the six conspecific populations of *T. sparrmanii* . Lane 1-6, restriction fragment profiles obtained of six individuals representing each of Wondergat, Molopo Oog, Malemani, Amalinda, Klerkskraal and Marico Oog populations ; lane7, pBR322/H*inf*I marker; lane 8-12, four individuals from the Amalinda population; lane 13, undigested cytochrome b/d-loop (0.9kb) fragment.

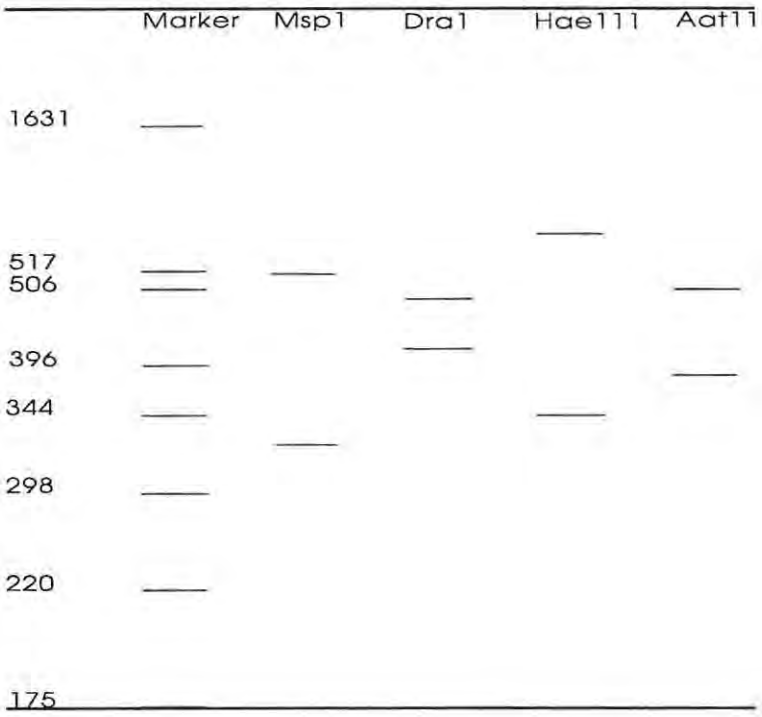


Figure 12. Diagrammatic representation of the restriction fragment profiles of the cytochrome b/d-loop segment obtained for the six conspecific populations of *T. sparrmanii* examined. Lane 1, pBR322/H*inf*I size marker; lanes 2-5, representative cytochrome b/d-loop restriction fragment profiles obtained from all the *T. sparrmanii* populations with each of the enzymes MspI, DraI, HaeIII and AatII respectively. These profiles were identical within and between the *T. sparrmanii* populations for each of the enzymes shown.

4.2.3 Temperature Gradient Gel Electrophoresis (TGGE).

Three of the PCR-amplified mitochondrial DNA gene segments were subjected to TGGE analysis; namely the 0.5kb cytochrome b fragment, the 0.4kb 12S RNA fragment and the 0.51 kb d-loop fragment. These fragments were amplified from both the conspecific *T. sparrmanii* and the five colour forms of *T. guinasana.* The fragments were electrophoresed in the gel so as to be able to detect both inter- and intra population variation. Only the 45-85oC gradient produced good quality resolution of the fragments and only the results thereof have been reproduced in this thesis.

In the analysis of the d-loop fragment of the colour forms, five different colour forms were run on the gel adjacent to each other, together with one or more samples comprising a single colour form eg. a dark blue, olive, light blue, blue stripe and olive-stripe forms were run with one or more other samples of the olive form. Adjacent to these were run six samples representing the six conspecifics of *T. sparrmanii.* The temperature gradient used was 45-85°C. Not only would this strategy have revealed any differences between the *T. guinasana* colour forms, but between the colour forms and the *T. sparrmanii* conspecifics. A similar strategy was followed in the analysis of the cytochrome b and the 12S RNA segments. In order to increase the ease of denaturation of the amplification products, and possibly enhance detection of sequence differences, 2µl of DMSO were mixed with 2µl of the amplified DNA, boiled for two minutes and then loaded onto the gel.

There were no apparent differences in the mobility of the amplified fragments in the temperature gradients used in the study. No differential migration was observed between fragments amplified from individuals from any of the populations/localities, nor were there any detected between the *T. sparrmanii* and *T. guinasana* (Figure 13). The lack of differential migration in amplified fragments of *T. sparrmanii* and *T. guinasana* was on the other hand, despite the loading approach of the samples onto the gel, which was designed to detect both intra- and interspecies differences in migration of amplicons in the TGGE gel. The inclusion of the 2µl of DMSO (Dimethylsulphoxide) and subsequent boiling of samples prior to loading also did not yield the expected results, in that it did not reveal any "hidden" differences not detectable in its absence.

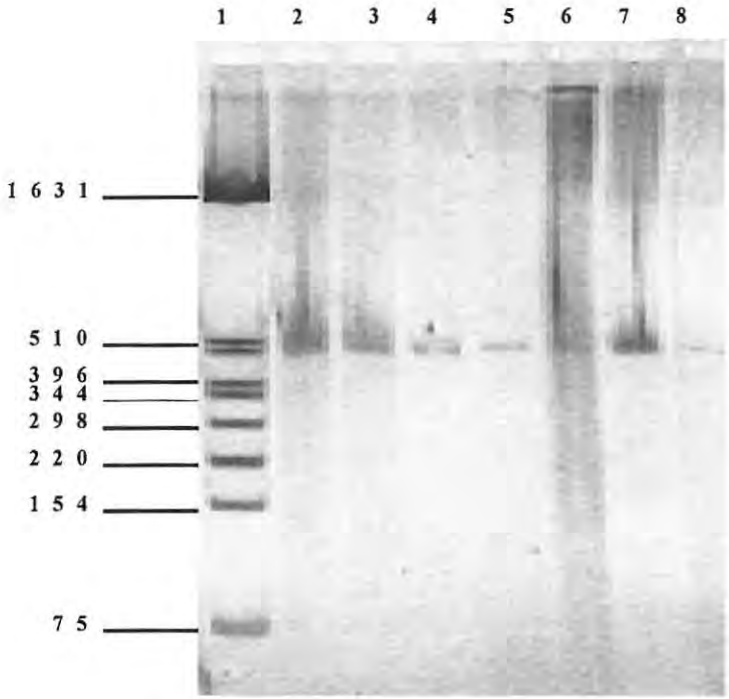


Figure 13. TGGE analysis of the amplified (± 500 bp) d-loop fragments from the conspecific *T. sparrmanii* populations and a *T. guinasana* colour form. Lane 1, pBR322/H*inf*I size marker; lane 2-7, TGGE migration of d-loop fragments of fish from Marico Oog, Wondergat, Amalinda, Malemani, Klerkskraal and Molopo Oog respectively; lane 8, TGGE migration of the d-loop fragment from a light blue form of *T. guinasana.* The fragments were electrophoresed in a 45-85°C gradient.

4.3 DISCUSSION.

4.3.1 PCR/RFLP analysis.

There are two forms of variation that mitochondrial DNA analysis can reveal, namely base substitutions (or restriction site polymorphisms) and length variants. Length variants are thought to occur as a result of tandem duplications of short 80-140 base pair sequences within the control or d-loop region. Base substitutions are the dominant form of variation although American shad (*Alosa sapidissima*) and striped bass (*Morone saxatilis*) exhibit a greater tendency toward length polymorphisms (Waldman & Wirgin, 1994).

Mitochondrial DNA accumulates mutations much more rapidly than single copy nuclear genes, with nucleotide substitutions generally occurring at a rate 5 to 10 times that of coding nuclear genes (Waldman & Wirgin, 1994). This means that it may provide markers of greater variability and which are sensitive to drift and are likely to indicate differences between populations and or species (Park & Moran, 1994). The rapid mutation rate exhibited by mitochondrial DNA gives it the potential to provide considerably more genetic divergence information over shorter evolutionary time scales (Waldman & Wirgin, 1994). Given that the *T. sparrmanii* populations in the various sinkholes are estimated to have occupied their habitats for only about 16 000 years, mitochondrial DNA analysis as a technique for studying variation in these populations was a rational choice..

PCR amplification, sequencing and restriction enzyme analysis of portions of the cytochrome b gene and the control region has been used successfully in fish population analysis to detect both intra- and inter population differentiation (Carr & Marshall, 1991; Bartlett & Davidson, 1991; Martin *et al.* 1992; Boulding *et al.* 1993; Innes *et al.*, 1998; Imsiridou *et al.*, 1998; Triantalfyllidis *et al.*, 1999; Chow *et al.*, 2000; Tabata & Taniguchi, 2000). Nelson *et al.*, (2000) found considerable genetic structuring among 11 different sites and populations of *Amphiprion ocellaris* using sequence analysis of the 5' end of the cytochrome b gene.

Cespedes *et al.*, (1999) applied PCR-RFLP analysis of a 359bp fragment of the cytochrome b gene successfully to discriminate between two closely related species of *Solea solea* and *Microchirus azevia*. The cytochrome b gene was one of three mitochondrial regions assayed

when Triantalfyllidis *et al.* (1999) differentiated between seven *Silurus glanis* populations and three populations of *Silurus aristotelis*. It was therefore not unreasonable to expect that restriction endonuclease analysis of the entire cytochrome b gene might reveal genetic variation between the *T. sparrmanii* conspecifics examined in this study. However, coding regions are, as pointed out earlier, under significant functional constraint and hence evolve slower, which could explain the lack of variation (in the gene) observed in this study.

The attraction of the control region has been the observation that it contains the most intraspecific mitochondrial DNA nucleotide diversity (Cann *et al.*, 1987; Martin *et al.*, 1992) and is therefore thought to evolve much faster than the coding regions (Moritz *et al.*, 1987). Studies of this region should therefore provide sufficient resolution for population analysis (Beckenbach, 1991). Saccone *et al.* (1987) ascribed the observed hypervariability of this region to frequent length mutations, and since the region is non-coding, the lack of functional constraint on sequence change. The lack of variation observed in the sixty individuals (representing six conspecific populations) assayed in this study can be ascribed to a number of factors.

First, the number of restriction enzymes used may have been too low to detect variation effectively. This seems unlikely as other studies have detected variation using comparable numbers of enzymes. In Chow *et al.*'s (1993) studies, this technique of PCR-RFLP proved useful in investigating genetic stock structure. It indicated sufficient intraspecific polymorphism between four of the species they assayed. In their PCR/RFLP analysis of ribosomal genes and the d-loop region of Amorhead (*Pseudopentaceros wheeleri*), Martin *et al.* (1992) used only three restriction enzymes in 87 individuals and detected a total of 10 distinct mitochondrial DNA types. Innes *et al.* (1998) used four restriction enzymes to conduct a PCR-RFLP analysis of the D-loop fragment and successfully distinguished between and identify six species of billfish found in Australian waters. Cespedes *et al.*, (1998) achieved equal success with a 359 bp cytochrome b fragment using four restriction enzymes for specific identification of sole (*Solea solea*), European plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*). If therefore the regions assayed in this study contained variation it should have been detected by the enzymes used.

In a study similar to this one, de Villiers *et al.* (1992) found that of twelve restriction enzymes they used to assay genetic variation in allopatric populations of *P. philander*, six produced fragment patterns that were common to all eight populations assayed and the other six produced variant fragment patterns. They determined that the maximum between-population sequence divergence was 0.0096, a value almost consistent with that expected between members of a single large panmictic population (de Villiers *et al.*, 1992). The assay of sixty individuals at two mitochondrial regions with six enzymes performed in this study is therefore in line with others, and the lack of variation observed can be attributed to a real lack of differentiation between the conspecific *T. sparrmanii* populations.

Second, the length of the control region amplified via PCR might have been too short. This seems unlikely in that Chow *et al.* (1993) could detect differences in mitochondrial DNA fragments of comparable size (cytochrome b = 355 bp and 12sRNA = 450 bp) in their PCR-RFLP analysis of thirteen western Atlantic snapper species. Using these regions they found enzymes with which they could identify eight of the species at one or both of the regions assayed. Other studies include Cespedes *et al.* (2000): 321 bp of 12sRNA fragment; Carrera *et al.*, 1999: 464 bp of the cytochrome oxidase subunit II (COII); Cespedes *et al.*, (1999): 358 bp of the cytochome b gene.

In conclusion, none of the enzymes assayed were diagnostic for any of the populations with the mitochondrial sequences used in this study. The populations from which the *T. sparrmanii* were sourced are relatively small and one would expect that fixation or loss of new mutations would be rapid in a uniparentally inherited molecule like mitochondrial DNA. The observed monomorphism should therefore be real. These results point in three main directions.

The first possibility is that the restriction sites observed are contained within the conserved areas on both the cytochrome b and the d-loop portions of the fragments amplified. However, it could be argued that the restriction sites could not have been located in the conserved regions of the control region, since it was the 5' domain that was examined. This domain is known for its variability.

A second possibility is that the populations of *T. sparrmanii* underwent a recent and possibly transient bottleneck resulting in loss of genetic variation. Although this scenario could explain lack of intrapopulation variation, it does not explain lack of differences between the populations. A combination of factors such as founder effects, inbreeding or bottlenecks may be responsible for this lack of variation.

Thirdly, it is possible that there was a single panmictic population of *T. sparrmanii* during wetter periods in the history of the area containing the populations, and the fish populations have not been separated for long enough to allow sufficient genetic differentiation at the mitochondrial level. As the drier periods set in, there would have been fragmentation of the populations into distinct units accommodated in the sinkholes and springs, as a result of terrestrial fragmentation. The suggested period of 11000 -16 000 years for the colonization of these habitats by fish (Twentyman-Jones, 1992) would have corresponded to one during which the waters subsided, giving rise to isolation of the fish populations. The period 16 000 - 20 000 years ago coincided with the peak of the glacial period, which was characterized by drier conditions. This wold have intensified or consolidated the isolation of the fish populations. However, this period is in an evolutionary sense not substantially long as to produce enhanced differentiation detectable by the techniques used in this study, between the fish populations. Hence the observed lack of inter-population differences as examined in this mitochondrial DNA study. The scenario of the populations having been only recently separated from a single panmictic population appears to be the most feasible.

A study narrowly focused on the hypervariable regions of the mitochondrial DNA, utilizing for example sequencing and RFLP analysis of the control region could perhaps yield more detailed answers about the genetic differentiation in these populations.

4.3.2 TGGE analysis.

Three regions of the mitochondrial DNA were assayed for differences in this study. These were the slower evolving 12S rRNA, the cytochrome b and the faster evolving d-loop/control region. Even if differences were not observed in the first two regions, the control region was expected, if not to detect geographical partitioning of the conspecific *T. sparrmanii* or chromatic polymorphism of the *T. guinasana* (see Chapter 6), to at least indicate sequence differences between *T. sparrmanii* and *T. guinasana.* No apparent differences were observed in the migration properties of the amplified fragments from the colour forms of *T. guinasana,* and the conspecific populations of *T. sparrmanii* samples. Nor were there any species-specific differences between the two *Tilapia* species. Slight mobility differences were observed only when the amplification products (d-loop) were denatured before being loaded on the gel (Figure 13).

Gel electrophoresis in parallel to the denaturing gradient has been used successfully to detect mutations in DNA fragments as well as single base-pair differences in sequences (Fischer & Lerman, 1980; Ke & Wartell *et al.*, 1995; Fischer ,1983).

As discussed in the introduction, TGGE is capable of detecting single base pair differences. The explanation for the lack of detectable variation in the sequences analyzed in this study may lie in the possibility that such variation as might have been present may reside elsewhere other than in the first melting domains of the molecules. DNAs of identical length but differing sequence will differ in the stability of their first melting domain (Ke & Wartell,1995). The identical retardation of migration of the sequences analysed in this study then probably indicates partial denaturation of regions of similar sequence (i.e not containing any mutations, insertions or deletions). Hence the lack of detectable differences.

In studies on the influence of single base pair mismatches on DNA stability (Ke & Wartell, 1995) and of neighbouring base pairs on the stability of single base bulges and base pairs in DNA fragments (Ke & Wartell, 1995) it has been shown that the degree of destabilization of the DNA molecule under study depends on, or is influenced by, the base type and its neighbouring base sequence.

It was shown that a single base bulge identical to the nearest neighbour base will generally induce less destabilization than one whose identity is different from its nearest neighbours. In the context of the analysis carried out in this study A-T and G-C changes in sequence are likely to create much less stability of the DNA helix than A-G and T-C changes and would therefore be likely to be detected. It would seem safe to suggest that if there were any sequence differences resident in the lowest melting domains of the DNA fragments analyzed in this study, they are most likely to have been ones that induced the least instability or destabilization and hence more difficult to detect in the absence of GC clamps or denaturing steeper gradients.

According to Sheffield *et al.*, (1989), if the entire DNA segment is melted, then the gel loses its resolving ability. Since no GC-clamps (a high melting temperature G+C-rich sequence) were attached to the DNA sequences analysed here (by virtue of the nature of the approach), this might be the case. The observation that short segments of DNA coding for restriction enzyme cleavage sites can be incorporated into the ends of PCR-amplified DNA fragments suggested a mechanism for attaching short GC clamps to genomic DNA fragments (Sheffield *et al.*, 1989). If a DNA fragment has two melting domains and the mutation/sequence variations are carried in the second higher temperature melting domain, TGGE would not detect such sequence differences. The attachment of a GC-clamp allows the detection of sequence changes in the second domain by TGGE. Sheffield *et al.* (1989) demonstrated using this approach that TGGE could detect single base substitutions contained in a second higher temperature domain (achieved using the GC-clamps) in the cloned mouse β^{major} globin promoter region. Their results showed that the PCR procedure could be used to attach a 40bp GC-clamp to cloned DNA fragments and that the GC-clamp allowed the detection of single base substitutions present at positions in the fragments that otherwise would not be detected. The attachment of GC-clamps therefore extends the usefulness of denaturing gradient gel electrophoresis for the detection of genetic variation. In retrospect, in a case like in this study, where the sites of sequence difference are unknown, this approach would have been particularly applicable. This would be supported by the absence of differences between *T. sparrmanii* and *T. guinasana* fragments. Although the two are recognized as being closely related, they are regarded as two separate species (Oosthuizen *et al.*, 1993).

Based on the results of TGGE as used in this study, the conspecific *T. sparrmanii* populations have not accumulated sequence differences which are detectable by the technique and approaches used here and are therefore, besides geographic separation, still a homogenous species. The populations probably originated from a single panmictic population that existed during wetter periods in their history and have only recently become separated.

CHAPTER 5.

RAPD FINGERPRINTING OF CONSPECIFIC POPULATIONS OF THE FISH *T. SPARRMANII* AND COMPARISON WITH A SUBSET OF PRIMERS BETWEEN *T. SPARRMANII* AND *T. GUINASANA*.

5.1. INTRODUCTION.

RAPD fingerprinting (Williams *et al.*, 1990) is beginning to find extensive use in the examination of the genetic structure of higher organisms. It appears to have found ready applications in providing answers on the genetic structure and variation of several plant species such as assessment of genetic diversity (N'goran *et al.*, 1994; Isabel *et al.*, 1995), assessment of genetic variation (Sulaiman & Hasnain, 1996; Paran *et al.*, 1997; Russell *et al.*, 1997), tracing migration in plants (Vellekoop *et al.*, 1996), and demonstration of the hybrid origin of rare species (Rossetto *et al.*, 1997). Other uses of RAPDs in population studies have involved population differentiation in honey bees (Suazo *et al.*, 1998) and genetic variability of the endangered light footed clapper rail (Nusser *et al.*, 1996).

RAPD fingerprinting has found wider application in the examination of the genetic structure of fish populations and species (Kubota *et al.*, 1992; Dinesh *et al.*, 1993, 1996; Bardakci & Skibinski, 1994). Other applications have involved assaying of genetic variation (Bilawski & Pumo, 1997), detection of differentiation in sea bass (Caccone *et al.*, 1997), genetic marker inheritance in lake trout (Stott, *et al.*, 1997), stock discrimination (Gomes *et al.*, 1998), identification and characterization of river catfish (Chong *et al.*, 2000), genetic heterogeneity in silver crucian carp (Zhou *et al.*, 2000), assessment of genetic variability in brown trout (Cagigas *et al.*, 1999), genetic differentiation among stationery and anadromous perch (Nesbo *et al.*, 1998), gene mapping and analysis of genetic variation in catfish (Liu *et al.*, 1999), identification of fish species (Partis, & Wells, 1996) as well as phylogenetic analysis of cichlid fishes (Sultmann *et al.*, 1995).

RAPDs have one major disadvantage and that is that due to dominance of the bands, it is impossible to distinguish the heterozygote band$^+$/band$^-$ from the homozygotes band$^+$/band$^+$ (Aagaard *et al.*, 1998). Thus allelic frequencies and heterozygosities cannot be directly calculated from RAPDs data (Isabel *et al.*, 1995).

This must be balanced against the fact that the generation of RAPD markers is:

- highly effective in terms of cost and time requirements
- does not require *a priori* knowledge of sequence details of the genomes under investigation and is
- feasible with minute amounts of DNA (Schierwater, 1995).

In the context of this study of *T. sparrmanii* and the later analysis of *T. guinasana*, the objectives posed in Chapter 1 could be answered using RAPDs without the need for further detail on the populations' genomic structures.

The primary objective of this study was to:

- use the method of RAPD fingerprinting to characterize genetically the various populations of *T. sparrmanii* from the sinkholes and springs of the North West Province, with respect to their genetic identity in relation to each other, and
- compare these populations to *T. guinasana* as an attempt to contribute to the understanding of the genetic relationships between the two species.

In this study RAPD fingerprinting was used to generate fingerprints of fish from six conspecific populations of *T. sparrmanii*. This is two more populations than were used in Nxomani *et al.* (1994). Nine 10-mer primers were used in the analysis. Within and between population average similarity based on the fingerprints was determined for each primer by formulae given in chapter 2. These were converted into values of genetic distance (D*ij*) which were then averaged and used to draw dendrograms to show the relationships between the populations. Three of the nine primers were used to compare *Tilapia guinasana*, a closely related Tilapia whose major distinction from *T. sparrmanii* is its polychromatism (Greenwood, 1992). The genetic relationships as determined from the data generated by the nine primers are shown and discussed in the results section.

5.2. RESULTS.

Each primer had a characteristic fingerprint pattern that remained constant for all the conspecific populations, and the levels of variability differed with different primers and between the populations (Figure 14). For the primers 1, 2, 7 and 10, only four samples per population were used in the final analysis while for the rest of the primers the number of profiles analysed averaged 7. This was the case because poorly amplified samples were not included in the final analysis and only reproducible patterns were considered for analysis.

The number of bands ranged from 20 to 35. Combination of primers did not reduce the number of bands, instead it led to loss of detectable intra- and inter variation. Each of the primers generated unique fingerprint patterns (Figure 15A and B). Similarity values within each of the populations were computed from data matrices based on presence and absence (designated "1" and "0" respectively) of fingerprint bands generated by each of the primers. This was done by making pairwise comparisons between all the individuals from each population, determining the number of shared bands n_{xy} and the total number of bands $(n_x + n_y)$ generated by the respective primer and using equation 1 from chapter 2 ($S_{xy} = 2n_{xy}/(n_x + n_y)$ to get the similarity index value. Within population similarity was therefore calculated as the average of S_{xy} across all possible comparisons between individuals within a population. The values of within population similarity calculated in this manner are shown in Table 6 and are represented graphically in Figure 16.

Between population similarity (S_{ij}) was corrected for within population similarity and determined using equation 2 as explained in chapter 2 ($S_{ij} = 1 + S'_{ij} - 0.5\ (S_i + S_j)$) . The ($S_{ij}$) values obtained for all the populations and across all nine primers are shown in Table 7 and are graphically represented in Figure 17. These values were converted into measures of average genetic distance (D_{ij}) between the populations using equation 3 from chapter 2 ($D'_{ij} = -\ln[S'_{ij}/\sqrt{(S_iS_j)}]$) and the resultant values are shown in Table 8 and are graphically represented in Figure 18. A subset of the primers (primers 3, 4 and 6) were used to investigate the genetic relationship between *T. sparrmanii* and *T. guinasana*. The similarity values obtained for the conspecific populations of *T. sparrmanii* and *T. guinasana* comparison are shown in Table 9 and the corresponding genetic distance estimates are shown in Table 10.

Genetic distance matrices from which dendrograms depicting the relationships between the conspecific populations of *T. sparrmanii* and *T. guinasana* were drawn are shown in Tables 11 and 12 respectively. The dendrograms drawn from these are shown in Figures 19 and 20 depicting the genetic relationships between the *T. sparrmanii* populations on their own and the comparison using the subset of primers with *T. guinasana*, respectively.

The highest value of intra population similarity (S) was obtained for the Marico Oog population at 0.951± 0.056 (Table 6 and Figure 16) and the lowest (0.836 ± 0.117) was obtained for Wondergat population, indicating minimal within population diversity in the former and the highest in the latter. Between population similarity was highest between the Molopo Oog and the Malemani populations and lowest between the Wondergat and Molopo Oog populations. The highest estimate of genetic distance was found to be between the Wondergat and Molopo Oog populations and lowest was between the Molopo Oog and Amalinda populations.

The clustering of the *T. sparrmanii* populations based on the estimated genetic distances is shown by the dendrogram in Figure 19. The dendrogram shows two major clusters with one consisting of two sub-clusters. The single major cluster involves the Marico Oog and Wondergat populations shown to be closely related to each other and more distinct from the rest of the populations. The second major clusters has two sub-clusters, one involving the Molopo Oog and Amalinda populations also shown to be closely related to each other while the other shows a close relationship between the Klerkskraal and Malemani populations.

T. guinasana is regarded as being a closely related species to *T. sparrmanii* (Greenwood, 1992; Oosthuizen *et al.*, 1993) and it was of interest to estimate the level of genetic relatedness between the two species. A subset of three primers was used to estimate the genetic relatedness between the two species. The resultant values from the computations of the genetic distance estimates are shown in Table 12 and they indicate a mean genetic distance of 0.371± 0.037 when *T. guinasana* is compared with all six conspecific populations of *T. sparrmanii*. In contrast, the average genetic distance between all of the *T. sparrmanii* conspecific populations had mean of only 0.139 ± 0.069. It is clear from this that the overall genetic distance between *T. guinasana* and *T. sparrmanii*, based on the analysis performed with the three primers, is almost three times that between the conspecific populations of *T. sparrmanii*. The implications of this are expanded on in the discussion section.

Direct measurements of gene diversity (such as allele frequencies and heterozygosities) cannot be inferred directly from RAPDs data because of the predominantly dominant nature of RAPD markers (Aagaard *et al.*, 1998). This is more critical in the case of allelic frequencies which can only be determined from RAPDs data by following various models based on assumptions that are quite often difficult to verify (Isabel *et al.*, 1995). To get a crude measure of the variability within the populations of *T. sparrmanii* examined in this study, the coefficient of variation (CV) (Fowler & Cohen, 1990) was determined using the data in Table 6. The CV is calculated as the ratio of the standard deviation to the mean, and expressed as a percentage by multiplying by 100. The value obtained gives a relative measure of the variability within samples from different locations.

The values of relative variability (or CV) were based on the assumption that the mean similarity values (Table 6) could be taken to reflect, in a converse manner, the degree of variability within each of the populations, since a high degree of within-similarity should in principle reflect a low degree of variability within a sample. The CV's for the Wondergat, Molopo Oog, Malemani, Klerkskraal, Marico and Amalinda populations indicated that the Wondergat population had the highest relative variability (13.99) while the least relative variability was obtained for the Molopo Oog population (4.83%). The Marico Oog and Malemani populations had a similar relative variability (5.88%). The Klerkskraal and Amalinda populations had relative variabilities of 8.29% and 4.95% respectively.

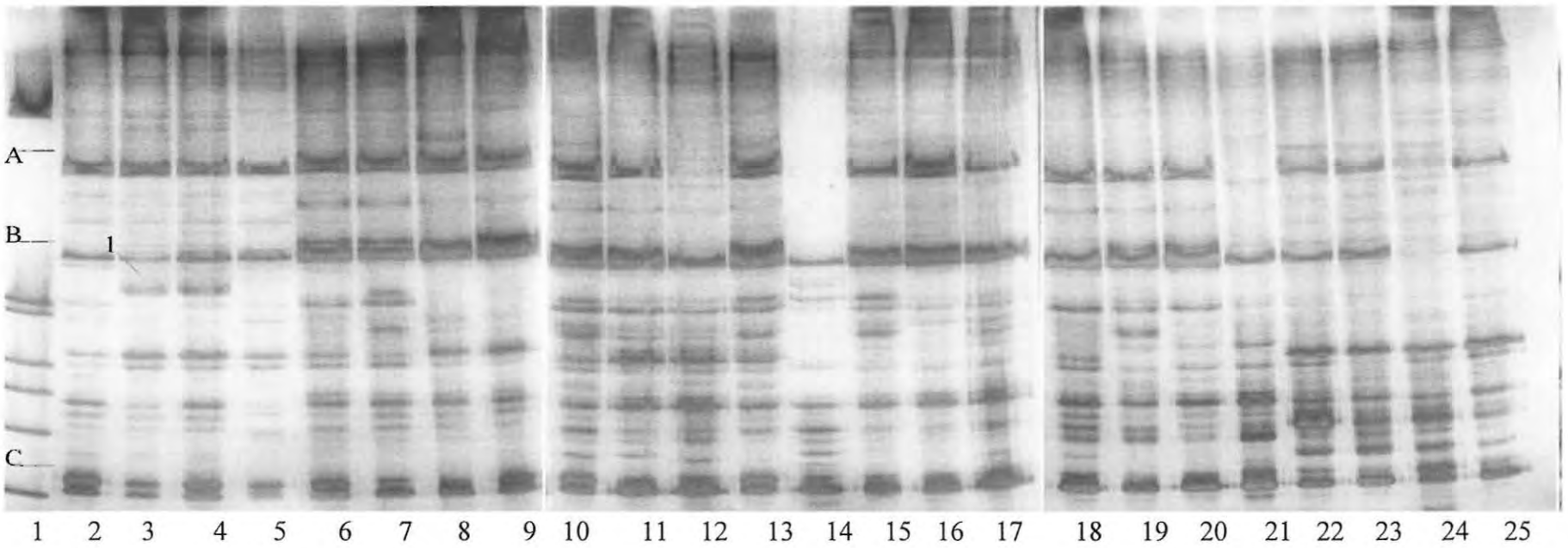


Figure 14. RAPD fingerprints generated by primer number 2. Lanes 1, pBR322/H*inf*1 size marker; lanes 2-5, 6-9, 10-13, 14-17, 18-21 and 22-25 are RAPD fingerprints generated by the primer for the Amalinda, Molopo Oog, Wondergat, Malemani, Klerkskraal and Marico Oog respectively.A, B and C indicate major bands that were common to all the populations. The band marked one indicates an example of variation in absence and presence of bands across the populations.

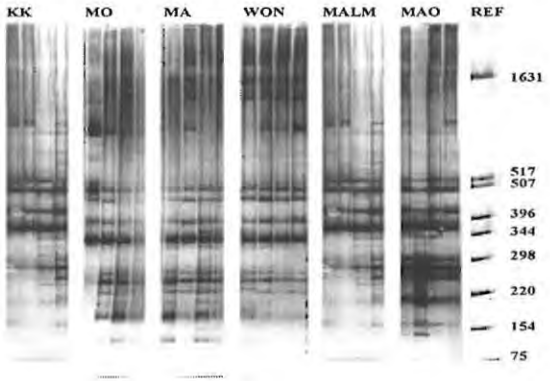


Figure 15. (A) RAPD fingerprints generated by the primer 3 for each of the *T. sparrmanii* populations. The RAPD patterns of four individuals from each of Klerkskraal (KK), Molopo Oog (MO), Amalinda (MA), Wondergat (WON, Malemani (MALM and Marico Oog (MAO) respectively are shown

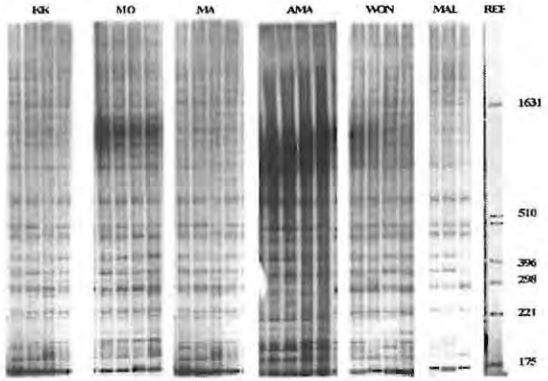


Figure 15. (B) RAPD fingerprints generated by the primer 6 for each of the *T. sparrmanii* populations. The RAPD patterns of four individuals from each of Klerkskraal (KK), Molopo Oog (MO), Marico Oog (MA), Amalinda (AMA), Wondergat (WON), and Malemani (MAL) respectively are shown.

Table 6. Average similarity (S) across all primers within the conspecific populations of *T. sparrmanii*.

Primer	Wondergat	Molopo	Malemani	Klerkskraal	Marico	Amalinda
1	0.972	0.988	0.957	0.939	0.976	0.984
2	1.000	0.929	0.983	0.983	1.000	0.941
3	0.854	0.869	0.909	0.952	0.889	0.882
4	0.881	0.941	0.929	0.978	0.992	0.904
6	0.669	0.852	0.875	0.758	0.902	0.948
7	0.898	0.966	0.818	0.952	0.991	0.866
10	0.738	0.962	0.969	0.969	0.969	0.899
11	0.693	0.923	0.948	0.853	1.000	0.948
12	0.818	0.954	0.871	0.863	0.852	1.000
Mean	0.836	0.932	0.918	0.916	0.952	0.930
SD	0.117	0.045	0.054	0.076	0.056	0.046

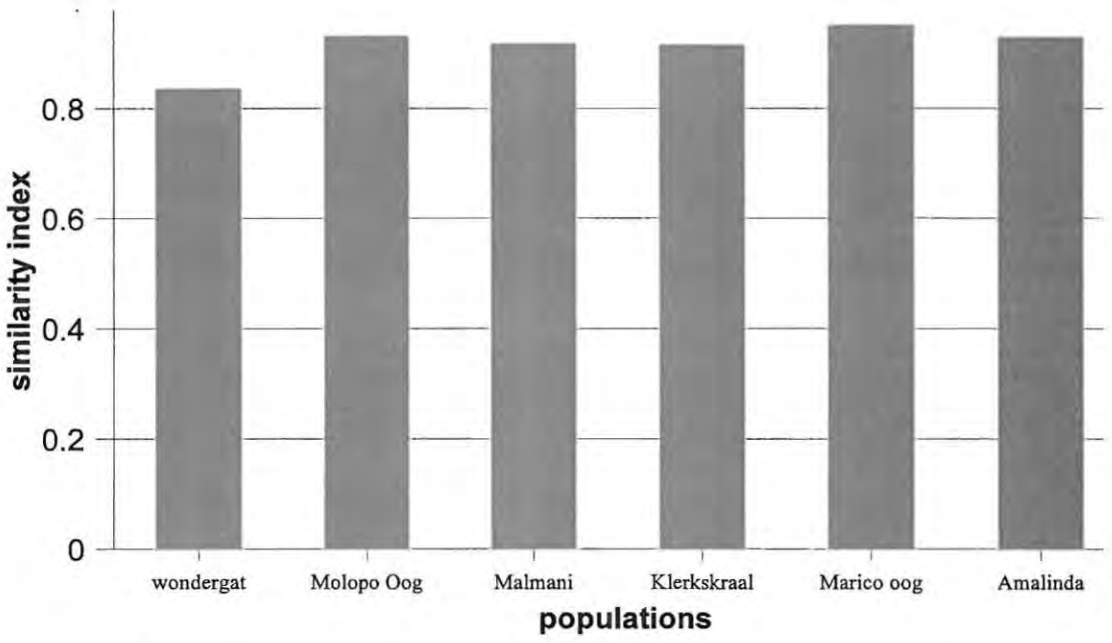


Figure 16. Graphic representation of the average similarity (S, from Table 5) across primers within the populations of *T. sparrmanii*. The Wondergat population had the lowest within population similarity (0.836) while the there were minimal differences in the values within the other populations, with within population similarity values ranging from 0.916 to 0.952.

Table 7. The estimated similarity (S_{ij}) for each primer between the conspecific populations of *T. sparrmanii*. Key: wo = Wondergat, ma = Malemani, kk = Klerkskraal, mao = Marico Oog, ama = Amalinda, mo = Molopo Oog.

Primer	**wo/mo**	**wo/ma**	**wo/kk**	**wo/mao**	**wo/ama**	**mo/ama**	**mo/kk**	**mo/mao**	**ma/ama**	**ma/kk**	**ma/mao**	**ama/kk**	**ama/mao**	**kk/mao**	**mo/ma**
1	0.866	0.921	0.891	0.872	0.945	0.860	0.959	0,864	0,952	0.937	0.995	0.961	0.905	1.000	0,962
2	0.869	0.759	0.884	0.912	0.905	0.898	0.669	0.828	0.705	0.767	0.884	0.788	0.822	0.758	0.877
3	0.818	0.905	0.883	1.022	0.774	0.982	0.839	1.049	0.782	0.962	0.958	0.797	0.829	0.973	0.861
4	0.867	0.910	0.923	0.879	0.848	0.849	1.003	0.774	1.047	0.973	0.892	0.911	0.978	0.867	0.917
6	0.776	1.120	1.002	0.715	1.013	0.923	0.874	0.944	0.803	0.851	0.969	0.789	0.896	0.956	0.923
7	0.863	0.979	0.984	0.761	0.845	0.947	0.859	0.817	0.915	0.979	0.845	0.909	0.913	0.938	0.858
10	0.765	1.069	0.685	1.069	0.719	0.993	0.958	0.958	0.912	0.954	0.954	0.835	0.835	0.954	0.958
11	0.662	1.029	0.977	0.904	0.899	0.965	0.862	0.789	0.752	0.974	0.826	0.799	0.766	0,974	1.462
12	0.892	0.794	0.688	0.887	0.785	0.801	0.953	1.063	0.704	0.853	0.977	0.818	0.824	0.976	1.06
Mean	0.819	0.942	0.899	0.891	0.859	0.913	0.886	0.898	0.841	0.917	0.922	0.845	0.863	0.933	0.986
SD	0.074	0.119	0.095	0.111	0.092	0.066	0.099	0.109	0.120	0.075	0.062	0.065	0.064	0.075	0.189

Table 8. Estimated average genetic distance (D_{ij}) across all primers between the conspecific populations of *T. sparrmanii*. Key: wo = Wondergat, ma = Malemani, kk = Klerkskraal, mao = Marico Oog,ama = Amalinda, mo = Molopo Oog.

Primer	**wo/mo**	**wo/ma**	**wo/kk**	**wo/mao**	**wo/ama**	**mo/ama**	**mo/kk**	**mo/mao**	**ma/ama**	**ma/kk**	**ma/mao**	**ama/kk**	**ama/mao**	**kk/mao**	**mo/ma**
1	0.146	0.086	0.121	0.111	0.058	0.010	0.043	0.149	0.153	0.068	0.006	0.050	0.004	0.041	0.102
2	0.146	0.279	0.125	0.092	0.103	0.137	0.425	0.196	0.115	0.271	0.125	0.366	0.279	0.249	0.203
3	0.238	0.114	0.137	0.025	0.301	0.169	0.193	0.107	0.021	0.042	0.107	0.278	0.029	0.249	0.215
4	0.157	0.104	0.086	0.137	0.187	0.093	0.003	0.267	0.124	0.029	0.119	0.049	0.145	0.099	0.022
6	0.343	0.153	0.003	0.051	0.278	0.094	0.169	0.018	0.181	0.293	0.036	0.243	0.051	0.278	0.119
7	0.158	0.228	0.017	0.291	0.193	0.169	0.159	0.208	0.043	0.021	0.183	0.085	0.066	0.014	0.097
10	0.315	0.088	0.452	0.088	0.415	0.045	0.045	0.045	0.008	0.049	0.049	0.098	0.049	0.1994	0.194
11	0.478	0.048	0.025	0.104	0.117	0.039	0.168	0.248	0.039	0.055	0.196	0.303	0.025	0.250	0.261
12	0.127	0.279	0.628	0.145	0.265	0.064	0.052	0.075	0.227	0.007	0.112	0.379	0.029	0.255	0.208
Mean	0.234	0.153	0.177	0.116	0.213	0.091	0.151	0.146	0.101	0.093	0.104	0.206	0.075	0.181	0.156
SD	0.120	0.087	0.216	0.076	0.113	0.057	0.123	0.089	0.077	0.109	0.064	0.135	0.086	0.102	0.076

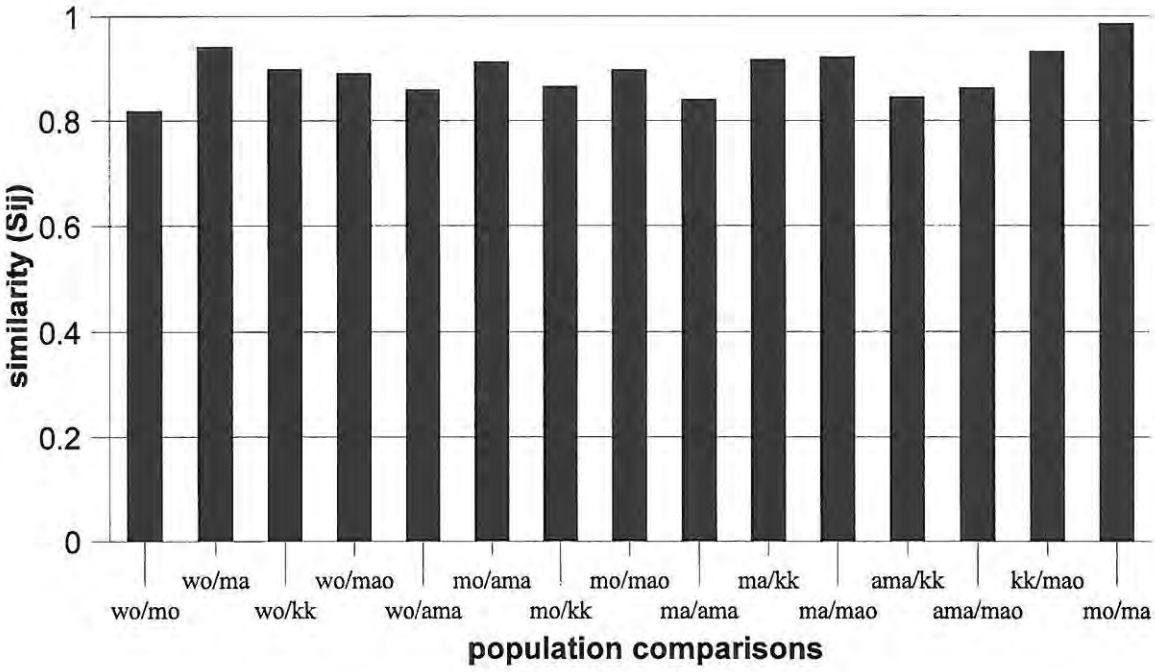


Figure 17. Between population similarity (S_{ij}) for the *T.sparrmanii* populations. The highest between population similarity was between the Molopo and Malmani populations with an S_{ij} value of 0.986, while the lowest was between the Wondergat and Molopo Oog populations (0.819). Key: wo = Wondergat, ma = Malemani, kk = Klerkskraal, mao = Marico Oog, ama = Amalinda, mo = Molopo Oog.

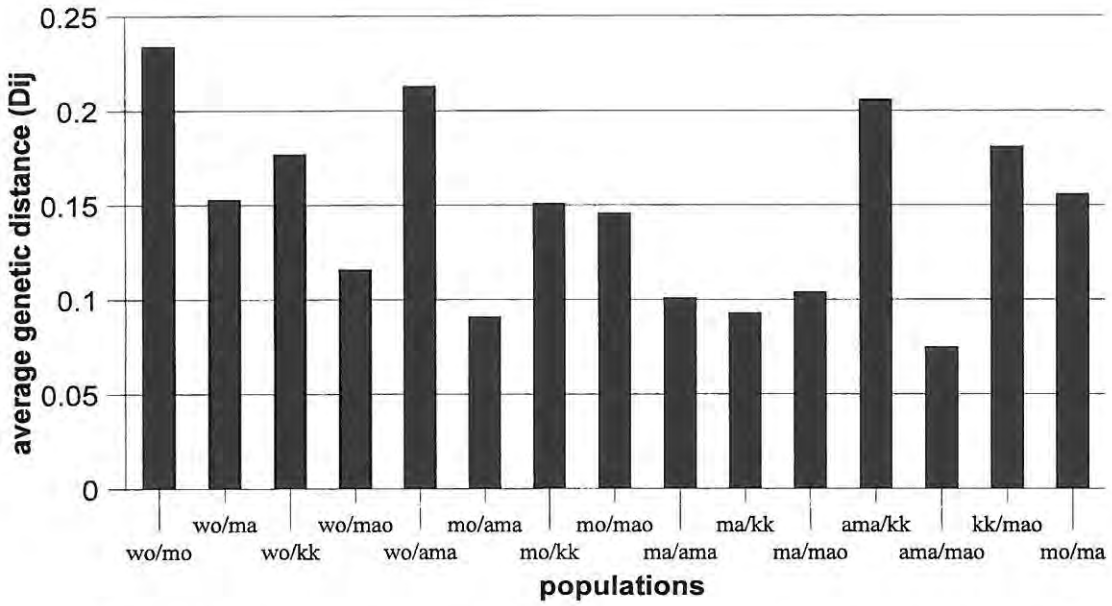


Figure 18. Graphical representation of the average genetic distance (D_{ij}) between the populations of *T. sparrmanii*. Key: wo = Wondergat, ma = Malemani, kk = Klerkskraal, mao = Marico Oog, ama = Amalinda, mo = Molopo Oog.

Table 9. Average similarity (S_{ij}) between the conspecific populations of *T. sparrmanii* and *T. guinasana*. Key: wo = Wondergat, mo =Molopo Oog, ma= Malemani, mao=Marico Oog, kk=Klerkskraal, ama= Amalinda, guin= T. *guinasana*

Primer	won/mo	won/ma	won/kk	won/mao	won/ama	won/guin	mo/ma	mo/kk	mo/mao	mo/ama	mo/guin	ma/kk	ma/mao	ma/ama	ma/guin	kk/mao
3	0.818	0.905	0.883	1.022	0.774	0.702	0.861	0.839	1.049	0.982	0.729	0.962	0.958	0.782	0.674	0.973
4	0.867	0.910	0.923	0.879	0.848	0.680	0.917	1.003	0.774	0.849	0.760	0.973	0.892	1.047	0.656	0.867
6	0.776	1.120	1.002	0.715	1.013	1.128	0.923	0.874	0.944	0.850	0.715	0.851	0.969	0.803	0.418	0.956
Mean	0.637	0.971	0.936	0.872	0.878	0.837	0.900	0.905	0.922	0.894	0.735	0.929	0.939	0.877	0.832	0.932
SD	0.045	0.123	0.061	0.154	0.122	0.253	0.034	0.086	0.139	0.077	0.063	0.067	0.042	0.147	0.143	0.057

Primer	kk/ama	kk/guin	mao/ama	mao/guin	ama/guin
3	0.797	0.760	0.829	1.023	0.938
4	0.911	0.595	0.978	0.879	0.705
6	0.789	0.904	0.896	0.689	0.809
Mean	0.832	0.753	0.901	0.864	0.817
SD	0.068	0.155	0.075	0.168	0.117

Table 10. Estimated average genetic distances (D_{ij}) across the primers used to RAPD fingerprint both *T. sparrmanii* and *T. guinasana*.
Key: wo = Wondergat, mo =Molopo Oog, ma= Malemani, mao=Marico Oog, kk=Klerkskraal, ama= Amalinda, guin= T. *guinasana*

Primer	**won/mo**	**won/ma**	**won/kk**	**won/mao**	**won/ama**	**won/guin**	**mo/ma**	**mo/kk**	**mo/mao**	**mo/ama**	**mo/guin**	**ma/kk**	**ma/mao**	**ma/ama**
3	0.238	0.114	0.137	0.025	0.301	0.442	0.169	0.193	0.107	0.021	0.387	0.042	0.107	0.278
4	0.157	0.104	0.086	0.137	0.187	0.431	0093	0003	0.267	0.124	0.294	0.029	0.119	0.049
6	0.343	0.153	0.003	0.051	0.278	0.449	0.094	0.169	0.018	0.426	0.293	0.036	0.243	1.202
Mean	0.246	0.124	0.075	0.071	0.255	0.441	0.119	0.122	0.131	0.109	0.369	0.121	0.087	0.190
SD	0.093	0.026	0.068	0.059	0.060	0.009	0.044	0.103	0.126	0.081	0.068	0.149	0.045	0.123

Primer	**ma/guin**	**kk/mao**	**kk/ama**	**kk/guin**	**mao/ama**	**mao/guin**	**ama/guin**
3	0.475	0.029	0.249	0.314	0.215	0.126	0.441
4	0.457	0.145	0.099	0.578	0.022	0.555.	0.244
6	1.202	0.051	0.278	0.132	0.119	0.456	0.385
Mean	0.342	0.075	0.208	0.341	0.119	0.379	0.357
SD	0.373	0.062	0.096	0.224	0.097	0.225	0.102

Table 11. Matrix of average similarity (above diagonal) and genetic distance (below diagonal) for the conspecific populations of *T. sparrmanii.*

	Wondergat	Molopo Oog	Malemani	Klerkskraal	Marico Oog	Amalinda
Wondergat	-	0.819	0.942	0.899	0.891	0.859
Molopo Oog	0.234	-	0.986	0.886	0.898	0.913
Malemani	0.153	0.156	-	0.917	0.922	0.841
Klerkskraal	0.177	0.151	0.093	-	0.933	0.845
Marico Oog	0.116	0.146	0.104	0.181	-	0.863
Amalinda	0.213	0.091	0.101	0.206	0.104	-

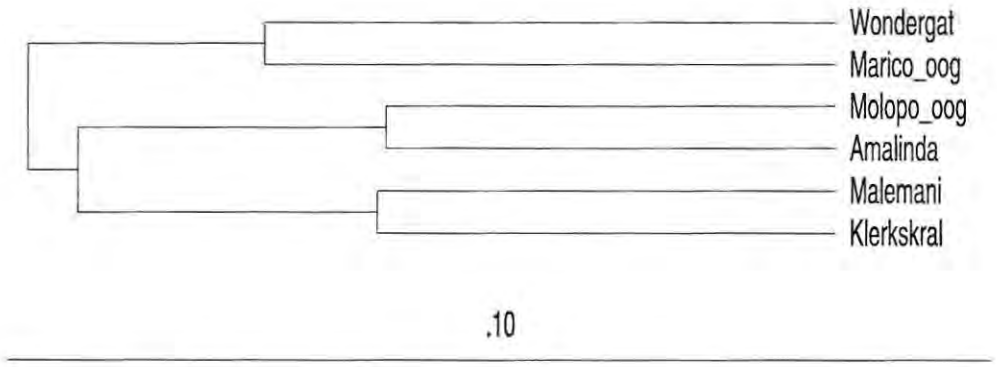


Figure 19. UPGMA dendrogram drawn from estimates of the genetic distances between the conspecific populations of *T. sparrmanii* calculated for all the primers (see Table 9 above).

Table 12. Average genetic distance (above diagonal) and similarity (below diagonal) between the conspecific populations of *T. sparrmanii* and *T. guinasana*..

	Wondergat	Molopo Oog	Malemani	Klerkskraal	Marico Oog	Amalinda	*T. guinasana*
Wondergat	-	0.246	0.124	0.075	0.071	0.255	0.441
Molopo Oog	0.637	-	0.119	0.103	0.131	0.109	0.369
Malemani	0.971	0.900	-	0.121	0.087	0.190	0.342
Klerkskraal	0.936	0.905	0.929	-	0.075	0.208	0.341
Marico Oog	0.872	0.922	0.939	0.932	-	0.119	0.379
Amalinda	0.878	0.894	0.877	0.832	0.901	-	0.356
T. guinasana	0.837	0.735	0.832	0.753	0.867	0.356	-

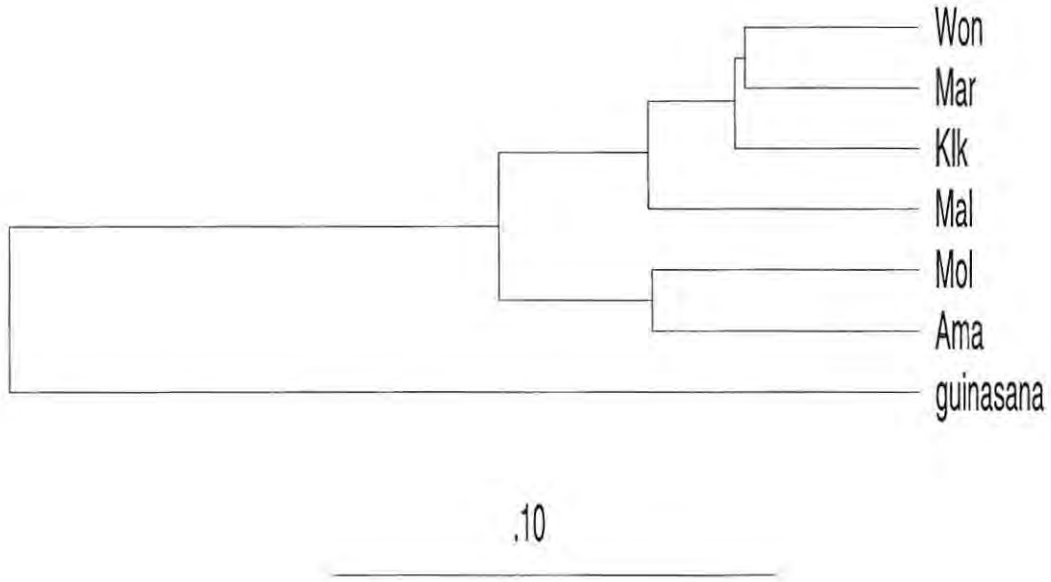


Figure 20. UPGMA dendrogram of genetic distance estimates between conspecific populations of *T. sparrmanii* and *T. guinasana*. The differentiation between the *T. sprrmanii* populations is indicated through the clustering together of these populations. The distinction between *T. guinasana* and *T. sparrmanii* is also clear from the clustering.

Key: Won = Wondergat, Mar = Marico Oog, Klk = Klerkskraal, Mol = Molopo Oog, Ama = Amalinda, guinasana = *T. guinasana*

From the data in Table 6 it was of interest to determine to what degree the primers were in agreement in the degree of similarity they detected between the conspecific populations of *T. sparrmanii*. This was done by determining the correlation coefficient between all possible primer pairs (36 in total). Fifty -eight percent (21) of the values were positive and they averaged 0.241±0.154. Only two of the values were significant even at the ten percent level, one negative (-0.619) and one positive (0.615). According to Fowler & Cohen (1990) values of correlation ranging between 0.20 and 0.39 indicate weak correlation. The correlation value of 0.241±0.154 falls within this range. This means that there was minimal agreement between the primers in the amount of similarity they detected between the populations of *T. sparrmanii*. The positive correlation values and the primer pairs for which they were determined are shown in Table 13 below: Because the genetic distances were determined from the similarity values it would be expected that the same situation would apply to them as well.

The highest correlation was found to be between primers 3 and 10 (0.615) and the lowest was between primers 2 and 12 (0.039). The lack of agreement could point to the fact that the primers assay genetic variation at different regions of the genome. It would be expected that different regions of the genome would contain different levels of genetic variation. The lack of correlation between the primers should be seen in this light. The combination of all nine primers therefore represents an indication of genetic variation in the regions of the genome covered by the primers.

Table 13. Primer pairs with positive correlation and the respective values.

Primer pair	Correlation values
1 and 4	0.366
1 and 6	0.181
1 and 7	0.111
1 and 10	0.054
1 and 11	0.216
1 and 12	0.196
2 and 3	0.231
2 and 6	0.045
2 and 11	0.199
2 and 12	0.039
3 and 6	0.077
3 and 10	0.615
3 and 11	0.119
3 and 12	0.447
4 and 7	0.345
6 and 7	0.410
6 and 11	0.344
7 and 11	0.125
10 and 11	0.273
10 and 12	0.408
11 and 12	0.269

To determine if the genetic distances in Table 8 between the conspecific populations of *T. sparrmanii* were significant , a two-way analysis of variance (two-way ANOVA) was performed on the data in Table 8 exactly as described in Chapter 6 . Only the values of the calculations are shown below. The key to abbreviations is as follows: CT = correction term, SS refers to sum of squares. A, B and T subscripts refer to the variables A and B and total sum of squares respectively. The summary statistics for the ANOVA analysis are shown in Table 14 below.

1. $CT = (19.694)^2 / 135 = 2.873$
2. $SS_T = 4.611 - 2.873 = 1.738$
3. $SS_A = 3.030 - 2.873 = 0.187$
4. $SS_B = 3.178 - 2.873 = 0.305$
5. $SS_{within} = SS_T - (SS_A + SS_B)$
   $= 1.738 - (0.187 + 0.305)$
   $= 1.738 - 0.492 = 1.246$

6. df for $SS_T = (n_T - 1) = 135-1 = 134$
   df for $SS_A = (r - 1) = 9 - 1 = 8$
   df for $SS_B = (c - 1) = 15 - 1) = 14$
   df for $SS_{within} = (c - 1) (r - 1) = 8 \text{ X } 14 = 112$

7. $S^2_T = 1.738 / 134 = 0.013$
   $S^2_A = 0.187 / 8 = 0.023$
   $S^2_B = 0.305 / 14 = 0.022$
   $S^2_{within} = 1.246 / 112 = 0.011$

8. $F_{8,134}$ (variable A) = Variance A / Variance within
   $= 0.023 / 0.011 = 2.091$

   $F_{14, 134}$ (variable B) = Variance B / Variance within
   $= 0.022 / 0.011 = 2.00$

Table 14. Summary table for ANOVA.

Source of variation	Sums of squares	Variance	degrees of freedom	F-value
variable A (primers)	0.187	0.023	8	2.091
variable B (populations)	0.305	0.022	14	2.00
within	1.246	0.011	112	
total	1.738	0.056	134	

Consultation of F distribution tables (Fowler & Cohen 1990), indicated that the critical values for degrees of freedom (df): 8, 34 was 1.938 (infinity value taken) and that for df:14,134 was between 1.666 and 1.754. Both values are below the tabulated values of 2.091 and 2.00 (Table 14) respectively. The objective of this ANOVA analysis was to determine if the primers differed significantly in estimating the genetic distances between the conspecific populations of *T. sparrmanii*. Secondly, it was to determine if the conspecific populations of *T. sparrmanii* were significantly genetically different. The Null Hypothesis was that there were no significant effects in both cases. Because the tabulated values obtained exceed the critical values for $F_{8,134}$ and $F_{14,134}$ ($P = 0.05$), the Null Hypothesis was rejected and the conclusion made that the primers differed in the extent to which they differentiated between the populations, and that the conspecific populations were significantly differentiated as determined by the data from the primers. The former conclusion was supported by the correlation test performed above in which only weak correlation was found between the primers in terms of the variation they detected between the populations of *T. sparrmanii.*

Considering the genetic distance values in the comparison of the conspecific populations of *T. sparrmanii* and *T. guinasana*, it was of interest to see whether or not the mean genetic distance between the conspecific populations was significantly smaller than the mean genetic distance between the conspecific populations and *T. guinasana*. This would serve to confirm the genetic distinction between *T. sparrmanii* and *T. guinasana*. To investigate this, the mean genetic distances between the conspecific populations were extracted from Table 10 (see also Table 12) as a separate data set and their mean value compared to that between the conspecific populations and *T. guinasana*. Table 15 below shows the extraction of the data sets and their organisation for statistical analysis.

Table 15. Comparison of the mean of genetic distances between *T. sparrmanii* conspecific populations and between the conspecific populations and *T. guinasana* (as obtained from Table 9)

Conspecific populations

Genetic distance

X	X^2
0.246	0.061
0.124	0.015
0.075	0.005
0.071	0.006
0.255	0.065
0.119	0.014
0.122	0.015
0.131	0.017
0.109	0.102
0.121	0.015
0.087	0.008
0.190	0.036
0.051	0.003
0.278	0.077
0.119	0.014

$\bar{x} = 0.139$

$\sum X_1 = 2.098$

$\sum X_1^2 = 0.363$

$s^2 = 0.005$, $s = 0.069$

T. sparrmanii / T. guinasana

Genetic distance

X_2	X_2^2
0.441	0.194
0.369	0.136
0.342	0.117
0.341	0.116
0.379	0.144
0.356	0.127

$\sum X_2 = 2.228$

$\sum X^2_2 = 0.834$

$s^2_2 = 0.001$

$s_2 = 0.037$

$\bar{x} = 0.371$

The genetic distances values shown under column X correspond (top to bottom) to the comparisons Wondergat and Molopo Oog, Malemani, Klerkskraal, Marico Oog and Amalinda respectively; Molopo Oog and Malemani, Klerkskraal, Marico Oog and Amalinda respectively; Malemani and Klerkskraal, Marico Oog and Amalinda respectively; Klerkskraal and Marico Oog, Amalinda respectively; Marico Oog and Amalinda.

The values under X_2 correspond (top to bottom) to the comparisons of each of Wondergat, Molopo Oog, Malemani, Klerkskraal, Marico Oog and Amalinda to *T. guinasana*. The difference between the means is given by $\bar{x}_1 - \bar{x}_2$ which was equal to 0.139 - 0.371 = - 0.232, indicating that the mean of the genetic distances between the *T. sparrmanii* conspecific populations was smaller than that between *T. sparrmanii* and
T. guinasana.

In order to determine the confidence level for the mean difference, the population variance must be computed, and it was estimated as:

$sp^2 = (n_1 - 1)\, s_1^2 + (n_2 - 1)\, s_2^2 / (n_1 + n_2) - 2$

$= (15 - 1)(0.005) + (6 - 1)(0.001) / (15 + 6) - 2$

$= 0.004$

$sp = \sqrt{0.004}$

$= 0.0632$

The 95% confidence level for the difference in the means was computed as follows:

$(\bar{x}_1 - \bar{x}_2) \pm t[.975]sp \sqrt{1/n_1 + 1/n_2}$ $(df = 21-2 = 19)$

$(0.139 - 0.371) \pm (2.093)(0.0632) \sqrt{1/15 + 1/6}$

$-0.232 \pm (0.132) \sqrt{(0.067 + 0.167)}$

$-0.232 \pm (0.484)(0.132)$

-0.232 ± 0.064 corresponding to a confidence interval of - 0.296 to - 0. 168.

The lower confidence limit for the mean genetic distance was -0.296 and upper limit was - 0.168. Because both confidence limits were negative, $\mu_1 - \mu_2$ therefore was negative. It was also confirmed by the calculation above that $\mu_1 - \mu_2$ was - 0.232. Based on this it could be concluded with 95% confidence that the mean genetic distance between the conspecific populations of *T. sparrmanii* was significantly smaller than that between *T. sparrmanii* and *T. guinasana*. This supported the separate species status of *T. guinasana* from *T. sparrmanii*.

5.3 DISCUSSION.

RAPD fingerprinting has proven to be a powerful tool for effective identification of taxonomic units, from the population up to the genus level. RAPD markers (limitations notwithstanding) presently provide an effective means for identification of operational taxonomic units with relatively high precision and without extensive effort (Schierwater, 1995).

The data obtained in this study in the examination of the genetic relationships between the conspecific populations of *T. sparrmanii* using RAPD fingerprinting indicated that there is genetic uniqueness between the populations, much as it is confined within the limits of conspecificity (as evidenced by relatively high similarity values between the populations). The levels of variation both within and between the populations differed with different primers. Measurement of correlation between the primers indicated lack of agreement (no significant correlation was found), thus supporting the foregoing observation, and indicating that the primers assayed different regions of the genome. Coefficient of variation measures indicated that the Wondergat population had the highest relative variability (13.99%), followed by the Klerkskraal (8.29%), Malemani (5.88%), Marico Oog (5.88%), Amalinda (4.95%) and the Molopo Oog (4.83%) populations respectively.

Cluster analysis of the relationships of the between the *T. sparrmanii* populations (Figure19) indicated that both Wondergat and Marico Oog populations are shown as being the most differentiated from the rest. The next most distantly related populations shown by the RAPD study were the Malemani and Klerkskraal (also indicated as closely related to each other) populations. Then came the Molopo Oog and Amalinda populations (shown as closely related to each other).

The mean genetic distance between all of the *T. sparrmanii* conspecific populations with all of the nine primers was 0.146. Using RAPD fingerprinting, Bardakci & Skibinski (1994) found that the technique could discriminate between three species of Tilapia and several sub-species of *Oreochromis niloticus*. From the dendrogram they drew, the values of genetic distances that they obtained for the subspecies ranged from just below 0.05 to just over 0.10 while that between the species ranged from just above 0.25 to approximately 0.34 (values read from the dendrogram in Figure 4 of Bardakci & Skibinski (1994). Even though Bardakci & Skibinski used different primer sequences, their values also place those found in this study in line with others that indicate populational sub-division below the species level.

The dendrogram in Figure 19 indicates that the conspecific populations of *T. sparrmanii* may possess, above the observed morphological differences (Greenwood, unpub. report) unique genetic characteristics, though confined within the limits of conspecificity as pointed out above.

In a morphological investigation of the relationships between the Wondergat, Molopo Oog, Marico Oog and Klerkskraal populations (Greenwood, unpub. rep) it was found that the Molopo Oog population possessed characteristics that were not shared by the other populations, nor were these characteristic of *T. sparrmanii* in general. In the RAPD study, apart from an indicated closer relationship with the Amalinda population, the Molopo Oog population was not shown as any more distinct than any of the other populations are from each other (Figure 19). Better distinction of these populations from the rest was shown in the analysis involving *T. guinasana* (Figure 20). Here the Molopo Oog and Amalinda populations are indicated as being closely related to each other and are more differentiated from the other populations. Greenwood (unpub. rep) found that the Marico Oog population closely resembled the Wondergat population. This closer relationship is also shown by this RAPD study in the analysis of both the *T. sparrmanii* on its own and its comparison to *T. guinasana*. In fact, the latter case illustrated this more clearly.

Fresh water habitats are often discrete and patchy leading to higher degrees of isolation. Isolation and the relative lack of migratory pathways between fragmented fresh waters carries implications for the extent of genetic divergence among fresh and anadromous fishes. The most important implication for this study is that there may be a high degree of genetic divergence (Carvalho *et al.*, 1991; Carvalho, 1993), even at a local level (possibly as a result of local adaptation). In this way, highly fragmented gene pools are produced with enhancement of opportunities for population differentiation. Gene flow and the extent to which it occurs is one of the micro evolutionary forces that influence population differentiation, quite often in cohort with selection and genetic drift (Carvalho, 1993). The population sub-division revealed by this RAPD study between the *T. sparrmanii* conspecific populations is probably due to the isolation in the sinkholes and springs, and consequent limitations to gene flow. The sub-division is probably a manifestation of local adaptation by the populations. The level of genetic divergence has not as yet become very high, as evidenced by the high levels of genetic similarity between the populations.

A two-way analysis of variance indicated that the differentiation between the fish populations was significant (P = 0.05) and the primers differed in the amount of differentiation they detected between the populations. The fact that the differentiation between the populations has not as yet reached the level of subspecies probably indicates that barriers to gene flow have only taken effect quite recently.

Translocation of fish is likely to have taken place in the period before the conservation authorities took an active interest in the conservation and management of these populations. Many of the sinkholes and springs are on private properties which may have previously given the owners free reign over activities around these sites. Some of the sinkholes, like Wondergat, are attractive dive sites, a factor that may have contributed to mixing of fish between sites. At the time the study was conducted there was only anecdotal evidence of fish introductions and translocations between the sinkholes and springs.

The populational differentiation between the *T. sparrmanii* fish shown by the RAPD study differs from the lack of differentiation observed with the SDS-PAGE study in Chapter 3. This could be explained in terms of RAPDs possessing higher resolution power than SDS-PAGE of proteins. They have been found to detect higher levels of genetic variation even when compared to enzyme loci (Cagigas *et al.*, 1999).

5.3.1 The relationship between *T. sparrmanii* and *T. guinasana*.

Morphological similarity between *T. guinasana* and *T. sparrmanii* has led to uncertainty about the validity of the former as a species (Oosthuizen *et al.*, 1993). Suggestions have ranged from it being an aberrant population of *T. sparrmanii* (mentioned by Greenwood, 1992) to it having evolved from a population of *T. sparrmanii* (mentioned by Oosthuizen *et al.*, 1993). As pointed out earlier, it was therefore of interest to estimate the degree of relatedness between the two species, even if from a subset of the primers used to compare the *T. sparrmanii* conspecific populations. This was not an attempt to delineate the taxonomic relationships of *Tilapia* species definitively, but only to estimate the level of genetic relatedness between the two species in question. In Figure 20 is shown an UPGMA dendrogram drawn from genetic distance estimates generated by a subset of three primers, between conspecific populations of *T. sparrmanii* and *T. guinasana*.

The conspecific status of the *T. sparrmanii* populations was confirmed by their close relatedness compared to that between them and *T. guinasana*. Also, even though only a subset of primers was used in the analysis, the relationships between the *T. sparrmanii* populations remained essentially the same, indicating the consistency of the RAPD analysis.

From Tables 10 and 15, the mean genetic distance between all the conspecific populations of *T. sparrmanii* was 0.139± 0.069 compared to that between *T. guinasana* and all the conspecific populations of *T. sparrmanii*, which was 0.374 ± 0.037. The average genetic distance between the two species of 0.374 falls just above the value of 0.25 to 0.34 for species differentiation obtained by Bardakci & Skibinski (1994) and may thus be taken as confirming separate species status of *T. guinasana*.

This is essentially the same finding as made with the SDS-PAGE results in Chapter 3 (in terms of confirmation of the separate species status of *T. guinasana* and *T. sparrmanii*). The results obtained in this study were in agreement with morphological work done by Greenwood (1992) in confirming the species status of *T. guinasana* as being separate from *T. sparrmanii*. In addition, the former species is readily distinguishable from the latter by its extensive polychromatism. Greenwood's (1992) work indicated that
T. guinasana is indeed a distinct species which is unique in several morphological features (Greenwood, 1992). Oosthuizen *et al.* (1993) found a genetic distance D (Nei, 1972) of 0.07 between *T. sparrmanii* and *T. guinasana* using allozyme analysis at 32 loci. The low value of genetic distance between the two species has to be seen against the fact that gene products are under functional constraints and therefore unlikely to evolve rapidly, and also the fact that RAPDs probably also assay genetic variation at non-coding regions of the genome. It may however be in line with the observed morphological similarity (Greenwood, 1992) between the two species.

Although not directly comparable with the RAPD s fingerprinting study here, Shaklee *et al.* (1982) using allozymes stated that the genetic distances at interspecies level range from 0.025 to 0.65 with an average of 0.30 for species of the same genus. This agrees well with similar estimates of Grant *et al.* (1988) of >0.025 and average of 0.40. The genetic distances calculated for the RAPDs fingerprinting in this study are therefore not very different from those obtained from allozymes.

Furthermore, many studies have found congruence between measures of population genetic variation and differentiation between allozymes and RAPDs. Isabel *et al.* (1995) found complete congruence between gene diversity estimates derived from genotypic data at enzyme loci and RAPD loci in black spruce.

Isozyme analysis and RAPD markers detected similarly low genetic variation in the gymnosperm species *Amentotaxus formosana* Li (Wang *et al.*, 1996). Similar agreement has been shown in Douglas-fir (Aagard *et al.* , 1998), in the dipteran species *Ceratitis capitata* (Baruffi *et al.*, 1995), in aspen (Liu & Furnier, 1993) and buffalo grass (Peakall *et al.*, 1995). The inter subspecies and species differences obtained by Bardakci & Skibinski (1994) using RAPDs are also in line with the above and those obtained in this study.

In conclusion, using RAPD fingerprinting, it has been shown in this study that the conspecific populations of *T. sparrmanii* are genetically unique, although this fact is circumscribed by the parameters of conspecificity. When these populations were compared to *T. guinasana*, their conspecificity was confirmed and *T. guinasana* was shown to be a separate species.

CHAPTER 6.

RAPD FINGERPRINTING OF COLOUR FORMS OF *TILAPIA GUINASANA*.

6.1 INTRODUCTION.

Tilapia guinasana is a tilapiine cichlid that is endemic to a single sinkhole, Guinas, (surface area ca 2800 m^2), situated in the northern part of Namibia. The surface area of the sinkhole gives *T. guinasana* the smallest area of endemicity for any known *Tilapia* species (Ribbink, *et al.*, 1991). However, it is now established in Lake Otjikoto together with another translocated tilapiine species, *Oreochromis mossambicus* (Greenwood, 1992; Skelton, 1993). *Tilapia guinasana* is unique among tilapiines because of its extensive polychromatism; a phenomenon that is particularly unexpected among substratum-spawning cichlids which are conservative with regard to colouration. *Tilapia guinasana*'s polychromatism is not sex limited, compared to haplochromine cichlids in which this phenomenon is sex limited.

Five distinct colour forms of the species have been identified. These are olive, olive striped, dark blue, blue striped and light blue. All are discrete except for the light blue form which was found to be highly variable, with elements of white, light blue, yellow and even black blotches in some individuals (Ribbink *et al.*, 1991; Greenwood 1992).

There is limited knowledge on polychromatism and how it evolves. This also applies to that which is exhibited by *T. guinasana*. Ribbink *et al.* (1991) list three possibilities that might explain *T. guinasana* 's polychromatism. Firstly, it might be due to a high level of individual chromatic variability with colour gradations linking the various forms. This however, would be highly unusual since colouration in cichlids is highly restricted. The second possibility is that *T. guinasana* might be exhibiting discrete sex-limited polychromatism, which would be atypical for the species and African sub-stratum spawners in general (Ribbink *et al.*, 1991).

The third possibility is related to the failure of taxonomists to recognise an important phenomenon, the existence of a complex of sympatric sibling species within a taxon (Ribbink *et al.* (1991). Their studies of *T. guinasana* in the Guinas sinkhole however, showed that it is a single species comprised of five principal colour forms and a rare and relatively larger sixth form, none of which were sex limited.

It has been further suggested that because of darkness in the sinkhole (1) there was no need for communication and because of absence of predators in the sinkhole (2) there was no selection for camouflaging colours (Perinth, 1978). Skelton (1988) has proposed that *T. guinasana* might have lost genetic "control" over colour when the sinkhole was still an underground cave. He further suggested that without competition or predation from other closely related fish species, selection or need for a distinctive colouration for purposes of mate recognition, general communication or camouflage was obviated. However, Greenwood (1992) points out that in Lake Malawi there exist equally un-camouflaged and bright coloured fish despite the presence of considerable predation, which is a fact that casts doubt on Skelton (1988)'s latter suggestion. Further, in other cave dwelling cichlids, the tendency is towards loss of or reduction of colouration rather than an elaboration of pigments and patterns (Greenwood, 1992). Skelton (1988)'s suggestion on the subterranean origin of the species, and hence the polychromatism does not, in the light of the previous sentence, seem to explain adequately the origin of *T. guinasana*'s polychromatism. Ribbink *et al.* (1991) give detailed descriptions of the five distinct colour forms of the species and a sixth non-breeding (according to their observations) form, black, which was larger than the others.

Three factors make *T. guinasana* one of the most unique (because of its polychromatism) but vulnerable vertebrates in southern Africa. These are i) a natural distribution that is limited to a single sinkhole that is found in an arid region of the continent, ii) fluctuations in water levels in the sinkhole and iii) environmental pollution. Our hypothesis was that, given the permanence and discrete nature of the colouration of *T. guinasana*, the colour polymorphs should be genetically distinct and we used RAPD fingerprinting to test this.

6.2 RESULTS.

Four primers (4, 6, 7 and 12, Table 16) of random sequence were used to generate RAPD amplification profiles for the five colour forms of *T. guinasana.* The number of samples used for the fingerprinting study ranged from three to ten depending on availability of the colour respective colour forms. The lower number resulted from the relative scarcity of especially the blue stripe and olive stripe forms.

Each primer/template combination produced constant fingerprint patterns for each colour form (see Figure 20) and each primer had a characteristic fingerprint pattern. Although there was a high level of similarity between RAPD profiles of the colour forms, both intra-colour form (Figure 21) and inter-colour form (Figure 22) differences could be detected. These are shown by means of arrows in the respective figures. Table 16 shows the estimated genetic distances obtained with each of the primers and their means for all the primers. This data was used to draw the UPGMA dendrogram (Figure 23).

The highest genetic distance between any of the colour forms of *T. guinasana* was between the dark blue and the olive forms (0.445) and the least genetically different were the olive stripe and blue stripe forms. Table 17 shows the genetic distances (and similarities) which were used to draw up a dendrogram (Figure 22) depicting the relatedness between the colour forms. The dendrogram indicates that the blue stripe and olive stripe forms are more closely related to each other with the light blue form being a closely related outlier. Both the dark blue and olive forms are indicated as being more distantly related to the other colour forms.

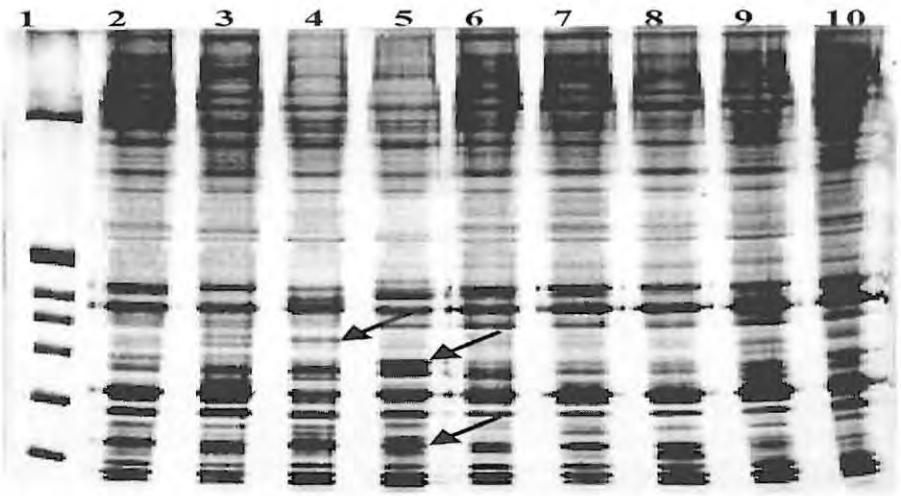


Figure 21. RAPD fingerprint patterns of the olive form of *T. guinasana* generated with primer 7. Lane 1, pBR322/H*in*f1 size marker; lane 2 - 10, RAPD fingerprints of the olive form of *T. guinasana* generated with primer 7. Arrows indicate areas of intra specific variation detected by the primer.

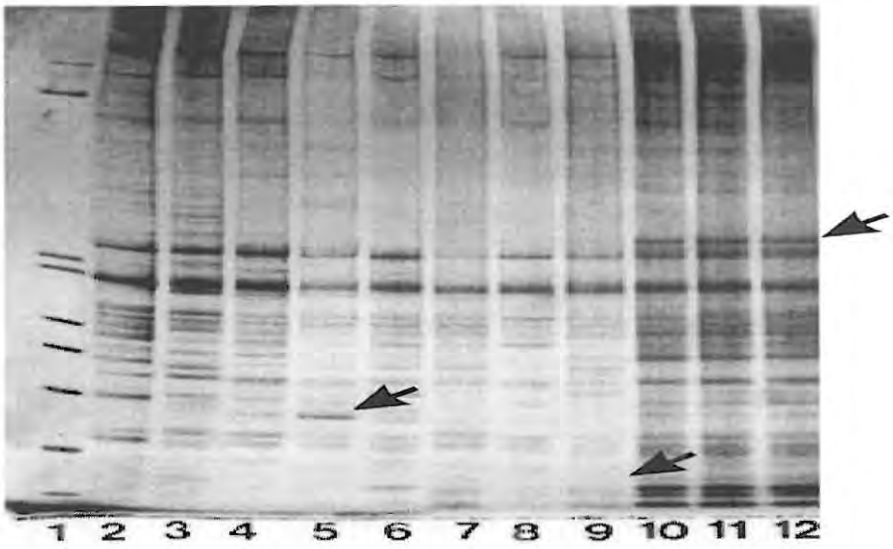


Figure 22. Amplification profiles of the five colour forms of *T. guinasana* with primer 12. Lane 1, pBR322/ H*inf*1 size marker; lane 2- 4, amplification profiles of three individuals of the dark blue form; lanes 5- 7, amplification profiles of three individuals of the striped blue form; lanes 8-9, amplification profiles of two individuals of the light blue form; lane 10, an amplification profile of one olive form; lanes 11- 12 show amplification profiles of two individuals of the olive striped form. Inter-colour form differences are shown by the arrows.

Table 16. Estimated genetic distances (D_{ij}) between the colour forms of *T. guinasana* across the four primers used. Key: db = dark blue, lb = light blue, sb = blue stripe, os = olive stripe and o = olive.

Primer	db/o	db/lb	db/sb	db/os	o/lb	o/sb	o/os	lb/sb	lb/os	sb/os
4	0.097	0.097	0.097	0.097	0.077	0.118	0.077	0.077	0.118	0.118
6	0.693	0.560	0.288	0.387	0.560	0.499	0.499	0.288	0.288	0.241
7	0.294	0.424	0.298	0.413	0.380	0.481	0.556	0.006	0.021	0.001
12	0.697	0.497	0.249	0.220	0.578	0.542	0.019	0.240	0.408	0.178
Mean	0.445	0.395	0.233	0.279	0.398	0.410	0.288	0.152	0.209	0.135
SD	0.299	0.205	0.093	0.148	0.232	0.196	0.278	0.133	0.172	0.102

Table 17. Matrix of similarity (above diagonal) and genetic distance (below diagonal) between the colour forms of *T. guinasana*. Key: db = dark blue, lb = light blue, sb = blue stripe, os = olive stripe and o = olive.

	Db	lb	sb	os	o
db	-	0.769	0.771	0.771	0.689
lb	0.395	-	0.841	0.822	0.704
sb	0.233	0.152	-	0.879	0.695
os	0.279	0.209	0.135	-	0.872
o	0.445	0.398	0.410	0.288	-

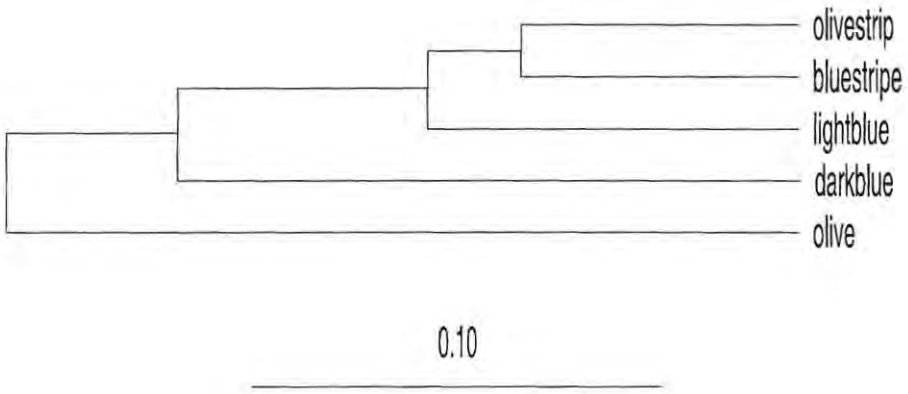


Figure 23. An UPGMA dendrogram drawn from the genetic distances between the colour forms of *T. guinasana* as obtained from the four primers used in this study. The dendrogram was drawn using Neighbour-joining in Phylip3.5.

The significance of the results obtained and recorded in Table 16 was determined to assess two aspects: (1) the significance of differences in the genetic distance detected by the primers between the colour forms and (2) the significance of genetic distances between the colour forms of *T. guinasana*.

A two-way analysis of variance (two-way ANOVA) was then performed on the data in Table 16 using the primers and colour forms as variables A and B respectively. The Null Hypothesis was that there were no significant differences due to either the primers or the colour forms. Because the genetic distance values represented single observations, the two-way ANOVA performed was one specifically designed for single observations. The difference between this type of two-way ANOVA and the normal one is that because there are only single values or observations per cell (in the ANOVA table) it is not possible to compute a between samples sum of squares. This also makes it impossible to compute or derive a sum of squares for interaction and that source of variability cannot be investigated. To perform two-way ANOVA using single observations, it has to be assumed that there is no interaction (Fowler and Cohen, 1990). In order to perform two-way ANOVA on the data in Table 16, the data was recast into an ANOVA table as in Table 18 below. Below the table are shown in detail all the calculation steps that need to be done to perform two-way ANOVA on single observations.

Table 18. Genetic distances between the colour forms of *T. guinasana* with column and row totals and sums of squares for rows and columns (data arrangement for ANOVA)

Variable A-primers	Variable B - colour forms												
	db/o	db/lb	db/sb	db/os	o/lb	o/sb	o/os	lb/sb	lb/os	sb/os	$\sum x$	$\sum x^2$	n
4	0.097	0.097	0.097	0.097	0.077	0.118	0.077	0.077	0.118	0.118	0.973	0.097	10
6	0.693	0.560	0.288	0.387	0.560	0.499	0.499	0.288	0.288	0.241	4.303	2.062	10
7	0.294	0.424	0.298	0.413	0.380	0.481	0.556	0.006	0.021	0.001	2.874	1.210	10
12	0.697	0.497	0.249	0.220	0.578	0.542	0.019	0.240	0.408	0.178	3.628	1.727	10
$\bar{x}$	0.445	0.395	0.233	0.279	0.398	0.410	0.288	0.152	0.209	0.135		$\bar{x}_T = 0.294$,	
$\sum x$	1.781	1.578	0.932	1.117	1.595	1.640	1.151	0.611	0.835	0.538		$\sum x_T = 11.778$	
$\sum x^2$	1.061	0.749	0.243	0.378	0.798	0.788	0.564	0.146	0.246	0.104		$\sum x^2{}_T = 5.096$	
n	4	4	4	4	4	4	4	4	4	4		$n_T = 40$	

The various steps in the performance of two-way ANOVA on single observations were as follows:

1. Correction term (CT): $CT = (\sum x_T)^2 / n_T = (11.778)^2 / 40 = 3.468$
2. Total sum of squares : $SS_T = \sum x^2{}_T - CT = 5.096 - 3.468 = 1.628$

3. Sum of squares for variable A: $SS_A = (\sum x_1)^2 / n_c + (\sum x_2)^2 / n_c + (\sum x_3)^2 / n_c + (\sum x_4)^2 / n_c - CT$

   where n_c is the number of columns (number of observations in a row) and subscripts 1- 4 refer to rows 1- 4.

$SS_A = (0.973)2/10 + (4.303)2/10 + (2.874)2/10 + (3.628)2/10 - 3.468 = 0.62$

4. Sum of squares for Variable B: $SS_B = (\sum x_1)^2 / n_r + ... + (\sum x_{10})^2 / n_r - CT$

   where n_r refers to the number of rows (number of observations in a column) and subscripts 1 - 10 refer to the rows 1 -10.

$SS_B = (1.781)^2 / 4 + (1.578)^2 / 4 + (0.932)^2 / 4 + (1.117)^2 / 4 + (1.640)^2 / 4 + (1.151)^2 / 4 + (0.611)^2 / 4 + (0.835)^2 / 4 + (0.533)^2 / 4 - 3.468 = 0.461.$

5. Within sum of squres: $Ss_{within} = SS_T - (SS_A - SS_B)$

   $= 1.628 - (0.620 + 0.461)$

   $= 0.547$

6. Degrees of freedom for each sum of squares:

   df for $SS_T = n_T - 1 = 40-1 = 39$

   df for $SS_A = r - 1 = 4 - 1 = 3$

   df for $SS_B = c - 1 = 10 - 1 = 9$

   df for $SS_{within} = (c - 1)(r -1) = (3)(9) = 27$

7. Estimation of variances:

$s^2_T = 1.628/39 = 0.042$

$s^2_A = 0.620/3 = 0.207$

$s^2_B = 0.461/9 = 0.051$

$s^2_{within} = 0.547/27 = 0.020$

8. The F- value for each variable:

$F_{2,27}$ (variable A) = Variance A/Variance within = 0.207/0.020 = 10.35

$F_{9,27}$(variable B) = Variance B / Variance within = 0.051/0.020 = 2.55

9. Summary table for ANOVA:

Table 19. ANOVA table for assessing the significance of genetic differences between the colour forms of *T. guinasana.*

Source of variation	sum of squares	degrees of freedom	variance	F-value
Variable A (primers)	0.620	3	0.207	10.35
Variable B (colour forms)	0.461	9	0.051	2.55
Within	0.547	27	0.020	
Total	1.628	39	0.278	

At this stage F- distribution tables are consulted to compare the tabulated values with the critical values in the distribution table. At P = 0.05 both the tabulated values of $F_{3,27} = 10.35$ and $F_{9,27} = 2.55$ exceed the critical values of 2.960 and 2.250 respectively. The Null Hypothesis supposed that there were no significant differences in both the genetic distances determined with the different primers nor were there any significant differences between the colour forms of *T. guinasana.*

This Null Hypothesis had to be rejected and the conclusion drawn was that the primers differentiated to different extents between the colour forms and that the colour forms are significantly genetically different. Based on this it could be concluded that each colour form is genetically unique, although there is as yet no permanent reproductive barrier between them, physical or biological.

6.3 DISCUSSION.

Ribbink *et al.* (1992) reported that there were detectable levels of assortative mating (mating between members of the same colour form) between the colour forms of *T. guinasana,* it being more evident among the light blue, olive and dark blue fish. However, this was only significant ($P = 0.001$) among the dark blue fish, and not complete, and the probability of mating between any colour forms appeared to depend on the average occurrence of the respective colour form(s) in the breeding population.

The dendrogram in Figure 22 shows that there is a degree of genetic differentiation between the colour forms of *T. guinasana*. There is no evidence that the colour forms have differentiated to such an extent that they can be considered to be separate species. Furthermore, *T. guinasana* exists as a single population of relatively few individuals in a single sinkhole. There are thus no physical barriers to breeding between the colour forms. The levels of genetic distinctiveness shown in the dendrogram may be a result of the assortative mating that has been observed between the colour forms.

Ribbink *et al.* (1991) observed significant levels of assortative mating with the dark blue forms and to some extent with the light blue and olive forms. The fact that the olive and the dark blue forms are shown in the dendrogram as being more distantly related to the other colour forms may be indicative of significant levels of assortative mating within these colour forms. From the table of genetic distance values (Table 17) it can be observed that the highest values are those between the dark blue forms and the olive forms on the one hand, and the other colour forms on the other. The close relationship shown on the dendrogram between the olive stripe and blue stripe forms may be indicative of a higher frequency of mating between the two colour forms.

It would not be unreasonable to think that the stripe patterns on these fish could blur the

distinction between the colour forms in terms of mate recognition.

Two-way ANOVA performed on the values of genetic distances between the colour forms indicates that there is significant differentiation between them ($F_{3,27}$ and $F_{9,27} > P = 0.05$), suggesting that each colour form may be genetically unique. The ANOVA result also suggested that the primers differentiated to between the colour forms different extents.

In conclusion, this RAPD fingerprinting study found that the colour forms of *T. guinasana* exhibit a significant degree of genetic differentiation, although they are still members of a single species. The observed assortative mating may be the primary source of such differentiation. The values of estimated genetic distances ranged from 0.135 to 0.445.

If the values of Bardakci & Skibinski (1994) are taken then the colour forms range from potential subspecies to potential species, and it may be argued that the differences between the colour forms may be indicative of an incipient species formation process, since the genetic distance values straddle the conspecific and congeneric species status levels. The average genetic distance (Nei, 1972) between the Tilapia species studied by Oosthuizen *et al.* (1993) was 0.356. Sodsuck & McAndrew (1991) obtained values of genetic distances (Nei, 1972) between Tilapia species that ranged from 0.007 to 0.661 with an average of 0.405.

Based on the data obtained from the four primers used in this study, and comparing them with the other studies or comparisons of Tilapia species as outlined above, it would appear that the genetic distances computed are indicative of the initial processes of species or sub-species formation, by the colour forms of *T. guinasana*, more so the dark blue and olive forms. It is not clear whether the assortative mating observed is a product of this incipient species formation process or a determinant thereof. A more extensive study, involving perhaps a larger number of primers and individuals would shed light as to the extent of this observed differentiation and what exactly the role of assortative mating is.
This work has been published in Nxomani *et al.* (1999), which is attached as Appendix C.

CHAPTER 7
DISCUSSION.

This study aimed to achieve three broad objectives:

(a) To assay the genetic uniqueness of allopatric populations of the fish *T. sparrmanii* that are found in the sinkholes and springs of the North West Province, South Africa.
(b) To examine the genetic relationships of *T. sparrmanii* with its polychromatic sister species, *T. guinasana*, which is endemic to the Guinas sinkhole in Namibia.
(c) To investigate whether or not the colour forms of *T. guinasana*'s are genetically distinct.

The study was prompted by the concern of conservation authorities in the Department of Environmental Affairs and its Nature Conservation agency about possible loss of unique or differentiated fauna and the ecosystems that they inhabit, in the event of extensive water abstraction. Such a threat was posed by the demand for the subterranean water in these systems for domestic, agricultural and commercial use. In addition these ecosystems are facing habitat destruction and introduction of alien species. These concerns and threats face both fish species.

This chapter serves to outline, interpret and discuss the findings of the study, and aims to achieve the following:

(a) Summarize the findings of this study
(b) Interpret the findings in the context of the techniques employed
(c) Discuss the implications of the findings, the current status of the fish populations and the long term implications within a conservation genetics perspective
(d) Outline specific recommendations for the management and conservation of the fish populations
(e) Recount the objectives of the study and indicate to what extent they have been achieved

7.1 Summary of the findings of this study

7.1.1 *SDS-PAGE of total cellular protein*

SDS-PAGE of total cellular proteins was used and investigated in this study as a fast and relatively inexpensive indicator of the genetic relatedness between the *T. sparrmanii* populations. It was the least sensitive of the techniques utilized in this study and it did not reveal any genetic partitioning of the conspecific populations of *T. sparrmanii* examined using manual analysis. Neither did it reveal any intrapopulational differences examined. This differed from the results of an earlier study, Nxomani *et al.*(1994), which found populational sub-division. The difference between the two studies can be ascribed to gel-to-gel variation enhancing the populational differentiation found in the earlier study.

The present study circumvented this problem through direct comparison of samples on the same gel. A further strength of the present study was the use of two additional *T. sparrmanii* populations which were not available to the earlier study (Nxomani *et al.* 1994). However, in the present study SDS-PAGE differentiated between *T. sparrmanii* and *T. guinasana*, as well as between the two species and unrelated fish species (Figure 4) thus demonstrating its utility as an indicator of genetic relatedness, albeit at a genus level. The fact that non-destructive sampling procedures such as biopsies, fin clippings and minimal blood samples provide sufficient material for PAGE to be run quickly and yield useable information about the degree of relatedness of species, holds promise for the technique to be used by under-resourced conservation managers. This takes on major significance in southern Africa, given the current resource limitations faced by the sub-region and its conservation management entities.

7.1.2 Mitochondrial DNA studies.

Restriction endonuclease analysis of PCR-amplified portions of the cytochrome b gene and the d-loop revealed no differentiation within and between the *T. sparrmanii* populations. TGGE analysis of the same DNA regions also did not detect genetic differences within and between the populations. The lack of intra-population subdivision correlates with that found with the SDS-PAGE study. Of particular interest was the fact that TGGE analysis did not detect differences between the *T. sparrmanii* and *T. guinasana* samples either. This lack of genetic partitioning despite habitat fragmentation points to a recent separation of the fish populations from a recent ancestral population.

7.1.3 RAPD fingerprinting study.

RAPD fingerprinting detected genetic variation within and between the conspecific *T. sparrmanii* populations. It was therefore more sensitive than mitochondrial DNA analysis in the detection of intra- and interspecific differentiation of the populations examined, an observation also made by Bardakci & Skibinski (1994) in their studies of Nile Tilapia *Oreochromis niloticus*. The technique showed (Figure 18) that there is genetic differentiation between the conspecific populations. From the dendrogram (Figure 18) the Wondergat and Marico Oog populations were shown to be more closely related to each other.

The Molopo Oog and Amalinda populations were shown to be distinct but closely related to each other, so were the Malemani and Klerkskraal populations. If geographic separation were to be used as a basis for defining genetic relationships based on the potential for gene flow, the Amalinda population would have been expected to be the most distinct from the others as it is derived from a hatchery in East London, more than a thousand kilometres away. Given its uncertain origin and the high probability of it originating from a number of localities, a distance correlation cannot be made.

RAPD fingerprinting not only showed differentiation between the *T. sparrmanii* conspecifics, but it indicated that the colour forms of *T. guinasana* are genetically distinct. The findings of this work were published in Nxomani *et al.* (1999) which is attached hereto as Appendix C. RAPD fingerprinting also indicated that the two are separate species and thus contributed to clarification of the question on the species status of *T. guinasana* previously posed (Oosthuizen *et al.*, 1993; also see Skelton (1988) and Ribbink *et al.* (1991)). The ability of the technique to indicate statistically significant levels of genetic differentiation between the colour forms of *T. guinasana* has important implications for both species.

7.2 Understanding the findings

7.2.1 *The position of T. sparrmanii in the Stenotopy-Eurytopy continuum as a determinant of the observed degree of differentiation between populations.*

Fishes are described as eurytopic if they have a broad habitat tolerance range, an altricial life history style, wide distribution, are highly mobile, largely consist of large populations under favourable conditions and generally have a large body size (Ribbink, 2001). Stenotopic fishes are characterized by a narrow habitat tolerance range, a precocial life history style, narrow distribution, a sedentary approach, usually small populations and have a relatively small body size. Rates of speciation and divergence will differ between them depending on their position in the stenotopy-eurytopy continuum (Ribbink, 2001).The explosive speciation and adaptive radiation of the Great Lakes cichlids (Fryer & Iles, 1972; Ribbink, 1987, Martens *et al.*, 1994; Sturmbauer, 1998) has been attributed to greater levels of stenotopy amongst them, compared to eurytopic cichlids and other fish families both in the lakes and in riverine systems. Patterns of species richness demonstrate that those species flocks with the most stenotopic members are larger than those that comprise eurytopic species (Ribbink, 1991, 1992)

Summarized, the stenotopy-eurytopy continuum concept holds that stenotopic fishes are highly specialized with regard to habitat utilization, such that small habitat discontinuities amount to geographic barriers to stenotopes, resulting in the isolation of gene pools and the promotion of allopatric speciation (Fryer 1959, Fryer & Iles, 1972; Ribbink *et al.*, 1983a). Small changes in habitat characteristics impose considerable selection pressures on narrow stenotopes leading to extinction or even speciation. In the case of eurytopes however, even large changes in habitat characteristics may not impose significant selection pressures to elicit great genetic and phenotypic responses to change (which could result in higher propensities to speciate). How does the stenotopy-eurytopy concept apply to the *T. sparrmanii* populations under discussion?

Life history style aspects of T. sparrmanii

Skelton (1993) explains that the distribution of freshwater fish in southern Africa originated from further north in Africa in the form of three "waves of invasion", with each wave progressing further southwards during periods of interconnectedness of the different river basins.

The first invasion is estimated to have taken place 2-3 million years ago, resulting in the Cape and Karoo fauna and reaching the Orange and Cape coastal rivers. A second invasion could have occurred in 1.8 - 2 million years ago during the existence of a connection between the Okavango-upper Zambezi and the Limpopo basin. Less than 1.8 million years ago an invasion occurred along the coastal region, thus linking the Zambezi and Limpopo basin. The more recent connections between the Orange and the Limpopo basins may explain the existence of species common to the two systems (Skelton, 1993).

Tilapia sparrmanii occurs from the Orange River and the Natal south coast northwards to the upper reaches of southern Zaire tributaries, Lake Malawi and the Zambezi system. It is also extensively translocated south of the Orange in the Cape. It is described as being tolerant of a wide range of habitats although preferring quiet or standing waters (Skelton, 1993). Its occurrence in the sinkholes and springs studied here is probable confirmation of this preference. The sinkholes are much deeper and more insular than the more open marsh-type springs. The species' wide habitat tolerance range and geographical distribution range allow it to occur comfortably despite these differences.

In explaining why riverine cichlids did not undergo the rapid speciation processes characteristic of the lacustrine cichlids the following argument has been suggested. With the exception of the Zaire/Congo river which has more lacustrine features, African rivers are seasonal with markedly different flow patterns, levels of turbidity, temperature, silt loads, oxygenation etc. between and during seasons (Ribbink, 2001). The riverine environment would therefore demand that organisms living in it would have to be able to tolerate harsh and rapidly changing conditions and have a broad habitat tolerance range and are generalists. Cichlids occupying rivers in the Africa context are eurytopic and have fewer opportunities to speciate (Ribbink 2001). *Tilapia sparrmanii* appears to be eurytopic.

Tilapia sparrmanii populations in the sinkholes and springs are unlikely to have differentiated with any rapidity despite the fragmentation of their habitat and isolation. A possible explanation for this is that the lacustrine cichlids tend to be narrowly stenotopic and highly specialized, whereas *T. sparrmanii* appears to be largely eurytopic, if its distribution is taken into account.

Apart from its broad habitat range tolerance, it is an omnivore that feeds on available foods ranging from algae, soft plants, small invertebrates such as insects and even small fish (Skelton, 1993). These attributes would allow *T. sparrmanii* to withstand changing environments and thus differentiate more slowly than if it were stenotopic. The observed lack of differentiation at protein and mitochondrial levels may be a reflection of the eurytopic attributes of the species and its populations.

There are, however, other considerations that must be noted. Protein-coding genes evolve much slower than non-coding genes which are under functional constraints (Grant *et al.*, 1988). In studying restriction enzyme variation in allopatric populations of another southern African cichlid species, *P. philander*, including fish obtained from Wondergat and Molopo Oog; de Villiers *et al.* (1992) found a maximum between-population sequence divergence of only 0.009 despite clear morphological differences (Twentyman-Jones, 1992; Twentyman-Jones *et al.*, 1997) They interpreted this value to be only slightly higher than intra population divergence. Studies by Seyoum and Kornfield (1992) have shown similar limitations of mtDNA analysis in fish populations and Bardakci and Skibinski (1994) outline further limitations. With respect to the TGGE analysis, a real possibility exists that genetic differences existed within higher domains of the sequences analyzed, not detectable by the gradients used in this study.

The fact that the isolation of the populations is likely to have taken place in the last 20 000 years and the suggestion of eurytopic attributes of *T. sparrmanii* outlined above, leads one to conclude that the results obtained in this study indicate a recent origin of the populations studied from a common ancestral population. The time span of 20 000 years may have been inadequate for a eurytope to differentiate to the point where species can be identified.

The RAPD fingerprinting study found minimal genetic variation within and significant genetic variation between the *T. sparrmanii* populations. It thus proved more sensitive than both the protein and mitochondrial DNA studies. Bardakci and Skibinski (1994) made similar findings with respect to the higher sensitivity of RAPD fingerprinting compared to mitochondrial DNA analysis. The differentiation detected by RAPD fingerprinting was circumscribed by the limits of conspecificity and indicated populational subdivision below the species level.

It reflects an adaptation to local conditions of unique ecosystems and therefore constitutes differences at a genetic level that are of ecological significance (Hindar *et al.*, 1991). The populations sizes that were dealt with in this study are too small to speak meaningfully of speciation. None of the existing theoretical models would be adequately addressed or met by the specific context of the *T. sparrmanii* population.

The physical locations of the sinkholes and springs appears to partly explain the clustering observed with the RAPD fingerprinting results. Consideration of Figure 1, shows that the Marico Oog sinkhole is part of the Limpopo river system and its drainage is North-easterly. Malemani (situated just off the Harts river, which branches from the Vaal river) and Klerkskraal (just off the Vaal river) are part of the Orange-Vaal river system whose drainage is southwards. The Wondergat sinkhole and the Molopo Oog spring appear to be part of the Molopo river system whose drainage is in a westerly direction. Probably, during the much wetter periods the populations from these river systems were once a single panmictic population and became fragmented during drier periods accompanied by geological changes that resulted in the presently described drainage systems (see Skelton, 1994) as indicated above.

The clustering of the Malemani and Klerkskraal fishes may be reflective of the fact that these springs are part of the same river system and may have only become recently effectively separated. Clustering of the Molopo Oog and Amalinda suggests that the latter population originated from the Molopo river system and was translocated. Molopo Oog and Wondergat appear to be part of the same river system, the Molopo river system. The distinction of Molopo Oog fish from that of Wondergat therefore may be due in part to the unique nature of the two systems. Molopo Oog is an open marsh-type environment in contrast to the Wondergat sinkhole which is confined and highly defined. The nature of the latter habitat may have enhanced micro-environmental effects to greater differentiation between the two as a result of local adaptation.

7.2.2 *The case of T. guinasana*

As other studies have done before (Greenwood 1992; Oosthuizen *et al.*, 1993), this study confirmed the independent species status of *T. guinasana*, from *T. sparrmanii*. The RAPD study indicated significant genetic differentiation between the colour forms of *T. guinasana* (Nxomani *et al.*, 1999). As speciation is a consequence of adaptive processes that are determined by the development of limitations or blocks to gene flow and existence of reproductive isolation mechanisms (Bush, 1975), the assortative mating exhibited by the species may have implications for its evolution and continued existence. In spite of the fact that the incomplete assortative mating observed in the species (Ribbink *et al.*,1991) could serve to maintain genetic diversity in the species, it could also have undesirable effects if it were to become complete. Meffe (1987) has commented on the possibility of the formation of smaller genetic demes, from processes that enforce populational sub-division, thus possibly decreasing the effective genetic population size. However, if such demes survive the possible bottlenecks, higher levels of genetic differentiation could be achieved by the species, including the formation of subspecies.

The origin of polychromatism in *T. guinasana* has been a matter of speculation (Skelton, 1988; Perinth, 1978) and the question that remains is why the species developed the colour forms in the first place? An interesting factor about *T. guinasana*'s polychromatism is that all juveniles are a uniform colour and fish only take on the different colour forms upon sexual maturation, except for the light blue form (Ribbink *et al.*, 1991 and personal communication). The fact that only sexually mature individuals exhibit the polychromatism indicates that there is a genetic switch for it. Regardless of the manner in which it has arisen, the incomplete assortative mating (Ribbink *et al.*, 1991) is likely to help maintain significant levels of variability in the species, which is critical for its continued existence in the face of threats to its environment and its ability (from a genetic point of view) to respond to changes therein.

There must have been a stimulus though in the past that encouraged the development of the polychromatism. Female cichlids (at least in the Great lakes of East Africa), are often of drab colours while males tend to be brilliantly coloured (Witte *et al.*, 1997; Stiassny & Meyer 1999). Such a colour dimorphism may therefore be related to a specific mate recognition system. *Tilapia guinasana* does not possess this sexual dimorphism (Ribbink *et al.*, 1991).

Stiassny and Meyer (1999) suggest that the colour morphs in the Great Lakes' cichlids may have arisen as a result of the preferences of the females. If that were to be true, then sexual selection, rather than pressure for physical survival, may have driven the diversification (Stiassny & Meyer, 1999). The fact that the polychromatism in *T. guinasana* is exhibited at sexual maturity probably indicates that colouration plays an important role in the breeding process.

7.3 The genetics of small populations and the implications for conservation and management of the *T. sparrmanii* and *T. guinasana* populations.

Conservation genetics concerns itself with the central question of loss of genetic variation resulting in erosion of evolutionary flexibility (Meffe, 1986). A multiplicity of factors are responsible for this decline in the ability of organisms to evolve, and they include genetic bottle-necks, genetic drift and inbreeding depression (Meffe, 1986). Genetic bottlenecks cause the sampling of a few individuals from a larger gene pool, resulting in a remnant population that contains little overall variation (Meffe, 1986). With genetic drift, small populations lose genetic variation faster than will larger populations (Lacy, 1987; Quinn & Hastings, 1988). Inbreeding results in the expression of deleterious recessive alleles (Meffe, 1990). The results of inbreeding are amongst others reduced fertility, high mortality of offspring, slow or aberrant development and hastened adult mortality (Ralls & Ballou, 1983, 1988; Lacy, 1988). The extent to which the effects of these factors sets in is enhanced by small population size. This section considers these factors and relates them to the situation of the *T. sparrmanii* populations as well as that of *T. guinasana*.

The ultimate result of all these factors (bottlenecks, genetic drift and inbreeding depression) is low genetic variability within, and even extirpation of, local populations (Pringle, 1997) as well as high genetic divergence between populations (Meffe & Vrijenhoek, 1988; Allendorf & Leary,1988; Vrijenhoek *et al.*, 1985, Meffe, 1986, 1987, 1990; Mesquita *et al.*, 2000). The immediate effect of this depletion of genetic variability is increasing levels of homozygosity of individuals in the population concerned.

In the absence of significant levels of genetic variation between individuals on which natural selection can act, a population is unable to adapt to changing environments; and thus becomes vulnerable to new predators, diseases, parasites, climatic conditions, to competitors and changing food supplies (Lacy, 1987). Both the random (drift and bottlenecks) and directional (inbreeding) forces driving genetic diversity loss in small populations reduce the effective genetic population size (Meffe, 1990).

The sinkholes and springs represent fragmented habitats. Habitat fragmentation and hence isolation of populations, especially when combined with low population numbers predisposes them to the deleterious effects listed above. Significant abstraction of water from the sinkholes and springs would cause bottlenecks due to possible periodic drying up of these water bodies or sections thereof. In the Portuguese nase, *Chondrostoma lusitanicum* (Collares-Pereira 1980), a small cyprinid that inhabits shallow streams of medium flow, low intra-population variation and high levels of divergence between isolated populations have been attributed to bottlenecks caused by significant reductions and fluctuations in the level of water in the water bodies inhabited by the fish (Coelho *et al.*, 1997). The *T. sparrmanii* populations therefore represent an important component of South Africa's biodiversity that may be at risk to ecological and genetic integrity compromised.

A decrease in genetic diversity among fragmented populations has been documented (Lacy, 1987; Van Dongen *et al.*, 1998; Pringle, 1997) and it has been pointed out that such loss of genetic diversity can lead to a reduction in fitness and even local extinction (Saccheri *et al.*, 1998). Furthermore, genetically homogeneous populations may exhibit reduced performance in the long term. It has been shown that heterozygous individuals perform better in fitness parameters such as survival, disease resistance, growth and physiological efficiency (Allendorf & Leary, 1986). In the Sonoran topminnow, *Poeciliopsis occidentalis occidentalis*, survival, growth, early fecundity and developmental stability were found to be greatest in laboratory reared fish from the most heterozygous natural population studied (Quattro & Vrijenhoek, 1989). These findings emphasize the need to ensure the genetic heterozygosity of populations through maintenance of population numbers that are viable from the point of view genetic variation.

The fluctuations in the population numbers of the *T. sparrmanii* in the sinkholes and springs (Ribbink, 1994, unpub report) may pose a serious threat to the continued existence of these fishes. The threat to *T. sparrmanii* is at a populational rather than at a species level. One of the biggest concerns regarding the conservation status of these fishes is that their habitats are fragmented and are subject to deterioration through overgrazing within the catchment, pollution from recreational and domestic sources as well as the introduction of alien species.

7.3.1 *Ecological threats faced by the T. sparrmanii and T. guinasana populations.*

Skelton (1990) notes that of the six classes of threats to vertebrate species identified by the IUCN, only two (habitat destruction and the effects of introduced species), seriously influence threatened fishes of southern Africa. These two threats (plus various extents of water quality deterioration, hybridization and over-harvesting) are also a reality for the fish populations in the sinkholes and springs, even though they apply at a population rather than species level. They are considered below in the context of the ecosystems under investigation in this study.

Water abstraction

Water abstraction is the greatest threat facing all of the fish fauna in the sinkholes and springs. In the shallower water bodies such as springs, the indigenous cichlid fishes are generally limited in their distribution to the edges of the water bodies, with juveniles and mouth brooding females being found in the shallower warmer waters and the associated reedbed cover (Ribbink *et al.* 1994, unpub). The abstraction of water from these water bodies will then deprive these fishes of their breeding sites and concentrate them in areas where they are vulnerable. These factors may expose the populations to possible extinction as a result of predation and lack of breeding sites.

Habitat degradation

The sinkholes and springs risk habitat degradation through alterations of water flow through the construction of weirs as well as grazing pressure on surrounding vegetation. All these factors have the potential to affect the ecosystems either in concert or in sequence; resulting in detrimental effects such as silting (from soil erosion as a result of overgrazing), degradation of water quality (from water surface usage and inflow of pollutants). Small water bodies are particularly susceptible to the influx of chemicals and nutrients through agricultural, industrial and domestic pollution due to their limited capacity to dilute out these factors.

Many of the sinkholes and springs are either found on private property and/or are surrounding by dwellings, thus predisposing them to these factors. There is evidence that nine out of ten possible extinctions of the cyprinodontiforms could be attributed in part to species inhabiting small pools and springs where the ecology was drastically changed if not entirely destroyed by these habitat degradation (Harrison & Stiassny, 1999). A similar threat faces the fish in the springs and sinkholes, albeit at a population level.

Alien introductions

The introduction of alien plants and animals can be as deleterious as habitat destruction in freshwater ecosystems (Moyle, 1997). Introduced fishes have the potential to affect the "new" environment in several ways that include: habitat changes, introduction of parasites and diseases to which the indigenous fauna have little or no resistance, competition for food with and predation on indigenous species, as well as hybridization if fish are closely related (Taylor *et al.*, 1984; Harrison & Stiassny, 1999). In southern Africa, the impact of introduced alien fish species affects 83% of threatened freshwater fishes and is a major threat to 58% of them (Skelton, 1990). Large mouth bass *Micropterus salmoides* (Lacepide, 1802) has rapidly colonized many South African waterways resulting in severe detrimental effects on the indigenous fish populations (Jubb, 1973; de Moor & Bruton, 1988). The sinkholes and springs have not been immune to this colonization, resulting in certain cases in dramatic declines of the indigenous fish populations. For example, the bass was introduced in 1928 for the purpose of aquaculture and sporting angling (Ribbink *et al.*, 1994, unpub report). Although the fishes in some localities are somewhat protected from the bass by reedbed cover, these alien fish still pose a significant threat to the long term sustainability of the indigenous fish populations. Examples abound worldwide of how introduced fishes have taken over habitats of indigenous fishes. The mosquitofish have over-run endemic tooth-carp in Spain (Garcia-Marin *et al.*, 1990) and the Nile Perch has compromised endemic cichlids in Lake Victoria (Barel *et al.*, 1985), are but a few examples. Once established, introduced fishes become very difficult to eradicate (Lowe-McConnell, 1990).

If these populations were to become extinct it would not be possible to effect recolonization of their habitats with identical populations. Their loss through the threats listed above would be detrimental, and would constitute loss of unique genetic resources.

Meffe (1987) has posed the question of what it is that gets lost when populations or alleles disappear, and whether or not lost genetic diversity can be recouped. The populations of *T. sparrmanii* in the ecosystems considered in this study number only in the several hundreds to several thousands (Ribbink *et al.*, 1994, unpub rep), but they constitute individual breeding populations and gene pools which could be lost for good if they are not protected from the threats they are facing. Leberg (1990) evaluated the effect of a reduction in genetic variation on characteristics of mosquitofish populations under field conditions. He found that a reduction of 25% in genetic variation resulted in a 56% reduction in population size (Leberg, 1990). Granted that mosquito fish may be exhibiting higher levels of sensitivity to heterozygosity reductions, this emphasized the fact that the effects on genetic variation-reducing factors such as inbreeding may be more severe in the field than in the laboratory.

Admittedly, threats to populations and species are usually a complex of factors acting singly, in concert or sequentially, thus compromising the ability of the population or species to cope with any one threat. Combinations of the above threats will predispose the fish populations examined by this study to extinction. The *T. sparrmanii* populations display detectable levels of differentiation that may be related to their adaptation to their unique environment and therefore have important ecological roles within unique but small ecosystems.

7.4 *Speciation modes and the cases of T. sparrmanii and T. guinasana*

Bush (1975) defines speciation as the process of splitting of lineages through which gene flow is sufficiently reduced between sister populations, such as to allow each to become irrevocably committed to different evolutionary paths.

The mechanisms by which speciation occurs are of fundamental interest to biologists as they underpin the entire process of macro-evolution (Shaw *et al.*, 2000). The origin of new species from geographically isolated populations of the same ancestral species (allopatry) is widely accepted, well documented and described (Coyne, 1992; Rice & Hostert, 1993). On the other hand, the alternative process whereby divergence and the formation of new species may occur without geographic isolation (sympatry) has often been dismissed because it has proved intractable theoretically (Dieckmann & Doebeli,1999; Shaw *et al.*, 2000).

The challenges inherent in finding good natural examples of sympatric speciation (Wilson *et al.*, 2000) has raised ongoing debates regarding its significance (Mayr, 1988; Bush,1994). This section does not aim to resolve these conflicts, but simply attempts to locate the genetic structuring found by this study in *T. sparrmanii* populations and in *T. guinasana* in this context.

7.4.1 *Allopatric speciation and Tilapia sparrmanii*

Tilapia sparrmanii populations examined in this study are isolated in a variety of water bodies, namely a sinkhole and several springs. Each of these water bodies is subject to its own complex of selection pressures over a wide geographic region and presents ideal opportunity for classic allopatric speciation. The study however detected only genetic differentiation commensurate with populational sub-division rather than speciation. In the African Great Lakes, allopatric mechanisms of speciation have been suggested to explain the observed rapid speciation and radiation of cichlid fishes (Fryer & Iles, 1972; Ribbink *et al.*, 1983) and these suggestions have been supported by molecular genetic evidence of genetic differentiation levels commensurate with speciation (Van Oppen *et al.*, 1997b; Arnergard *et al.*, 1999; Moran *et al.*, 1994).

Given that the *T. sparrmanii* populations have been isolated in their current habitats for more than 16 000 years and have shown little speciation; while the Great Lakes cichlids have rapidly speciated within several hundred to a few thousand years (Wilson *et al.*, 2000 quote 12 400 years for Lake Victoria), the difference requires some consideration. Similar levels of populational differentiation below the species level was found in highly distributed populations of the riverine cichlid *P. philander* (de Villiers *et al.*, 1992; Twentyman-Jones *et al.*,1997). In explaining the lack of speciation in *P. philander* compared to cichlids of the African lakes, Twentyman-Jones *et al.* (1997) argued that the species was eurytopic and therefore was bound to speciate slowly relative to the stenotopic lacustrine cichlids.

This thesis has invoked the same argument as an explanation for the lack of high levels of genetic differentiation in *T. sparrmanii* (see section 7.2). The eurytopy of *T. sparrmanii*, the small population sizes and shorter separation period are factors that explain the lack of speciation in the populations examined by this study.

7.4.2 *T. guinasana and sympatric speciation*

The case of *T. guinasana* presents a potential scenario for the existence of sympatric speciation (evolution of intrinsic barriers to gene flow in the absence of extrinsic barriers). *Tilapia guinasana* exists in the Guinas sinkhole as a single population that exhibits five distinct colour polymorphs that are non-sex-limited, within what is regarded as the smallest area of endemicity for any known Tilapia (Ribbink *et al.*, 1991). This polychromatism is exhibited (except for the light blue form) at sexual maturity. There are no physical barriers to gene flow within the sinkhole and Ribbink *et al.* (1991) did not report any habitat preferences associated with mating behaviour. What they noted was that *T. guinasana*'s colour polymorphs exhibited significant levels of assortative mating (Ribbink *et al.*, 1991) and these were later shown to be correlated with significant levels of genetic distinction (Nxomani *et al.*, 1999).

A similar phenomenon has been observed in the Neotropical Midas cichlid *Amphilophus citrinellum* wherein *A. citrinellum* individuals start out as normal protectively coloured black-and-white striped juveniles. Some of the normal morphs assume a gold colour during their ontogeny and such a colour assumption is not sex limited, while others only assume the gold colour upon sexual maturation (Wilson *et al.*, 2000). Wilson *et al.* (2000) showed that the assortative mating was correlated with genetic structuring of populations on the basis of colouration. In both cases (*T. guinasana* and *A. citrinellum*) assortative mating appears to play an important role in the divergence within the populations concerned.

Assortative mating (Kondrashov & Mina, 1986) and resource polymorphism (Meyer, 1993b; Smith & Skulason, 1996) have been suggested as important factors in sympatric speciation. Only the former phenomenon on the basis of colouration has been observed in *T. guinasana* (Ribbink *et al.*, 1991) while both phenomena have been observed in *A. citrinellum,* on the basis of both colouration and trophic polymorphism (Wilson *et al.*, 2000). Non-random mating (as is the consequence of assortative mating, this author's emphasis) is a prerequisite for sympatric speciation (Kondrashov & Shpak, 1998). In order for sympatric speciation to be complete, there has to be selection against and elimination of the intermediate genotype, and non-random mating alone is capable of eliminating the intermediate genotype even in the absence of selection (Kondrashov & Shpak, 1998).

Even though the assortative mating in *T. guinasana* was not complete, Ribbink *et al.* (1991) observed only discrete colour polymorphs and no intermediate colouration in the Guinas sinkhole. This possibly indicates lack of fitness of hybrids produced from inter-colour matings thus implying the beginnings of sympatric speciation, with assortative mating being the driver thereof.

This scenario is supported by observations and molecular genetic data in *A citrinellum* (Wilson *et al.*, 2000). In all four lakes from which they sampled the colour polymorphs of *A. ctrinellum* (lakes Nicaragua, Apoyo, Jiloa and Masaya) they observed heterozygote deficiencies. They took this to suggest that non-random mating was having a significant impact on the genetic structuring of *A citrinellum* in Nicaraguan lakes, thereby promoting species-level divergence through sympatric speciation (Wilson *et al.*, 2000). The polychromatism of *T. guinasana* appears therefore to indicate the incipient stages of a sympatric speciation process that is being driven by assortative mating. The origin of the assortative mating remains a matter of speculation.

7.5 Recommendations and scenarios for conservation and management of the *T. sparrmanii* populations and *T. guinasana*.

The primary focus of (fish) conservation genetics is the maintenance of the capacity of fishes to genetically adapt or evolve, in the face of environmental changes. Conservation programmes therefore of necessity aim to maintain the capacity of organisms to evolve (Meffe, 1986). Addressed in combination, these factors become critical for the maintenance of species in perpetuity (Frankel & Soulé, 1981). Genetic variation is the outcome of long periods of evolution and constitutes the evolutionary legacy of a species. In this sense, understanding the population genetic structure of a species assists in the formulation of a genetically rational conservation programme (Garcia-Marin *et al.*, 1990)

Extensive, non-invasive molecular assessment of genetic diversity within and between the populations of *T. sparrmanii* and their comparison to populations from other localities, is urgently required. Particular focus needs to be on the assessment of variability within the species in the rest of its distribution. Populations from the Orange/Vaal and/or Limpopo river systems would strengthen the value and strength of the analyses.

It has been suggested that population comparisons including those from other parts of the species' distribution could provide a bench-mark against which to understand the levels of isolation and variability within the systems examined in this study. Results obtained from these studies would then provide information as to the most appropriate mechanisms for conservation and management of the populations. If such results indicate high levels of genetic distinction between the sinkhole and spring populations from riverine or other *T. sparrmanii* populations from elsewhere, captive propagation may be essential.

If not, then they should be conserved as resources important for the maintenance of genetic diversity of the species. Such an approach will also be consistent with the new paradigm of conserving not only species but populations, subspecies and the ecosystems they inhabit (Meffe, 1987). Using allozyme electrophoresis for example, it is possible to determine the level of genetic diversity and see whether it lies between or within populations (Meffe, 1987). This information could be used to found captive populations for complementation of the numbers of fish in the sinkholes, in terms of ensuring that these "new colonists" would be representative of the gene pool of the *T. sparrmanii*.

If allozyme electrophoresis indicates greater within-population genetic diversity, these founding members can be obtained solely from any one of the populations. If on the other hand, the genetic diversity is found to be higher between populations, then the founding members must be obtained from as many sources as possible in order to maintain that genetic integrity (Carvalho & Hauser, 1994). In this manner levels of genetic diversity would be maximised in the founding/captive population and the potential for local adaptation may in this way be possible to assess (Nelson & Soule, 1987). Apart from allozyme electrophoresis, any techniques or methods that allow the measurement of heterozygosity in populations and at a single locus would be appropriate for this analysis. Any further assessment of the genetic variability of these populations should take this recommendation into serious consideration.

This study was preceded by one that looked at allopatric populations of *P. philander* in similar environments as the *T. sparrmanii* populations (Twentyman-Jones, 1992). That study found that the populations of *P. philander* were morphologically and genetically different and were unique and worthy of conservation.

Most important however, was the finding that captive propagation of threatened fish populations and the release of captive bred individuals into their natural habitat can lead to the recovery of that population (Ribbink & Twentyman-Jones, 1989). Lessons learned from that study will be instructive in attempts to map out conservation scenarios for the *T. sparrmanii* populations.

One such lesson is that where captive propagation is used to restock diminishing populations, the problem which was responsible for the reduction of the population in the first place should be removed before reintroduction of captive bred fish into their natural habitat. In the face of fluctuations in population numbers as a result of water abstraction and alien introductions, this is a significant lesson that must be heeded. As Carvalho (1993) put it, it is not only the maintenance or conservation of genetic diversity that is important, but the actual preservation of those genetic resources. The emphasis on the quantity of that diversity should not fool people into thinking that it is possible to recoup or compensate for losses. What is important is the employment of integrated management strategies that combine continual assessment of genetic diversity, measures to preserve it and a global approach to the implications of evolutionary processes which will be impacted upon by all such measures, no matter how good or well intentioned they may be.

Breeding experiments of the colour forms of *T. guinasana* as a means to determine its genetic basis have failed to yield any positive results in the past (A. Ribbink, personal communication), but they are the most appropriate way of determining the heritability or otherwise of the polychromatism and perhaps may lead to identification of the genetic characters that encode it. In such experiments the role of the assortative mating should be investigated. The entire species is in danger of being lost unless specific conservation measures are taken to protect the fish and its habitats. One such measure is a comprehensive study of its biology and ecology (Skelton, 1990) in order to gain a sound understanding of the species and its interactions with the ecosystem that it inhabits.

Meffe and Vrijenhoek (1988) have emphasized the need to incorporate experimental studies of population genetics and fitness into management of endangered fishes, in order to better predict the long term success and survival of remaining populations.

Given the fact the species has the smallest area of endemicity for any known Tilapia (Ribbink *et al.*, 1991) and the hazards associated with environmental pollution of the sinkhole and water abstraction; it comes as no surprise that it was listed in as critically endangered (IUCN Red List; Baillie & Groombridge, 1996). Its status should remain exactly thus, until conditions change in the long-term. The observed genetic differentiation between the conspecific *T. sparrmanii* populations on the one hand, and the *T. guinasana* colour forms on the other, require that those charged with the management of these populations adopt conservative strategies in their protection. Each *T. sparrmanii* population might therefore be unique, having changed phenotypically and genetically from the parental population (A. Ribbink, personal communication). They should be maintained as separate populations as they contribute to southern Africa's biodiversity and may lay the foundation to the development of new species.

It would be unwise to allow random and unmonitored interference with these populations. Equally unwise would be any step to effect withdrawal of the water from the sinkholes and springs, as this might result in the destruction of an initial process of differentiation / speciation. Total withdrawal of water would certainly lead to extinction of these fishes. They are clearly unique and must be conserved as part of the country's heritage. Specific recommendations for the conservation and management of the fish populations and the ecosystems they inhabit are as follows:

(a) That this study has shown significant levels of genetic differentiation between the populations, and they and the habitats and ecosystems that support them should be protected from all perturbations by human interference, especially movement of fish from one location to another.

(b) That any changes in the landscape surrounding these populations should be monitored as this may lead to extermination of the small populations by the introduction of toxic substances from run-offs.

(c) That the fish and their habitats/ecosystems should be the subject of ongoing research and investigation (using non-invasive techniques) to gain a more thorough understanding and knowledge of the different aspects and dynamics that obtain therein. Integral to that investigation should be a thorough assessment of the temporal and spatial distribution of genetic variation in both species. This will go a long way in shaping a long-term conservation management strategy for these and other similar situations. Prudent and informed decisions affecting the future of a species cannot be made in the absence of a basic knowledge of the amount and distribution of its genetic variation (Meffe, 1987). For the *T. sparrmanii* and *T. guinasana* populations, this study is the beginning and it should be built upon.

(d) That all alien fish species be removed from these systems and no further introductions should be allowed, as they are one of the principal drivers of extirpation of populations and species.

(e) That proactive management programmes suggested above are urgently required to ameliorate the anthropogenic effects at all the sites investigated.

7.6 Conclusion

This study set out to answer the following questions, which are expressed by paraphrasing the objectives outlined at the beginning:

1. Are the populations of *T. sparrmanii* found in the sinkholes and springs of the North West Province of South Africa genetically distinct?

Using RAPD fingerprinting it has been demonstrated by this study that these populations are genetically distinct. The study was in general agreement with morphological findings by Greenwood (unpub. Rep). Greenwood found that the Molopo Oog population exhibited characteristics not shared by the other populations, and that the Marico Oog population resembled the Wondergat population. RAPD fingerprinting showed the Wondergat and the Marico Oog populations to be more closely related to each other and to be the most divergent of the populations. Although indicated as closer to the Amalinda population (a possible indication that this population comes from the Orange river system), the Molopo Oog population was shown as significantly distinct from the others by the RAPD study.

The genetic distance values obtained indicate that the various populations of *T. sparrmanii* are becoing genetically differentiated. Much as it would be presumptuous to accord them subspecies status, this study demonstrated statistically significant genetic distinction of these populations, and recommends that (from a conservation / management point of view) they should be treated as such.

SDS-PAGE and mitochondrial DNA analysis of the populations failed to detect inter-population differences. This result indicates that the populations may have been a single panmictic population during wetter periods in their history, and have only been recently separated / isolated. The differentiation detected by the RAPD fingerprinting study therefore may be indicator of local adaptation by the populations to their environments.

This study represents the first examination of the genetic architecture of these populations. de Villiers *et al.* (1992) examined mitochondrial DNA restriction enzyme variation of populations of *P. philander* inhabiting similar environments, and Twentyman-Jones (1992) investigated similar populations of *P. philander* mostly from a morphological perspective.

2. Is *T. guinasana* genetically distinct from *T. sparrmanii* and what is the species status of the former?

Using a subset of three oligonucleotide primers to analyse fingerprint patterns of the two species this study demonstrated that *T. guinasana* is significantly ($P = 0.05$) genetically distinct from *T. sparrmanii*. The average genetic distance separating the two Tilapia fishes was found to be in line with species-level differences (see Bardakci & Skibinski, 1994). This served to confirm the separate species status of *T. guinasana* as held by Greenwood (1992) based on morphological examination of the two fishes and by Oosthuizen *et al.* (1993) using allozyme data. SDS-PAGE analysis also indicated differences between *T. guinasana* and *T. sparrmanii*. The failure of TGGE analysis to distinguish the two species can be ascribed to the sensitivity of the techniques used in the study, rather than true lack of distinction between the as they are formally recognized as separate species.

3. Are the colour polymorphs of *T. guinasana* genetically distinct?

The colour forms of *T. guinasana* were shown in this study using RAPD fingerprinting with four decamers to be genetically distinct from each other (Nxomani *et al.*, 1999). The colour polymorphism in conjunction with the assortative mating observed in the species (and any selective pressures exerted by the environment) may play a complimentary and facilitative role in speeding up this process. This study represents the first contribution from a genetic perspective towards the understanding of the origin and extent of the colour polymorphism of *T. guinasana*.

4. What conservation measures can be taken in conserving the genetic diversity of these isolated and threatened populations?

Because we do not know what exactly the role of biodiversity is in the normal functioning of biological communities and ecosystems; nor do we know what the required minimum diversity is for the functioning of organisms and their ecosystems (Solbrig, 1991), whatever detectable diversity there is should be protected in the best way possible. This is the attitude that should be adopted towards the conspecific populations examined by this study and the ecosystems that they inhabit. Meffe (1987) considers this a "conservative approach to conservation genetics". Conservation of genetic diversity that is neither beneficial nor harmful to the organisms involved is far better than the potential loss of a population or a species. Through adoption of this conservative approach, the probability of inflicting irreparable harm to systems that we do not fully understand is minimized (Meffe, 1987). Specific recommendations have been outlined in section 7.4)

APPENDICES:

APPENDIX A. Data and similarity matrix tables for RAPD fingerprint data.

Table A1. Data and similarity matrix from fingerprints generated with primer 1 for each of the populations of *T. sparrmanii*.

24 26

MO11	11111111011111111011111000	1.000
MO3	11111111011111111011111000	1.000 1.000
MO12	11111111011111111011111000	1.000 1.000
MO14	11111111011111111011110000	0.962 0.962 0.962 1.000
KK34	11111111011111111011101100	1.000
KK12	11111111011111011011101100	0.962 1.000
KK16	11111111011111111011101100	1.000 0.962 1.000
KK3	11111111011111111011101100	1.000 0.962 1.000 1.000
A22	11111111011111111111101100	1.000
A16	11111111011111111111101101	1.000 0.960
A21	11111111011111111111101100	1.000 0.960 1.000
A24	11111111011111111111101100	1.000 0.960 0.960 1.000
MAO3	11111111011111111111101100	1.000
MAO19	11111111011111111111101000	0.960 1.000
MA09	11111111011111111111101000	0.960 1.000 1.000
MAO6	11111111011111011111101000	0.920 0.960 0.960 1.000
MA25	11111111011111111111110100	1.000
MA22	11111111011111111011111100	0.920 1.000
MA23	11111111011111111011111100	0.920 1.000 1.000
MA17	11111111011111111111101100	0.890 0.920 0.920 1.000
W12	11111111111111111111111110	1.000
W16	11111111111111111111111100	0.960 1.000
W18	11111111111111011111111100	0.920 0.960 1.000
W22	11111111111111011111111100	0.890 0.920 0.920 1.000

Table A2. Data and similarity matrix from fingerprints generated with primer 2 for each of the populations of *T. sparrmanii*.

MAO9	11101110111011111111111	1.000
MAO19	11101110111011111111111	1.000 1.000
MAO3	11101110111011111111111	1.000 1.000 1.000
MAO6	11101110111011111111111	1.000 1.000 1.000 1.000
MO12	11100110110000101111011	1.000
MO3	11101110111001011111011	0.875 1.000
MO14	11101110110001011011011	0.875 0.916 1.000
MO11	11101110111001011011111	0.792 0.917 0.917 1.000
A24	11100110111011111111111	1.000
A22	11100110111001111111011	0.912 1.000
A21	11100110110001111111011	0.875 0.958 1.000
A16	11100110110101111111011	0.833 0.875 0.958 1.000
W12	11101110110011101111111	1.000
W16	11101110110011101111111	1.000 1.000
W18	11101110110011101111111	1.000 1.000 1.000
W22	11101110110011101111111	1.000 1.000 1.000 1.000
KK12	11011110110011101110111	1.000
KK16	11011111110011101110111	0.958 1.000
KK34	11011111110011101110111	0.958 1.000 1.000
KK3	11011110110011101110111	1.000 0.958 0.958 1.000
MA17	11001110111011011111011	1.000
MA25	11001110111011011111011	1.000 1.000
MA23	11001110011011011111011	0.958 0.958 1.000
MA22	11001110011011011111011	1.000 0.958 0.958 1.000

Table A3. Data and similarity matrices
from fingerprints generated by primer 3
for each of the *T. sparrmanii* populations

A19*	11000001110100000000000000000	1.000
A20	01000001010100000100000000000	0.893 1.000
A19	11000001010000000000000000000	0.928 0.893 1.000
A16	11110001110111000100000000000	0.821 0.786 0.750 1.000
A22	01010001110111010100000000000	0.786 0.821 0.714 0.893 1.000
A26	11110001110111010100000000000	0.750 0.750 0.714 0.929 0.893 1.000
A21	11010001110110000100000000000	0.893 0.857 0.821 0.929 0.893 0.893 1.000
MA30	01000001110111001101001011 01	1.000
MA25	01000001010111001101000011 01	0.929 1.000
MA22	01000001010111000101000011 01	0.893 0.964 1.000
MA23	00000001110111001101001011 01	0.964 0.893 0.857 1.000
MA17	11000011110111001101000011 00	0.857 0.857 0.821 0.821 1.000
MA20	00000001111111001101001011 01	0.928 0.857 0.821 0.964 0.876 1.000
MA26	01000001111111001101001011 01	0.893 0.929 0.857 0.929 0.857 0.786 1.000
MO5	10000001110111001110000011 00	1.000
MO12	11100001110111001101000010 00	0.821 1.000
MO3	10000001110111001101000000 00	0.857 0.893 1.000
MO10	11000001110111001101000000 00	0.893 0.821 0.964 1.000
MO11-2	10000001111110110101001011 01	0.679 0.643 0.714 0.643 1.000
M11	01000001110111001101001011 00	0.929 0.857 0.821 0.857 0.714 1.000
MO14	01000001110111001101001011 00	0.857 0.857 0.821 0.857 0.750 1.000 1.000
KK34	01000001110111001101001011 00	1.000
KK8	01000001110101001101000000 00	0.857 1.000
KK16	01000001110101001101000000 00	0.857 1.000 1.000
MAO8	01000001110111001101000011 00	1.000
MAO10	01000001110111001101000011 00	1.000 1.000

Table A3 continued

MAO7	0100000111010000110100001100	0.929 0.929 1.000
MAO6	0000000111000000010100001100	0.821 0.892 0.892 1.000
MAO12	0100000111011100110100001100	1.000 1.000 1.000 0.821 1.000
MAO4	0100000111011100110100000100	0.964 0.964 0.928 0.857 0.964 1.000
MA9	0100000111011100110101000000	0.893 0.893 0.821 0.714 0.893 0.928 1.000
MAO3	0011110111011111110101001100	0.786 0.786 0.679 0.607 0.678 0.678 0.714 1.000
W9	0000110110111011111110100001	1.000
W15	0100010111011000110100001101	0.876 1.000
W22	0110010111011101110100001100	0.929 0.857 1.000
W8	0010111111111101110100001100	0.857 0.821 0.857 1.000
W12	0010011111011101110100001101	0.821 0.893 0.929 0.893 1.000
W20	0110111111111101110100001100	0.678 0.786 0.893 0.929 0.857 1.000
W14	0000000111110000110000011100	0.714 0.750 0.679 0.679 0.678 0.642 1.000
LB1	0000011010110000000100011010	1.000
SB1	0000110011010011110000010000	0.607 1.000
OS13	0100110111010000110000010000	0.607 0.857 1.000

Table A4. Data and similarity matrices from fingerprints generated by primer 4 for each of the *T. sparrmanii* populations.

47 27

KK34	11110001111111001011011011	1.000
KK19	11111001111111001011000011	0.889 1.000
KK3	11111001111111001011000011	0.889 1.000 1.000
KK6	11111001111111001011000011	0.926 1.000 1.000 1.000
KK16	11111001111111001011000011	0.889 1.000 1.000 1.000 1.000
KK12	11111001111111001011000011	0.889 1.000 1.000 1.000 1.000
KK14	11111001111111001011000011	0.889 1.000 1.000 1.000 1.000 1.000
MO12	11111001111111001011000011	1.000
MO8	11111001111111001111000011	0.963 1.000
MO10	11111001111111001111000011	0.963 1.000 1.000
MO14	11111001111111001111000011	0.963 1.000 1,000 1,000
MO3	11111001111111001111000011	0.963 1.000 1.000 1.000 1.000
MO11-2	11111001000011001111000011	0.852 0.852 0.852 0.852 0.852 1.000
MO4	11111001100011001011000011	0.889 0.852 0.852 0.852 0.852 0.9261.000
MAO1	11111001111111001111110111	1.000
MAO4	11111001111111001111110111	1.000 1.000
MAO3	11111001111111001111110111	1.000 1.000 1.000
MAO9	11111001111111001111110111	1.000 1,000 1,000 1.000
MAO10	11111001111111001111110111	1.000 1.000 1.000 1.000 1.000
MAO7	11111001111111001111110111	1.000 1.000 1,000 1,000 1,000 1,000
MAO6	11111001111111001111111111	0.963 0.963 0.963 0.963 0.963 0.963 1.000
A19	11111001110011001111111111	1.000
A21	11111001101111001111111011	0.852 1.000
A26	11111001100011001111011011	0.889 0.889 1.000
A24	11111001101111001111011011	0.815 0.963 0.926 1.000
A16	11111001101111001011011011	0.778 0.889 0.889 0.926 1.000

Table A4 continued

A22	11111001100011100101100l011	0.815 0.815 0.926 0.852 0.889 1.000
A20	11111001111111100111100l011	0.815 0.889 0.852 0.926 0.889 0.815 1.000
MA25	11111101111111100111100l011	1.000
MA22	11111101111111100111000011	0.963 1.000
MA23	11111101111111100111000011	0.963 1.000 1.000
MA30	11111101110011100111010011	0.852 0.889 0.889 1.000
MA20	11111001111111100111010011	0.889 0.926 0.926 0.889 1.000
MA17	11111001110011100111010011	0.815 0,852 0.852 0.852 0.926 1.000
MA26	11111001111111100111010011	0.926 0.926 0.926 0.852 1.000 0.926 1.000
W18	11111001100111100001101011l	1.000
W9	11111001100111100001101011l	1.000 1.000
W17	11111001100111100101101001l	0.926 0.926 1.000
W22	11111001100111100101100001l	0.889 0.889 0.926 1.000
W20	11111001100111100001100101l	0.889 0.889 0.889 0.926 1.000
W16	11111001100111100001100101l	0.889 0.926 0.926 0.926 1.000 1.000
W15	11110010000010110101101101l	0.519 0.556 0.740 0.667 0.667 0.667 1.000
LB5	11110010000010110101110011l	1.000
BS12	11110010000010110111110111l	0.926 1.000
OS17	11111010000010111011101111	0.889 0.889 1.000
O4	11111010000010111111101111	0.926 0.889 0.926 1.000

Table A5. Data and similarity matrices from fingerprints generated by primer 6 for each of the populations of *T. sparrmanii*.

KK6	10011100010110000000000010000011	1.000
KK1	10011100010000000000100010000010	0.857 1.000
KK19	10010100000101000111000011000011	0.714 0.714 1.000
KK13	10011101011101000110100011000111	0.679 0.607 0.750 1.000
KK9	10111101010101010000011011100011	0.679 0.607 0.607 0.714 1.000
KK14	10111001011101010000000011100010	0.643 0.607 0.571 0.679 0.821 1.000
KK8	10000001011000000000000011000010	0.679 0.750 0.607 0.643 0.571 0.750 1.000
W17	10000100110100000100001111100011	1.000
W11	10000000000100000101000100000010	0.714 1.000
W7	10010000000111000100000100011011	0.607 0.750 1.000
W8	11010000001010000010000000010011	0.464 0.607 0.642 1.000
W4	10010001001100000010000000000000	0.500 0.714 0.607 0.750 1.000
W1	10011100111101111010100111100111	0.571 0.321 0.393 0.357 0.357 1.000
W9	10011100111101111011101111100111	0.571 0.357 0.286 0.321 0.929 0.929 1.000
MA22	10011100111101111011100111101111	1.000
MA26	10011100111101111111111111101111	0.893 1.000
MA20	10011100111101111111111111101111	0.893 1.000 1.000
MA25	10011101011101111111101111100111	0.857 0.857 0.857 1.000
MA23	10011101011101111101100111100111	0.821 0.786 0.786 0.929 1.000
MA24	10011100111101111001100111000111	0.893 0.857 0.786 0.786 0.857 1.000
MA30	10011100001101111010100111000111	0.821 0.679 0.714 0.786 0.786 0.856 1.000
MAO4	10011100011101111001100111000110	1.000
MAO8	10011100001101111011100111000111	0.821 1.000
MAO6	10011100011101111011100111000111	0.929 0.964 1.000
MAO9	10011100011101111001100111000111	0.964 0.929 0.964 1.000
MAO11	10011100111101111010100100000111	0.821 0.857 0.893 0.857 1.000

Table A5 continued

MAO3	10011100001101111010000010000110	0.821 0.857 0.821 0.786 0.857 1.000
MAO10	10011100001101111000000010000111	0.821 0.857 0.786 0.857 0.857 0.929 1.000
MO12	11011100001100111001000010000111	1.000
MO2	10010100011100010001111010110111	0.678 1.000
MO10	10011100001100011111110010000111	0.821 0.714 1.000
MO11-2	10011100001100011011110010000111	0.857 0.786 0.964 1.000
MO6	10010100111101011001000110000111	0.750 0.714 0.714 0.786 1.000
A26	10010100111101011011000110000111	1.000
A22	10010100101101010001000110000111	0.893 1.000
A24	10010100101101110001000110000111	0.857 0.964 1.000
A20	10010100101101110001000100000111	0.857 0.964 0.964 1.000
A19*	10010100101101110001000100000111	0.821 0.929 0.964 1.000 1.000
O3	10011100100101110100001000100111	1.000
SB3	10011100000000000000000000000000	0.607 1.000
OS17	10011111000101010000000010000000	0.607 0.786 1.000
LB2	10011110001101000001100000000001	0.571 0.750 0.750 1.000
DB10	10011010001100000001100000000010	0.500 0.750 0.679 0.857 1.000

Table A6. Data and similarity matrices from fingerprints generated by Primer 7 for each of the populations of *T. sparrmanii*.

24 44

MAO12	11111100111011000111111111110111101010001111	1.000
MAO10	11111100111011000111111111110111101010001111	1.000 1.000
MAO19	11111100111001000111111111110111101010001111	0.977 0.977 1.000
MAO11	11111100111001000111111111110111101010001111	0.977 0.977 1.000 1.000
MA23	11010100111111100011111010110110110010001111	1.000
MA17	11010110100011100101111111110111000000001111	0.927 1.000
MA25	11111011101010010110111111110111100010001111	0.636 0.727 1.000
MA26	11111001111001010111110101110111101010001111	0.636 0.636 0.818 1.000
KK16	11111100111001000111110000110111101010001110	1.000
KK34	11111100101001000111110101110111101010001111	0.932 1.000
KK12	11111101111001000111110000110111101010001110	0.977 0.909 1.000
KK18	11111111111011000111110101110111101010001110	0.886 0.909 0.909 1.000
WON18	11111011101010000111110101110111101010001111	1.000
WON16	11111011101010010011010101010111100010001111	0.886 1.000
WON19	11111011101010010111110101011111101110101111	0.886 0.864 1.000
WON12	11110011001110100101010101011110100110001111	0.750 0.818 0.772 1.000
AMA24	11111111111101000111110101111110101010001111	1.000
AMA21	11011011110011001011111111111110101111011111	0.727 1.000
AMA20	11111010111011000110110101111110101010000111	0.864 0.727 1.000
AMA18	11111010110011100111110101110111101010001110	0.795 0.705 0.841 1.000
MO6	11111010111001100111110101110111101010001111	1.000
MO8	11111010111001110111110101110111101010001111	0.977 1.000
MO3	11111010111001110011010101010111101010001111	0.909 0.932 1.000
MO4	11111010111001110011010101010111101010001111	0.909 0.932 1.000 1.000

Table A7. Data and similarity matrices from fingerprints generated by primer 10 for each of the populations of *T. sparrmanii*.

24 13

KK12	1111111011110	1.000
KK34	1111111011111	0.923 1.000
KK18	1111111011111	0.923 1.000 1.000
KK16	1111111011110	1.000 0.923 1.000
MO4	1111111011110	1.000
MO12	1111111011110	1.000 1.000
MO5	1111111011111	0.923 0.923 1.000
MO8	1111111011111	0.923 0.923 0.923 1.000
MA23	1111111011111	1.000
MA26	1111111011111	1.000 1.000
MA25	1111111011110	0.923 0.923 1.000
MA17	1111111011110	0.923 0.923 1.000 1.000
MAO12	1111111011110	1.000
MAO11	1111111011111	0.923 1.000
MAO3	1111111011110	1.000 0.923 1.000
MAO9	1111111011111	0.923 1.000 0.923 1.000
W18	1110111011111	1.000
W16	1000110011110	0.692 1.000
W22	1000010111110	0.538 0.846 1.000
W12	0011010100111	0.385 0.385 0.538 1.000
A24	0011111011110	1.000
A21	1111111011111	0.769 1.000
A22	1111111011110	0.846 0.923 1.000
A20	1111111111111	0.692 0.923 0.846 1.000

Table A8. Data and similarity matrices from fingerprints generated by primer 11 for each of the populations of *T. sparrmanii*.

41 20

MAO10	11111011111011001001	1.000
MA09	11111011111011001001	1.000 1.000
MAO3	11111011111011001001	1.000 1.000 1.000
MAO7	11111011111011001001	1.000 1.000 1.000 1.000
MAO19	11111011111011001001	1.000 1.000 1.000 1.000 1.000
MA012	11111010111011011001	1.000 1.000 1.000 1.000 1.000 1.000
MAO6	11111010111011011001	1.000 1.000 1.000 1.000 1.000 1.000 1.000
MA30	11111110111011111001	1.000
MA28	11111110110011111011	0.900 1.000
MA26	11111110111011111011	0.960 0.950 1.000
MA17	11111110111011111001	0.950 0.950 1.000 1.000
MA22	11111110111011111001	1.000 0.900 0.950 0.950 1.000
MA23	11101110111011111011	0.900 0.900 0.900 0.950 0.950 1.000
MA25	11101111111011111011	0.900 0.900 0.950 0.950 0.900 0.850 1.000
KK12	11101101101011011001	1.000
KK14	11111101001011001000	0.800 1.000
KK19	11111010101011001001	0.600 0.800 1.000
KK34	11111011101011011001	0.650 0.750 0.750 1.000
KK6	11111011101011001001	0.700 0.800 0.800 0.950 1.000
KK16	11111011101011011001	0.700 0.800 0.800 0.950 1.000 1.000
A28	11111010101011011001	1.000
A26	11111010110011011001	0.900 1.000
A22	11111110110011111001	0.800 0.900 1.000
A16	11111110111011111001	0.850 0.850 0.950 1.000
A20	11111110111011111001	0.850 0.858 0.950 1.000 1.000
A24	11111110011011111001	0.800 0.800 0.900 0.950 0.950 1.000

Table A8 continued

A21	11111110110010001001	0.750 0.850 0.850 0.800 0.800 0.750 1.000
W1	11101110110011111001	1.000
W11	01101000000001101001	0.650 1.000
W9	00000000000000101001	0.450 0.800 1.000
W8	00001001000000001001	0.400 0.750 0.850 1.000
W4	01001100000100101001	0.550 0.800 0.750 0.750 1.000
W17	11111110101111011001	0.750 0.500 0.300 0.350 0.500 1.000
W6	11111110111111111001	0.850 0.500 0.300 0.250 0.500 0.900 1.000
MO3	11111110111111101001	1.000
MO10	11111110111111011001	0.900 1.000
MO2	11111110111111111001	0.950 0.950 1.000
MO1	11111110111111111001	0.950 0.950 1.000 1.000
MO11	11111110011110111101	0.800 0.800 0.850 0.850 1.000
MO8	11111110101110111101	0.850 0.800 0.850 0.850 0.900 1.000
MO11-2	11111110111110111001	0.900 1.000 0.950 0.950 0.900 0.900 1.000

Table A9. Data and similarity matrices from fingerprints generated by primer 12 for each of the *T. sparrmanii* populations.

KK13	11110100001110011101000000000001000	1.000
KK5	11110110001110011101000100000011000	0.917 1.000
KK8	11110110001110011101000000000011000	0.944 0.972 1.000
KK9	01110100000110001101000000000001010	0.889 0.833 0.833 1.000
KK4	10110000000110001001000000000001010	0.833 0.750 0.778 0.889 1.000
KK34	01110100000100011010100000000001000	0.806 0.722 0.750 0.833 0.750 1.000
KK2	00110110000100011010100000000001000	0.750 0.722 0.750 0.750 0.750 0.944 1.000
A26	00110110000100011010100000000001000	1.000
A27	00110110000100011010100000000001000	1.000 1.000
A19	00110110000100011010100000000001000	1.000 1.000 1.000
A21	01110110000100011010100000000001000	1.000 1.000 1.000 1.000
A28	01110110000101011010100000000001000	1.000 1.000 1.000 1.000 1.000
A20	01110110000101111010100000000001000	1.000 1.000 1,000 1,000 1,000 1,000
MA23	01110110000101111010100001000001010	1.000
MA30	01110110001100111010100001000001010	0.944 1.000
MA20	01110110001100011010100000000001000	0.861 0.944 1.000
MA25	01110000001100011011100000000001000	0.778 0.806 0.917 1.000

Table A9 continued

MA17	01110000000110011001100000000001000	0.750 0.750 0.833 0.917 1.000
MA28	01110000000110001000100000000001000	0.778 0.750 0.833 0.861 0.944 1.000
MA22	01100100000110001001000000000001000	0.694 0.694 0.750 0.806 0.889 0.889 1.000
MO4	01100100001110001001000000000001000	1.000
MO8	01100100001110011001000000000001000	0.972 1.000
MO6	01000100001100011001000000000001000	0.917 0.944 1.000
MO10	01000100001100011001000000000001000	0.917 0.972 1.000 1.000
MO11	01110000001110011001000000000001000	0.917 0.944 0.889 0.861 1.000
M09	01110100001110011001000000000001000	0.972 0.972 0.917 0.917 0.972 1.000
MO1	01110000001110011001000000000001000	0.917 0.944 0.917 0.889 1.000 0.972 1.000
MAO4	01110100001110011001000000000001000	1.000
MAO10	01110000001110011001000000000001000	0.972 1.000
MAO12	01110000001111011001000100000001000	0.917 0.944 1.000
MAO6	01110100001111011001000100000001000	0.944 0.917 0.944 1.000
MA011	01100100001111111001000100000001000	0.889 0.861 0.972 0.944 1.000
MAO3	01010100101000100100001000001010100	0.667 0.611 0.583 0.611 0.611 1.000
MA08	01100000000110100101100000000000100	0.778 0.806 0.833 0.722 0.722 0.611 1.000
W9	01100000001110010010000100000000101	1.000
W8	01100000001110100001100100100000101	0.833 1.000

Table A9 continued

W11	01000100010000100000010000010010101	0.639 0.639 1.000
W10	01000001001111101000110000010000101	0.694 0.750 0.667 1.000
W6	01001001001111101000110010010000101	0.639 0.694 0.611 0.944 1.000
W12	01000001001111101000110010010000101	0.667 0.722 0.639 0.972 0.944 1.000
W1	01000000001110100000000000000000101	0.861 0.861 0.778 0.778 0.778 0.806 1.000

Table A10. Data and similarity matrices from
fingerprints generated by primer 7
for each of the colour forms of *T. guinasana*.

17 36

DB7	11111110011110000111011010101110000	1.000
DB4	11111110011010000111011010101110010	0.944 1.000
DB8	11100010010111111011011010101111100	0.694 0.639 1.000
DB6	11000000011011000011011001010111000	0.750 0.750 0.750 1.000
O5	10000000011111000011011011111110000	1.000
O2	10000000011110000011011001000111011	0.778 1.000
O1	10000000011110000011011001000111001	0.833 0.944 1.000
O9	10000000011110000011011001100111001	0.944 0.944 0.944 1.000
LB2	11000000010110000011011011100111111	1.000
LB8	11010001110110000111111111111101111	0.750 1.000
LB11	10010001110110000111111111111101111	0.722 0.972 1.000
LB10	10110001110110000111111111111101111	0.944 0.944 0.972 1.000
SB5	10110001110110000111111111011101111	1.000
SB7	10110001110110000111111111010110010 1	0.917 1.000
SB6	11110001110110000111111111000110111 1	0.917 0.889 1.000
SB3	11110001010110000111111111011101111 1	0.072 0.861 0.917 1.000
OS1	11110001110110000111111111011101111 1	1.000

Table A11. Data and similarity matrices from fingerprints generated by primer 12 for each of the colour forms of *T. guinasana*.

13 29

DB10	11111100111001101111111111000	1.000
DB14	11111100111111101111101110000	0.862 1.000
DB8	11111100111011101111111111001	0.931 0.862 1.000
DB15	11011100111001101111101110000	0.897 0.897 0.828 1.000
SB7	01011100111001101111101110000	1.000
SB4	00001100111101101111111111001	0.793 1.000
LB9	00001000101000001111101110001	1.000
O7	00000111011010111101010110111	1.000
O6	00000111011010111111010110111	0.966 1.000
O8	00011100111010000110100011001	0.448 0.483 1.000
O10	11111000111011011111101110101	0.488 0.483 0.586 1.000
OS13	11011000111011011110101111001	1.000
OS17	11111000111001011110101111000	0.897 1.000

Appendix B: Nxomani *et al.* (1994)

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DIFFERENTIATION OF ISOLATED, THREATENED FISH POPULATIONS IN DOLOMITIC WATERS OF THE TRANSVAAL, SOUTH AFRICA, BY POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF TOTAL CELLULAR PROTEINS

C. D. Nxomani, A. J. Ribbink

J. L. B. Smith Institute of Ichthyology, Somerset Street, Private Bag 1015, Grahamstown 6140, Republic of South Africa

&

R. Kirby

Department of Biochemistry and Microbiology, Rhodes University, PO Box 94, Grahamstown 6140, Republic of South Africa

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Abstract

Isolated, small ecosystems associated with springs and sinkholes in semi-arid regions of the western Transvaal, South Africa, are under threat as there is a growing need to tap the dolomitic waters which maintain these systems. Part of a programme to demonstrate that these systems are unique and worthy of conservation is to characterize fish populations genetically and morphologically. The data presented here indicate that the populations of Tilapia sparrmanii *from the Malemani and Molopo Oog springs and the Wondergat sinkhole differ from each other genetically when examined using polyacrylamide gel electrophoresis (PAGE) of total soluble protein. The outgroups in the analysis were* Tilapia guinasana, *a closely related substratum-spawning cichlid endemic in Guinas sinkhole, Namibia, and* Pseudocrenilabrus philander, *a distantly related mouthbrooder of the haplochromine lineage. The findings of this study were (a) that PAGE can distinguish between species and between conspecific populations and (b) that the isolated populations of the dolomitic ecosystems of the western Transvaal are unique and should be conserved.*

Keywords: Cichlidae, *Tilapia*, southern Africa, evolution.

INTRODUCTION

Molecular studies are becoming increasingly valuable in providing the guidance necessary for wise management decisions in conservation. In this paper we test the applicability of a molecular technique to resolving problems in systematics and conservation. The study was conducted to determine whether isolated, small, threatened populations of *Tilapia sparrmanii*, a threatened substratum-spawning cichlid fish in the dolomitic springs and sinkholes of western Transvaal, South Africa, are different from each other. This is a widespread species in southern Africa. Allopatric populations of *T. sparrmanii* show some morphological differentiation (P. H. Greenwood, pers. comm.) suggesting that each population may have unique phenotypic and genetic characteristics. The sinkholes and springs are tiny water bodies (Ribbink *et al.*, 1991) which accommodate small, diminishing populations of fish which are threatened by the growing need to tap the underground waters for agriculture, industry and domestic use in these relatively dry regions.

Out of concern for the apparently unique ecosystems, we were commissioned by the Department of Environment Affairs (South Africa) to characterize the small allopatric fish populations in each water body, the underlying rationale being that if these populations are morphologically and genetically unique, then steps should be taken to ensure that they, and the unique ecosystem to which each of these populations contributes, should be conserved. Polyacrylamide gel electrophoresis (PAGE) of total soluble proteins from muscle tissue was used in this study. PAGE has been used extensively in the classification, grouping and identification of micro-organisms (Kersters & De Ley, 1975; Owen & Jackman, 1982; Vera Cruz *et al.*, 1984; Jackman & Pelczynska, 1986; Qhobela & Claflin, 1988, 1992; Murphy *et al.*, 1990; Qhobela *et al.*, 1991) but despite this and its potential value as an effective tool in systematics and conservation genetics of higher organisms, it has not been used to any extent in the characterization of vertebrates.

In this study PAGE was used as an indicator of the genetic relatedness of isolated conspecific populations of *Tilapia sparrmanii*. The technique involves separa-

tion of proteins with uniform charge according to their molecular weights, in a gel matrix that acts as a molecular sieve. Proteins are reacted with the anionic detergent sodium dodecylsulphate (SDS) to form negatively charged complexes. The binding of the proteins to the SDS denatures and solubilizes them and the complexes form rods of a length roughly proportional to the proteins' molecular weights (Smith, 1984).

MATERIALS AND METHODS

Sample collection

Thirty-five individuals of *T. sparrmanii* were collected from the Amalinda Hatchery, in the eastern Cape. This sample was used as the standard population of *T. sparrmanii*, against which the genetic variation of others was determined. *T. sparrmanii* sample populations of

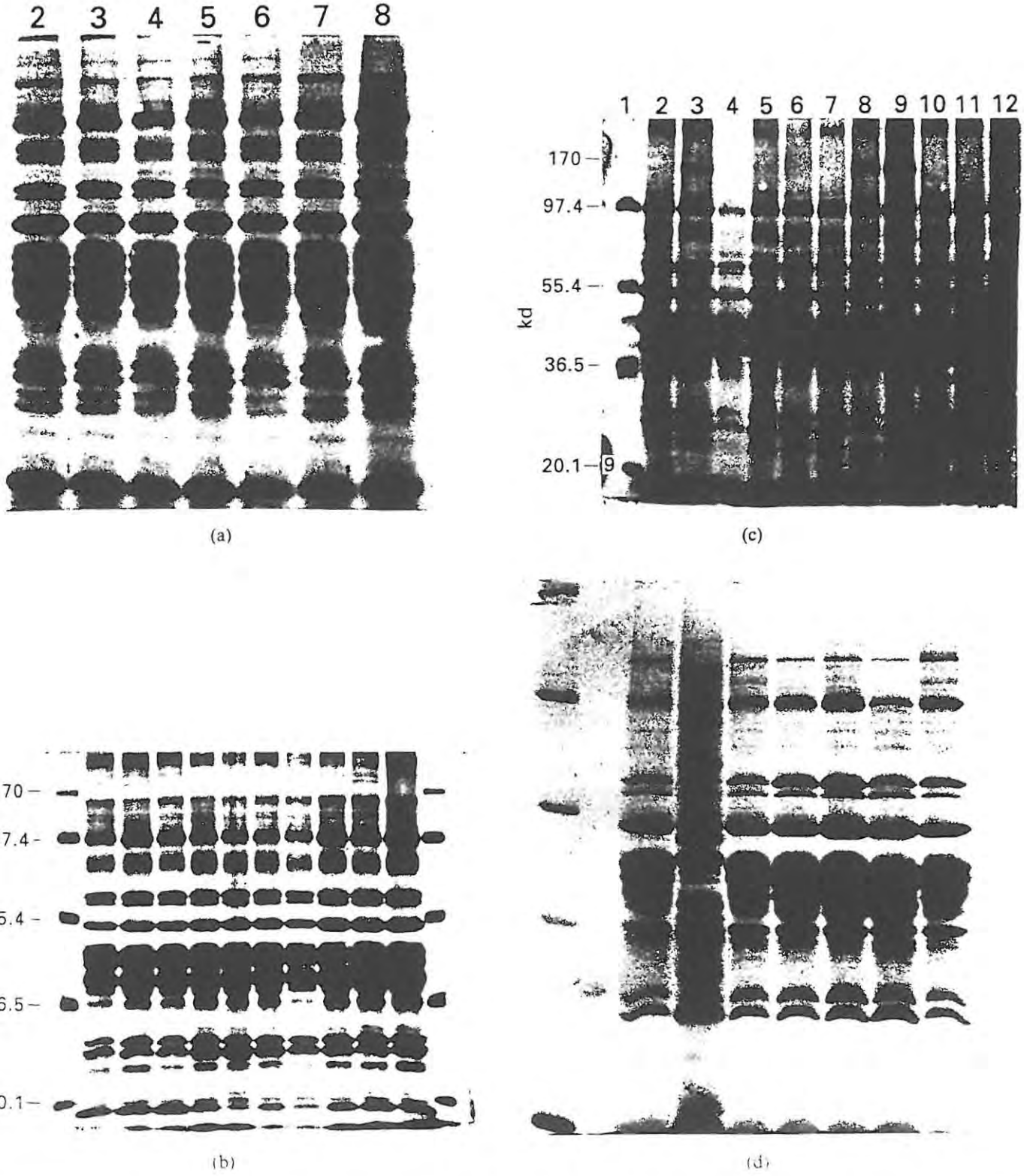


Fig. 1. SDS-PAGE protein patterns of *T. sparrmanii* from (a) Amalinda – protein markers not shown; (b) Wondergat – protein markers in outermost tracks; (c) Malemani – protein markers in first lane; (d) Molopo Oog – protein markers in first lane.

similar size were collected from Wondergat sinkhole, and the Malemani and Molopo Oog springs, in the western Transvaal. Genetic similarity of these was compared with that of the eastern Cape form, using PAGE of total soluble protein from muscle tissue.

The fishes chosen for the outgroup analysis were *Tilapia guinasana*, a species which is endemic to the Guinas sinkhole in Namibia and which is closely related to *T. sparrmanii* (Trewavas, 1936; Ribbink *et al.*, 1991; Greenwood, 1992) and *Pseudocrenilabrus philander*, a distantly related cichlid of the haplochromine lineage.

Sample preparation for PAGE analysis

After immersion in liquid nitrogen fish were stored at -20°C. Approximately 1 g of muscle tissue was crushed with a pestle and mortar in 1 ml of 0.1M phosphate buffer, pH 7.0. The tissue-buffer emulsion was then decanted into 1.5ml Eppendorf tubes and centrifuged for 10 min at 13000 rpm. The supernatant was decanted into fresh tubes and stored at 4°C until used for electrophoresis. The amount of protein in each sample extract was determined using Bradford's method of protein assay (Hammond & Kruger, 1988).

Electrophoresis and analysis of samples

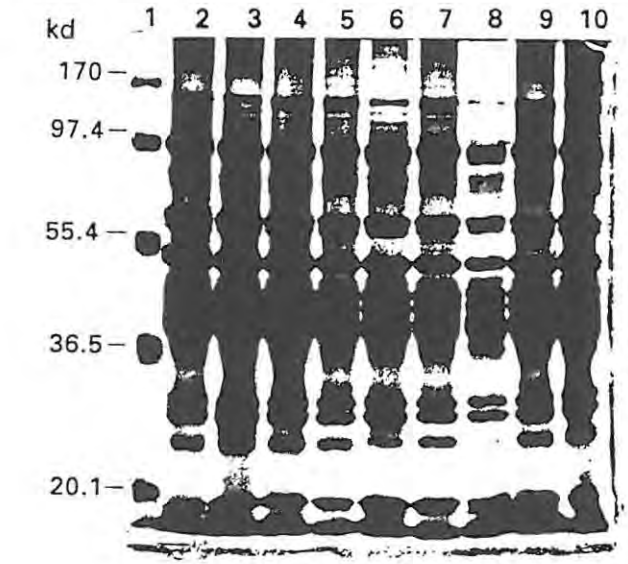
Polyacrylamide slab gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE) was performed on the samples using the buffer system of Laemmli (1970). The protein extract from each sample was loaded on the gel. Protein standards (from Boeringer Mannheim (Germany), Combitek Calibration proteins, Cat. no. 750115(1991)) were included on all gels to facilitate gel-to-gel comparison and determination of molecular weights of the proteins in each sample. The markers were alfa$_2$-macroglobulin, 170 kD; phosphorylase b, 97.4 kD; glutamate dehydrogenase, 55.4 kD; lactate dehydrogenase, 36.5 kD and trypsin inhibitor, 20.1 kD.

The gels were run for 8 h at 150 V with water cooling or at 40 V for 16 h. For visualization of proteins, the gels were stained in 45% methanol, 44.8% distilled water, 10% glacial acetic acid and 0.2% Coomasie Brilliant Blue G-250 (Fluka Chemica, Switzerland) for 12–16 hours. They were destained in 45% methanol, 7% glacial acetic acid and 48% distilled water for 4–6 h. The gels were dried under vacuum for 3 h and then photographed. Protein patterns obtained using PAGE were subjected to numerical analysis using the GelManager program (Version 1.01). This is a fully automated program that involves no manual manipulations of data. It performs pairwise comparisons of protein absorbance profiles, draws similarity matrices and generates dendograms by use of Pearson's product moment correlation coefficient and the Unweighted Pair Group Method with Averages (UPGMA).

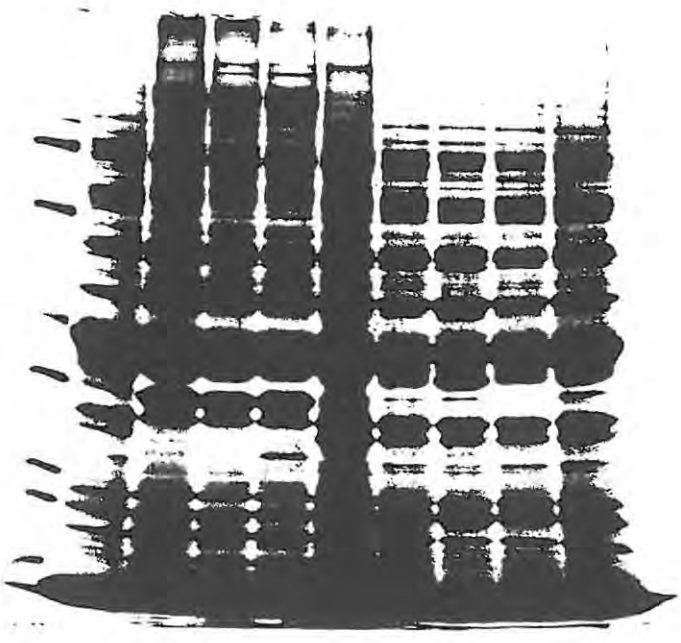
RESULTS AND DISCUSSION

The representative protein banding patterns obtained are shown in Fig. 1(a)–(d) and Fig. 2(a) and (b). Clustering of the populations using the GelManager program is shown in Fig. 3. The dendogram (Fig. 3) shows that the *T. sparrmanii* from Malemani and Amalinda are more closely related to *Tilapia guinasana* than to the other conspecific populations from Wondergat and Molopo Oog. This is an unexpected finding as *T. guinasana* is considered to be a closely related but different species (Trewavas, 1936; Ribbink *et al.*, 1991; Greenwood, 1992) and was used here as an outgroup. This finding suggests that a more thorough examination of the systematics of *T. guinasana* may be necessary.

The Wondergat and Molopo Oog populations are the most distinct, such that on the basis of this study only they could be considered distinct species. The dis-



(a)



(b)

Fig. 2. SDS-PAGE protein patterns of (a) *T. guinasana*—protein markers in first lane; (b) *P. philander*—protein markers in first lane.

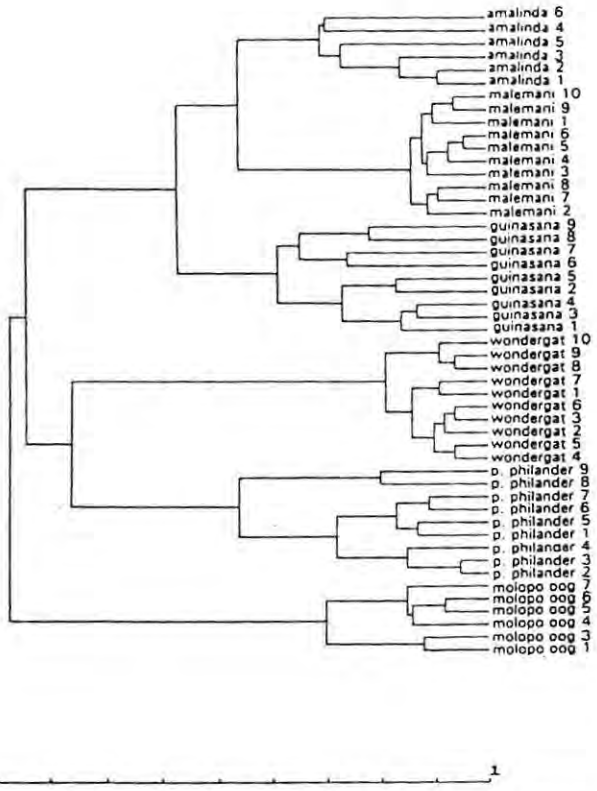


Fig. 3. Dendogram based on the protein patterns of *Tilapia sparrmanii* from Amalinda, Malemani, Wondergat and Molopo Oog, *Tilapia guinasana (T. guinasana)* from Namibia and *Pseudocrenilabrus philander (P. philander)* from Namibia. The dendogram was generated by the GelManager program using Pearson's correlation coefficient and UPGMA clustering.

tant relationship of *P. philander* is confirmed by the low similarity (25%) to the *Tilapia* populations.

The protein data indicate that: (1) the *T. sparrmanii* populations are distinct from each other (based on the PAGE results); (2) Malemani and Amalinda *T. sparrmanii* are closely related to each other and to *T. guinasana*, suggesting that the systematics of *T. guinasana* should be re-evaluated; (3) Molopo Oog and Wondergat *T. sparrmanii* have differentiated significantly from the other conspecifics to a level where they may be considered distinct species.

CONCLUSION

Each population of *T. sparrmanii* is genetically distinct and may also be recognized by population-specific morphological characteristics (P. H. Greenwood, pers. comm.). The uniqueness of each population therefore makes it clear that together with other unique components of their ecosystems (Ribbink *et al.*, 1991; de Villiers *et al.*, 1992), they contribute to systems which should be conserved. Equally, none of the populations should be allowed to interbreed with other populations in any of these ecosystems and none from any other system should be introduced into these waters. Also, a mixing of populations in captive propagation, with a view to restocking the different water bodies with the progeny, should not take place.

The success of the PAGE technique in the differentiation of geographically isolated populations of *T. sparrmanii* demonstrates that its value is not restricted to micro-organisms and can also be used with fish. The applicability of the technique to other vertebrates is untested, but its use here suggests that it may prove to be a quick, simple and relatively inexpensive method for at least preliminary examination of the genetic taxonomy of a variety of vertebrates.

The value of this technique for making management decisions regarding fishes is clear from our study. It also has potential for managers of reserves and parks whose responsibility for small populations includes making decisions regarding the introduction of new genes into their breeding populations. Non-destructive sampling, such as biopsies, will provide sufficient material for PAGE to be run quickly, giving answers regarding the degree of relatedness of the populations concerned, and the information on which management decisions should be based.

ACKNOWLEDGEMENTS

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Appendix C: Nxomani *et al.* (1999)

Clifford Nxomani[1]
Antony J. Ribbink[2]
Ralph Kirby[1]

[1]Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, South Africa
[2]J. L. B. Smith Institute of Ichthyology, Rhodes University, Grahamstown, South Africa

DNA profiling of *Tilapia guinasana*, a species endemic to a single sinkhole, to determine the genetic divergence between color forms

Northwestern South Africa and Namibia contain a number of sinkholes in the dolomitic rock formations found in this area. These contain isolated populations of *Tilapia*. Most contain *Tilapia sparmanii*, but the one in Namibia, Guinas, is of particular interest as it contains the endemic species, *Tilapia guinasana*, which exhibits none sex-limited polychromatisms, which is unique for *Tilapia*. This sinkhole is under environmental threat, particularly as a result of being a recreational diving site. This study, using randomly amplified polymorphic DNA sequences (RAPDs), when analyzed using analysis of variance (ANOVA), shows that the colour forms of *Tilapia guinasana* are genetically distinct. This confirms previous evidence that assortative mating between color forms takes place. The various possible hypotheses for the occurrence and genetic stability of the color polymorphism are discussed. Further, a new hypothesis is put forward based on a need to maximize outbreeding in fully isolated population with no possibility of increase in size above the maximum and limited carrying capacity of the sinkhole.

Keywords: Speciation / DNA profiling / Color forms / *Tilapia guinasana* / Randomly amplified polymorphic DNA
EL 3469

1 Introduction

Tilapia guinasana is a tilapine cichlid that is endemic to a single sinkhole, Guinas (surface area ~ 2800 m^2), situated in the northern part of Namibia. The surface area of the sinkhole gives *T. guinasana* the smallest area of endemicity for any known *Tilapia* species. However, it is now together with another translocated tilapine species *Oreochromis mossambicus*, established in Lake Otjikoto [1]. *T. guinasana* is unique among tilapine because of its extensive polychromatism, a phenomenon that is particularly unexpected among substratum-spawning cichlids which are conservative with regard to coloration. *T. guinasana*'s polychromatism is not sex-limited, compared to other Haplochromine cichlids which are sex-limited. Five distinct color forms of the species have been identified. These are olive, olive striped, dark blue, blue striped and light blue. All are discrete except for the light blue form which was found to be highly variable, with elements of white, light blue, yellow and even black blotches in some individuals [1–3].

Little is known about polychromatism and how it evolves, and this has also been the case with *T. guinasana*. Ribbink *et al.* [2] list three possibilities that might explain *T. guinasana*'s polychromatism. First, it might be due to a high level of individual chromatic variability with color gradations linking the various forms. This however, would be unusual since color in cichlids is highly restricted. Second, *T. guinasana* might be exhibiting discrete sex-limited polychromatism, which would be atypical for the species and African substratum spawners in general. The third possibility is related to the failure of taxonomists to recognize an important phenomenon, the existence of a complex of sympatric sibling species within a taxon. However, their studies of *T. guinasana* in the Guinas sinkhole showed that it is a single species comprised of five principal color forms and a rare and relatively larger sixth form, none of which were sex-limited.

It has been further suggested that because of darkness in the sinkhole, there was no need for communication; and because of absence of predators in the sinkhole, there was no selection for camouflaging colors [3]; therefore, *T. guinasana* might have lost genetic 'control' over color when the sinkhole was still an underground cave. Thus, without competition or predation from other closely related fish species, selection or need for a distinctive coloration for purposes of mate recognition, general communication, or camouflage was obviated. However, Greenwood [1] points out that in Lake Malawi there exist equally uncamouflaged and bright colored fish despite the presence of considerable predation, which is a fact that casts doubt on this hypothesis. Further, in other cave-dwelling cichlids, the tendency is towards loss or reduction of colo-

Correspondence: Professor R. Kirby, Department of Biochemistry and Microbiology, Rhodes University, PO Box 94, Grahamstown, 6140, South Africa
E-mail: mirk@giraffe.ru.ac.za
Fax: +46-6223984

Abbreviation: ANOVA, analysis of variance

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ration rather than an elaboration of pigments and patterns [1] and thus a subterranean origin of the species, and hence the polychromatism does not seem to explain adequately the origin of *T. guinasana*'s polychromatism.

Four factors make *T. guinasana* one of the most vulnerable vertebrates in southern Africa: its unique polychromatism, a natural distribution that is limited to a single sinkhole that is found in an arid region of the continent, frequent fluctuations in water levels in the sinkhole, and environmental pollution. Our aim was to test the hypothesis that the color polymorphs of *T. guinasana* are genetically distinct and we used randomly amplified DNA (RAPD) sequence profiling to test this. RAPD sequences offered the simplest way of measuring the genetic differences between and within the color forms [4–6] and were used successfully on a number of fish species [7–11].

2 Materials and methods

2.1 Collection and handling of fish samples

T. guinasana was collected from the sinkhole using scuba and transported to the laboratory in aerated plastic bags and placed in prepared tanks at the J. L. B. Smith Institute of Ichthyology, Grahamstown, Eastern Cape. Four each of dark blue, light blue, blue stripe and olive were collected. Only one olive stripe could be collected. This sample made up between 3% and 5% of the total fish population in the sinkhole and was considered the maximum sample possible. Fish considered to be under stress were immediately frozen in liquid nitrogen and kept at −20°C until processed.

2.2 DNA extraction

DNA was extracted from muscle tissue by the method of Gold and Richardson [12] and quantified using absorbance on a Shimadzu UV-160 spectrophotometer at 260 nm. Ten microliters of each sample were electrophoresed in a 1% agarose gel to check the quality of the DNA in the extracts.

2.3 DNA amplification

DNA amplification was performed in an automated JDI (Cape Town, South Africa) water-cooled thermocycler. Each reaction tube (0.5 mL Eppendorf tubes were used) was supplemented with 100 ng template DNA, 100 pM of the respective primer, 2.5 mM of each of the deoxynucleotides, 16 mM magnesium chloride, 1 × *Taq* polymerase buffer, and 1 unit of *Taq*. The reactions were carried out in a total volume of 50 μL (made up to volume with sterile distilled water). Each reaction mixture was overlaid with 50 μL of sterile paraffin oil. The samples were subjected to the following cycle: initial denaturation at 94°C for 270 s, 30 cycles of denaturation at 94°C for 30 s, primer annealing at 36°C for 30 s, and primer extension at 72°C for 60 s. This was followed by a final extension step at 72°C for 240 s. Four primers of random sequence (shown in Table 1) were used to generate RAPD amplification profiles for the five color forms of *T. guinasana*. Following amplification, samples were either directly subjected to electrophoretic separation or stored at 4°C.

Table 1. Primers used in this study

Primer 4	CTTCGTACAC
Primer 6	AACCGATGCT
Primer 7	ACGTAGCACT
Primer 12	ACCTGCGTTA

2.4 Electrophoretic analysis of amplification products

Amplification products were separated electrophoretically in 5% and 10% discontinuous PAGE using the buffer systems of Laemmli [13]. The stacking gel was prepared in 0.5 M Tris-HCl, pH 6.8, and contained 0.4% SDS. The resolving or separation gel was prepared in 1.5 M Tris-HCl, pH 8.8, and also contained 0.4% SDS. The amplified samples were initially run on Hoefer Scientific Instruments SE 280 electrophoresis units (San Francisco, CA, USA) for 2.5 h to check for the success and extent of the amplification. Successfully amplified samples from different populations were electrophoresed adjacent to each other in a 50-well Owl electrophoresis unit overnight at 70–80 V. The running/tank buffer was 1 × Tris-borate-EDTA. Once electrophoresis was completed, gels were stained with silver nitrate. The gels were placed in glass/plastic trays and covered with buffer A (10% v/v ethanol, 0.5% v/v acetic acid) and incubated twice for 3 min. This was followed by incubation in 0.1% w/v silver nitrate solution for 10 min. Gels were then washed twice in double distilled water for 1 min. Developing solution B (1.5% w/v sodium hydroxide, 0.01% w/v sodium borohyride, 0.15% v/v formaldehyde; in a total volume of 300 mL) was added and the gels left indefinitely until the stain had developed. Amplification products were visualized as brown bands on a light yellowish-brown background. The gels were photographed for permanent record.

2.5 Numerical analysis of gel images

Comparison of RAPD patterns was done within and between color forms of the fish. Amplification products were scored for presence and absence, respectively. An index of similarity, S_{xy}, within and between colors was

determined. Sij was converted to a measure of genetic distance (Dij) [14]. D_{ij} values obtained were used to construct the dendrogram using the unweighted pair-group method of analysis (UPGMA) in NEIGHBOR of the Phylip package (Felsenstein, 1993). This statistic was calculated for each of the primers and an average estimate determined, which was then used to construct the dendrogam.

3 Results

3.1 Genetic variation within and between color forms

The highest genetic distance between any of the color forms of *T. guinasana* was between the dark blue and the olive forms (0.445) and the least genetically different were the olive stripe and blue stripe forms (0.135). Table 2 shows the genetic distances (and similarities) which were used to draw up a dendrogram depicting the relatedness between the color forms. The dendrogram indicates that the blue stripe and olive stripe forms are more closely related to each other with the light blue form being a closely related out group. Both the dark blue and olive forms are indicated as being more distantly related to the other color forms.

3.2 Analysis of genetic differences between color forms using ANOVA

In order to determine the significance of these results or, to put it differently, to determine if there is significant difference in the genetic makeup of the color forms, a two-way analysis of variance (two-way ANOVA) was performed using the primers and color forms as the variables. The null hypothesis was that there are no significant differences due to either the primers or the color forms. Because the genetic distance values represented single observations, the two-way ANOVA performed was one specifically designed for single observations [15]. At $P = 0.05$ the F values exceed the critical values and the null hypothesis was rejected. It was therefore concluded that the primers differentiated to different extents between the color forms and that the color forms are significantly genetically different. Based on this it could be concluded that there is a genetic basis for *T. guinasana*'s polychromatism and that each is genetically unique.

4 Discussion

4.1 Genetic basis for genetically distinct nature of the color forms

Ribbink *et al.* [2] reported that there were detectable levels of assortative mating (mating between members of the same color form) between the color forms of *T. guinasana*, it being more evident among the light blue, olive and dark blue fish. However, this was only significant ($P = 0.001$) among the dark blue fish, and not complete, and the probability of mating between any color forms appeared to depend on the average occurrence of the respective color form(s) in the breeding population. The dendrogram in Fig. 1 shows that there is a degree of genetic differentiation between the color forms of *T. guinasana*. There is no evidence that the color forms have differentiated to such an extent that they can be considered to be separate species, but the genetic distinctness of the color forms supports the assortative mating hypothesis. Furthermore, *T. guinasana* exists as a single population of relatively few individuals in a single sinkhole. There are thus no physical barriers to breeding between the color forms and, in fact, random mating under such circumstances would be expected as the norm. The levels of genetic distinctiveness shown in the dendrogram could result from the assortative mating that has been observed between the color forms. Ribbink *et al.* [2] observed sig-

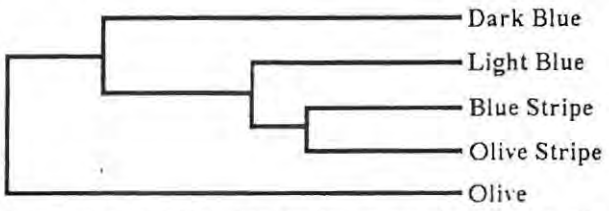

Figure 1. A UPGMA dendrogram drawn from the genetic distances between the color forms of *T. guinasana* as obtained from the four primers used in this study

Table 2. Estimated genetic distances (D_{ij}) between the color forms of *T. guinasana* across the four primers used

Primer	db/o	db/lb	db/sb	db/os	o/lb	o/sb	o/os	lb/sb	lb/os	sb/os
4	0.097	0.097	0.097	0.097	0.077	0.118	0.077	0.077	0.118	0.118
6	0.693	0.560	0.288	0.387	0.560	0.499	0.499	0.288	0.288	0.241
7	0.294	0.424	0.298	0.413	0.380	0.481	0.556	0.006	0.021	0.001
12	0.697	0.497	0.249	0.220	0.578	0.542	0.019	0.240	0.408	0.178
Mean	0.445	0.395	0.233	0.279	0.398	0.410	0.288	0.152	0.209	0.135
SD	0.299	0.205	0.093	0.148	0.232	0.196	0.278	0.133	0.172	0.102

b, blue; lb, light blue; sb, blue stripe; os, olive stripe; o, olive

Table 3. Matrix of similarity (above diagonal) and genetic distance (below diagonal) between the color forms of *T. guinasana*

	db	lb	sb	os	o
db	–	0.769	0.771	0.771	0.689
lb	0.395	–	0.841	0.822	0.704
sb	0.233	0.152	–	0.879	0.695
os	0.279	0.209	0.135	–	0.872
o	0.445	0.398	0.410	0.288	–

db, dark blue; lb, light blue; sb, blue stripe; os, olive stripe; o, olive

nificant levels of assortative mating with the dark blue forms and to some extent with the light blue and olive forms. The fact that the olive and the dark blue forms branch out in the dendrogram as being more distantly related to the other color forms may be indicative of significant levels of assortative mating within these color forms. From the genetic distance values it can be observed that the highest values are those between the dark blue forms and the olive forms on the one hand, and the other color forms on the other.

The close relationship shown on the dendrogram between the olive stripe and blue stripe forms may be indicative of a lower frequency of assortative mating between the two color forms. It would not be unreasonable to think that the stripe patterns on these fish could blur the distinction between the color forms in terms of mate recognition. In a similar manner, the higher genetic distances between color forms are detected between the dark blue/olive (0.445), striped blue/olive (0.410), olive/light blue (0.398) and dark blue/light blue (0.395) pairs of color forms compared to the remainer which have genetic distances in the range 0.135–0.279. The former are empirically the easiest color forms to distinguish and this would suggest a mechanism by which they would exhibit the highest level of assortative mating. This is confirmatory to the results of Ribbink *et al.* [2] where assortative mating was highest among the light blue, olive, and dark blue fish.

Two-way ANOVA performed on the values of genetic distances between the color forms supports the hypothesis that there is significant genetic differentiation between the color forms, and that there is a genetic basis for the polychromatism exhibited by this species. The ANOVA result also suggested that the primers differentiated to different extents between the color forms. Notwithstanding the above results and conclusions, these results provide no genetic basis for the stable establishment of multiple color forms of a species in a single isolated small sinkhole. It has been postulated that the *Tilapia* found in this and other sinkholes in this dolomitic geological area originated during the warmer and wetter conditions of 11–16 000 years ago *via* flash floods with fragmentation of the populations during the subsequent drier periods (Twentyman-Jones, M. Sc. Thesis, Rhodes University). Obviously, this could result in a significant founder effect and speciation but does not provide a possible origin for the polychromatism. These results do not support the hypothesis that *T. guinasana* might have lost genetic 'control' over color. Such an event would not result in genetic differentiation of the color forms as has been shown. Neither is the polychromatism sex-limited as shown by Ribbink *et al.* [2]. The genetic distinctness of the color forms also excludes the color gradation hypothesis of Ribbink *et al.* [2].

It is necessary to consider the third possibility of Ribbink *et al.* [2], that a complex of sympatric sibling species within a taxon exists in Guinas in more detail. The RAPD fingerprinting study found that the color forms of *T. guinasana* exhibit a significant degree of genetic differentiation, although they are still members of a single species. The observed assortative mating may be the primary source of such differentiation. The values of estimated genetic distances ranged from 0.135 to 0.445. If the criteria of Shaklee *et al.* [16] are taken into account, it may be argued that the differences between the color forms may be indicative of an incipient species formation process, since the genetic distance values straddle the conspecific and congeneric species status levels. The interspecies genetic distance values obtained by Bardakci and Skibinski [17] in their RAPD study of Tilapia species and subspecies, ranged from 0.1 to around 0.35. The average genetic distance between the Tilapia species studied by Oosthuizen *et al.* [18] was 0.356. Sodsuk and McAndrew [19] obtained values of genetic distances between Tilapia species that ranged from 0.007 to 0.661 with an average of 0.405. The values obtained in this study seem to fit more comfortably into the lower end of this range.

Based on the data obtained from the four primers used in this study, and comparing them with the other studies or comparisons of Tilapia species as outlined above, it would appear that the genetic distances computed are indicative of the initial processes of species or subspecies formation, by the color forms of *T. guinasana*, more so the dark blue and olive forms. It is not clear whether the assortative mating observed is a product of this incipient species formation process or a determinant thereof. A more extensive study, involving perhaps a larger number of primers and individuals would shed light on the extent of this observed differentiation and what exactly the role of assortative mating is in this system.

Thus, we do not have a species complex *per se*, but perhaps one in the making. What could be driving this phe-

nomenon? We would like to suggest that the various color forms are part of a species complex with limited inter color form gene flow allowing a maximization of the overall genetic diversity of the population as a whole. Because the population is small, isolated, endemic to this sinkhole only, and without any introduction of new genes from any other population, the genetic diversity of such a population would tend to decrease due to inbreeding. As it stands, the genetic diversity of the overall population is quite high because it is made up of the individual color forms. Such a balanced polymorphism could be established as a means of maximizing genetic diversity and therefore individual survival once a color difference has occurred within the population. Such an initial color difference could occur simply by founder effect. Whether such a polychromatic population complex within a species is moving towards speciation is unclear but if such a population was released from the sinkhole, then it would seem likely that speciation would occur. It would be interesting to compare the Guindas population with the exotic population of *T. guinasana* found in Lake Otikoto where the effects of assortative mating in a much greater water volume should speed up speciation. Because of the endangered status of the species, which limits the number of samples that can be used for studies, sequencing of rapidly evolving genes of the representative color forms would perhaps yield better answers to the questions of evolution and taxonomy of *T. guinasana* and its relationship with other recognized closely related Tilapia species.

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