PRIORITISING NATIVE FISH POPULATIONS FOR CONSERVATION USING GENETICS IN THE GROOT MARICO CATCHMENT, NORTH WEST PROVINCE, SOUTH AFRICA

BY

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ABSTRACT

The Groot Marico catchment in the North West Province is a National Freshwater Ecosystem Priority Area (NFEPA) because it represents unique landscape features with unique biodiversity that are considered to be of special ecological significance. Three native freshwater species *Amphilius uranoscopus*, *Chiloglanis pretoriae* and *Barbus motebensis*, have high local conservation importance and *B. motebensis* is endemic to the catchment and is IUCN-listed as vulnerable. The main objective of this study is to contribute towards the effective conservation of these three species in the Groot Marico River system by assessing their genetic structure to determine whether tributary populations of the three species comprise of one genetic population or whether they are divided into genetically distinct subpopulations, in order to prioritise areas for conservation. The central null hypothesis was that there is no genetic differentiation between tributary populations (i.e., panmixia) of *B. motebensis*, *A. uranoscopus* and *C. pretoriae* in the Groot Marico catchment, North West Province.

In total, 80 individuals per species were collected, targeting at least 10 individuals per population from a total of eight populations (seven tributaries and the Groot Marico main stem) and across the study area. Samples were collected by electrofishing and specimens were euthanized using an overdose of clove oil. A sample of muscle tissue was removed for genetic evaluation and the remainder of the specimens served as voucher specimens. For the genetic evaluation, mitochondrial (ND2, cyt *b*) and nuclear (S7) genes were used. Genetic techniques used were DNA extraction, polymerase chain reaction (PCR), purification and sequencing. From the 240 individuals collected, 123 sequences for *B. motebensis*, 111 sequences for *A. uranoscopus* and 103 sequences for *C. pretoriae* were analysed across all three genes. Statistical analysis included looking at cleaned sequences in order to obtain models using MODELTEST (version 3.06). Population structuring and phylogeographic analysis was performed in Arlequin (version 2000), TCS (version 1.2.1) and PAUP*.

Results indicated that for *B. motebensis* the null hypothesis could be rejected as there were two distinct lineages (the Draai and Eastern lineages) that demonstrated significant divergence in both the ND2 and S7 genes, suggesting historical isolation. The low divergence in the mitochondrial cytochrome *b* gene ($0\% < D < 0.8\%$) suggests that this isolation is not very old and is probably not comparable to species level differentiation. The null hypothesis was also rejected for *A. uranoscopus* as there were also significant levels of differentiation between tributary populations resulting in the identification of two lineages (the Ribbok and Western lineages). However, for *C. pretoriae*, the null hypothesis could not be rejected as there was no genetic differentiation between tributary populations i.e., one panmictic population.

Therefore, due to each species showing different genetic structuring within the tributary populations, more than one priority area for conservation needs to be implemented. These priority areas of conservation where therefore evaluated based on the current conservation status of the species (*B. motebensis* being vulnerable on the IUCN Red List), the number of Evolutionary Significant Units for each species and the overall genetic diversity of all three species in the Groot Marico catchment. In total, four tributary populations were conservation priorities areas, these were the Draai, Vanstraatens, Ribbok and Kaaloog tributaries.

The Draai, Vanstraatens and Kaaloog tributaries were selected as priority areas for *B. motebensis* (*B. motebensis* is considered to be the most vulnerable of all three species). The Draai tributary was selected due to the *B. motebensis* population within the tributary showing isolation from the rest of the tributary populations. In order to conserve *B. motebensis* from the Southern lineage, the Vanstraatens and Kaaloog tributaries were selected. Reasons for selecting these two specific tributaries within the Southern lineage were that the Vanstraatens tributary had unique alleles (three Evolutionary Significant Units) for *B. motebensis* and the Kaaloog tributary had high genetic diversity (H_D = 0.889, ND2 gene) when compared to the other tributary populations.

The Ribbok and Vanstraatens tributaries were selected as priority areas for the conservation of *A. uranoscopus*. The Ribbok tributary was selected as it showed isolation from the rest of the tributary populations, as seen with the Draai tributary (*B. motebensis*) and the Vanstraatens tributary was selected to represent the Western lineage as it had the highest diversity for both genes (ND2 and S7). The Ribbok tributary has the highest prioritisation when compared to the Vanstraatens tributary.

Chiloglanis pretoriae occurs within the Draai, Vanstraatens, Ribbok and Kaaloog tributaries, therefore by prioritising these tributaries for conservation, *C. pretoriae* will in turn be conserved.

DECLARATION

I, the undersigned, hereby declare the work contained in this thesis is my own original work. It has not been submitted before for the award of any other degree at any other university

Signature ..... Date .....

DEDICATION

This thesis is dedicated to my parents, Mike and Penny Van der Walt, and my sister

Amy-Leigh Van der Walt

For all their support and encouragement throughout my studies

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CHAPTER 1

1.1. Thesis introduction

Freshwater ecosystems are among the richest and most diverse ecosystems on earth, and freshwater fish account for a large part of biodiversity (Hermoso *et al.* 2009). These ecosystems and especially the headwaters of rivers are key areas for conservation because they contain both unique biodiversity and provide extensive ecosystem services (Abell *et al*. 2007; Moilanen *et al.* 2008).

South Africa is no exception. A recent assessment of threats to aquatic biodiversity (Darwall *et al*. 2009) demonstrated elevated levels of threat to freshwater-dependent fish, molluscs, dragonflies, crabs and plants, when compared to other countries in Southern Africa. In an attempt to better conserve aquatic ecosystems, South Africa has recently categorised its river systems according to set ecological criteria (i.e. ecosystem representation, water yield, connectivity, unique features, and threatened taxa) to identify National Freshwater Ecosystem Priority Areas (NFEPAs) (Driver *et al*. 2011). The NFEPAs are intended to be conservation support tools and envisioned to guide the effective implementation of measures to achieve the National Environment Management Biodiversity Act (NEMBA) biodiversity goals (Nel *et al*. 2011).

The Groot Marico catchment in the North West Province (Figure 1.1) is a NFEPA because the Marico River is the only free-flowing river in the North West Province (Nel *et al*. 2011), and because the catchment contains unique landscape features of special ecological significance (Roux, 2010). These unique landscape features include dolomitic eyes, which are ground-water fed aquifers that arise from fractures at the contact zones between the underlying dolomite intrusions and sedimentary or igneous rock (Wellington, 1995). The permanent water flow provided by these aquifers to headwater tributaries of the river is of special ecological significance in this arid region. As a result, these streams are prioritised in national-level conservation plans (e.g. Provincial River Health Programme Progress Report, 2011) and are NFEPA-listed as fish sanctuary areas (Nel *et al*. 2011).

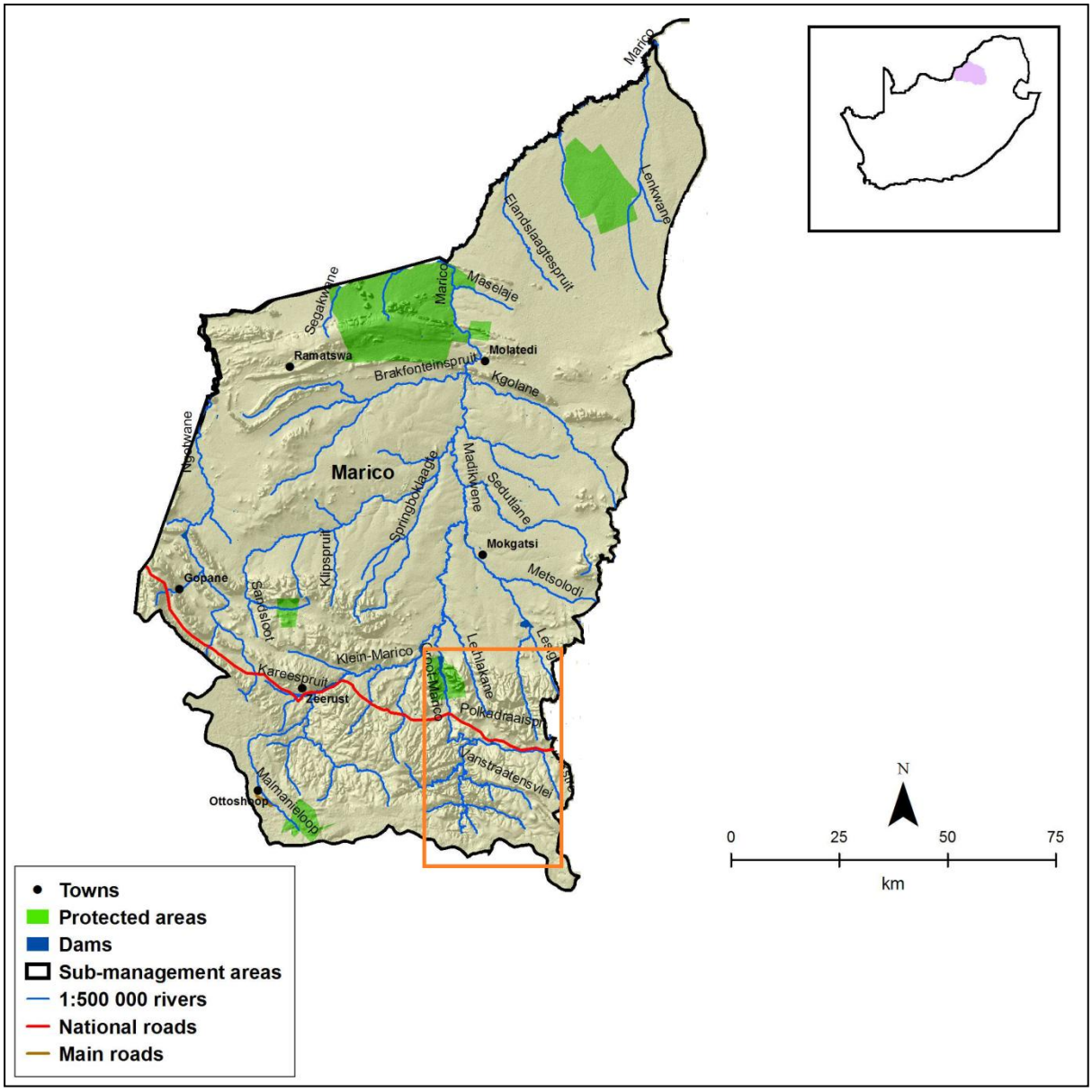

Figure 1.1. Map illustrating the Groot Marico catchment in the North West Province, South Africa. The study area within the Groot Marico catchment is outlined by the orange rectangular box (taken from A systematic conservation plan for freshwater biodiversity of the Crocodile (West) and Marico Water Management Area Report (Smith-Adao *et al*. 2006).

The Groot Marico catchment and Crocodile catchment as a whole contains 29 fish species (Smith-Adao *et al*. 2006). In the assessment of the systematic conservation plan for freshwater biodiversity of the Crocodile (West) and Marico Water Management Area Report, Smith-Adao *et al*. (2006) listed six freshwater fish species as having high local conservation importance. These were the Marico barb, *Barbus motebensis* (Steindachner, 1894), Johnston's topminnow, *Aplocheilichthys johnstoni* (Günther, 1894), Stargazer mountain

catfish, *Amphilius uranoscopus* (Pfeffer, 1889), Snake catfish, *Clarias theodorae* (Weber, 1897), Shortspine suckermouth catfish, *Chiloglanis pretoriae* (Van der Host, 1931) and the canary kurper, *Chetia flaviventris* (Trewavas, 1961) (Table 1.1). As is the case for many South African freshwater fishes (see Tweddle *et al*. 2009), the primary threats to these species include water extraction for irrigation, habitat alteration, competition with and predation by introduced alien species (Habitat Integrity Assessment Report of selected Rivers in the North West Province, 2007).

Table 1.1. Species of special concern in the Groot Marico catchment, North West Province and their current status and habitat preferences. Habitat preference: A = Fast flow (> 0.3 m/s) with rocky substrate; B = slow flow (< 0.3 m/s) with marginal vegetation.

Species	IUCN Red List Status (2013)	Isolated populations	Type localities	Habitat preference	
				A	B
B. motebensis	Vulnerable	X			X
A. johnstoni	Special Concern	X			X
A. uranoscopus	Special Concern	X		X	
C. pretoriae	Special Concern		X	X	
C. flaviventris	Special Concern		X		X
C. theodorae	Special Concern	X			X

Water from the Groot Marico main stem and its tributaries is primarily used for agricultural irrigation and water supply to farms and livestock (Department of Water Affairs and Forestry, 2004) and several aquifers are considered over-extracted (Department of Water Affairs and Forestry, 2003). Although headwater tributaries in the Groot Marico catchment flow in deeply incised gorges that are not necessarily impacted by agriculture (Grobler *et al*. 2007), many are impacted by alien vegetation and slate mining (Roux, 2010) which may be responsible for accelerating riverbank erosion, increased sedimentation and reduced stream flow (Vertessy, 2000).

The lower reaches of the main stem and several tributaries also contain weirs and dams to facilitate water extraction (Smith-Adao *et al.* 2006). These dams not only modify river flows but may also be sources for alien fish invasions into headwater streams (Johnson *et al*. 2008). In the Groot Marico system these include the largemouth bass, *Micropterus salmoides* (Lacepède, 1802), a species which, through predation has been shown to fragment stream fish populations (Ellender *et al*. 2011) and impact on invertebrate communities (Weyl *et al*. 2009). Conversely, man-made and natural barriers such as dams (Figure 1.2) and waterfalls (Figure 1.3) may also prevent invasions by inhibiting upstream movements of alien fishes

(Weyl *et al*. 2009; Wassermann *et al*. 2011) and maintaining such barriers may be important conservation tools.

Figure 1.2. The Twyfelspoort Dam wall on the Polkadraai tributary, Groot Marico catchment, North West Province, South Africa.

Figure 1.3. Natural barrier within the Groot Marico catchment, North West Province, South Africa near the confluences of the Kaaloog and Vanstraatens tributaries.

Effective conservation of Marico headwater fishes will therefore require targeted interventions to alleviate catchment degradation, ensure water flows and prevent invasions. Such conservation action will need to be guided not only in terms of species but also in terms of the area. Recently, such recommendations have included information on the genetic structure of populations. For example, an assessment of the genetic diversity of *Pseudobarbus afer* in the Sundays River catchment, Addo elephant National Park, revealed

that all riverine populations of *P. afer* hold unique genetic characteristics and that each river represents an important conservation unit (Weyl *et al.* 2008). Therefore, in terms of conservation the entire genetic diversity of the Sunday River *P. afer* stocks were considered important (Weyl *et al*. 2008). This study therefore shows the relevance of Evolutionarily Significant Units (ESUs). ESUs allow evolutionary processes that could have shaped intraspecific diversity to continue (Crandall *et al*. 2000; Moritz, 1999; Moritz *et al.* 2002). They are further defined as historically isolated and divergent populations and are diagnosed as sets of populations showing reciprocal monophyly of mitochondrial DNA combined with significant divergence of allele frequencies at nuclear loci (Moritz, 1994). Therefore, if gene flow between tributary populations is significantly low among the species within the Groot Marico catchment as compared to *P. afer* (Weyl *et al.* 2008), conservation measures will need to be implemented according to their ESUs.

1.2. Conservation species

Of the six conservation priority fish species listed in Smith-Adao *et al.* (2006), three species, *B. motebensis*, *A. uranoscopus* and *C. pretoriae* were the focus of the current study because: (1) *B. motebensis* is IUCN red-listed as vulnerable; (2) all three species have smaller body sizes compared to the other species in the catchment, potentially making them vulnerable to predation by the non-native largemouth bass, *Micropterus salmoides*; (3) the Marico catchment is the type locality for *B. motebensis* and *C. pretoriae*; and lastly (4) comparing the effects of all three species having similar body sizes but different body forms would be interesting. A review on the current knowledge of these species follows.

1.2.1. Marico barb, *Barbus motebensis*

The genus *Barbus* forms one of the largest fish genera in the world (Collares-Pereira & Madeira, 1990). There are approximately 300 species in Africa and approximately 50 species in southern Africa, therefore making it the largest genus in the sub-region (Vlok, 2005). *Barbus motebensis* (Figure 1.4) is one of the soft-rayed barbs in the chubbyhead barb group (Skelton, 2001). The chubbyhead barbs are endemic to cooler regions of South Africa. This group of barbs is considered to be of ecological significance since they are predominantly the only fish species present in small headwater streams because they are often found isolated in small stretches of river above waterfalls (Engelbrecht, 1996). The chubbyhead barb group consists of 4 described species: chubbyhead barb, *Barbus anoplus* (Weber, 1897); the red-tail barb, *B. gurneyi* (Günther, 1868); Marico barb, *Barbus motebensis* (Steindachner, 1894);

Amatola barb, *Barbus amatolicus* (Skelton, 1990) and the shorthead barb, *Barbus breviceps* (Trewavas, 1936) as well as several undescribed species (Skelton, 2001, Engelbrecht, 1996).

Barbus motebensis is a small (80 mm SL) cyprinid fish characterised by numerous conical tubercles on the snout, forehead and lower jaw and two pairs of barbels (Jubb, 1968; Skelton, 2001) (Figure 1.4). It is endemic to the upper catchments of the Marico, Crocodile and Steelpoort branches of the Limpopo River catchment in the North West and Gauteng Provinces of South Africa (Engelbrecht & van der Bank, 1997) (Figure 1.5). In the Groot Marico catchment, this species has a small area of occupancy (< 2000 km^2) and is restricted to headwater streams (Engelbrecht, 1996). It was first described by Steindachner (1894) from specimens sampled from the Marico district. The species was classified taxonomically by Skelton (2001) and *Barbus motebensis* is its valid species name.

Figure 1.4. Photograph of the Marico barb, *Barbus motebensis*, 70 mm total length (TL).

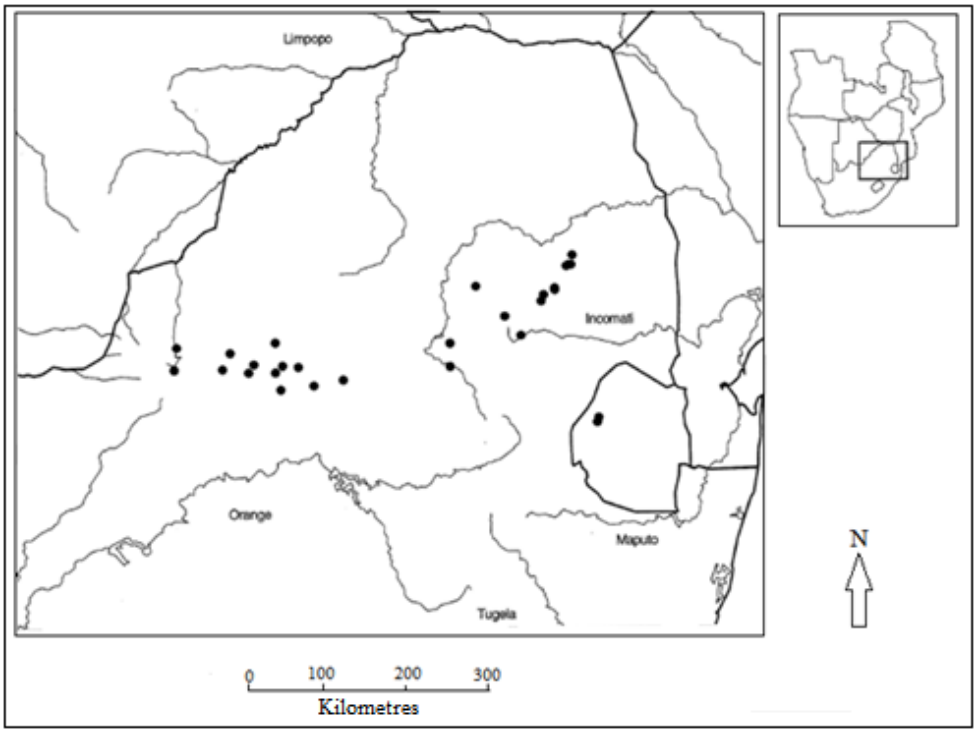

Figure 1.5. Species distribution map of *Barbus motebensis* in the North West and Gauteng provinces of South Africa (modified from the Atlas of Southern African freshwater fishes, Scott *et al.* 2006).

There is little population genetic information available for *B. motebensis*. What is known is based on the allozyme electrophoresis conducted by Engelbrecht and Van der Bank (1997) to determine whether *B. anoplus* and *B. motebensis* represent one or more species and to what extent their populations differed. The study was conducted because previous researchers suggested that *B. anoplus* and *B. motebensis* may be synonymous (Groenewald, 1958; Gaigher, 1969, 1973, 1976). Engelbrecht and Van der Bank (1997) demonstrated a large degree of genetic divergence within the chubby-head barbs and proposed two genetically different groups: (1) a chubbyhead *B. anoplus* group and (2) a tubercle barb *B. motebensis* group. This is based on the large genetic distances between the groups of populations studied and the presence of obvious tubercles on breeding males of *B. motebensis.* No comparisons of catchment level differences in populations have however been undertaken.

1.2.2. Stargazer mountain catfish, *Amphilius uranoscopus*

Catfishes are extremely diverse, with about 31 families, 400 genera and more than 2200 described species (Skelton, 2001). In South Africa, there are 6 families of catfishes with 12 genera and 39 described species (Skelton, 2001). The catfish family, Amphiliidae (Regan, 1911), is widely distributed and consists of 12 genera and 66 species (Ferraris, 2007). The stargazer mountain catfish, *Amphilius uranoscopus* (Pfeffer, 1889) (Figure 1.6) is widespread in eastern, central and southern Africa ranging from the Mkuze and Pongola Rivers in Natal northwards to the Limpopo River and the Zambezi all the way up to the Tana River in Kenya, and also in the Okavango River, which flows through Angola, Namibia and Botswana (Seegers, 2008; Skelton, 1984; Van Oosterhout *et al.* 2009) (Figure 1.7).

Figure 1.6. Photograph of the stargazer mountain catfish, *Amphilius uranoscopus*, 130 mm total length (TL).

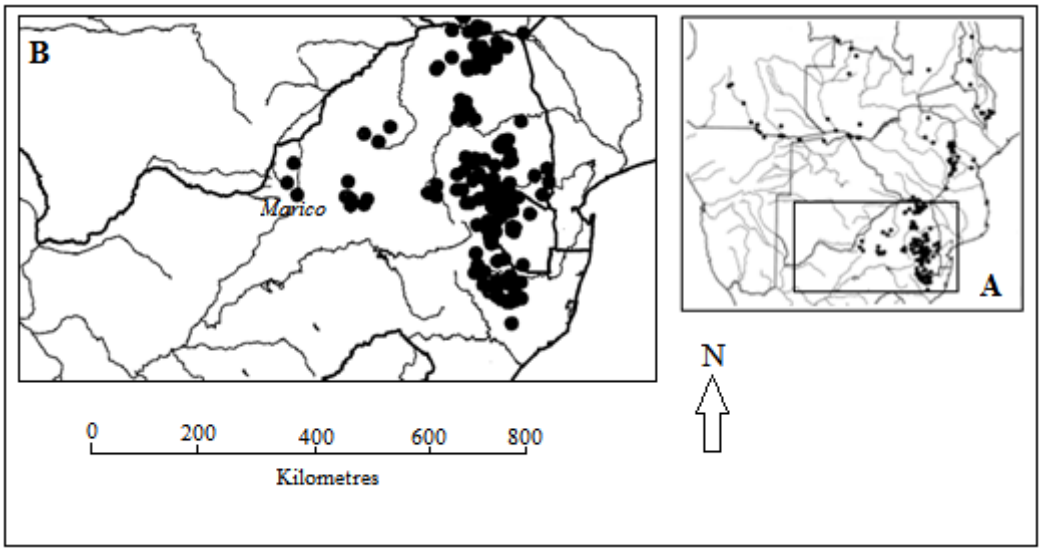

Figure 1.7. Species distribution map of *Amphilius uranoscopus* in central and East Africa (A); and species distribution of *A. uranoscopus* in the Groot Marico catchment, South Africa (B) (modified from the Atlas of Southern African freshwater fishes, Scott *et al*. 2006).

Pfeffer (1889) described *A. uranoscopus* from the upper ranges of the Wami River in Tanzania. In 1984, Skelton revised the genus *Amphilius* from eastern and southern Africa and identified two groups. The first group was from western Africa and the second group from eastern and southern Africa. *Amphilius uranoscopus* therefore belongs to the eastern and southern Africa group (Thomson, 2007).

The species name *uranoscopus* refers to the eyes which are positioned on the top of the head, hence the common name, stargazer mountain catfish (also called common mountain catfish) (Van Oosterhout *et al*. 2009). Species of the *Amphilius* genus are typically found in headwater streams which consist of fast-flowing, clear and cool water that is rich in oxygen with rocky substrates (Skelton, 1986; Moyle & Cech, 1996; Van Oosterhout *et al*. 2009; Ngugi *et al*. 2009). *Amphilius uranoscopus* is often the first species to be found near the source of a river (Van Oosterhout *et al*. 2009) and is therefore characterised as a headwater specialist (Skelton, 1986).

Morphological adaptations for these preferred habitats include expanded pectoral and pelvic fins with an expanded first ray, depressed body, upwardly directed eyes and a reduced gas bladder (Skelton, 1986; Walsh *et al*. 2000). The large pelvic fins have been observed to form a feeble sucking disc, which in conjunction with the body enable fish to cling to the rocky substrate (Jackson, 1961; Burgess 1989). *Amphilius uranoscopus* attains 170 mm in total length (TL), they are nocturnal and predominantly feed on aquatic insects (scraped from the surface) and other small animals such as crustaceans and small tadpoles (Ngugi, 2009). The South African species breeds during the summer season (Marriott *et al.* 1997). Breeding coincides with the rainy season and spawning commences in response to environmental cues such as changes in water quality and increased water flow rate as well as changes in water quality following rainy periods (Marriott *et al.* 1997). However, there is little information available on the phylogenetics of *Amphilius* species.

1.2.3. Short-Spine suckermouth catfish, *Chiloglanis pretoriae*

The short-spine suckermouth catfish, *Chiloglanis pretoriae* (Figure 1.8) belongs to the largest African catfish family Mochokidae, which consists of 10 genera and approximately 170 species, all of which are endemic to tropical Africa and the Nile Valley (Berra, 1981) (Figure 1.9). The genus *Chiloglanis* comprises 34 species of which eight occur in Southern Africa. One of these eight species is the catfish species, *C. pretoriae* (Van der Host, 1931) which has a widespread distribution in the eastern parts of central Africa. This includes the Incomati,

Limpopo, middle and lower Zambezi, Pungwe and Buzi systems (Figure 1.9) (Jubb & Le Roux, 1969).

Figure 1.8. Photograph of the short-spine suckermouth catfish, *Chiloglanis pretoriae*, 90 mm total length (TL).

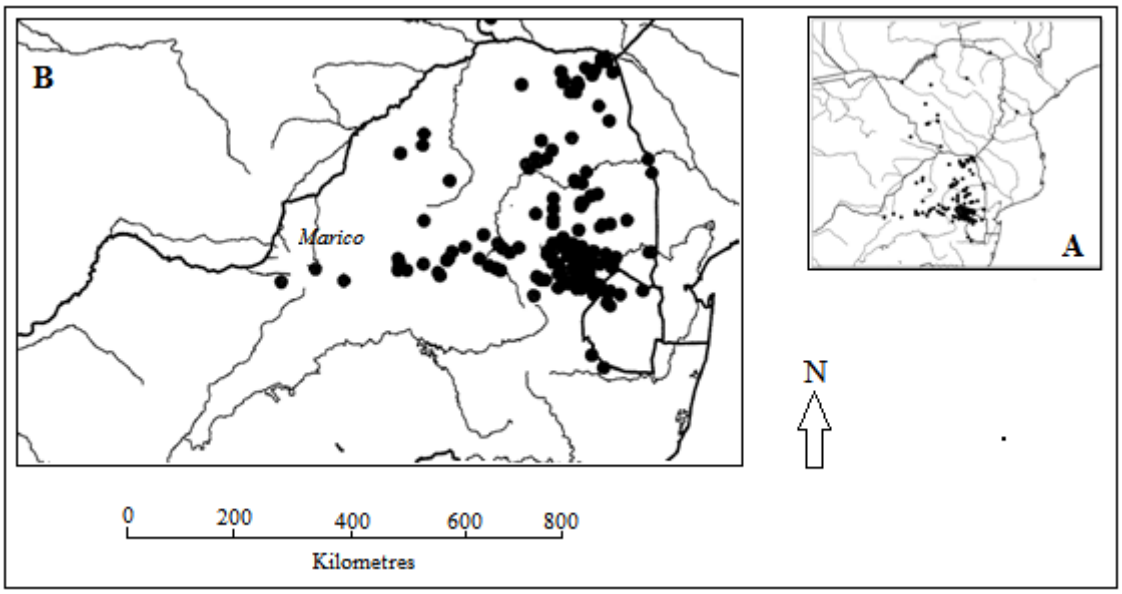

Figure 1.9. Species distribution map of *Chiloglanis pretoriae* in central and East Africa (A); and species distribution of *C. pretoriae* in the Groot Marico catchment, South Africa (B) (modified from the Atlas of Southern African freshwater fishes, Scott *et al.* 2006).

Chiloglanis pretoriae was first identified and classified by Van der Horst (1931) within the Crocodile River, Pretoria District, and Transvaal which is now recognised as Gauteng. This species habitat preference is quite specialised and is common to the region in which it occurs (Skelton, 2001). It prefers rocky rapids and riffles to such an extent that when flow ceases it may disappear from stretches of a river (Gaigher 1973; Kleynhans 1984).

This species of catfish has a well developed sucker-like mouth which it uses to suck and attach itself to rocky substrates (De Villiers, 1991). This adaptation allows this species to withstand fast flowing streams and rivers. The colouration of this species is olive brown with olive yellow markings. The dorsal fin has one spine and five to six soft rays which have dark bands across them (Jubb, 1968). The body is depressed anteriorly and weakly compressed posteriorly. The head is strongly depressed with small eyes present as well as three pairs of barbels. The mouth is inferiorly positioned allowing for the sucker-like mouth and is surrounded by highly modified papillae (Burgess, 1989). Its diet includes aquatic insects. The species breeds within the summer months of the South African climate (Skelton, 2001).*Chiloglanis pretoriae* faces major threats such as habitat degradation. The species has disappeared from its type locality around Pretoria, South Africa. This can be counteracted by improved conservation (Skelton, 2002).

Little population genetic or phylogeographic information is available for *C. pretoriae*. Research done thus far, includes the use of allozyme electrophoresis for determining the genetic structure of populations of suckermouths (see Grant *et al*. 1988; Van der Bank, 1996; Engelbrecht & Mulder, 2000). Engelbrecht and Mulder (2000) determined the allozyme variation in *C. pretoriae* and *C. paratus* from the Limpopo River system, South Africa and demonstrated that there was extensive genetic differentiation between the congeneric species.

1.3. Population genetic models and their application to the Marico headwater fish communities

Lack of gene flow among stream-fish populations tends to cause high levels of genetic structuring of these populations compared to populations of marine and estuarine taxa (Gyllensten, 1985). This is a consequence of smaller effective population sizes and the isolation of populations by terrestrial or marine barriers which decrease dispersal and increases structuring (Ward *et al.* 1994; DeWoody & Avise, 2000). Within the context of conservation planning it is therefore important to identify not only genetic isolation in target species but also extrinsic and intrinsic factors responsible for isolation and the mechanisms that may be responsible for such structuring.

Within riverine environments, population structuring is best explained by five models reviewed by Meffe and Vrijenhoek (1988): (1) panmixia; (2) isolation by distance; (3) Stream/Hierarchy; (4) the discrete subpopulation and (5) the headwater.

1.3.1. Panmixia

Panmixia is the simplest model of population structure and is the default model for assessments of population structure (Humphries & Walker, 2013). It occurs when all subpopulations are genetically mixed within a catchment area or across several major drainage divisions (Richardson *et al*. 1986). For example, populations of common galaxias (*Galaxias maculates*) in New Zealand are a single well-mixed stock showing high levels of genetic connectivity (Waters *et al*. 2000).

1.3.2. Isolation by distance

For mobile species, but ones whereby dispersal is limited by distance, panmixia is apparent only within a single genetic neighbourhood, isolation by distance model applies (Humphries & Walker, 2013). Often, genetic isolation by distance is the only spatial pattern that can be expected (Hughes *et al*. 2009) and under this model there are no definable subpopulations but a continuum of overlapping neighbourhoods (Humphries & Walker, 2013) in which long distances between habitats can limit gene flow (Hughes *et al*. 2009). Furthermore, this occurs in connected populations if an individual dispersal distance is less than the range of the species (Slatkin, 1993). An example of where this model applies was shown by Wilcock *et al*. (2003) where the European caddisfly, *Plectrocnemia conspersa* showed little among-stream or among-basin genetic structure within a range of 20 kilometres (km) although genetic distance increased significantly over longer geographical distances (Hughes *et al*. 2009). Another example that showed similar effects of the model occurred in Australian bass, *Percalates novemaculeata* on the east coast (Jerry, 1997) and barramundi, *Lates calcarifer* in the north (Chenoweth *et al.* 1998).

1.3.3. Stream hierarchy model

The Stream hierarchy model summarises the relationship among populations within and among drainages due to rivers having a hierarchical structure and proposes that the possibility of connectivity is related to the position of a population in a particular drainage. Therefore populations within the same stream would be genetically more similar to each other compared to populations in a different subcatchment (McGlashan, 2000; Meffe &Vrijenhoek, 1988). A discrete subpopulation model applies when two or more genetically and geographically distinguishable subpopulations are present in a species (Richardson *et al*.

1986). These groups of subpopulations are separated by a physical or biological barrier, which limits gene flow and potentially results in isolation (Humphries & Walker, 2013).

1.3.4. Headwater model

The headwater model applies to organisms whose biological requirements limit them to the headwaters of stream systems and are likely to display little or no gene flow with subpopulations of the same species in other headwaters within a catchment (Hughes *et al*. 2009). This is a result of geographic fixation (Humphries & Walker, 2013) which in turn isolates populations in the uppermost reaches (Hughes *et al*. 2009).

It has been proposed that more than one model may apply to individual species. Understanding which models best explain observed genetic distributions is an important component of conservation planning because the use of these models may aid in allocating priority areas for conservation according to the species preferred habitat and dispersal abilities.

1.4. Thesis structure, aims and hypothesis

The overall objective of this thesis is to contribute towards the effective conservation of freshwater fishes in a NFEPA fish sanctuary area by assessing the genetic structure of *B. motebensis*, *A. uranoscopus* and *C. pretoriae* in headwater tributaries of the Groot Marico catchment, North West Province, South Africa.

The specific aim of the thesis is to assess whether there is evidence for genetic isolation among populations of the three species in different headwater tributaries, and to use catchment morphology information to propose which of the five above mentioned models best fit the observed genetic distributions. The central null hypothesis is that there is no genetic differentiation between tributary populations (i.e., panmixia) of *B. motebensis*, *A. uranoscopus* and *C. pretoriae* in the Groot Marico catchment. A secondary objective is to attempt to prioritise tributaries for conservation action based on the genetic structure of populations and level threat to the three fish species.

To address these objectives the thesis is divided into 6 chapters. Following this introduction (Chapter 1), the study area, sampling design, location of collection sites, laboratory methods and statistical analyses are described in Chapter 2. Chapters 3-5 examine the genetic diversity and structuring of *B. motebensis*, *A. uranoscopus* and *C. pretoriae*, respectively. In addition,

these species-specific chapters assess which genetic population model (panmixia, isolation by distance, stream hierarchy, discrete population, headwater) best fits each species, and discuss what extrinsic and intrinsic factors may have been responsible for the observed patterns. Chapter 6 discusses the overall patterns of genetic structuring displayed for all three species in terms of gene flow and isolation, and uses the information to identify Evolutionarily Significant Units (ESU's) that require management to conserve genetic diversity of the Groot Marico headwater fish community and to prioritise areas for immediate, medium and long term conservation.

CHAPTER 2

2.1. Study area

The Groot Marico catchment has a total area of 13 439.69 km^2. The Groot Marico catchment originates south of the Groot Marico town, which is considered as the aquifer plateau region of the North West Province and feeds into the Limpopo River in the north, ultimately forming the south western headwaters of the Limpopo River. The Marico River flows in a northerly direction, outlining the border between Botswana and South Africa, before turning north – east to join the Crocodile River which originates from the Witwatersrand (Wellington, 1955). The Crocodile and Marico River then give rise to the Limpopo River at their confluence, which has an international catchment spanning Botswana and Zimbabwe (King, 1951). The Marico River has a total length of 250 km and an altitudinal variation of 700 m descending at an average slope of 1:357 (Grobler *et al*. 2007). The Groot Marico catchment has a climate which is generally semi-arid, due to the variation in mean annual precipitation ranging from 650 mm in the upper reaches to 526 mm in the lower drier reaches (Department of Water Affairs and Forestry, 2004). This is a result of the rainfall in the area being highly seasonal with precipitation occurring predominantly as thunderstorms in the season of summer.

The perennial tributaries of the Groot Marico River catchment originate in dolomitic eyes (springs) which provide a constant flow to the river. These dolomitic eyes and tributaries are predominant within the upper catchment and cut through the mountains south of Groot Marico (Roux, 2010). A study conducted by the JLB Smith Institute (now the South African Institute for Aquatic Biodiversity) recognized and acknowledged the dolomitic eye ecosystem and biota as well as the associated tributaries within the Groot Marico catchment of exceptionally significant conservation value (Skelton *et al.* 1994). Dolomitic eyes are systems driven by aquifers. Aquifers are water bodies fed by groundwater and are formed from fractures in the underlying dolomite. These fractures are also referred to as dolomitic eyes or springs. The eyes which feed the Marico River occur on contact zones between the sedimentary and igneous rock and the dolomites of the Malmani subgroup, forming part of the Transvaal Super group. The Molemane, Molopo and Marico Rivers originate from unique dolomitic eyes and associated wetland systems (Roux, 2010).

2.1.1. Tributaries

Within the Study area of the upper Groot Marico Catchment, there are 7 tributaries of importance to this study (Figure 2.1). The two primary sources of the Groot Marico catchment are the Grootfontein dolomitic eye which gives rise to the Kaaloog tributary and another eye feeding into the Riet tributary. Secondary sources include the Draai and Polkadraai tributaries (Table 2.1).

The Grootfontein dolomitic eye and the Kaaloog tributary have been identified as special ecological features for the purpose of biodiversity protection (Smith-Ado *et al*. 2006). The Riet tributary originates in a dolomitic eye with a perennial stream and wetland area in the upper reaches, and then flows through a deeply incised gorge area (Smith-Ado *et al*. 2006). The Draai tributary is also of dolomitic origin whereas the Polkadraai tributary is one of the few tributaries within the catchment that is not of a dolomitic origin but rather from an extensive wetland area (North West Province Department of Agriculture, Conservation and Environment, 2006).

Other tributaries within the study area of the Groot Marico catchment include the Bokkraal, Ribbok and Vanstraatens tributaries. The Bokkraal tributary is a perennial tributary of the Groot Marico River with dolomitic origin. The Ribbok tributary originates in the south-east of the study area and has been identified as a special feature for the purpose of aquatic biodiversity conservation (Smith-Ado *et al*. 2006). The Vanstraatens tributary originates in the north-east of the study area and is of dolomitic origin (Smith-Ado *et al*. 2006).

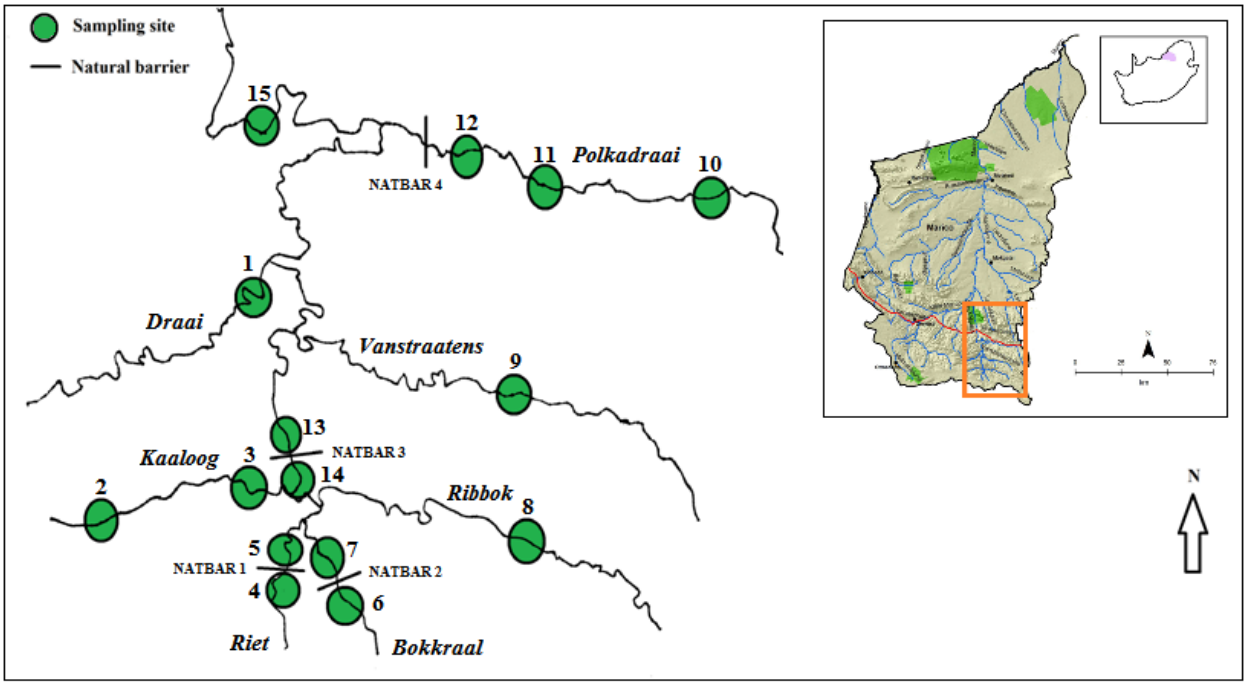

Figure 2.1. Map illustrating the tributaries and15 sampling sites (green dots) in which all three species were collected and analysed across the upper Groot Marico catchment, North West Province. There are 4 major natural barriers in the study area, indicated by perpendicular lines (NATBAR = natural barrier).

Table 2.1. The main tributaries within the study area of the Groot Marico River catchment, including flow rates, functions and importance (Roux, 2010).

Tributary	Location and function
Riet	Found 10m down from a dolomitic eye. It contributes approximately 0.29 m^3/s as measured by DWA (2009)
Kaaloog	DWA flow determination is 0.22 m^3/s
Bokkraal	Situated on the river is an active Tufu waterfall, and in 2010 a conservation area was launched identifying the importance of the waterfall. Contributes 0.29 m^3/s flow measured by DWA
Ribbok	Highly seasonal flow
Draai	Perennial tributary, dependant on high flow rates
Vanstraatens	Negatively impacted by invasive plants and dairy farming within the area
Polkadraai	Negatively impacted by farming and alien invasive plant vegetation

2.1.2. Natural barriers

Within the study area of the Groot Marico catchment, four predominant natural barriers within the tributaries was assessed (Figure 2.1). However, it should be noted that more natural barriers may exist in sections of the Groot Marico River and its tributaries that were not investigated during the two surveys (Table 2.2).

Table 2.2. Approximate heights of the natural barriers in the Groot Marico catchment, North West Province.

Natural Barrier	Height (m)
1	1.5
2	8
3	1
4	2

2.1.2.1. Natural Barrier 1

This barrier consists of a cascading waterfall in the upper reaches of the Riet tributary (Figure 2.2). The largest cascade is approximately 1.5 m high and would form a partial barrier to the upstream migration of native fish species within the Groot Marico catchment. This waterfall also forms a barrier to the upstream migration of largemouth bass, *Micropterus salmoides.* Therefore the upper reach of the Riet tributary may serve as a refuge for native species such as *Barbus motebensis*.

Figure 2.2. Natural Barrier 1 in the upper reaches of the Riet tributary in the form of a cascading waterfall.

2.1.2.2. Natural barrier 2

Within the upper reaches of the Bokkraal tributary a large waterfall is present, namely the Tufa waterfall (Figure 2.3). This is one of the few active Tufa waterfalls present in the world. A Tufa waterfall is formed when water running over dolomite rock absorbs calcium. Mosses which grow on the rocks in the stream extract carbon dioxide during photosynthesis which causes the calcium in the water to precipitate deposit as layers of tufa on the surface of the waterfall. This is a process which is thought to takes millions of years. Due to the height of the Tufa waterfall it forms a natural barrier within the Bokkraal tributary, preventing the upstream migration of all fish species.

Figure 2.3. Natural barrier 2 in the upper reaches of the Bokkraal tributary in the form of a Tufa waterfall.

2.1.2.3. Natural Barrier 3

In the upper reaches of the Groot Marico main stem between the confluences of the Kaaloog and Vanstraatens tributaries is a third natural barrier (Figure 2.4). This barrier is approximately 1 m in height and is a partial barrier to the upstream migration of native species such as *B. motebensis* and the non-native *M. salmoides*, due to its height.

Figure 2.4. Natural barrier 3, a waterfall in the main stem of the Groot Marico River, near the confluences of the Kaaloog and Vanstraatens tributaries.

2.1.2.4. Natural Barrier 4

Within the middle reach of the Polkadraai tributary, there is a natural waterfall (Figure 2.5). This waterfall is approximately 2 m in height and is considered a natural barrier to the upstream migration of all fish species within the Groot Marico catchment. However, this waterfall is considered irrelevant due to its location approximately 100 m from the Twyfelspoort Dam wall. The Twyfelspoort Dam is a large impoundment and the dam wall is approximately 6 m in height, forming a complete barrier to upstream migration of all fish species.

Figure 2.5. Natural barrier 4 consists of a natural barrier in the middle reaches of the Polkadraai tributary. However, the importance of this barrier is irrelevant due to its location being in close proximity to the Twyfelspoort Dam wall as seen in the far background.

2.2. Sample Collection

Sampling was carried out during the summer season (March and November 2012) and samples were collected by single pass electrofishing and seine netting. Locality co-ordinates of each site were taken and all fish were treated according to the standards outlined in the SAIAB Animal Ethics Policy.

Specimens were euthanized by an overdose concentration of clove oil. Small pieces of muscle tissue from each specimen was then removed by use of a dissection blade and placed into 1.5 ml vials containing 98% ethanol. Photographs and a variety of different cuts of the specimens were taken for identification as well as linking it to the relevant tissue sample. The remaining specimens were fixed in formalin and served as voucher specimens. Voucher specimens were accessioned into the National Collection of the South African Institute of Aquatic Biodiversity in Grahamstown, South Africa. The tissue vials were then stored in a freezer at -20 ℃ for DNA extraction and once all laboratory work was completed, the tissue was transferred to SAIAB's Biomaterial Bank for long term storage in ultra deep freezers at -70 ℃.

2.3. Sample Size

In total, 240 individuals were collected across all three headwater species (80 individuals per species), ten individuals per population across the study area for a total of eight populations (seven tributaries and the Groot Marico main stem) (Figure 2.1). From these 240 individuals, only 158 individuals were analysed for the mitochondrial ND2 gene, 25 individuals were analysed for the mitochondrial cytochrome *b* gene and 154 individuals were analysed for the nuclear S7 gene (Table 2.3).

Table 2.3. Total number of individuals used for genetic analysis for all three headwater species across the Groot Marico catchment.

	ND2	cytochrome b	S7
Barbus motebensis	68	25	30
Amphilius uranoscopus	51	-	60
Chiloglanis pretoriae	39	-	64
Total	158	25	154

In total, 88 sites were sampled across the catchment (Appendix A). These 88 sites were further sub-divided into a total of 15 sampling reaches (Appendix B). This was done in order to minimise the number of sites to reaches in which genetic samples were collected and used for analysis (Figure 2.1, Table 2.4).

Table 2.4. Localities in which all three headwater species were collected for genetic analysis.

Map	Locality
1	Lower Draai
2	Upper Kaaloog
3	Lower Kaaloog
4	Above Riet waterfall
5	Below Riet waterfall
6	Above Bokkraal waterfall
7	Below Bokkraal waterfall
8	Upper Ribbok
9	Middle Vanstraatens
10	Upper Polkadraai
11	Middle Polkadraai
12	Lower Polkadraai
13	Groot Marico Sonop
14	Below Groot Marico waterfall
15	Groot Marico Riverstill

2. 4. Laboratory Methods and Materials

2.4.1. DNA extraction, polymerase chain reaction (PCR), purification and sequencing

For each specimen, total genomic DNA was isolated from muscle tissue using the nucleic acid and protein purification extraction kit, Nucleospin Tissue (Macherey-Nagel, Germany). A fragment of the mitochondrial ND2 gene was amplified using the forward primer ND2-F (CTA CCT GAA GAG ATC AAA AC) and the reverse primer ND2-R (CGC GTT TAG CTG TTA ACT AA) (Kocher *et al.* 1995) in all three species. The first intron of the nuclear S7 gene was amplified for all three species using the forward primer S7RPEX1F (TGG CCT CTT CCT TGG CCG TC) and the reverse primer S7RPEX3R (GCC TTC AGG TCA GAG TTC AT) (Chow & Hazama, 1998). In addition, a fragment of the mitochondrial cytochrome *b* (cyt b) gene was amplified for *B. motebensis* using the forward primer Gcyt-Glu (GAA AAA CCA CCG TTG TTA TTC A) and the reverse primer Gcyt-Thr (CGA CTT CCG GAT

TAC AAG ACC) (Waters & Wallis, 2001) to allow comparison to published sequences of species in the chubbyhead barb group (Tsigenopoulos *et al.* 2002).

Polymerase chain reactions (PCRs) followed the same methods for all three species for ND2 and S7 and were performed with a Veriti 96 well thermal cycler (Applied Biosystems, North America). Amplification was performed in 25 µl volumes, each containing 1 X reaction buffer, 2.5 mM $MgCl_2$, 0.8 mM of DNTPs, 0.2 mM of each primer, 1 U Super-Therm Taq polymerase, 100-200 ng of template DNA and the final volume of the reaction was adjusted using H_2O in order to obtain a 25 µl volume reaction. PCR cycling conditions for the ND2 mtDNA reactions involved an initial denaturation of 3 minutes at 95℃, followed by 35 cycles of 45 seconds at 95 ℃, 45 seconds at 50℃ and 1 minute at 72℃ and a final extension of 5 minutes at 72℃. PCR cycling conditions for the nuclear S7 intron reactions involved an initial denaturation of 1 minute at 95℃, followed by 30 cycles of 30 seconds at 95 ℃, 1 minute at 60℃ and 2 minutes at 72℃ and a final extension of 10 minutes at 72℃. In the case of cyt *b* for *B. motebensis*, PCR cycling conditions included an initial denaturation of 2 minutes at 94℃, followed by 35 cycles of 30 seconds at 94℃, 45 seconds at 59℃ in order to anneal the DNA and 1 minute at 72℃ and a final extension of 5 minutes at 72℃. All PCR products were loaded in wells of agarose gel and then electrophoresed. If DNA was present, it was sent away to Macrogen (South Korea) for purification and sequencing. All three gene regions were sequenced with only the forward primers using an ABI 370xl DNA Analyzer (Applied Biosystems).

2.5. Population genetic analysis

2.5.1. Sequence editing and alignment

Sequences were cleaned and trimmed manually to equal lengths, using the program SEQMAN 10.2.1 (DNASTAR, Madison, WI) for all genes. Alignment of corrected sequences was done using ClustalX (Thompson *et al.* 1997). Unique haplotypes were identified using DnaSP 5.10 (Librado & Rozas, 2009). Due to the presence of diploid individuals, the S7 intron data was phased using the program Phase as incorporated into DnaSP.

2.5.2. Substitution models of sequence evolution and genetic distance measures

Prior to analysis, the best fitting model of molecular evolution for each gene (ND2, cyt *b* and S7) was estimated using Bayesian Information Criterion (BIC) (Burnham & Anderson, 2002) in ModelTest 3.7 (Posada & Crandall, 1998).

2.5.3. Genetic structuring

Analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.5 (Excoffier *et al.* 2005) was performed to test for genetic structuring among fish populations in the Groot Marico catchment. This analysis was done for genes ND2 and S7. Distance model parameters were used for the AMOVA analysis.

The AMOVA was carried out using 10000 permutations to assess the significance of differences between estimated values and those expected when haplotypes are randomly assigned to populations. Three predefined hierarchical structures were tested for both ND2 and S7 to assess which one explained most of the variation for each species.

For both the ND2 and S7 genes, pairwise Φ_{ST} values and pairwise F_{ST} values were calculated in ARLEQUIN to determine the genetic differentiation among populations.

2.5.4. Phylogenetic analysis

Parsimony networks were constructed using the program, TCS 1.2.1 (Clement *et al*. 2000) for both the mitochondrial ND2 and nuclear S7 genes. Ambiguous branches in the parsimony networks were removed according to the rules described by Templeton *et al*. (1987). For *B. motebensis*, the cytochrome *b* gene was used to conduct a maximum likelihood analysis (Felsentein, 1981) which was done in PAUP* (Swofford, 2002) and a Bayesian analysis which was done in MrBayes 3.1 (Huelsenbeck & Roquist, 2001) to compare the lineage divergence found in *B. motebensis* to species level divergences found between two other species in the chubbyhead group. *Barbus gurneyi* was used as the outgroup.

CHAPTER 3

Genetic structuring of the Marico barb, *Barbus motebensis*, in the Groot Marico Catchment, North West Province, South Africa

3.1. Introduction

The Marico barb, *Barbus motebensis* Steindachner, 1894, is a small cyprinid fish that is endemic to the upper catchments of the Marico, Crocodile and Steelpoort branches of the Limpopo River System in the North West and Gauteng provinces of South Africa (Engelbrecht & Van der Bank, 1997). It has been assessed according to IUCN Red List criteria as Vulnerable, because it is thought to be impacted by water abstraction and general habitat degradation (IUCN, 2013). Taxonomically, *B. motebensis* belongs to the Chubbyhead barb group (Engelbrecht, 1996; Skelton, 2001). According to Skelton (2001), the other members of this group are *B. anoplus* Weber, 1897 (chubbyhead barb), *B. gurneyi* Günther, 1868 (redtail barb), *B. amatolicus* Skelton, 1990 (Amatola barb) and *Barbus breviceps* Trewavas, 1936 (shorthead barb).

Previous studies suggest that members of the Chubbyhead barb group are typically restricted to upper catchments of rivers and are often isolated in headwaters of rivers which results in extensive genetic differentiation between populations (Engelbrecht & Van der Bank, 1994, 1996; Engelbrecht, 1996). In the upper reaches of the Groot Marico catchment, *B. motebensis* is limited to the headwater streams of the study area, with a small area of occupancy (< 2000 km^2) (Engelbrecht, 1996). This restricted distributional range may be due to various extrinsic and intrinsic factors. Extrinsic factors include climate changes or geological barriers such as waterfalls (e.g. Swartz *et al.* 2007, 2009; Chakona *et al*. 2013; Maturuse, 2013;). Intrinsic factors include the species' specific dispersal ability (e.g. Chakona *et al*. 2013), habitat preference (e.g. Chakona *et al*. 2012) and physiological adaptations (e.g. Chakona *et al*. 2011). In fishes, such factors may isolate populations and limit population sizes, which may result in higher levels of genetic structuring among fish populations in freshwater habitats compared to the more connected marine environment (Gyllensten, 1985; Ward *et al.* 1994).

Conservation plans should therefore be adapted to specific environments and should aim to allow evolutionary processes that could have shaped intraspecific diversity to continue (Moritz, 1999; Crandall *et al*. 2000; Moritz *et al*. 2002). It is important, however, to first identify the diversity that has to be conserved. This has been done effectively in the past by

identifying Evolutionarily Significant Units (ESUs). Moritz (1994) defines ESU's as historically isolated and divergent populations and are diagnosed as sets of populations showing reciprocal monophyly of mitochondrial DNA combined with significant divergence of allele frequencies at nuclear loci.

An example of the use of ESUs to determine conservation priorities is the Maloti Minnow Conservation Project (Skelton *et al.* 2001). The project was carried out for the Lesotho Highlands Development Authority to conserve *Pseudobarbus quathlambae* (Barnard, 1938), and is a model for how other threatened species programs could be approached (Darwall *et al*. 2009). *Pseudobarbus quathlambae* was threatened by the development of inter-basin water transfer schemes of the Lesotho Highlands Water Project as well as by the construction of large reservoirs (Skelton *et al*. 2001). Genetic analysis of individuals from the known populations revealed two ESUs (Mohale and Eastern populations) within this Critically Endangered species (Swartz, 2005). Conservation plans were subsequently developed to not only allow the survival of these two ESUs, but also to maintain the isolation between them.

Since *B. motebensis* is considered to be of conservation concern, it is important to assess its intraspecific genetic diversity and to determine if it has separate ESUs. The aims of this chapter are therefore to: (1) assess the genetic diversity o f *B. motebensis* in the Groot Marico catchment; (2) determine whether genetic isolation and structuring has occurred; (3) use these data to develop hypotheses on how the ecology of this species, natural landscape and climatic processes may have influenced the genetic structure of populations. These aims will be achieved by testing the null hypothesis that there is no genetic differentiation between tributary populations of *B. motebensis*.

3.2. Materials and Methods

3.2.1. Specimens

Specimens of *B. motebensis* for genetic analysis were analysed from 11 sampling sites across six different tributaries and in the Groot Marico main stem of the Groot Marico catchment (Figure 3.1). For the mitochondrial ND2 gene, *B. motebensis* were analysed from the Draai, Kaaloog, Bokkraal, Ribbok and Vanstraatens tributaries as well as the Groot Marico River main stem. For the nuclear S7, *B. motebensis* was analysed from the Draai, Kaaloog, Riet, Bokkraal, Ribbok and Vanstraatens tributaries and the Groot Marico River main stem (see Table 3.1 for sample sizes). No specimens of *B. motebensis* were collected from the Polkadraai tributary and the lower Groot Marico main stem, suggesting that they have been extirpated from these sections of the catchment (Figure 3.1, Table 3.1).

3.2.2. Laboratory and statistical analysis

Total genomic DNA extraction, amplification of the ND2, S7 and cyt *b* fragments and sequencing of resulting products were followed according to the methods outlined in Chapter 2 (see Section 2.4.1). Sequence editing, alignment (see Section 2.5.1) and statistical analysis (see Section 2.5.3) followed the methods outlined in Chapter 2. AMOVA analysis (see Section 2.5.3) was performed on three predefined hierarchical structures for both the mitochondrial ND2 and nuclear S7 genes (Table 3.2, Table 3.3). As there were no published mitochondrial ND2 gene sequences available for closely related *Barbus* species for comparative purposes, four individuals from the Bokkraal tributary, Vanstraatens tributary and the Groot Marico main stem and three individuals from the Riet, Ribbok and Draai tributaries (total= 21) were sequenced for the cytochrome *b* gene to compare to the two species in the chubbyhead barb group that have already been sequenced for this gene (Tsigenopoulos *et al*. 2002). Maximum likelihood analysis (Felsentein, 1981) was done in PAUP* (Swofford, 2002) and Bayesian analysis was performed in MrBayes 3.1 (Huelsenbeck & Roquist, 2001) to compare the lineage divergence found in *B. motebensis* to species level divergences found between two other species in the chubbyhead group. *Barbus gurneyi* was used as the outgroup (see Section 2.5.4).

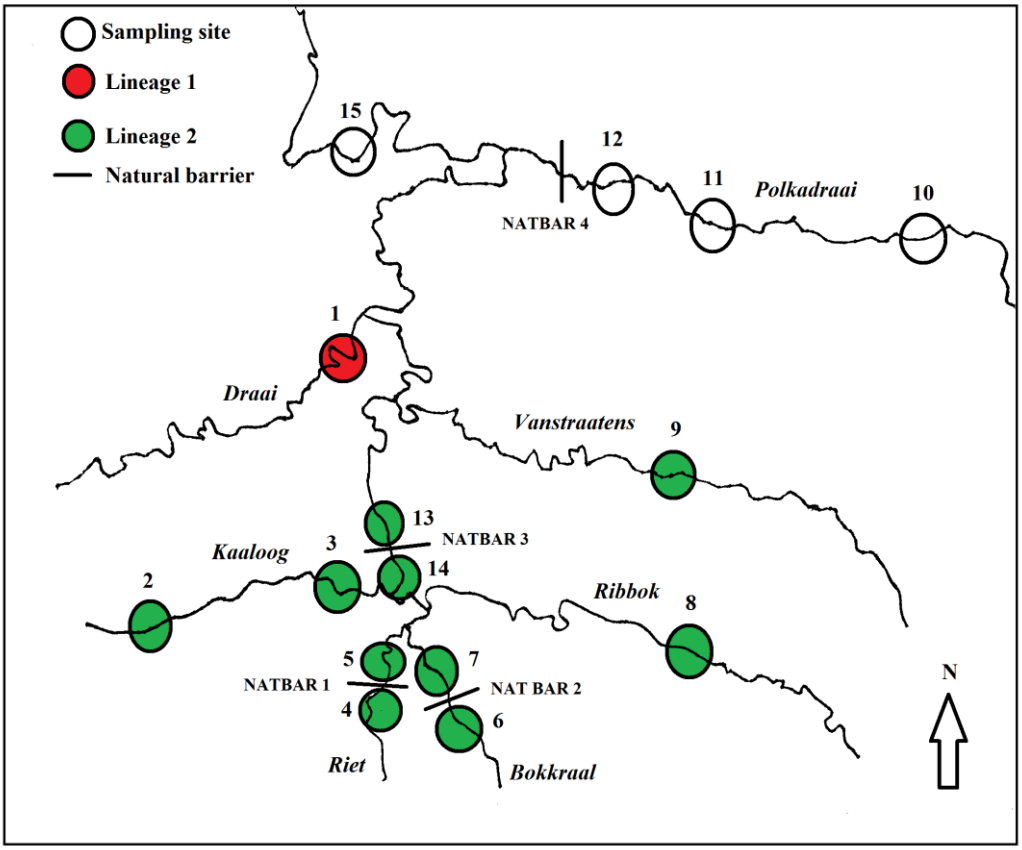

Figure 3.1. Map illustrating the 11 sites where *B. motebensis* specimens were collected and analysed across the upper Groot Marico catchment, North West Province. There are 4 major natural barriers in the study area, indicated by perpendicular lines (NAT BAR = natural barrier). From the genetic analysis, both the mitochondrial ND2 and nuclear S7 genes had 2 major genetic lineages. The first lineage (Draai lineage, red) only occurs in the Draai tributary. The second lineage (Southern lineage, green) occurs in the Kaaloog, Riet, Bokkraal, Ribbok and Vanstraatens tributaries and the Groot Marico main stem. *Barbus motebensis* were not recorded in the Polkadraai tributary (Sites 10, 11 and 12) or the lower Groot Marico main stem (site 15). Not all the samples from all sites were analysed successfully for both genes (see Table 3.1 for presence/absence and the samples that were analysed successfully).

Table 3.1. Localities (see Figure 3.1) and sample sizes (*n*) analysed for the mitochondrial ND2 gene and the nuclear S7 gene of *Barbus motebensis.*

Site	Locality	Locality Code	Presence/ Absence	ND2 analysis (*n*)	S7 analysis (*n*)
1	Lower Draai	LD	yes	7	2
2	Upper Kaaloog	UK	yes	0	1
3	Lower Kaaloog	LK	yes	9	2
4	Above Riet waterfall	ARW	no	0	0
5	Below Riet waterfall	BRW	yes	0	10
6	Above Bokkraal waterfall	ABW	yes	11	3
7	Below Bokkraal waterfall	BBW	yes	12	2
8	Upper Ribbok	UR	yes	12	4
9	Middle Vanstraatens	MV	yes	4	2
10	Upper Polkadraai	UP	no	0	0
11	Middle Polkadraai	MP	no	0	0
12	Lower Polkadraai	LP	no	0	0
13	Groot Marico Sonop	GMS	yes	5	0
14	Below Groot Marico waterfall	BGMW	yes	8	4
15	Groot Marico Riverstill	GMR	yes	0	0
Total				68	30

Table 3.2. Different structures used in the analysis of molecular variance (AMOVA) for populations of *Barbus motebensis* based on the mitochondrial ND2 gene.

	Structure 1	Structure 2	Structure 3
Group 1	Draai	Draai	Draai
Group 2	Kaaloog Bokkraal Ribbok	Kaaloog Bokkraal	Kaaloog Bokkraal Groot Marico
Group 3	Vanstraatens Groot Marico	Ribbok Vanstraatens Groot Marico	Ribbok Vanstraatens

Table 3.3. Different structures used in the analysis of molecular variance (AMOVA) for populations of *Barbus motebensis* based on the nuclear S7 gene.

	Structure 1	Structure 2	Structure 3
Group 1	Draai	Draaifontein	Draaifontein
Group 2	Kaaloog	Kaaloog	Kaaloog
	Riet	Riet	Bokkraal
	Bokkraal	Bokkraal	Riet
	Ribbok		Groot Marico
Group 3	Vanstraatens	Vanstraatens	Ribbok
	Groot Marico	Groot Marico	Vanstraatens

3.3. Results

3.3.1. Mitochondrial DNA analysis

3.3.1.1. ND2 diversity

For the mitochondrial ND2 gene, TrN+G (Tamura & Nei, 1993) was the substitution model that best fit the data, with a gamma value of 0.128. Analysis of 68 individuals from six populations for 947 base pairs of the mitochondrial ND2 gene resulted in 24 alleles defined by 38 variable sites (Ti:Tv ratio = 3.399) (Table 3.4). No sequences were obtained for the Riet tributary (Figure 3.1, Table 3.1). The overall nucleotide diversity was low (π = 0.007) and no significant deviations from those expected under predictions of neutrality were found (D = -0.497, P > 0.10 (Tajima, 1989); F = -0.829, P > 0.10 (Fu & Li, 1993)). Overall haplotype diversity was high (H_D = 0.883) and ranged from 0.439 (Site 7 in the Bokkraal tributary, Figure 3.1) to 0.900 (site 13 in the Groot Marico main stem, Figure 3.1) (Table 3.4).

3.3.1.2. ND2 allele distribution

Eighteen of the 24 alleles (75%) were private to single localities (Table 3.4). Neither the Vanstraatens (alleles 1, 2 and 3) nor Draai populations (alleles 18, 19 and 20) shared any of their alleles with other tributaries. There were other private alleles in the upper Ribbok (site 8, alleles 6 and 7), lower Kaaloog (site 3, allele 9, 10 and 11), Groot Marico main stem below the waterfall (site 14, allele 13, 14 and 15), Groot Marico main stem on the Sonop farm (site 13, alleles 16 and 17) and above the waterfall on the Bokkraal tributary (site 6, alleles 23 and 24) (see Table 3.1). A further three alleles had very restricted distributions. Alleles 21 and 22 were restricted to the Bokkraal tributary, but were found above and below the waterfall (sites 6 and 7). Allele 12 was restricted to two localities in the Groot Marico main stem (sites 13 and 14) (see Table 3.1) (Table 3.4).

Only three alleles had wide distributions. Individuals from the lower Kaaloog, Bokkraal (above waterfall) and upper Ribbok shared allele 5. Allele 8 was restricted to the lower Kaaloog and Groot Marico main stem above the waterfall. The most widespread allele (allele 4) was found in the lower Kaaloog, Bokkraal (above and below waterfall), Ribbok and the Groot Marico main stem (above waterfall) (Table 3.4).

Table 3.4. The geographic distribution and frequency of mtDNA (ND2) alleles among sampled localities of *Barbus motebensis.* Samples sizes (N) and haplotype diversity (H_D) are included (See Table 3.1 for locality descriptions). LD = Lower Draai; LK = Lower Kaaloog; ABW = Above Bokkraal Waterfall; BBW = Below Bokkraal Waterfall; UR = Upper Ribbok; MV = Middle Vanstraatens; GMS = Groot Marico Sonop; BGMW = Below Groot Marico Waterfall.

Allele Number	N	1 LD	3 LK	6 ABW	7 BBW	8 UR	9 MV	13 GMS	14 BGMW
1	2	-	-	-	-	-	2	-	-
2	1	-	-	-	-	-	1	-	-
3	1	-	-	-	-	-	1	-	-
4	21	-	3	6	9	1	-	1	1
5	8	-	2	1	-	5	-	-	-
6	4	-	-	-	-	4	-	-	-
7	2	-	-	-	-	2	-	-	-
8	4	-	1	-	-	-	-	-	3
9	1	-	1	-	-	-	-	-	-
10	1	-	1	-	-	-	-	-	-
11	1	-	1	-	-	-	-	-	-
12	3	-	-	-	-	-	-	2	1
13	1	-	-	-	-	-	-	-	1
14	1	-	-	-	-	-	-	-	1
15	1	-	-	-	-	-	-	-	1
16	1	-	-	-	-	-	-	1	-
17	1	-	-	-	-	-	-	1	-
18	2	2	-	-	-	-	-	-	-
19	4	4	-	-	-	-	-	-	-
20	1	1	-	-	-	-	-	-	-
21	3	-	-	1	2	-	-	-	-
22	2	-	-	1	1	-	-	-	-
23	1	-	-	1	-	-	-	-	-
24	1	-	-	1	-	-	-	-	-
Total	68	7	9	11	12	12	4	5	8
H_D	0.883	0.667	0.889	0.727	0.439	0.742	0.833	0.900	0.893

3.3.1.3. ND2 genetic structuring

The parsimony network revealed two different lineages (Figure 3.2). The first lineage comprising alleles 18, 19 and 20 from the Draai tributary and a second "Southern" lineage comprising 21 alleles found in all other tributary populations and the Groot Marico main stem (Figure 3.2). Genetic distance estimates based on the TrN+G (Tamura & Nei, 1993) substitution model shows relatively low divergence among alleles of the Draai lineage ($0\% < D < 0.3\%$). The range of genetic distance estimates was much larger among alleles of the Southern lineage ($0\% < D < 2\%$). The genetic distances between alleles from the Draai and Southern lineages was relatively large ($0.9\% < D < 1.9\%$), but this divergence was not reflected in the cytochrome *b* data (see Section 3.3.1.4). One ambiguous branch, consisting of six mutational steps (between allele 3 and the missing alleles connected to allele 19) was broken in order to keep the branch between allele 3 and the missing alleles connected to allele 9, which had fewer (three) mutational steps (following the rules of Templeton *et al.* 1987) (Figure 3.2).

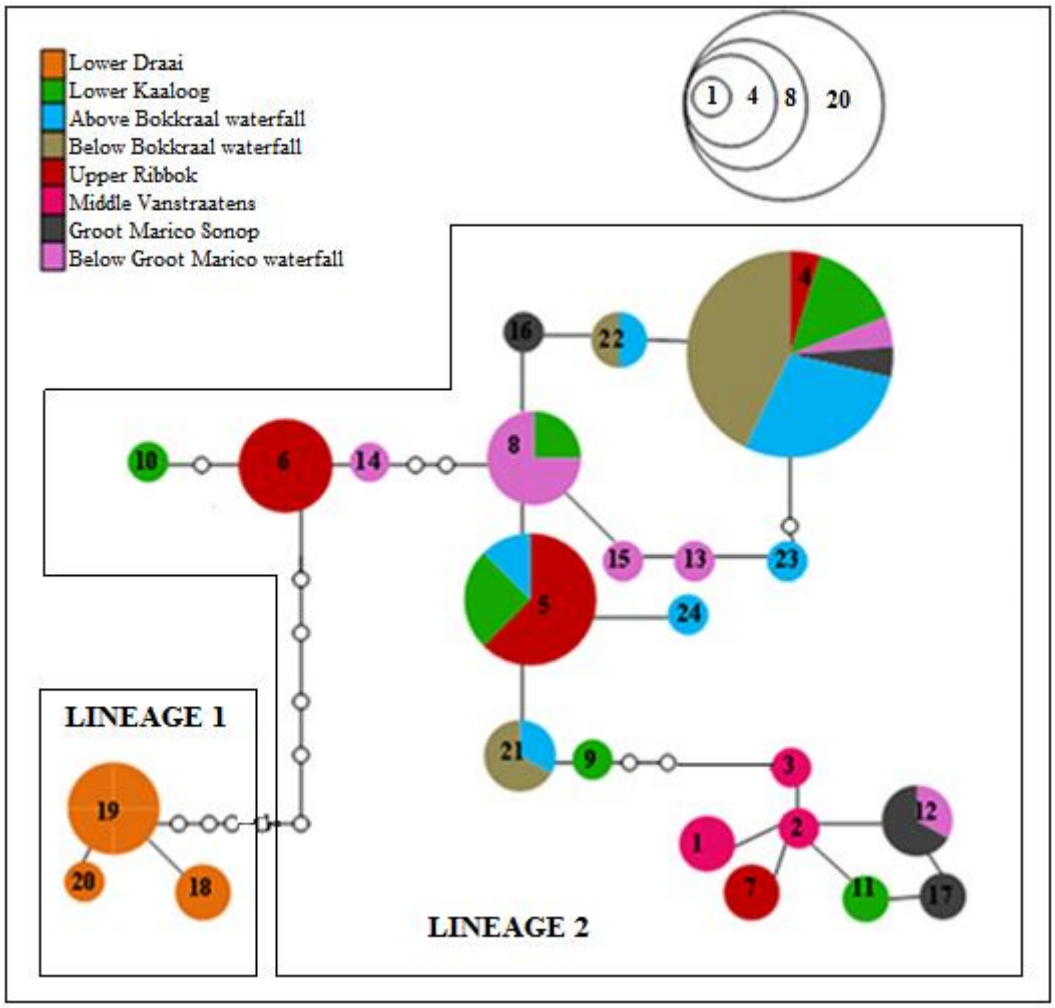

Figure 3.2. Parsimony network with 95% plausible set of mitochondrial ND2 allele connections (numbered circles) constructed with the program TCS 1.2.1 (Clement *et al*. 2000) for *Barbus motebensis* (See Table 3.1). The size of the circles indicates the relative frequency of the alleles (total N= 68) (See Table 3.4). Small open white circles that are not numbered indicate missing alleles. Each line in the network represents one mutational change. Lineages are indicated by rectangular lined boxes. Lineage 1 is referred to as the Draai lineage. Lineage 2 is referred to as the Southern lineage. Within the Southern lineage, the Vanstraatens tributary does not share any of its alleles with other populations (alleles 1, 2 and 3). Genetic distances within the Draai tributary (0.1% < *D* < 0.3%) show low divergence. Whereas, the genetic distance within the Southern lineage (0.1% < *D* < 2%) and between the Draai and Southern lineage (0.9% < *D* < 1.9%) show high divergence.

Pairwise Φ_{ST} values which take genetic distances into consideration, revealed significant ($P < 0.05$) levels of differentiation for 80% of the pairwise population comparisons. There were no significant differences between the Kaaloog and Ribbok, Kaaloog and the Groot Marico main stem and between the Ribbok and the Groot Marico main stem populations (Table 3.5).

Table 3.5. Pairwise Φ_{ST} values for *Barbus motebensis* from 6 populations in the Groot Marico catchment based on the mitochondrial ND2 gene using the Tamura and Nei model with a gamma value of 0.128. The comparisons that were significant ($P < 0.05$) are indicated by asterisks.

	1	2	3	4	5	6
1) Draai						
2) Kaaloog	0.615*					
3) Bokraal	0.902*	0.238*				
4) Ribbok	0.666*	-0.036	0.379*			
5)Vanstraatens	0.935*	0.531*	0.921*	0.634*		
6)Groot Marico	0.630*	-0.018	0.321*	0.119	0.513*	

Pairwise F_{ST} values, which do not take genetic distance among alleles into consideration, also revealed significant ($P < 0.05$) levels of differentiation for 80% of the pairwise population comparisons. Pairwise population comparisons between alleles of Kaaloog and Bokkraal, Kaaloog and Groot Marico main stem and between Ribbok and Vanstraatens were significant (Table 3.6).

Table 3.6. Pairwise *FST* values for *Barbus motebensis* from 6 populations in the Groot Marico catchment based on the mitochondrial ND2 gene. The comparisons that were significant ($P< 0.05$) are indicated by asterisks.

	1	2	3	4	5	6
1) Draai						
2) Kaaloog	0.216*					
3) Bokkraal	0.396*	0.078				
4) Ribbok	0.291*	0.076	0.305*			
5) Vanstraatens	0.265*	0.134	0.353*	0.224*		
6) Groot Marico	0.197*	0.025	0.194*	0.162*	0.119*	

The AMOVA analysis indicated that all variance components had significant levels of genetic structuring ($P < 0.001$) (Table 3.7). The analysis also indicated that most of the genetic variation was partitioned within tributary populations (Φ_{ST} = ~ 45%) for all three structures (see Table 3.2), however there was also significant variation among groups of tributaries (Φ_{CT} = ~ 35%) and among populations within groups of tributaries (Φ_{SC} = 27%) for all three structures. For Φ_{CT}, structure 1 had a slightly higher percentage variation (34.47%) compared to structure 2 (26.83%) and structure 3 (31.54%) (Table 3.7). All three

structures for Φ_{CT} had very similar results due to the divergence between the Draai and Southern lineages (Figure 3.2, Table 3.7).

Table 3.7. Results of the analysis of molecular variance (AMOVA) for the populations of *Barbus motebensis* showing the Φ-statistics and the percentage of variance components for the mitochondrial ND2 gene.

Variance Component		Structure 1	
	% Variance	Phi-value	*P*- value
Among groups	34.47	CT= 0.34	<0.001
Among populations within groups	22.42	ΦSC= 0.34	<0.001
Within populations	43.11	ΦST= 0.56	<0.001
		Structure 2	
Among groups	26.83	ΦCT= 0.27	<0.001
Among populations within groups	27.42	ΦSC= 0.37	<0.001
Within populations	45.74	ΦST= 0.54	<0.001
		Structure 3	
Among groups	31.54	ΦCT= 0.32	<0.001
Among populations within groups	25.08	ΦSC= 0.37	<0.001
Within populations	43.38	ΦST= 0.57	<0.001

3.3.1.4. Cytochrome b *phylogenetic analysis*

The Tamura and Nei model (Tamura & Nei, 1993) best fitted the cytochrome *b* data. Genetic distances based on this substitution model shows a high divergence between *B. gurneyi* and *B. motebensis* (8% < *D* < 9%) and between *B. anoplus* and *B. motebensis* (6% < *D* < 7%) (Figure 3.3). The divergence between the Draai and Southern lineages of *B. motebensis* for cytochrome *b* (0% < *D* < 0.8%) was much lower compared to the mitochondrial ND2 gene.

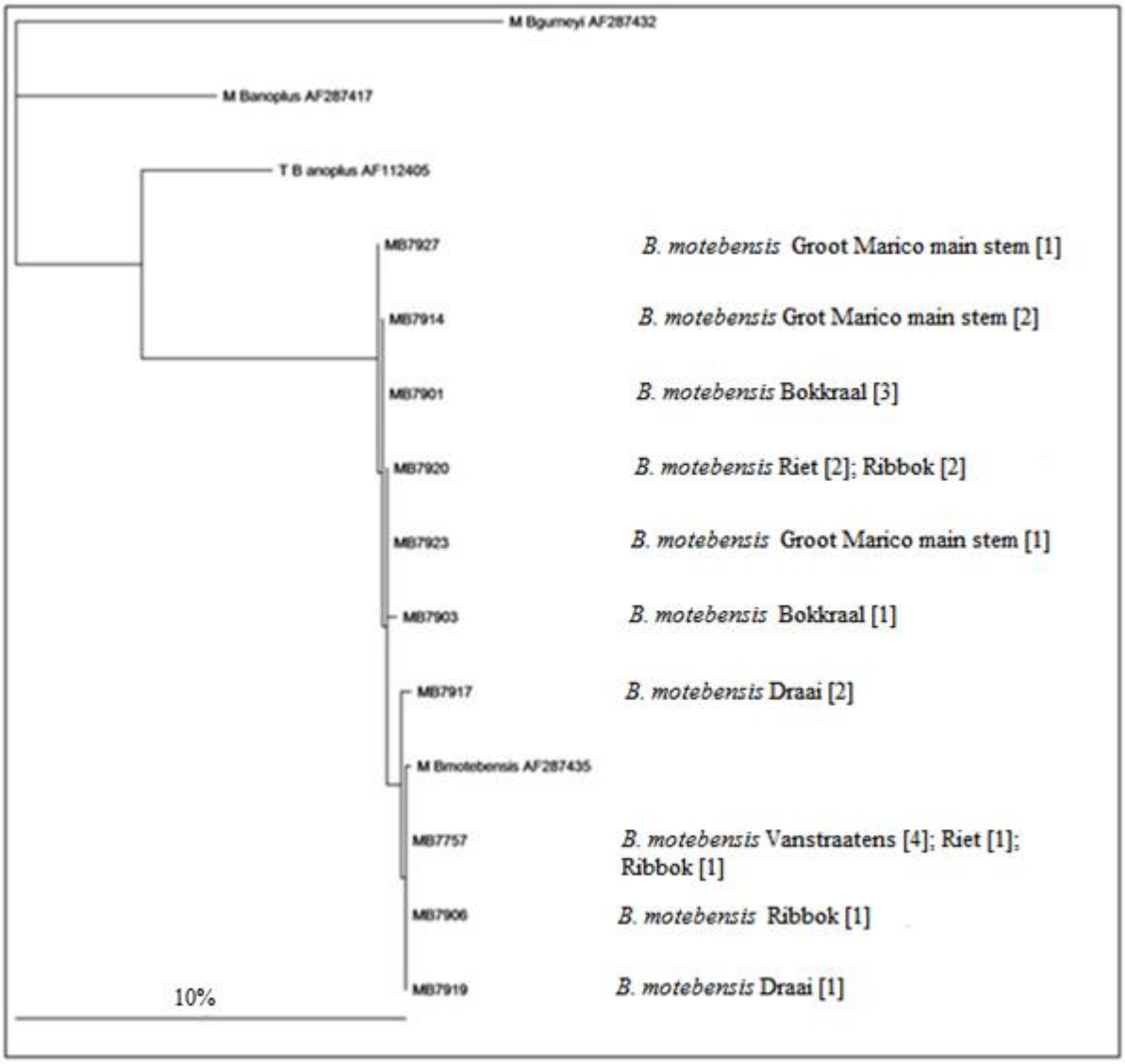

Figure 3.3. Maximum Likelihood tree based on mitochondrial cytochrome *b* sequences (816 base pairs) of *Barbus motebensis* from the Groot Marico catchment (present study; N=21) and published sequences of *B. motebensis*, *B. anoplus* and *B. gurneyi*. The tree is based on the TrN+1 substitution model (Tamura and Nei, 1993). *Barbus gurneyi* was chosen as the outgroup based on the analysis conducted by Engelbrecht and Van der Bank (1996). Brackets indicate the number of individuals. The scale bar represents 10% sequence divergence.

3.3.2. Nuclear S7 intron

3.3.2.1. S7 diversity

For the nuclear S7 gene, the substitution model selected was F81 (Felsenstein, 1981) with a gamma value of 0.000. Analysis of 30 individuals (60 sequences after phasing) from seven populations for 358 base pairs revealed nine alleles defined by 40 variable sites (Table 3.8). No significant deviations from those expected under predictions of neutrality were observed (D = -1.609, P > 0.10 (Tajima, 1989); F = -0.003, P > 0.10 (Fu & Li, 1993)). Overall haplotype diversity was moderate (H_D= 0.540). Haplotype diversity ranged from 0.250 to 1.00 for the Ribbok, Vanstraatens, below Riet waterfall, Draai and the upper Kaaloog. However, haplotype diversity was 0 for the lower Kaaloog and above and below the Bokkraal waterfall (Table 3.8).

3.3.2.2. Allele distribution

As was the case with the mitochondrial DNA results, the Draai population tributary did not share any of its alleles (alleles 8 and 9) with other tributaries (Table 3.8). Five alleles were private to single tributaries, namely in the Vanstraatens (allele 2), Riet (below waterfall) (allele 3), upper Ribbok (allele 4), upper Kaaloog (allele 5) tributaries and the Groot Marico main stem (below waterfall) (allele 7). Allele 6 was restricted to the upper Kaaloog and Groot Marico main stem (below waterfall). Allele 1 was the most widespread and found in six tributaries, namely the lower Kaaloog, above and below Riet waterfall, below Bokkraal waterfall, upper Ribbok, Vanstraatens and below the Groot Marico main stem waterfall (Table 3.8).

Table 3.8. The geographic distribution and frequency of S7 nuclear DNA alleles among sampled localities of *Barbus motebensis*. Sample sizes (N) and haplotype diversity (H_D) are included. (see Table 3.1 for locality descriptions). LD = Lower Draai; UK = Upper Kaaloog; LK = Lower Kaaloog; BRW = Below Riet Waterfall; ABW = Above Bokkraal Waterfall; BBW = Below Bokkraal Waterfall; UR= Upper Ribbok; MV = Middle Vanstraatens; BGMW = Below Groot Marico Waterfall.

Allele Number	N	1 LD	2 UK	3 LK	5 BRW	6 ABW	7 BBW	8 UR	9 MV	14 BGMW
1	40	-	-	4	12	6	4	7	3	4
2	1	-	-	-	-	-	-	-	1	-
3	8	-	-	-	8	-	-	-	-	-
4	1	-	-	-	-	-	-	1	-	-
5	1	-	1	-	-	-	-	-	-	-
6	3	-	1	-	-	-	-	-	-	2
7	2	-	-	-	-	-	-	-	-	2
8	2	2	-	-	-	-	-	-	-	-
9	2	2	-	-	-	-	-	-	-	-
Total	60	4	3	4	20	6	4	8	4	8
H_D	0.540	0.667	1.000	0.000	0.505	0.000	0.000	0.250	0.500	0.714

3.3.2.3. Genetic structuring

The parsimony network constructed for the S7 gene revealed two lineages that could not be connected with 95% confidence (Figure 3.4), similar to the mitochondrial ND2 data. The first lineage comprised only alleles from the Draai tributary (alleles 8 and 9). The seven remaining alleles (from all the sites excluding the Draai tributary) belonged to a second lineage (hereafter the "Southern lineage" equivalent to the Southern lineage inferred from the mitochondrial ND2 data). Genetic distances based on the F81 substitution model (Felsentein, 1981), showed relatively high levels of divergence within the Draai and Southern lineages (both were $0\% < D < 1\%$). However, the divergence between these lineages was exceptionally high ($9\% < D < 11\%$). One ambiguous branch (between allele 4 and the missing allele connected to allele 6) was broken to keep branches elsewhere in the haplotype network (Figure 3.4) following the rules of Templeton *et al*. (1987).

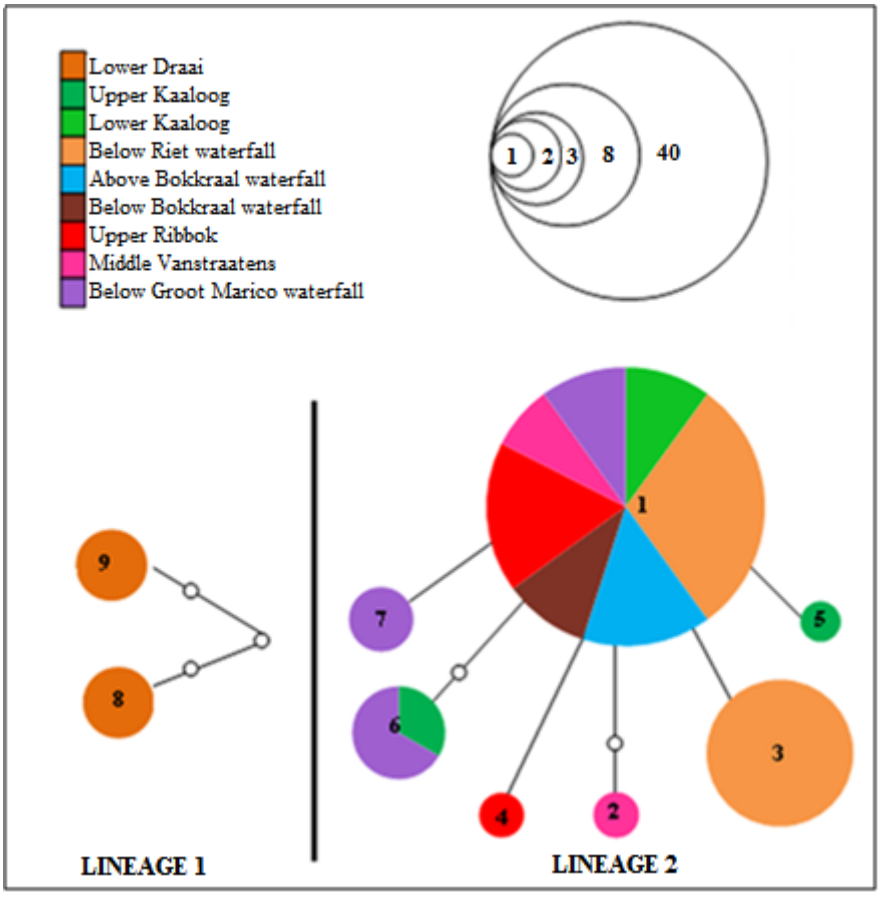

Figure 3.4. Parsimony network for the S7 nuclear gene alleles (numbered circles) of *Barbus motebensis* (See Table 3.1). Allele1 is the central allele. The size of the circles indicates the relative frequency of the alleles (See Table 3.8). Small open white circles that are not numbered indicate missing alleles. Each line in the network represents one mutational change. The thick line represents a separation within the parsimony network, indicating the two different lineages. Genetic distances showed that within lineage divergence (0% < *D* < 1%) was much lower than between lineage divergence (9% < *D* < 11%).

Pairwise Φ_{ST} values between populations that do take genetic distance into consideration showed significant levels of differentiation ($P < 0.05$) between 57% of the comparisons (Table 3.9). Pairwise F_{ST} values that do not take the genetic distance into consideration revealed significant levels of differentiation ($P < 0.05$) between 66% of the pairwise comparisons (Table 3.10). Both the F_{ST} and Φ_{ST} pairwise population comparisons therefore suggest genetic structuring among most of the *B. motebensis* populations.

Table 3.9. Pairwise Φ_{ST} values for *Barbus motebensis* from seven populations in the Groot Marico catchment based on the nuclear S7 gene. The comparisons that were significant ($P < 0.05$) are indicated by asterisks.

	1	2	3	4	5	6	7
1) Draai							
2) Kaaloog	0.964*						
3) Bokkraal	0.985*	0.200*					
4) Riet	0.985*	0.227*	0.126				
5) Ribbok	0.981*	0.136*	0.014	0.105			
6) Vanstraatens	0.967*	0.136	0.126	0.111	0.048		
7) Groot Marico	0.965*	0.136	0.145*	0.188*	0.098*	0.057	

Table 3.10. Pairwise F_{ST} values for *Barbus motebensis* from seven populations in the Groot Marico catchment based on the nuclear S7 gene. The comparisons that were significant ($P < 0.05$) are indicated by asterisks.

	1	2	3	4	5	6	7
1) Draai							
2) Kaaloog	0.397*						
3) Bokkraal	0.827*	0.325*					
4) Riet	0.606*	0.153*	0.126*				
5) Ribbok	0.714*	0.203*	0.014	0.067			
6) Vanstraatens	0.589*	0.094*	0.126	0.021	-0.023		
7) Groot Marico	0.353*	-0.004	0.333*	0.177*	0.224*	0.116	

The AMOVA analysis indicated that there was significant structuring ($P < 0.001$) for each variance component (Table 3.11) in all three structures (see Table 3.3). The degree of differentiation was at its highest within populations (F_{ST}) for all three structures. All three structures had significantly high variation for F_{CT}, but structure 1 had a slightly higher percentage variation (29.25%) compared to structure 2 (20.29%) and structure 3 (26.02%) (Table 3.11). All three pre-defined structures have similar results due to the lack of alleles being shared between the Draai and Southern lineages (Figure 6), which also explains the high variation among groups (F_{CT}). Structure 2 had the highest F_{ST} value (66.63%) but the lowest F_{CT} value (20.29%) (Table 3.11).

Table 3.11. Results of the analysis of molecular variance (AMOVA) for the populations of *Barbus motebensis* showing the *F*-statistics and the percentage of variance for the nuclear S7 gene.

Variance Component	Structure 1	
	% Variance	*F*-value
Among groups	29.25	$F_{CT} = 0.29$
Among populations within groups	9.21	$F_{SC} = 0.13$
Within populations	61.53	$F_{ST} = 0.38$
	Structure 2	
Among groups	20.29	$F_{CT} = 0.20$
Among populations within groups	13.09	$F_{SC} = 0.16$
Within populations	66.63	$F_{ST} = 0.33$
	Structure 3	
Among groups	26.02	$F_{CT} = 0.26$
Among populations within groups	11.31	$F_{SC} = 0.15$
Within populations	62.67	$F_{ST} = 0.37$

3.4. Discussion

The results indicate genetic differentiation among *B. motebensis* populations. Thus, the species does not consist of one panmictic population. Instead, the populations in the upper Groot Marico catchment belong to two distinct genetic lineages that show significant divergence for both the mitochondrial ND2 and nuclear S7 genes. Panmixia can also be rejected within the Southern lineage, since most of the mitochondrial ND2 alleles (90%) were restricted to only one or two tributaries. Therefore, the overall null hypothesis that there is no genetic differentiation between tributary populations can be rejected. This situation is not uncommon in small barbs. Relatively high levels of within–species divergence has been reported for other members of the chubbyhead barb group (Engelbrecht & Van der Bank, 1994, 1996) which should be taken into consideration when conservation management plans are designed for this group of fishes.

The major divergence in the mitochondrial ND2 gene between the Draai and Southern lineages was not reflected in the mitochondrial cytochrome *b* or nuclear S7 datasets. This is probably a result of slower mutation rates (in the case of cytochrome *b*) or possibly due to a different gene history (in the case of S7). Ward *et al*. (2005) found that the average K2P genetic distance within Australian fish species for the mitochondrial CO1 gene was 0.39%. Given that cytochrome *b* has a similar or slightly faster mutation rate compared to CO1 (Kartavtsev & Lee, 2006), the divergence between the Draai and Southern lineages of *B. motebensis* in cytochrome *b* ($0\% < D < 0.8\%$) is more typical of within-species differentiation than between-species divergence. This suggests that the isolation between these two lineages occurred relatively recently. The mutation rates detected for mitochondrial ND2 was much faster than cytochrome *b*, suggesting that it is a much better mitochondrial gene for population genetic studies in *Barbus* species.

The geographic distribution of genetic diversity in *B. motebensis* may have been influenced by extrinsic factors associated with different areas of the Groot Marico catchment (Figure 3.5). These different areas can be defined as the upper, middle and lower reaches of the study area. The extrinsic factors that could have played a role include stream structure, physical barriers to dispersal, isolation by distance, habitat differences or the presence of dolomitic eyes. In addition, intrinsic factors such as the dispersal ability and habitat preference of *B. motebensis* may have played a role.

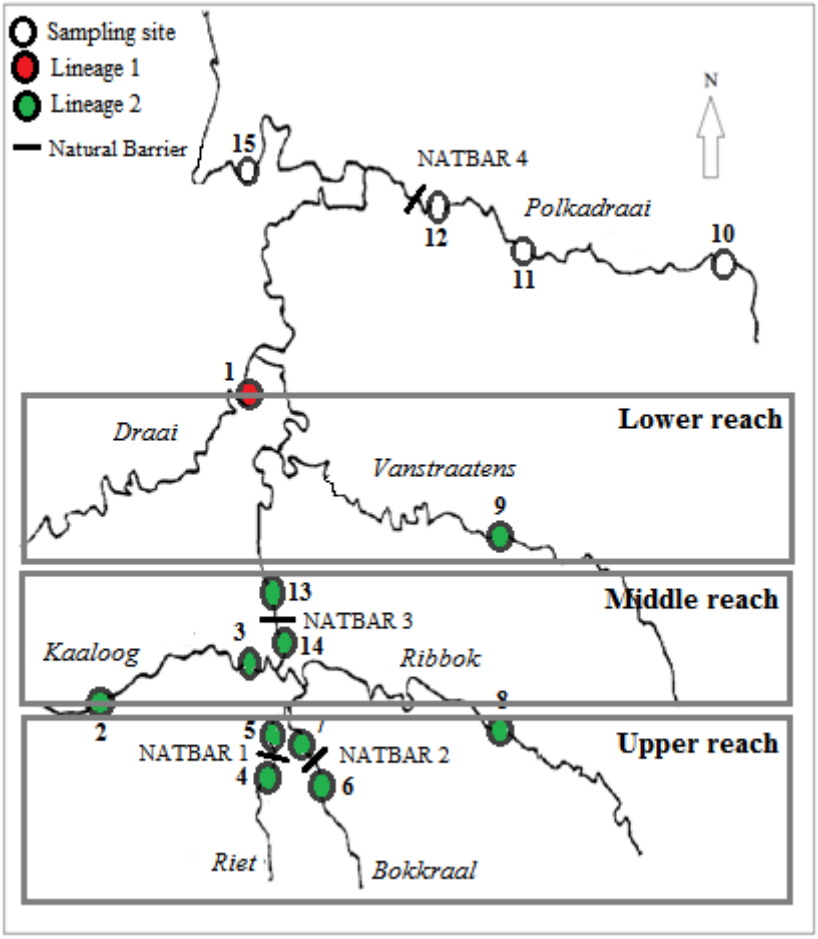

Figure 3.5. Map illustrating the 15 sites of the study area within the upper Groot Marico catchment, North West Province, where *B. motebensis* were collected and used for genetic analysis and how the different tributaries are divides into different reaches (upper, middle and lower) to aid the discussion of results. (NATBAR = natural barrier).

The upper reaches which includes the Riet and Bokkraal tributaries receive water from dolomitic sources, making them perennial tributaries (Provincial River Health Programme Progress Report, 2011). These tributaries have large natural barriers in the form of the approximately 8 m Tufa waterfall on the Bokkraal tributary and a 1.5 m cascading waterfall on the Riet tributary (see Figures 2.4 and 2.5 in Chapter 2). The presence of these waterfalls allows for downstream migration of *B. motebensis* into the lower reaches of the catchment, but likely prevents upstream migration. Although unlikely, *B. motebensis* may have been introduced by humans above these waterfalls. It is also possible that the source population of *B. motebensis* is above the Tufa waterfall and that the population below the waterfall is maintained through downstream migration.

In-stream barriers have been demonstrated to be effective in influencing population genetic structure in fishes (McGlashan & Hughes, 2000). Mataruse (2013) suggested that a small waterfall in the Granaat tributary of the Gamtoos River system, prevented upstream migration of the Joubertina galaxias, causing isolation of a population above the waterfall. In addition, Swartz (2005) further demonstrated that a waterfall in the lower reaches of the Tradou tributary within the Breede River system prevented upstream migration of both the Joubertina galaxias and Tradou redfin. Downstream migration might have been possible, but no introgression of mtDNA haplotypes between the Tradou and Breede lineages was found in the present study. This suggests that the Tradou lineage was either not in contact with the other lineages in areas where their distribution overlapped or alien fishes have had an influence in limiting their distribution and effectively removing potential historical distributions. However, in the present study, *B. motebensis* in the Bokkraal tributary showed little differentiation compared to other populations suggesting that complete isolation has not yet taken place or that isolation is too recent to be reflected in the genetic markers investigated. Furthermore, during surveys, *B. motebensis* was only found below the Riet River waterfall, suggesting that *B. motebensis* has been unable to move across this barrier. This is likely, as the waterfall is made up of a number of cascading steps (see Figure 2.4, Chapter 2). Although ND2 and S7 were variable enough to reject overall panmaxia, further research based on larger sample sizes and more variable markers are required to determine what influence the barriers have on fine scale gene flow patterns in *B. motebensis*.

The middle reach of the study area does not have natural barriers, making it open to gene flow between the Kaaloog and Ribbok tributaries. However, only two of the eight ND2 alleles that occur in these two tributaries are shared between them. The Kaaloog tributary is of dolomitic origin making it a perennial tributary. The Ribbok tributary is not of dolomitic origin, but is on a low gradient wetland with high seasonal flow (Provincial River Health Programme Progress Report, 2011). These factors may be playing a role in gene flow patterns between these two tributaries, but higher sample sizes are required to assess whether gene flow has indeed been affected. Below the confluence of these two tributaries is a waterfall on the Groot Marico main stem. This barrier may have an influence on genetic structuring, since both the Vanstraatens and Draai tributary populations have become isolated. However, the waterfall is only approximately 1 m in height, making it possible that this barrier may be breached during periods of flooding and it does not explain why downstream migration has apparently not occurred.

The lower reaches of the study area can be defined to include the Draai and Vanstraatens tributaries. Both of these tributaries are of dolomitic origin (Provincial River Health Programme Progress Report, 2011) and they do not have major waterfalls that would restrict gene flow. Given the lack of clear barriers to dispersal, the large genetic divergence between the Draai and the other tributary populations as well as the Groot Marico main stem for both the mitochondrial ND2 and nuclear S7 genes is surprising. Furthermore, the Vanstraatens population within the Southern lineage does not share its three ND2 alleles with any other population. The reasons for these two populations becoming isolated (and divergent in the case of the Draai) are not clear, but potential reasons may include habitat preference, flow conditions or isolation by distance.

The Vanstraatens and Draai tributaries have a lower flow volume and lower gradient compared to other tributaries in the Groot Marico catchment (Roux, 2010), which may have resulted in a disruption of gene flow or local adaption, especially in the case of the Draai population that has evolved divergent alleles. The faster flowing Groot Marico main stem may negatively affect *B. motebensis* migration between the Draai and Vanstraatens. However, the species is present in the main stem habitat in the upper reaches of the Groot Marico catchment and one would expect occasional migrants to cross the lower Marico main stem region, especially during floods.

If the Groot Marico main stem in the lower reaches of the study area is not suitable for *B.* motebensis, it is possible that isolation by distance played a role in isolating the Draai and Vanstraatens populations. Isolation by distance occurs when organisms have limited dispersal ability, which increases genetic differentiation between sites with increasing geographic distance between them (Wright, 1943). Slatkin (1993) further suggested that in highly connected populations, isolation by distance will occur if the dispersal distance of an individual is less than the range of the species. It may therefore be possible that *B. motebensis* individuals have small home ranges or very strong preference for tributary habitats and are able to maintain their position in tributaries during floods.

These patterns of genetic structuring can be used to prioritise conservation areas for *B. motebensis* in the Groot Marico catchment. The highest priority would be to conserve at least three populations to represent the Draai lineage (Draai tributary), the isolated alleles of the Vanstraatens tributary and at least one Southern tributary to represent as much of the rest of the Southern lineage's genetic diversity as possible. Further analysis of microsatellite loci or

a similar variable genetic marker will not only provide a better understanding of gene flow patterns and direction across the waterfall, but also allow more effective prioritisation to conserve genetic diversity.

CHAPTER 4

Genetic structuring of the Stargazer mountain catfish, *Amphilius uranoscopus*, in the Groot Marico Catchment, North West Province, South Africa

4.1. Introduction

The catfish species, *Amphilius uranoscopus* (Pfeffer, 1889), is widespread across southern, eastern and central Africa (Skelton, 1984; Seegers, 2008). In South Africa it occurs from the Mkuze and Pongola rivers in Kwa-Zula Natal northwards to the Limpopo River (Van Oosterhout *et al.* 2009). Although, *A. uranoscopus* is a widespread species, the ecology and distribution patterns of this species in the Groot Marico catchment are unknown. Furthermore, invasive species are a threat to *A. uranoscopus*. Kadye *et al*. (2008) found that *A. uranoscopus* occur in fragmented populations in the presence of alien trout, *Oncorhynchus mykiss*, in rivers of the montane plateau of Malawi. The non-native largemouth bass, *Micropterus salmoides* found in the upper reaches of the Groot Marico catchment may have a similar predatory impact on *A. uranoscopus*.

Besides the introduction of non-native species, tributary populations of *A. uranoscopus* may be genetically fragmented as a result of various extrinsic and intrinsic factors, similar to the factors affecting *Barbus motebensis* in Chapter 3. Compared to *B. motebensis*, *A. uranoscopus* should be better at dispersing across barriers due to its morphological adaptations (see section 1.2.2, Chapter 1). These adaptations could also have contributed to the much wider distribution of *A. uranoscopus* globally compared to *B. motebensis*.

As described in Chapter 3, it is important to identify Evolutionarily Significant Units to allow the conservation of evolutionary processes. Thus the aims of this chapter are therefore to: (1) assess the genetic diversity of *A. uranoscopus* in the Groot Marico catchment; (2) determine whether genetic isolation and structuring has occurred; (3) use these data to develop hypotheses on how the ecology of this species, natural landscape and climatic processes may have influenced the genetic structure of populations. As was the case with *B. motebensis* (Chapter 3), these aims will be achieved by testing the null hypothesis that there is no genetic differentiation between tributary populations of *A. uranoscopus*.

4.2. Materials and methods

4.2.1. Specimens

Specimens of *A. uranoscopus* for genetic analysis were collected from 11 sampling sites from six different tributaries and the Groot Marico main stem of the Groot Marico catchment (Figure 4.1) and analysed as described for *B. motebensis* in Chapter 3. For the mitochondrial ND2 gene, *A. uranoscopus* was analysed from the Kaaloog, Bokkraal, Ribbok and Vanstraatens tributaries and the Groot Marico River main stem. For the nuclear S7 gene, *A. uranoscopus* was analysed from the Draai, Kaaloog, Riet, Bokkraal, Ribbok and Vanstraatens tributaries and the Groot Marico River main stem (see Table 4.1 for sample sizes). No specimens of *A. uranoscopus* were found in the Polkadraai tributary (Figure 4.1, Table 4.1).

4.2.2. Laboratory and statistical analysis

Total genomic DNA extraction, amplification of the ND2 and S7 fragments and sequencing of resulting products followed the methods described in Chapter 2 (see Section 2.4.1). Furthermore, sequence editing, alignment (see Section 2.5.1) and statistical analysis (see Section 2.5.3) followed the methods outlined in Chapter 2. The AMOVA analysis (see Section 2.5.3) was done on three predefined hierarchical structures for both the mitochondrial ND2 and nuclear S7 genes (Table 4.2, Table 4.3).

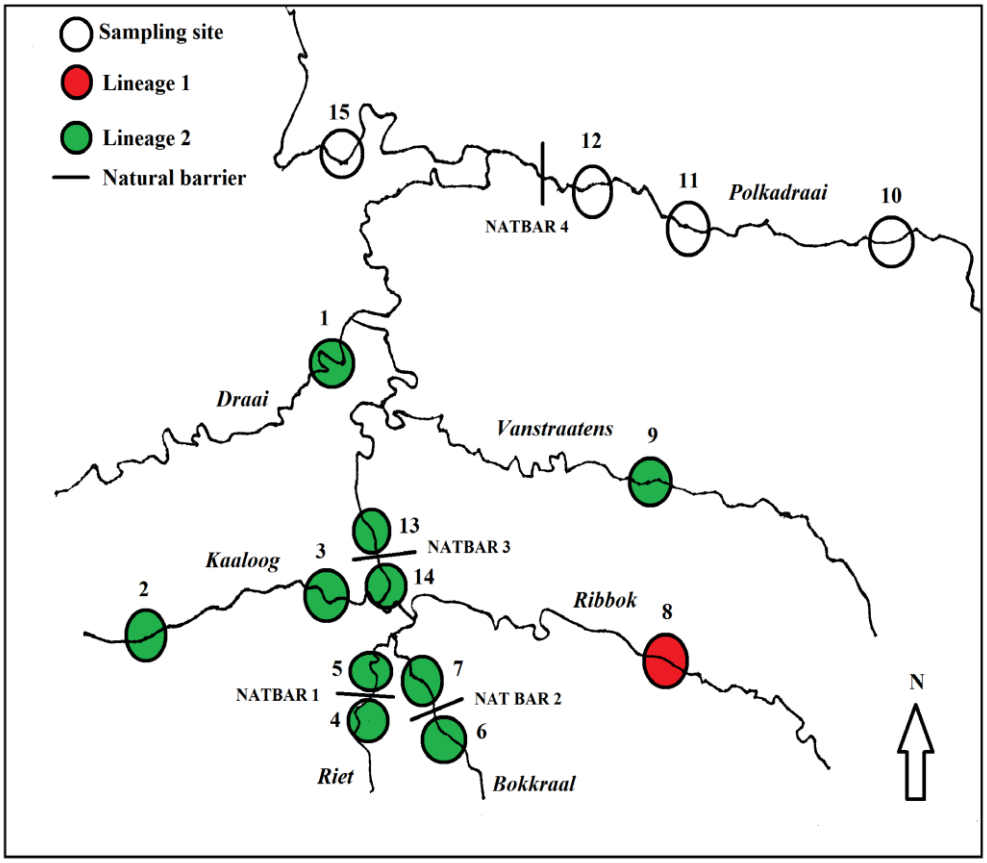

Figure 4.1. Map illustrating the 11 sites where *A. uranoscopus* specimens were collected and analysed across the upper Groot Marico catchment, North West Province. There are 4 major natural barriers in the study area, indicated by perpendicular lines (NATBAR = natural barrier). From the genetic analysis, the mitochondrial ND2 gene had 2 main lineages. The first lineage (Ribbok lineage, red) only occurs in the Ribbok tributary. The second lineage (Western lineage, green) occurs in the Draai, Kaaloog, Riet, Bokkraal and Vanstraatens tributaries and the Groot Marico main stem. The nuclear S7 gene had no distinct lineages and displayed panmaxia. *Amphilius uranoscopus* were not recorded in the Polkadraai tributary (Sites 10, 11 and 12). Not all the samples from all sites were analysed successfully for both genes (see Table 4.1 for presence/absence and the samples that were analysed successfully).

Table 4.1. Localities (see Figure 4.1) and sample sizes (*n*) analysed for the mitochondrial ND2 gene and the nuclear S7 gene of *Amphilius uranoscopus.*

Map	Locality	Locality Codes	Presence/ Absence	ND2 analysis (*n*)	S7 analysis (*n*)
1	Lower Draai	LD	yes	0	6
2	Upper Kaaloog	UK	yes	1	0
3	Lower Kaaloog	LK	yes	8	4
4	Above Riet waterfall	ARW	no	0	0
5	Below Riet waterfall	BRW	yes	0	2
6	Above Bokkraal waterfall	ABW	yes	7	8
7	Below Bokkraal waterfall	BBW	yes	2	0
8	Upper Ribbok	UR	yes	8	18
9	Middle Vanstraatens	MV	yes	11	8
10	Upper Polkadraai	UP	no	0	0
11	Middle Polkadraai	MP	no	0	0
12	Lower Polkadraai	LP	no	0	0
13	Groot Marico Sonop	GMS	yes	4	0
14	Below Groot Marico waterfall	BGMW	yes	8	14
15	Groot Marico Riverstill	GMR	yes	2	0
Total				51	60

Table 4.2. Different structures used for the analysis of molecular variance (AMOVA) for the populations of *Amphilius uranoscopus* for the mitochondrial ND2 gene.

Groups	Structure 1	Structure 2	Structure 3
1	Kaaloog	Kaaloog Bokkraal	Kaaloog Bokkraal
2	Bokkraal Ribbok	Ribbok	Ribbok Vanstraatens
3	Vanstraatens Groot Marico	Vanstraatens Groot Marico	Groot Marico

Table 4.3. Different structures used for the analysis of molecular variance (AMOVA) for the populations of *Amphilius uranoscopus* for the nuclear S7 gene.

Groups	Structure 1	Structure 2	Structure 3
1	Draai	Draai	Draai
2	Kaaloog Riet Bokkraal	Kaaloog Riet Bokkraal	Kaaloog Riet Bokkraal Ribbok Vanstraatens
3	Vanstraatens Groot Marico	Ribbok Vanstraatens Groot Marico	Groot Marico

4.3. Results

4.3.1. Mitochondrial DNA analysis

4.3.1.1. ND2 diversity

For the mitochondrial ND2 gene, F81 (Felsenstein, 1981) was determined as the substitution model that best fits the data. Analysis of 51 individuals from 5 populations for 534 base pairs of the mitochondrial ND2 gene resulted in 4 alleles defined by 10 variable sites (Ti:Tv ratio = 1.506) (Table 4.4). The overall nucleotide diversity was low (π = 0.004) and no significant deviations from those expected under predictions of neutrality were found (D = -0.1828, P > 0.10 (Tajima, 1989); F = 0.0593, P > 0.10 (Fu & Li, 1993)). Overall haplotype diversity was low (H_D = 0.395) and ranged from 0 to 1.000 (Table 4.4).

4.3.1.2. ND2 Allele distribution

Two of the four alleles were private to single localities (Table 4.4). The Ribbok tributary did not share its single allele (allele 4) with the other tributary populations. Individuals from the Vanstraatens tributary (site 9) and the Groot Marico main stem close to the Riverstill farm (site 15) shared allele 3 (see Table 4.1). Allele 1 was by far the most common allele and was shared among all sites except for the Ribbok tributary (Table 4.4). Allele 2 was only found in the Vanstraatens tributary (site 9).

Table 4.4. The geographic distribution and frequency of mtDNA (ND2) alleles among sampled localities of *Amphilius uranoscopus*. Samples sizes (N) and haplotype diversity (HD) are included (See Table 4.1 for locality descriptions. UK = Upper Kaaloog; LK = Lower Kaaloog; ABW = Above Bokkraal Waterfall; BBW = Below Bokkraal Waterfall; UR = Upper Ribbok; MV = Middle Vanstraatens; GMS = Groot Marico Sonop; BGMW = Below Groot Marico Waterfall; GMR = Groot Marico Riverstill.

Allele number	N	2 UK	3 LK	6 ABW	7 BBW	8 UR	9 MV	13 GMS	14 BGMW	15 GMR
1	39	1	8	7	2	-	8	4	8	1
2	1	-	-	-	-	-	1	-	-	-
3	3	-	-	-	-	-	2	-	-	1
4	8	-	-	-	-	8	-	-	-	-
Total	51	1	8	7	2	8	11	4	8	2
HD	0.395	0.000	0.000	0.000	0.000	0.000	0.473	0.000	0.000	1.000

4.3.1.3. Genetic structuring

The parsimony network revealed two different lineages (Figure 4.2). The first lineage comprised allele 4 from the Ribbok tributary and a second "Western" lineage comprising three alleles found in the Kaaloog, Bokkraal and Vanstraatens tributaries and the Groot Marico main stem (Figure 4.2). Genetic distances based on the F81 substitution model (Felsentein, 1981) showed low divergence (0.2% < *D* < 0.5%) within the Western lineage. The divergence between the Ribbok and Western lineages was relatively minor (1.3% < *D* < 1.7%; Figure 4.2) compared to those observed in Chapter 3 for *B. motebensis.*

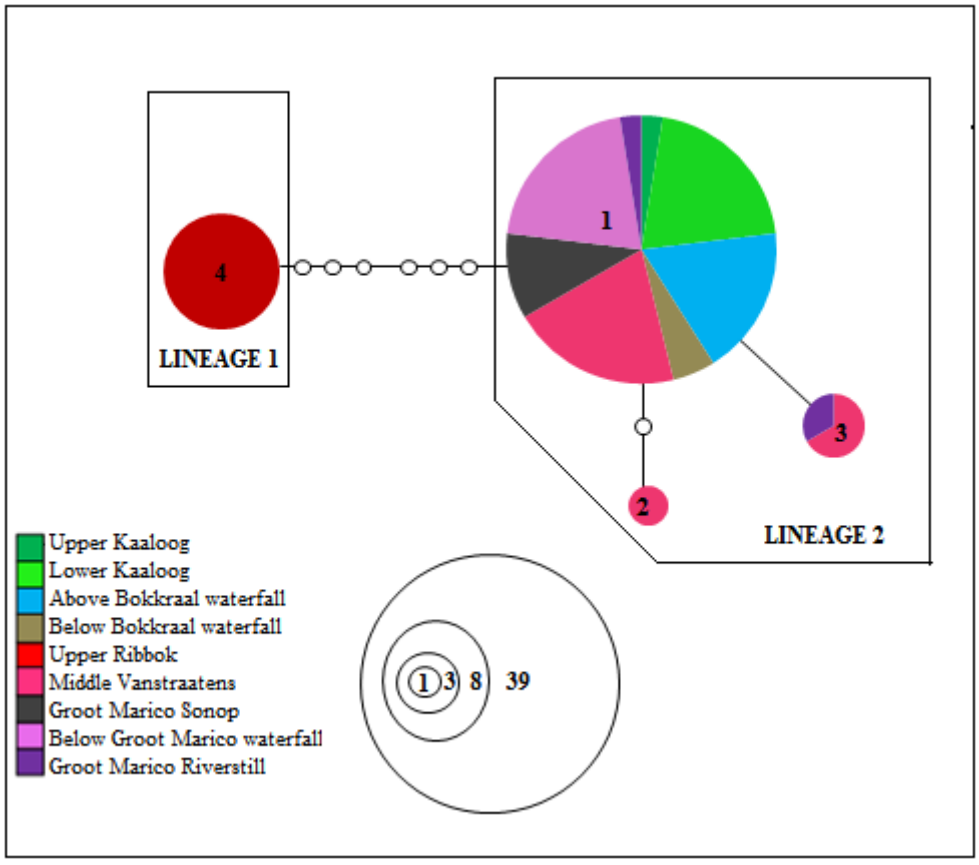

Figure 4.2. Parsimony network with 95% plausible set of mitochondrial ND2 allele connections (numbered circles) constructed with the program TCS 1.2.1 (Clement *et al.*2000) for *Amphilius uranoscopus* (see Table 4.1). The size of the circles indicates the relative frequency of the alleles (total N=51) (see Table 4.4). Small open circles that are not numbered indicate missing alleles. Each line in the network represents one mutational stage. Lineages are indicated by rectangular lined boxes. Lineage 1 is referred to as the Ribbok lineage. Lineage 2 is referred to as the Western lineage. The Western lineage consists of the Kaaloog, Bokkraal and Vanstraatens tributaries and the Groot Marico main stem. Genetic distances within the Ribbok lineage were 0%. Within the Western lineage, genetic distances indicate that divergence was low (0.2% < *D* < 0.5%) and between the Ribbok lineage and the Western lineage, the divergence was relatively high (1.3% < *D* < 1.7%).

Pairwise Φ_{ST} values which take genetic distances into consideration, revealed non-significant ($P > 0.05$) levels of differentiation for 60 % of the pairwise population comparisons. The only significant differences were comparisons that involved the Ribbok lineage compared to populations of the Western lineage (Table 4.5). Pairwise F_{ST} values which do not take genetic distance among alleles into consideration showed significant ($P < 0.05$) levels of differentiation among the same populations as the pairwise Φ_{ST} analysis (Table 4.6).

Table 4.5. Pairwise Φ_{ST} values for *Amphilius uranoscopus* from 5 populations in the Groot Marico catchment based on the mitochondrial ND2 gene using the F81 substitution model (Felsentein, 1981). The comparisons that were significant ($P < 0.05$) are indicated by asterisks.

	1	2	3	4	5
1) Kaaloog					
2) Bokkraal	0.000				
3) Ribbok	1.000*	1.000*			
4) Vanstraatens	0.027	0.027	0.946*		
5) Groot Marico	-0.035	-0.035	0.987*	-0.006	

Table 4.6. Pairwise *FST* values for *Amphilius uranoscopus* from 5 populations in the Groot Marico catchment based on the mitochondrial ND2 gene using the F81 substitution model (Felsentein, 1981). The comparisons that were significant ($P< 0.05$) are indicated by asterisks.

	1	2	3	4	5
1) Kaaloog					
2) Bokkraal	0.000				
3) Ribbok	1.000*	1.000*			
4) Vanstraatens	0.106	0.106	0.731*		
5) Groot Marico	-0.035	-0.035	0.908*	0.025	

The AMOVA analysis indicated that all variance components had non-significant levels of genetic structuring ($P > 0.001$) (see Table 4.2) (Table 4.7). Structure 2 best captures the genetic structuring in *A. uranoscopus* from the Groot Marico catchment, since most of the variation was found between groups (Φ_{CT} = ~ 94%). This is a result of the divergence between the Ribbok and Western lineages (Figure 4.2, Table 4.7).

Table 4.7. Results of the analysis of molecular variance (AMOVA) for the populations of *Amphilius uranoscopus* showing the Φ-statistics and the percentage of variance for the mitochondrial ND2 gene.

Variance Component	Structure 1	
	% Variance	Phi-value
Among groups	-27.34	ΦCT= -0.27
Among populations within groups	119.46	ΦSC= 0.94
Within populations	7.88	ΦST= 0.92
	Structure 2	
Among groups	94.21	ΦCT= 0.94
Among populations within groups	-0.04	ΦSC= -0.01
Within populations	5.83	ΦST= 0.94
	Structure 3	
Among groups	-63.48	ΦCT= -0.63
Among populations within groups	155.27	ΦSC= 0.95
Within populations	8.2	ΦST= 0.92

4.3.2. Nuclear S7 intron

4.3.2.1. S7 diversity

For the nuclear S7 gene, the best substitution model selected was F81 (Felsentein, 1981). Analysis of 30 individuals (60 sequences after phasing) from seven populations for 631 base pairs revealed four alleles defined by eight variable sites (Table 4.8). No significant deviations from those expected under predictions of neutrality were observed (D = -1.7896, P > 0.10 (Tajima, 1989); F = -1.4274, P > 0.10 (Fu & Li, 1993)). Overall haplotype diversity was low (H_D = 0.129). Haplotype diversity ranged from 0 for the lower Kaaloog, below the Riet waterfall, above the Bokkraal waterfall and upper Ribbok sites to 0.733 for the lower Draai site (Table 4.8).

4.3.2.2. S7 allele distribution

Three of the four alleles were private to single tributaries, namely the Vanstraatens (allele 2) and Draai tributaries (allele 3 and allele 4). Allele 1 was the most widespread and was found in all the tributaries that were analysed (Table 4.8).

Table 4.8. The geographic distribution and frequency of S7 nuclear DNA alleles among sampled localities of *Amphilius uranoscopus*. Sample sizes (N) and haplotype diversity (HD) are included (See table 4.1 for locality descriptions). LD = Lower Draai; LK = Lower Kaaloog; BRW = Below Riet Waterfall; ABW = Above Bokkraal Waterfall; UR = Upper Ribbok; MV = Middle Vanstraatens; BGMW = Below Groot Marico Waterfall.

Allele number	N	1 LD	3 LK	5 BRW	6 ABW	8 UR	9 MV	14 BGMW
1	56	3	4	2	8	18	7	14
2	1	-	-	-	-	-	1	-
3	1	1	-	-	-	-	-	-
4	2	2	-	-	-	-	-	-
Total	60	6	4	2	8	18	8	14
HD	0.129	0.733	0.000	0.000	0.000	0.000	0.250	0.000

4.3.2.3. S7 Genetic structuring

The parsimony network constructed for *A. uranoscopus* with 95% plausible set of nuclear S7 allele connections (Figure 4.3) did not reveal distinct lineages. However, the most divergent alleles (alleles 3 and 4) were both from the Ribbok tributary which supports the ND2 results that suggests isolation of this population.

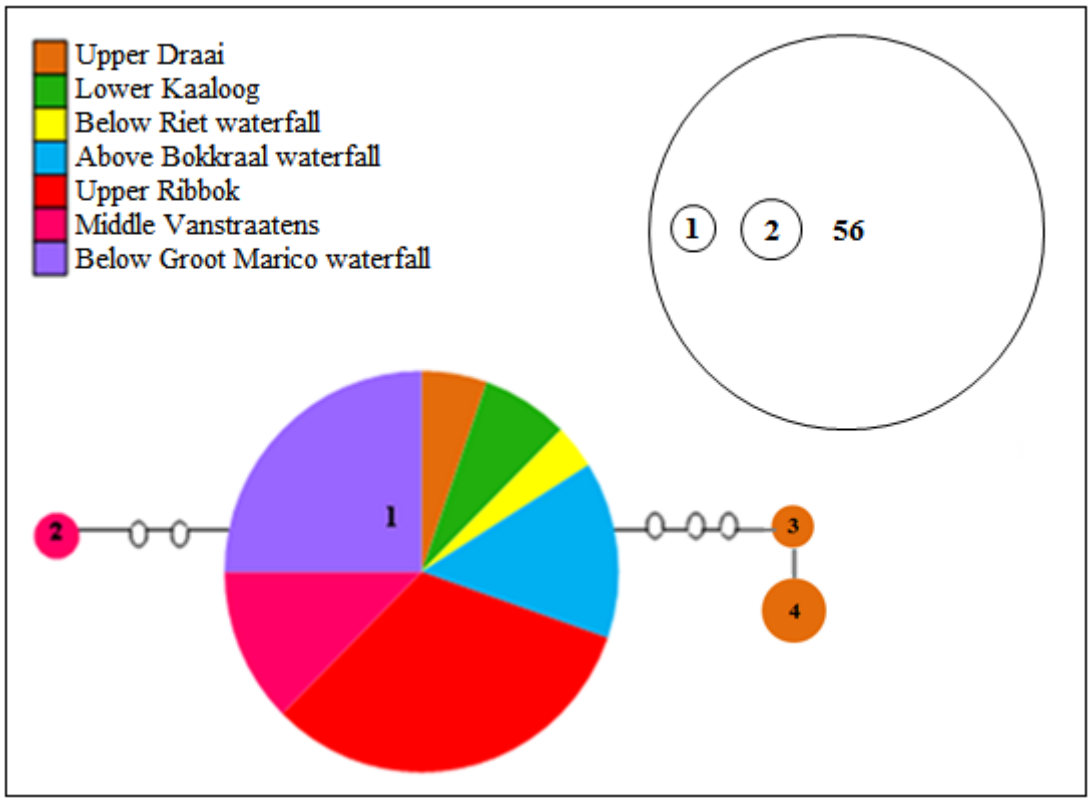

Figure 4.3. Parsimony network for the S7 nuclear gene alleles (numbered circles) of *Amphilius uranoscopus* (See Table 4.). Allele 1 is the central allele. The size of the circles indicates the relative frequency of the alleles (See Table 4.8). Small open white circles that are not numbered indicate missing alleles. Each line in the network represents one mutational change.

Pairwise Φ_{ST} values between populations that do take genetic distances into consideration showed no significant levels of differentiation ($P > 0.05$) between 100% of the comparisons (Table 4.9). Pairwise F_{ST} values that do not take genetic distance into consideration revealed significant ($P < 0.05$) levels of differentiation between 10% of the pairwise comparisons (Table 4.10). This is a result of the Draai tributary (alleles 3 and 4) being significantly different from the other tributary populations.

Table 4.9. Pairwise Φ_{ST} values for *Amphilius uranoscopus* from seven populations in the Groot Marico catchment based on the nuclear S7 gene. The comparisons that were significant ($P < 0.05$) are indicated by asterisks.

	1	2	3	4	5	6	7
1) Draai							
2) Kaaloog	0.115						
3) Riet	0.015	0.000					
4) Bokkraal	0.209	0.000	0.000				
5) Ribbok	0.352	0.000	0.000	0.000			
6) Vanstraatens	0.171	-0.050	-0.134	0.000	0.055		
7) Groot Marico	0.303	0.000	0.000	0.000	0.000	0.037	

Table 4.10. Pairwise *FST* values for *Amphilius uranoscopus* from seven populations in the Groot Marico catchment based on the nuclear S7 gene. The comparisons that were significant ($P < 0.05$) are indicated by asterisks.

	1	2	3	4	5	6	7
1) Draai							
2) Kaaloog	0.071						
3) Riet	-0.026	0.000					
4) Bokkraal	0.158	0.000	0.000				
5) Ribbok	0.289*	0.000	0.000	0.000			
6) Vanstraatens	0.065	-0.05	-0.135	0.000	0.055		
7) Groot Marico	0.243*	0.000	0.000	0.000	0.000	0.036	

The AMOVA analysis indicated that there was significant structuring for most ($P < 0.001$) variance components in all three structures (see Table 4.2) (Table 4.11). The degree of differentiation was at its highest within populations (F_{ST}) for all three structures (see Table 4.3). Structure 3 best describes the genetic structuring, since it has the highest *FST* and *FCT* values (Table 4.11).

Table 4.11. Results of the analysis of Molecular Variance (AMOVA) for the populations of *Amphilius uranoscopus* showing the *F*- statistics and the percentage of variance for the nuclear S7 gene.

Variance Component	Structure 1	
	% Variance	Phi-value
Among groups	20.65	FCT= 0.21
Among populations within groups	-3.76	FSC= -0.05
Within populations	83.10	FST= 0.17
	Structure 2	
Among groups	21.97	FCT= 0.22
Among populations within groups	-2.8	FSC= -0.03
Within populations	80.83	FST= 0.19
	Structure 3	
Among groups	22.74	FCT= 0.28
Among populations within groups	-3.31	FSC= -0.04
Within populations	80.57	FST= 0.19

4.4 Discussion

The overall null hypothesis that there is no genetic differentiation between tributary populations can be rejected because two distinct lineages (Ribbok and Western) were found with the mitochondrial ND2 data. The nuclear S7 data did not show a similar divergence, but two of the three S7 alleles that occurred in the Ribbok tributary were not shared with the other populations. The different results between the two genes may be due to differences in mutation rate (see Birky *et al*. 1989) or gene history.

The divergence between the two ND2 lineages ($1.3\% < D < 1.7\%$) is relatively recent. This divergence is not comparable to species level divergence and is more typical of within species level differentiation. The ND2 gene mutation rate is faster than the CO1 gene generally which exhibits a 2% divergence between species (Wolstenholme & Clary, 1985). The Ribbok lineage was only represented by one ND2 allele, but the population had the highest diversity of S7 alleles (N =3). The ND2 divergence in the Western lineage was low ($0.2\% < D < 0.5\%$), suggesting that gene flow may be taking place between these populations.

Previous studies have shown that the morphological adaptations of *A. uranoscopus* may assist in its dispersal ability, allowing for upstream migration. Pfeffer (1889) and Bell-Cross (1976) suggested that the strong first rays of the pectoral and pelvic fins of *A. uranoscopus* may assist it in crossing obstructing rocks. Furthermore, Van Oosterhout *et al.* (2009) observed this previously mentioned movement and found in addition that it uses its teeth on the upper jaw to maintain its position and its mouth and ventral side of the body forms a sucking disc for attachment to slippery rocks (Figure 4.4). These characteristics are assumed to be important to enable *A. uranoscopus* to survive in headwater streams where it may be faced with numerous waterfalls with steep obstructing rocks in shallow water. This may allow *A. uranoscopus* to interact with upstream populations (Van Oosterhout *et al*. 2009). This may be the case for the Bokkraal and Riet waterfalls, since both tributaries are part of the Western lineage and they share the most common ND2 and S7 alleles above and below these two waterfalls. However, more variable genetic markers will have to be analysed to test the possibility of upstream migration across such barriers.

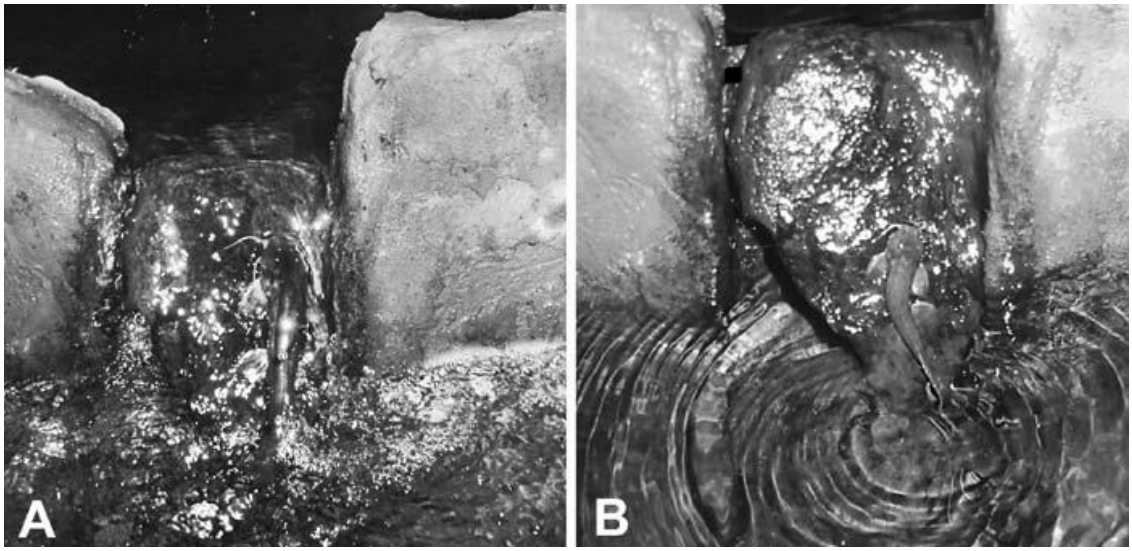

Figure 4.4. Two methods for *Amphilius uranoscopus* to migrate upstream over obstacles: **A** Swimming rapidly into the water flow, **B** In a walking motion, using fins and mouth (taken from Van Van Oosterhout *et al*. 2009).

However, the relatively large genetic divergence between the Ribbok tributary and populations of the Western lineage for the mitochondrial ND2 gene ($1.3\% < D < 1.7\%$) is not consistent with the proposed dispersal ability of *A. uranoscopus.* The reason for the relatively recent isolation of the Ribbok population is not clear, because there does not seem to be a natural barrier that could be playing a role. Potential reasons for this isolation may therefore include habitat preference, isolation by distance or it is possible that increased sample sizes may reveal more alleles that are more widespread. Isolation by distance is probably an unlikely explanation, due to the species dispersal ability (Scott *et al*. 2006) and its morphological adaptations outlined above.

Differences in ecology between the Ribbok and other tributaries and the Groot Marico main stem may have contributed to population fragmentation. The upper reaches of the Ribbok tributary has highly seasonal flow across a low gradient resulting in a wetland/ floodplain area (Habitat Integrity Assessment Report of selected Rivers in the North West Province, 2007). The Groot Marico main stem has a higher flow volume and a steeper gradient. It may be possible that populations of *A. uranoscopus* may be isolated as a result of the highly seasonal flow in the Ribbok tributary.

These patterns of genetic structuring can assist in assigning priority populations for the conservation of *A. uranoscopus* in the Groot Marico catchment. The highest priority would be to conserve at least two tributary populations which represent the Ribbok and Western lineages. In the case of the Western lineage, the Vanstraatens tributary would be preferable, because it has the highest diversity of both mitochondrial ND2 and nuclear S7 alleles.

Chapter 5

Genetic structuring of the Short-spine suckermouth catfish, *Chiloglanis pretoriae*, in the Groot Marico Catchment, North West Province, South Africa

5.1. Introduction

Chiloglanis pretoriae, van der Host, 1931, commonly known as the shortspine suckermouth catfish, is widely spread throughout the eastern parts of central Africa. In South Africa, it occurs from the Limpopo, Incomati and Pongola River systems (Jubb & Le Roux, 1969). When analysing this species distribution patterns in more detail, Bell-Cross (1972) documented that *C. pretoriae* does not occur in high-altitude systems within the Limpopo River system. However, Hecht and Scholtz (1983) documented the distributional range of *C. pretoriae* in the Steelpoort tributary of the Olifants River (Limpopo River system) to be from 1750 m above sea level to 457 m above sea level. This suggests that *C. pretoriae* has a wider altitudinal range when compared to most catfish species and prefers fast flowing rocky streams or rapids (Skelton, 2001).

Alternatively, *C. pretoriae* has a well developed sucker-like mouth, which it uses to suck and attach itself to rocky substrates allowing it to inhabit fast flowing streams or rivers (De Villiers, 1991). This species may therefore be better adapted than *Barbus motebensis* (Chapter 3) or even *Amphilius uranoscopus* (Chapter 4) to overcome natural barriers to dispersal in upper tributary streams. In addition, *C. pretoriae* is useful as an indicator species and has been used for this purpose in the Olifants catchment in the Limpopo Province (Rashleigh *et al*. 2009).

Despite the wide distribution of this species and its importance in conservation studies, there is limited information available on genetic diversity. This chapter investigates the patterns of genetic diversity of *C. pretoriae* in the Groot Marico catchment to determine whether this area has one panmictic population or discrete subpopulations (following Slatkin, 1985). Furthermore, it is important to determine which extrinsic or intrinsic factors (see Chapters 1, 3 and 4) underlie observed genetic patterns, so that populations can be prioritised for conservation management actions and whether these overlap with other species in the catchment (see Chapters 3 and 4).

Thus the aims of this chapter are to: (1) assess the genetic diversity of *C. pretoriae* in the Groot Marico catchment; (2) determine whether genetic isolation and structuring has occurred; (3) use these data to develop hypotheses on how the ecology of this species, natural landscape and climatic processes may have influenced the genetic structure of populations. As was the case with *B. motebensis* (Chapter 3) and *A. uranoscopus* (Chapter 4), these aims will be achieved by testing the null hypothesis that there is no genetic differentiation between tributary populations of *C. pretoriae*.

5.2. Materials and methods

5.2.1. Specimens

Specimens of *C. pretoriae* for genetic analysis were collected and analysed from 11 sampling sites from five different tributary populations and the Groot Marico main stem of the Groot Marico catchment (Figure 5.1) and analysed as described for *B. motebensis* in Chapter 3 and *A. uranoscopus* in Chapter 4. For the mitochondrial ND2 gene, *C. pretoriae* was analysed from the Draai, Kaaloog, Ribbok and Polkadraai tributary populations as well as the Groot Marico River main stem. For the nuclear S7 gene, *C. pretoriae* was analysed from the Draai, Kaaloog, Riet, Ribbok and Polkadraai tributary populations and the Groot Marico main stem (see Table 5.1 for sample sizes). No *C. pretoriae* were found in the Bokkraal and Vanstraatens tributaries. (Figure 5.1, Table 5.1).

5.2.2. Laboratory and statistical analysis

DNA extraction, amplification and sequencing of the ND2 and S7 fragments followed the methods outlined in Chapter 2 (see Section 2.4.1). Furthermore, sequence editing, alignment (see Section 2.5.1) and statistical analysis (see Section 2.5.3) followed the methods outlined in Chapter 2. AMOVA analysis (see Section 2.5.3) was done on three predefined hierarchical structures for both the mitochondrial ND2 and nuclear S7 genes (Table 5.2, Table 5.3).

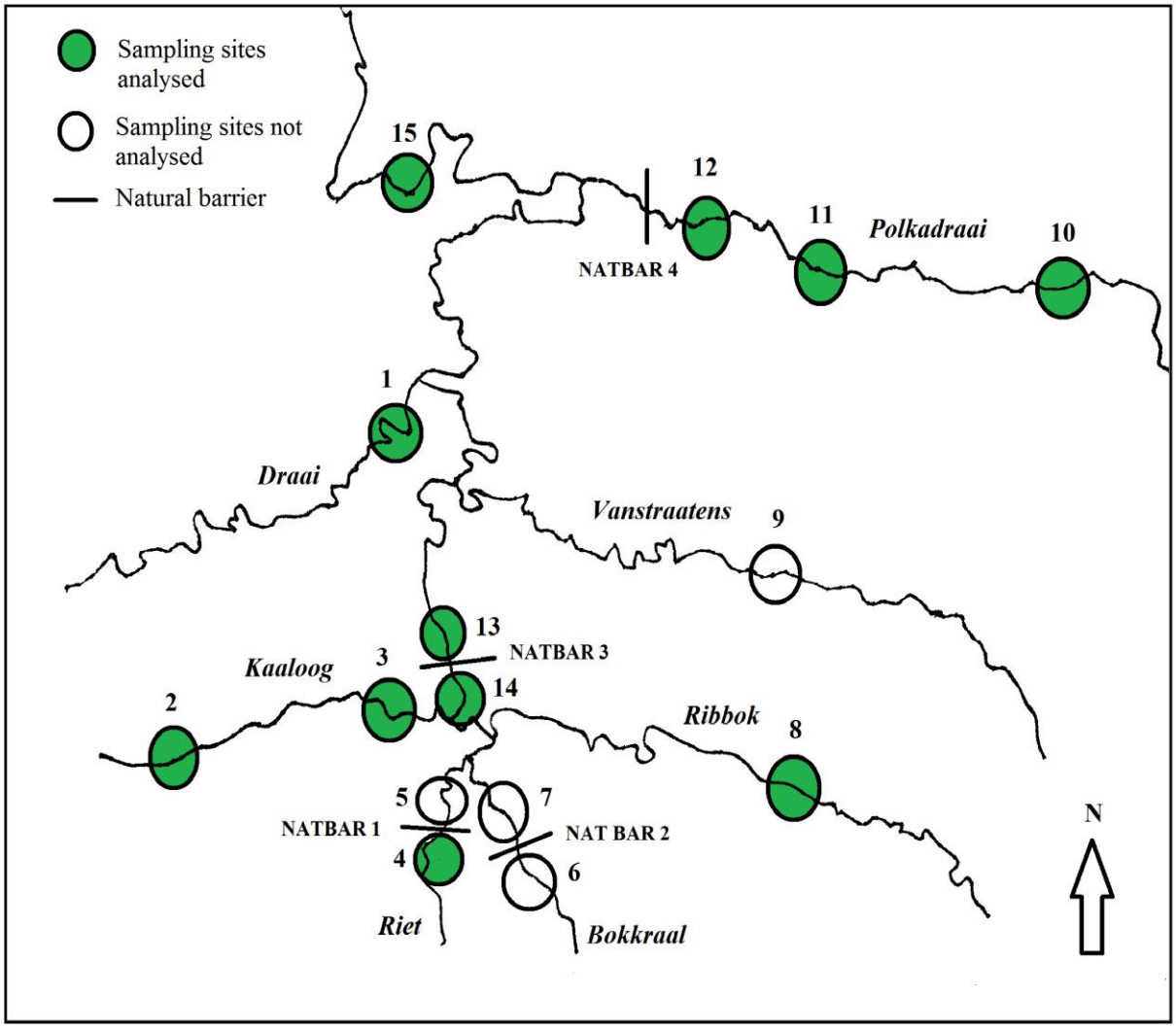

Figure 5.1. Map illustrating the 11 sites where *C. pretoriae* specimens were collected and analysed (green dots) across the upper Groot Marico catchment, North West Province. There are 4 major natural barriers in the study area, indicated by perpendicular lines (NATBAR = natural barrier). From the genetic analysis, both the mitochondrial ND2 and nuclear S7 genes revealed no distinct lineages of *C. pretoriae* populations. Samples of *C. pretoriae* were not found from the Vanstraatens tributary (Site 9), Bokkraal (Site 6 and 7) tributary and below the waterfall of the Riet tributary (site 5).

Table 5.1. Localities (See figure 5.1) and sample sizes (*n*) analysed for the mitochondrial ND2 gene and the nuclear S7 gene of *Chiloglanis pretoriae.*

Map	Locality	Locality Codes	Presence/ Absence	ND2 analysis (*n*)	S7 analysis (*n*)
1	Lower Draai	LD	Yes	3	4
2	Upper Kaaloog	UK	Yes	1	-
3	Lower Kaaloog	LK	Yes	6	6
4	Above Riet waterfall	ARW	Yes	-	9
5	Below Riet waterfall	BRW	Yes	-	-
6	Above Bokkraal waterfall	ABW	Yes	-	-
7	Below Bokkraal waterfall	BBW	Yes	-	-
8	Upper Ribbok	UR	Yes	9	20
9	Middle Vanstraatens	MV	Yes	-	-
10	Upper Polkadraai	UP	Yes	2	-
11	Middle Polkadraai	MP	Yes	3	4
12	Lower Polkadraai	LP	Yes	1	2
13	Groot Marico (Sonop)	GMS	Yes	4	-
14	Below Groot Marico waterfall	BGMW	Yes	4	19
15	Groot Marico (Riverstill)	GMR	Yes	6	-
Total				39	64

Table 5.2. Different structures used for the analysis of molecular variance (AMOVA) for the populations of *Chiloglanis pretoriae* for the mitochondrial ND2 gene.

	Structure 1	Structure 2	Structure 3
Group 1	Draai	Draai Kaaloog	Draai Kaaloog
Group 2	Kaaloog Ribbok	Ribbok Groot Marico	Ribbok
Group 3	Groot Marico Polkadraai	Polkadraai	Groot Marico Polkadraai

Table 5.3. Different structures used for the analysis of molecular variance (AMOVA) for the populations of *Chiloglanis pretoriae* for the nuclear S7 gene.

	Structure 1	Structure 2	Structure 3
Group 1	Draai	Draai Kaaloog	Draai Kaaloog
Group 2	Kaaloog Ribbok Riet	Ribbok Riet Groot Marico	Ribbok Riet
Group 3	Groot Marico Polkadraai	Polkadraai	Groot Marico Polkadraai

5.3. Results

5.3.1. Mitochondrial DNA analysis

5.3.1.1. ND2 diversity

For the mitochondrial ND2 gene, HKY (Hasegawa *et al.* 1985) was determined as the substitution model that best fit the data. Analysis of 39 individuals from five populations for 468 base pairs of the mitochondrial ND2 gene resulted in 8 alleles defined by 9 variable sites. (Table 5.4). The overall nucleotide diversity was low (π = 0.004) and no significant deviations from those expected under predictions of neutrality were found (D = -0.226, P > 0.10 (Tajima, 1989); F = -0.507, P > 0.10 (Fu & Li, 1993)). Overall haplotype diversity was high (H_D = 0.786) and ranged from 0 (sites 2 and 12) to 0.867 for the lower Kaaloog site (site 3) (Table 5.4).

5.3.1.2. ND2 allele distribution

Three of the eight alleles (38%) were private to single localities (Table 5.4). Allele 6 was only recorded from the lower Draai tributary (site 1) and the Groot Marico main stem close to the Sonop farm (site 13). Alleles 1, 2, 3 and 4 were more widespread and occurred in at least three different sites of the Groot Marico catchment (Table 5.4).

Table 5.4. The geographic distribution and frequency of mtDNA (ND2) alleles among sampled localities of *Chiloglanis pretoriae*. Sample sizes (N) and haplotype diversity (HD) are included (See Table 5.1 for locality descriptions). LD = Lower Draai; UK = Upper Kaaloog; LK = Lower Kaaloog; UR = Upper Ribbok; UP = Upper Polka; MP = Middle Polka; LP = Lower Polka; GMS = Groot Marico Sonop; BGMW = Below Groot Marico Waterfall.

Allele number	N	1 LD	2 UK	3 LK	8 UR	10 UP	11 MP	12 LP	13 GMS	14 BGMW	15 GMR
1	10	-	-	2	4	-	2	1	-	1	-
2	4	-	-	-	2	-	-	-	1	1	-
3	14	2	1	1	3	1	1	-	1	-	4
4	6	-	-	2	-	1	-	-	-	2	1
5	1	-	-	1	-	-	-	-	-	-	-
6	2	1	-	-	-	-	-	-	1	-	-
7	1	-	-	-	-	-	-	-	1	-	-
8	1	-	-	-	-	-	-	-	-	-	1
Total	39	3	1	6	9	2	3	1	4	4	6
H_D	0.786	0.667	0.000	0.867	0.722	1.000	0.667	0.000	1.000	0.833	0.600

5.3.1.3. ND2 genetic structuring

The parsimony network did not reveal geographically isolated lineages (Figure 5.2). Neither pairwise population Φ_{ST} values (which takes genetic distance into consideration) nor pairwise population F_{ST} values (which do not take genetic distances among alleles into consideration) revealed any significant ($P > 0.05$) results (Table 5.5 and Table 5.6)

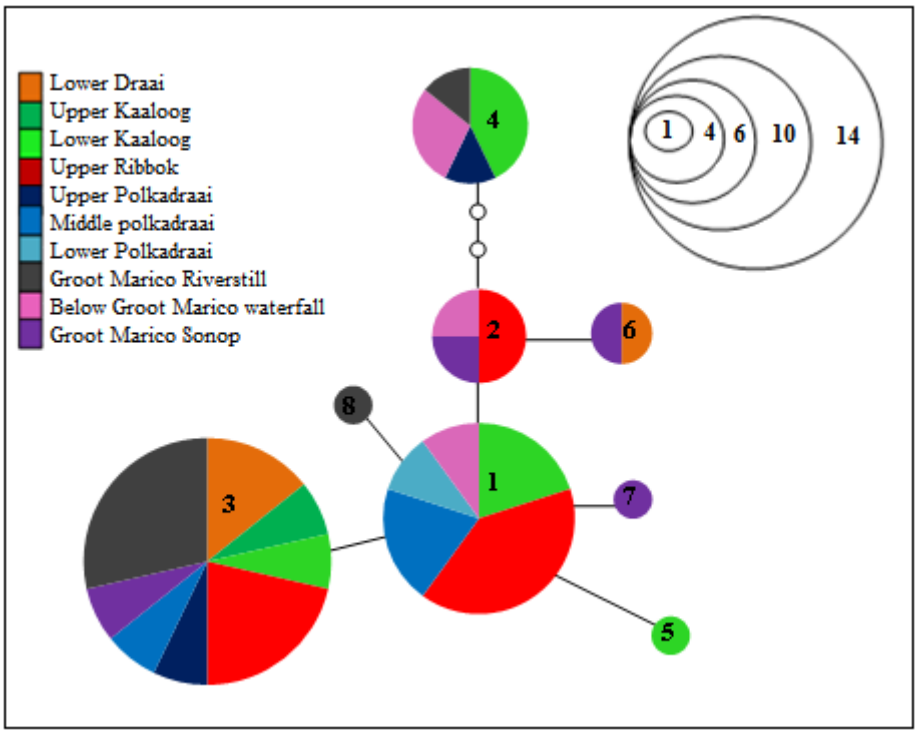

Figure 5.2. Parsimony network with 95% plausible set of mitochondrial ND2 allele connections (numbered circles) constructed with the program TCS 1.2.1 (Clement *et al.*2000) for *Chiloglanis pretoriae* (See Table 5.1). The size of the circles indicate the relative frequency of alleles (total N = 39) (See Table 5.4). Small open circles that are not numbered indicate missing alleles. Each line in the network represents one mutational stage.

Table 5.5. Pairwise Φ_{ST} values for *Chiloglanis pretoriae* from 5 populations in the Groot Marico catchment based on the mitochondrial ND2 gene. The comparisons that were significant ($P < 0.05$) are indicated by asterisks.

	1	2	3	4	5
1) Draai					
2) Kaaloog	-0.031				
3) Ribbok	-0.011	0.041			
4) Groot Marico	-0.077	-0.084	0.011		
5) Polkadraai	-0.081	-0.137	-0.076	-0.086	

Table 5.6. Pairwise *FST* values for *Chiloglanis pretoriae* from 5 populations in the Groot Marico catchment based on the mitochondrial ND2 gene. The comparisons that were significant ($P < 0.05$) are indicated by asterisks.

	1	2	3	4	5
1) Draai					
2) Kaaloog	0.032				
3) Ribbok	0.097	-0.012			
4) Groot Marico	-0.055	-0.042	0.037		
5) Polkadraai	0.090	-0.115	-0.091	0.017	

None of the variance components in the AMOVA analysis were significant ($P > 0.001$) (Table 5.7). The analysis also indicated that most of the variation was found within populations for all three hierarchical structures (see Table 5.2). This is as a result of all tributary populations as well as the Groot Marico main stem sharing alleles.

Table 5.7. Results of the analysis of molecular variance (AMOVA) for the populations of *Chiloglanis pretoriae* showing the Φ-statistics and the percentage of variance for the mitochondrial ND2 gene.

Variance Component	Structure 1	
	% Variance	Phi-value
Among groups	-1.88	CT= -0.02
Among populations within groups	-3.79	ΦSC= -0.04
Within populations	105.68	ΦST= -0.06
	Structure 2	
Among groups	-8.31	ΦCT= -0.08
Among populations within groups	0.9	ΦSC= 0.008
Within populations	107.4	ΦST= -0.07
	Structure 3	
Among groups	-0.72	ΦCT= -0.01
Among populations within groups	-4.59	ΦSC= -0.05
Within populations	105.31	ΦST= -0.05

5.3.2. Nuclear S7 gene

5.3.2.1. S7 diversity

For the nuclear S7 gene, the substitution model selected was HKY (Hasegawa *et al*. 1985). Analysis of 32 individuals (64 after phasing) from six populations for 543 base pairs revealed 17 alleles defined by 9 variable sites. No significant deviations from those expected under predictions of neutrality were observed (D =0.9816, P > 0.10 (Tajima, 1989); F =1.4360, P > 0.10 (Fu & Li, 1993)). Overall haplotype diversity was high (H_D = 0.882) and ranged from 0.742 for upper Ribbok (site 8) to 1.000 for the lower Kaaloog (site 3) and lower Polkadraai tributaries (site 12).

5.3.2.2. S7 allele distribution

Nine of the 17 alleles (53%) were private to single tributaries (Table 5.8). However, six of these were only one mutation step away from more common and widespread alleles. Allele 11 was only found in the Riet (above waterfall) and Kaaloog (lower reaches) tributaries. All the remaining alleles were found in at least three sites. Allele 4 had the most widespread distribution across four tributary populations and the Groot Marico main stem (Table 5.8).

Table 5.8. The geographic distribution and frequency of nuclear DNA (S7) alleles among sampled localities of *Chiloglanis pretoriae*. Sample sizes (N) and haplotype diversity (HD) are included (See Table 5.1 for locality descriptions). LD = Lower Draai; LK = Lower Kaaloog; ARW = Above Riet Waterfall; UR = Upper Ribbok; MP = Middle Polka; LP = Lower Polka; BGMW = Below Groot Marico Waterfall.

Allele Number	N	1 LD	3 LK	4 ARW	8 UR	11 MP	12 LP	14 BGMW
1	5	-	-	1	1	1	-	2
2	1	-	-	1	-	-	-	-
3	4	-	1	2	-	-	-	1
4	13	-	1	4	3	1	1	3
5	13	-	-	-	5	2	1	5
6	11	-	-	1	9	-	-	1
7	4	2	-	-	1	-	-	1
8	3	1	-	-	1	-	-	1
9	1	-	1	-	-	-	-	-
10	1	-	1	-	-	-	-	-
11	2	1	1	-	-	-	-	-
12	1	-	1	-	-	-	-	-
13	1	-	-	-	-	-	-	1
14	1	-	-	-	-	-	-	1
15	1	-	-	-	-	-	-	1
16	1	-	-	-	-	-	-	1
17	1	-	-	-	-	-	-	1
Total	64	4	6	9	20	4	2	19
HD	0.882	0.833	1.000	0.861	0.742	0.833	1.000	0.936

5.3.2.3. S7 Genetic structuring

The parsimony network with 95% plausible set of nuclear S7 allele connections for *C. pretoriae* did not reveal any distinct lineages (Figure 5.3). Pairwise Φ_{ST} values that do not take genetic distance into consideration showed no significant differences ($P > 0.05$) between 66% of the pairwise comparisons. Significant differences were recorded in all population comparisons involving the Draai population (Table 5.9). Similarly, pairwise F_{ST} values that do not take the genetic distance into consideration did not reveal significant differences ($P > 0.05$) between 53% of the pairwise population comparisons. Significant levels of differentiation were shown for all population comparisons involving the Draai population, as well as between the Ribbok and Kaaloog and between the Ribbok and Riet populations (Table 5.10). Four ambiguous branches (between allele 3 and allele 13; between allele 2 and allele 1; between allele 3 and allele 11; and between allele 5 and allele 14) were broken to

keep branches elsewhere in the parsimony network (Figure 5.3) following the rules of Templeton *et al.* (1987)

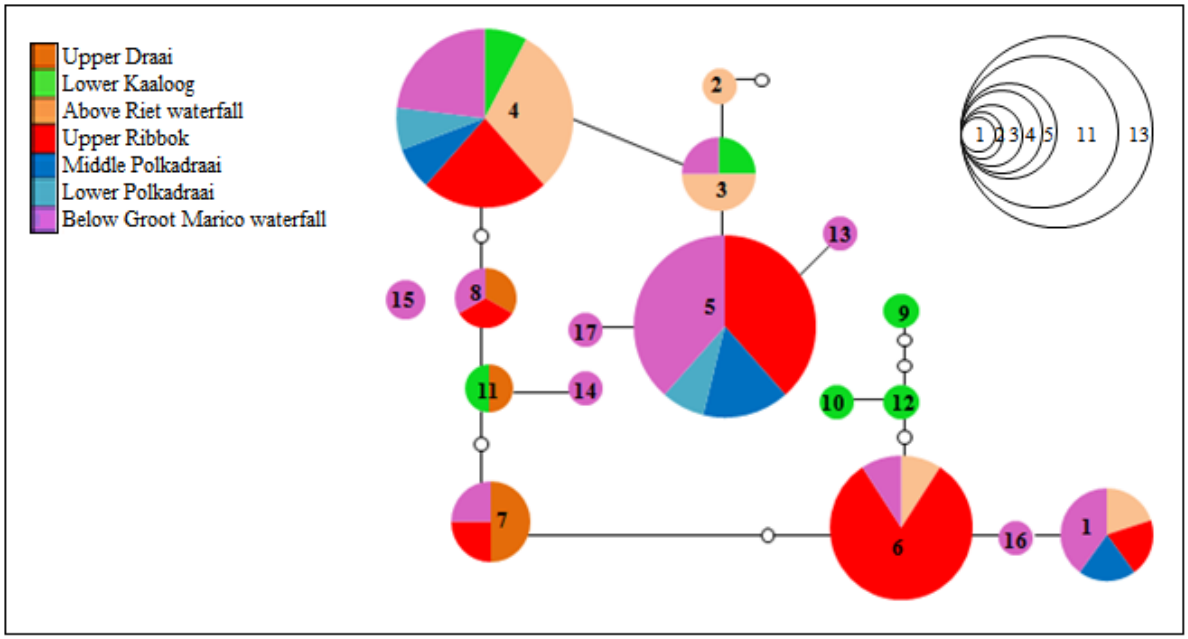

Figure 5.3. Parsimony network with 95% plausible set of nuclear S7 allele connections (numbered circles) of *Chiloglanis pretoriae* (See Table 5.1 for locality descriptions). The size of the circles indicates the relative frequency of the alleles (See Table 5.8). Small open white circle that are not numbered indicate missing alleles. Each line in the network represents one mutational change.

Table 5.9. Pairwise Φ_{ST} values for *Chiloglanis pretoriae* from six populations in the Groot Marico catchment based on the nuclear S7 gene. The comparisons that were significant ($P < 0.05$) are indicated by asterisks.

	1	2	3	4	5	6
1) Draai						
2) Kaaloog	0.245*					
3) Ribbok	0.347*	0.051				
4) Riet	0.484*	0.093	0.015			
5) Groot Marico	0.256*	0.020	0.004	0.024		
6) Polkadraai	0.491*	0.077	0.028	-0.019	0.012	

Table 5.10. Pairwise F_{ST} values for *Chiloglanis pretoriae* from six populations in the Groot Marico catchment based on the nuclear S7 gene in parsimony. The comparisons that were significant ($P < 0.05$) are indicated by asterisks.

	1	2	3	4	5	6
1) Draai						
2) Kaaloog	0.128*					
3) Ribbok	0.199*	0.069*				
4) Riet	0.233*	0.007	0.045*			
5) Groot Marico	0.097*	0.022	0.022	0.023		
6) Polkadraai	0.213*	0.024	0.002	-0.007	-0.012	

Similarly to the ND2 data, none of the variance components in the S7 AMOVA analysis were significant (Table 5.11). Most of the variation was found within populations for all three hierarchical structures (see Table 5.3).

Table 5.11. Results of the analysis of Molecular Variance (AMOVA) for the populations of *Chiloglanis pretoriae* showing the *F*- statistics and the percentage of variance for the nuclear S7 gene.

Variance Component	Structure 1	
	% Variance	Phi-value
Among groups	3.14	FCT=0.03
Among populations within groups	2.5	FSC=0.02
Within populations	94.36	FST=0.05
	Structure 2	
Among groups	0.75	FCT=0.01
Among populations within groups	4.34	FSC=0.04
Within populations	94.91	FST=0.05
	Structure 3	
Among groups	0.93	FCT=0.01
Among populations within groups	4	FSC=0.04
Within populations	95.07	FST=0.04

5.4. Discussion

Chiloglanis pretoriae is not demonstrating significant genetic differentiation between tributary populations and the Groot Marico main stem as measured with the mitochondrial ND2 and nuclear S7 genes. Therefore, the overall null hypothesis that there is no genetic differentiation between tributary populations could not be rejected with the present analysis. However, more variable genetic markers and more samples will have to be investigated to better understand gene flow patterns and the geographic distribution of genetic diversity before panmixia can be accepted.

Extrinsic (natural barriers) factors do not seem to influence the geographic distribution of genetic diversity in *C. pretoriae*. Intrinsic factors such as morphological adaptations may allow *C.* pretoriae to disperse across natural barriers in the Groot Marico catchment. Particularly its sucker-like mouth may allow this species to climb waterfalls, but it is unclear if such a phenomenon has ever been observed. Van Oosterhout *et al.* (2009) observed that *A. uranoscopus* makes use of its mouth and ventral side of the body to form a sucking disc to attach itself to slippery rocks and potentially move across barriers. It may be possible that *C. pretoriae* makes use of its sucker-like mouth to move across barriers in the Groot Marico catchment in a similar way. Periods of flooding in the Groot Marico catchment (which takes place in winter) may also assist in the dispersal of *C. pretoriae* by creating side channels that may bypass natural barriers. The species would nonetheless have to be a strong swimmer and/or require adaptations to make use of such opportunities.

Since very little genetic differentiation between populations of *C. pretoriae* was observed, population prioritisation for conservation actions is not as critical in this species compared to *B. motebensis* (Chapter 3) and *A. uranoscopus* (Chapter 4). The Groot Marico main stem sites carried most of the genetic diversity of *C. pretoriae* and although much less than the main stem, the Kaaloog had the most diversity compared to other tributary populations if both genes were taken into consideration.

CHAPTER 6

General discussion and recommendations for future research

6.1. Thesis conclusions

Genetic assessments are an important component of conservation because they allow conservation planners to develop strategies to maintain evolutionary potential (Leberg, 1990). In this thesis the genetic structuring of *Barbus motebensis* (Marico barb), *Amphilius uranoscopus* (Stargazer mountain catfish) and *Chiloglanis pretoriae* (Short-spine suckermouth catfish) was assessed to make recommendations for conserving their genetic diversity in headwater tributaries of the Groot Marico catchment, North West Province.

Population structuring was assessed using mitochondrial (ND2) and nuclear (S7) DNA analyses (see Chapter 2). This revealed high levels of genetic structuring for *B. motebensis* (Chapter 3) and *A. uranoscopus* (Chapter 4), but not for *Chiloglanis pretoriae* (Chapter 5). The genetic structuring found in *B. motebensis* and *A. uranoscopus* may have been influenced by extrinsic factors such as geological barriers. Intrinsic factors such as species specific dispersal ability, habitat preference and behavioural adaptations may have allowed *C. pretoriae* to maintain higher levels of dispersal compared to *A. uranoscopus* and *B. motebensis.*

For *B. motebensis*, the genetic analysis revealed two distinct lineages (the Draai and Southern lineages, Figure 3.1) which showed significant divergence (Chapter 3). The cytochrome *b* divergence between the Draai and Southern lineages of *B. motebensis* was more typical of within-species differentiation than between-species differentiation when compared to divergences for other members of the chubbyhead barb group.

In the case of *A. uranoscopus*, a major divergence was found between the Ribbok tributary and other populations (Ribbok and Western lineages, Figure 4.1). The nuclear S7 data did not show a similar divergence, but two of the three S7 alleles that occurred in the upper Ribbok were not shared with other tributary populations. The reason for the isolation of the Ribbok tributary is not clear but natural barriers do not seem to have had a major influence because the Ribbok tributary is not isolated by natural barriers and genetic differentiation was not observed in areas that were potentially isolated by major barriers.

Since at least three of the unique lineages found in the Groot Marico catchment may have very restricted distribution, they may require conservation actions to allow their survival. Conservation managers should also aim to conserve as much of the overall genetic diversity of all three species as possible.

6.2. Existing impacts

The Groot Marico catchment has been impacted by a number of factors such as the excessive abstraction of water, seepage from mines via dolomitic groundwater, land conversion and the threat of non-native species (Smith-Adao *et al.* 2006). The main threat has been the introduction of largemouth bass, *Micropterus salmoides*, into the Groot Marico catchment Especially *B. motebensis* is of concern as it has been listed as Vulnerable on the IUCN Red list (IUCN, 2013).

The impacts of *M. salmoides* in South Africa are mostly restricted to the main stems areas of rivers, but *M. salmoides* has invaded several headwater stream environments (De Moor & Bruton, 1988). Results from field surveys in the Groot Marico catchment suggest that *B. motebensis* were absent from pools where *M. salmoides* occurred. Similarly, Skelton (1993) and Ellender (2011) showed that a redfin minnow, *Pseudobarbus afer* was also absent in headwater pools occupied by *M. salmoides* in the Blindekloof stream, a tributary of the Swartkops River, Eastern Cape. This suggests that the invasive impacts of *M. salmoides* in the Groot Marico catchment may result in the eventual extirpation of *B. motebensis* if conservation actions are not implemented. It is not clear whether *M.* salmoides can cause the disappearance of *A. uranoscopus* or *C. pretoriae*, but will likely have a continued impact on them as well.

6.3. Conservation recommendations

Due to the impacts on *B. motebensis*, *A. uranoscopus* and *C. pretoriae* and their unique lineages in the Groot Marico catchment and because resources are limited, priority areas for conservation needs to be determined. Since the three species showed different genetic structuring patterns, multiple tributaries will have to be prioritised to conserve most of the genetic diversity found in the present study.

The priority areas for conservation were selected based on the following considerations (listed from highest to lowest priority):

1) The current conservation status of the species. Since *B. motebensis* is listed as Vulnerable on the IUCN Red list, its lineages received the highest priority.

2) The number of Evolutionarily Significant Units (ESUs) and their distribution, with the most restricted ESUS receiving the highest priority

3) The geographic distribution of genetic diversity for all three species in the tributary populations of the Groot Marico catchment, with the populations with the highest diversity receiving the highest priority.

Barbus motebensis is considered the highest priority species, because it is listed as Vulnerable (IUCN, 2013). The Draai, Vanstraatens and Kaaloog tributaries were selected for this species. The Draai tributary was selected to conserve the Draai lineage. Two populations of the Southern lineage were chosen to capture as much of its diversity as possible. The Kaaloog tributary had the highest genetic diversity when compared to the other tributary populations within the Groot Marico catchment. The Draai tributary is the highest priority, followed by the Kaaloog tributary.

For *A. uranoscopus*, the Ribbok tributary was selected because it showed isolation from the rest of the populations (Ribbok lineage). The Vanstraatens tributary was selected to represent the more widespread Western lineage as it had the highest genetic diversity for both genes (ND2 and S7). The Ribbok tributary is a high priority compared to the Vanstraatens tributary.

Chiloglanis pretoriae is not critical for prioritisation, because it occurs in all four tributaries that were prioritised for *B. motebensis* and *A. uranoscopus* (Figure 6.1) and is widespread throughout the Groot Marico catchment. For *C. pretoriae*, the Kaaloog tributary had almost as much diversity as the main stem sites and is therefore a good compromise to allow overlap with the areas chosen for the other two species. Four tributary populations were therefore selected as priority areas for conservation of all three species and their ESUs: the Draai, Vanstraatens, Ribbok and Kaaloog tributaries (Figure 6.1).

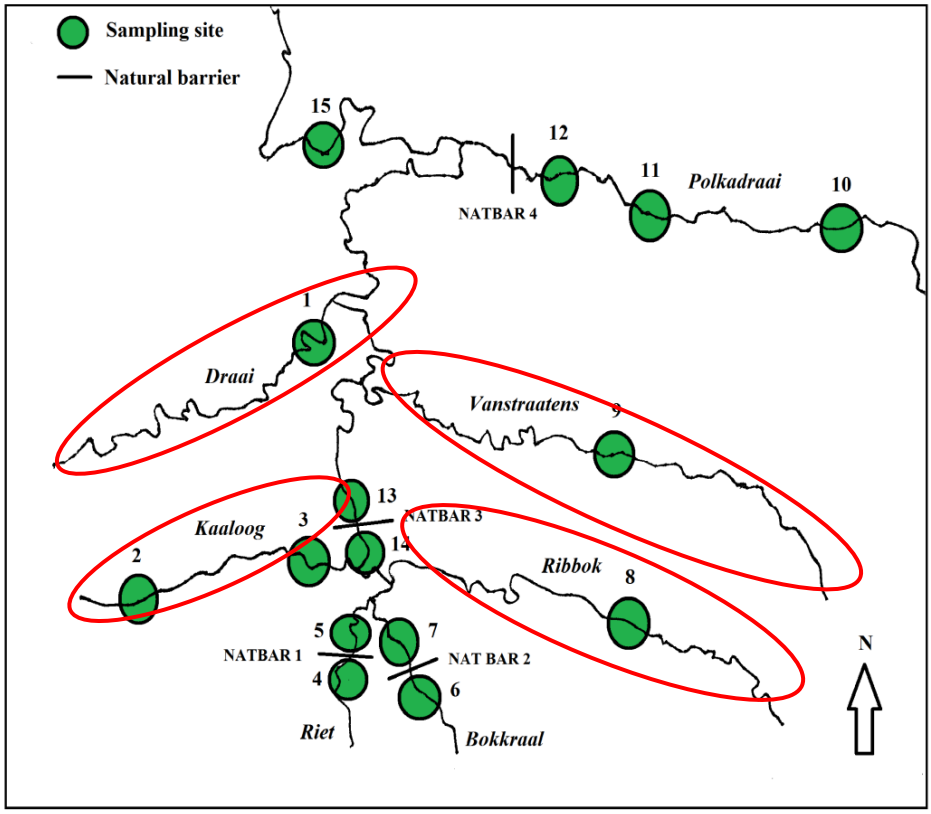

Figure 6.1. Priority areas for conservation for *Barbus motebensis*, *Amphilius uranoscopus* and *Chiloglanis pretoriae* in the Groot Marico catchment, North West Province.

6.4. Recommendations for future research

In this present thesis, lineage discovery and the assessment of the distribution of these lineages allowed initial conservation prioritisation. Therefore, the use of mitochondrial and nuclear DNA markers proved helpful in allowing conservation authorities to better focus their efforts towards the conservation of evolutionary processes of these three species. However, future research should include larger sample sizes and more variable genetic markers such as microsatellites to provide a better understanding of gene flow patterns and the direction of migration across waterfalls. This will further assist in effective prioritisation to conserve genetic diversity of these three species.

The direction of fish movement across waterfalls will provide a better understanding of whether populations above barriers are viable. Apart from the more variable genetic markers, individuals of the three fish species can also be tagged from above and below the barriers (see Chapter 2) to physically test their movement (capture-mark-recapture methods). However,

the small size of the three fish species could make tagging difficult to implement and a method that will be effective for long term monitoring may have to be developed.

In addition, ecological and behavioural studies will help to understand demographic changes in all three species. Wider geographic sampling should also be done to identify areas that may be vulnerable to the invasion of non-native fishes.

Such further studies will improve conservation management plans for all three species and may further assist in testing the following hypotheses with regards to their dispersal abilities:

1) *Barbus motebensis* has weak upstream dispersal across natural barriers
2) *Amphilius uranoscopus* has moderate to strong upstream dispersal abilities and use their morphological adaptations to cross (climbing) natural barriers
3) *Chiloglanis pretoriae* has strong upstream dispersal abilities and use their sucker-like mouth to climb across natural barriers.

Nonetheless, the existing information from the present study provides an adequate framework for the prioritisation of initial conservation action that will ensure the survival of *B. motebensis* and the maintenance of healthy *A. uranoscopus* and *C. pretoriae* populations.

APPENDICES

Appendix A. List of the 88 sites sampled in the upper Groot Marico catchment, North West Province, South Africa

ID	Date	Site ID	River Tributary	Section of tributary	GPS-S	GPS-E	Flow	Comments
1	2012/03/20	1	Kaaloog	High	25.78928	26.36646	None	Large eye
2	2012/03/21	2	Bokkraal	Middle	25.79623	26.44899	Slow	below waterfall
3	2012/03/21	3	Bokkraal	Middle	25.7958	26.44903	Slow	below waterfall
4	2012/03/21	5	Bokkraal	Middle	25.79583	26.44905	Fast	below waterfall
5	2012/03/21	4	Bokkraal	Middle	25.79583	26.44892	Slow	below waterfall
6	2012/03/21	6	Bokkraal	Middle	25.79501	26.44991	Slow	Below waterfall
7	2012/03/21	7	Bokkraal	Middle	25.79534	26.44961	Fast	Below waterfall
8	2012/03/21	8	Bokkraal	Middle	25.79542	26.44958	Slow	Below waterfall
9	2012/03/21	9	Bokkraal	Middle	25.79569	26.44934	Turbulent	Below waterfall
10	2012/03/21	10	Bokkraal	High	25.79663	26.44888	Slow	Above waterfall,
11	2012/03/21	11	Bokkraal	High	25.79993	26.45379	Slow	Above waterfall
12	2012/03/21	12	Bokkraal	High	25.80043	26.44956	Medium	Above waterfall
13	2012/03/21	13	Bokkraal	High	25.8005	26.44964	Fast	Above waterfall
14	2012/03/21	14	Groot Marico	Low	25.6445	26.42876	Slow	Angling
15	2012/03/22	o15	Kaaloog	Middle	25.78009	26.4317	Slow	
16	2012/03/22	o16	Kaaloog	Middle	25.78012	26.43176	Fast	
17	2012/03/22	o17	Kaaloog	Middle	25.77976	26.43227	Slow	
18	2012/03/22	o18	Kaaloog	Middle	25.77968	26.43222	Fast	
19	2012/03/22	o19	Kaaloog	Middle	25.77962	26.43231	Fast	
20	2012/03/22	o20	Kaaloog	Middle	25.77807	26.43211	Slow	Tree over hanging
21	2012/03/22	o21	Kaaloog	Middle	25.77734	26.43205	Fast	
22	2012/03/22	o22	Kaaloog	Middle	25.77744	26.43204	Stagnant	
23	2012/03/22	o23	Kaaloog	Middle	25.77571	26.43266	Slow	

24	2012/03/22	o24	Kaaloog	Middle	25.77569	26.43301	Very fast	
25	2012/03/22	o25	Kaaloog	Middle	25.77592	26.43327	Slow	
26	2012/03/22	o26	Kaaloog	Middle	25.77645	26.43375	Medium	
27	2012/03/22	p01	Kaaloog	High	25.78352	26.37984	Slow	
28	2012/03/22	p02	Kaaloog	High	25.78358	26.37953	Fast	Above waterfall
29	2012/03/22	p03	Kaaloog	High	25.78449	26.37571	Slow	
30	2012/03/23	Do1	Riet	High	25.79444	26.44356		
31	2012/03/23	OS1	Riet	Middle	25.79428	25.44362		
32	2012/03/23	Do2	Riet	High	25.79406	26.44256		
33	2012/03/23	Do3	Riet	High	25.79387	26.44224		
34	2012/03/23	Os2	Riet	High	25.79323	26.44126		
35	2012/03/23	Os3	Riet	High	25.79306	26.44001		
36	2012/03/23	Do5	Bokkraal	Low	25.78782	26.44979		
37	2012/03/23	Do4	Bokkraal-Riet	Middle	25.78707	26.45001		
38	2012/03/23	Os4	Bokkraal-Riet	Middle	25.78581	26.45013		
39	2012/03/24	Do9	Groot Marico	Low	25.64559	26.42888		
40	2012/03/24	Do11	Groot Marico	Low	25.64555	26.42891		
41	2012/03/24	Do10	Groot Marico	Low	25.64551	26.42913		
42	2012/03/24	Do8	Groot Marico	Low	25.64165	26.43193		
43	2012/03/24	Do7	Groot Marico	Low	25.64177	26.4318		
44	2012/03/24	Do6	Groot Marico	Low	25.64223	26.43154		
45	2012/03/24	OV1	Groot Marico	Low	25.6445	26.42876		
46	2012/03/23	P4	Riet	Middle	25.79376	26.44505		
47	2012/03/25	Do12	Draai	Middle	25.69851	26.42939		Snorkelling not feasible
48	2012/03/25	Do13	Draai	Middle	25.69854	26.42924		
49	2012/03/25	Do14	Draai	Middle	25.6984	26.42935		
50	2012/03/25	Do15	Draai	Middle	25.69825	26.42938		

51	2012/03/25	Po5	Draai	Middle	25.70137	26.42319		
52	2012/03/25	Po6	Draai	Middle	25.70059	26.42264		
53	2012/03/25	OS5	Draai	Middle	25.7008	26.42272		
54	2012/03/25	OS6	Draai	Middle	25.70033	26.42262		
55	2012/03/25	OS7	Draai	Middle	25.7002	26.42253		
56	2012/03/25	Do16	Draai	High	25.74079	26.34834		
57	2012/03/25	Do17	Draai	High	25.74089	26.3481		
58	2012/03/25	Do18	Draai	High	25.74097	26.34798		
59	2012/03/26	Do19	Polkadraai	Middle	25.6472	26.48914		Below Twyfelspoort
60	2012/03/26	Do20	Polkadraai	Middle	25.64732	26.48903		Below Twyfelspoort
61	2012/03/26	Do21	Vanstraatens	Middle	25.6668	26.53992		
62	2012/03/26	Do22	Vanstraatens	Middle	25.66673	26.53988		
63	2012/03/26	Do23	Vanstraatens	Middle	25.66654	26.53986		
64	2012/03/26	Do24	Polkadraai	Middle	25.66714	26.61758		Degraded by cattle, heavily silted
65	2012/03/26	Do25	Polkadraai	Middle	25.66729	26.61786		Degraded by cattle, heavily silted
66	2012/03/26	Do26	Polkadraai	Middle	25.66751	26.61788		Degraded by cattle, heavily silted
67	2012/03/26	Do27	Polkadraai	Middle	25.66762	26.61807		Degraded by cattle, heavily silted
68	2012/03/26	Do28	Vanstraatens	High	25.7396	26.53446		
69	2012/03/26	Do29	Vanstraatens	High	25.73963	26.5247		
70	2012/03/26	Do30	Vanstraatens	High	25.73964	26.52501		
71	2012/03/27	Po7	Ribbok	Middle	25.79067	26.52009		
72	2012/03/27	Po8	Ribbok	Middle	25.7909	26.52016		
73	2012/03/27	Po9	Groot Marico	Low	25.69443	26.43608		
74	2012/03/28	Do31	Groot Marico	Low	25.73518	26.4319		
75	2012/03/28	Do32	Groot Marico	Low	25.73499	26.43183		
76	2012/03/28	Do33	Groot Marico	Low	25.73481	26.43185		

77	2012/03/28	Do34	Groot Marico	Low	25.64706	26.40857		Lower 20 meters of Riet (very large pool)
78	2012/03/29	P11	Groot Marico	Middle	25.76697	26.44118		
79	2012/03/29	P12	Groot Marico	Middle	25.76747	26.4415		
80	2012/03/29	Do35	Groot Marico	Middle	25.76769	26.44179		
81	2012/03/29	OS11	Groot Marico	Middle	25.76877	26.44198		
82	2012/03/29	DS1	Groot Marico	Middle	25.76597	26.43933		
83	2012/03/29	DS2	Groot Marico	Middle	25.76637	26.44074		
84	2012/03/29	OS8	Groot Marico	Middle	25.76004	26.44147		
85	2012/03/29	OS9	Groot Marico	Middle	25.75914	26.44001		
86	2012/03/29	OS10	Groot Marico	Middle	25.76877	26.44198		
87	2012/03/29	DS3	Groot Marico	Middle	25.76105	26.44253		
88	2012/03/29	P14	Groot Marico	Middle	25.76004	26.44147		

Appendix B. Table indicating the 15 sampling reaches that were subdivided from 88 sites (Appendix A) in order to minimise the number of sites to reaches in which genetic samples were collected and used for analysis.

Sampling Reaches	Name	Cluster of ID Sites from Appendix A
1	Lower Draai	47 - 58
2	Upper Kaaloog	1; 27 -29
3	Lower Kaaloog	15 - 26
4	Above Riet waterfall	30; 32; 33; 35
5	Below Riet waterfall	3; 34; 46
6	Above Bokkraal waterfall	10 -13
7	Below Bokkraal waterfall	2; 3; 4 - 9
8	Upper Ribbok	71 - 72
9	Middle Vanstraatens	68-70
10	Upper Polkadraai	64 - 67
11	Middle Polkadraai	61 - 63
12	Lower Polkadraai	59 - 60
13	Groot Marico Sonop	78 -83; 86
14	Below Groot Marico waterfall	84 – 85; 87 -88
15	Groot Marico Riverstill	14; 39 -45; 77

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