SYSTEMATICS AND PHYLOGEOGRAPHY OF SUCKERMOUTH SPECIES (*CHILOGLANIS*) WITH EMPHASIS ON THE LIMPOPO RIVER SYSTEM AND IMPLICATIONS FOR WATER MANAGEMENT PRACTICES.

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

The genus *Chiloglanis* includes 45 species of which eight are described from southern Africa. The genus is characterized by jaws and lips that are modified into a sucker or oral disc used for attachment to a variety of substrates and feeding in lotic systems. The suckermouths are typically found in fast flowing waters but over varied substrates and water depths. This project focuses on three species, namely *Chiloglanis pretoriae* van der Horst 1931, *C. swierstrai* van der Horst 1931 and *C. paratus* Crass 1960, all of which occur in the Limpopo River System. The suckermouth catfishes have been extensively used in aquatic surveys as indicators of impacts from anthropogenic activities and the health of the river systems. Where the suckermouth catfishes occur naturally in the river systems, they are used in the determination of environmental flows and also as part of the fish assemblage assessment. The main aims of this project were as follows:

- Updating records on the distribution of the three *Chiloglanis* species in the Limpopo and associated river systems.
- Assessing within and between *Chiloglanis* species variation based on morphological and genetic analyses.
- Formulating management recommendations to water resource managers based on our results and analyses.

We used morphological and molecular methods as well as rapid habitat assessment to characterize the degree of genetic and morphological variation within and between populations of *Chiloglanis* and to update records on the distribution of the three *Chiloglanis* species in four primary catchments of the Limpopo River System. Where material was available the Limpopo ("in-group") populations were compared with those from the species' extreme geographical limits and these included populations from Swaziland, Botswana and Zimbabwe. Morphological data were analyzed using Principal Component Analysis (PCA). We sequenced the cytochrome *b* gene of the mitochondrial DNA (mtDNA) in specimens collected from the Limpopo River System and a population from the Sabie River was included for comparison. A rapid habitat assessment was carried out in the Olifants River where *Chiloglanis* species were collected both during the current and historical surveys.

The results on the distribution of the three species in the Limpopo River System reflected patterns typical of flow dependent primary freshwater fish species. The genetic and morphological results point towards isolation and divergence of tributary populations, which suggests little or no connection between these populations. Our analyses suggest that there must have been historical inter-connections that existed between the Limpopo River System and adjacent river systems that might have influenced the current

distribution of the *Chiloglanis* species. The molecular and morphological variation within and between populations from different systems is significant, suggesting that the genus *Chiloglanis* is a good surrogate for studying fragmentation and impacts on aquatic systems.

It is recommended that the Limpopo and Inkomati River Systems must continue to be managed separately avoiding Inter-basin Transfers (IBTs) where possible until the two genetically distinct populations of *C. swierstrai* are well understood and such patterns are confirmed using other aquatic species that inhabit both systems. We further recommend that a detailed mapping of environmental impacts across the Limpopo River System should be undertaken. A follow-up study is necessary to include all outlying populations of these three species as well as comprehensive analyses of other *Chiloglanis* species and should again be based on a combination of genetic and morphological approaches.

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LIST OF ACRONYMS

ABL	Anal fin Base Length
BD	Body Depth
CFF	Caudal Fin Fork
CFL	Caudal Fin Lower lobe
CFU	Caudal Fin Upper lobe
CPD	Caudal Peduncle Depth
CPL	Caudal Peduncle Length
cyt *b*	Cytochrome *b*
DBL	Dorsal Base Length
DFL	Dorsal Fin Length
DNA	Deoxyribo Nucleic Acid
DO	Dissolved Oxygen
DSL	Dorsal Spine Length
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
EC	Electrical Conductivity
EWR	Ecological Water Requirements
GDP	Gross Domestic Product
H_D	Haplotype Diversity
HL	Head Length
IBT	Interbasin Water Transfer
IOW	Inter-Orbital Width
KNP	Kruger National Park
MBL	Maxillary Barbel Length
MDG	Millennium Development Goal
Mg/l	Milligram per litre
$MgCl_2$	Magnesium Chloride
min	minutes
mM	milliMolar
MnBL	Mandibular Barbel Length
MnTW	Mandibular Tooth pad Width
mS/m	milliSiemens per meter
mtDNA	Mitochondrial Deoxyribo Nucleic Acid
MTL	Maxillary Tooth patch Length
MTW	Maxillary Tooth patch Width
MW	Mouth Width
ng	nanogram
N-OL	Posterior Nostril to Orbit Length

NW	Nostril Width
NWA	National Water Act
^{o}C	Degrees Celsius
OL	Orbit Length
PAL	Preanal Length
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDL	Predorsal Length
PFL	Pectoral Fin Length
PGW	Pectoral Girdle Width
pH	power Hydrogen ion
pmol	picomolar
PPL	Prepelvic Length
ppm	parts per million
PSL	Pectoral Spine Length
RHAM	Rapid Habitat Assessment Model
RHP	River Health Programme
SAIAB	South African Institute of Aquatic Biodiversity
SD	Standard deviation
SDL	Sucker Disc Length
SDW	Sucker Disc Width
sec	seconds
SL	Standard Length
SnL	Snout Length
S-NL	Snout to Anterior Nostril Length
T	Temperature
TDS	Total Dissolved Solids
TL	Total Length
U	units
VR	Velocity Rod
WMA	Water Management Area
µl	micro litre
π	Nucleotide Diversity

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1. INTRODUCTION

The threats to the future of aquatic biodiversity are many and well documented in a number of publications (Skelton, 1987; James and Barber, 1991; Hughes and Noss, 1992; Angermeier and Karr, 1994; Kleynhans and James, 1995; Schulz, 1996 and Kleynhans 1997). Ehrlich and Pringle (2008) reported that threats to the future of aquatic biodiversity require interventions in terms of strategies, if global aquatic biodiversity is to persist in an increasingly industrialized world. Such strategies have to be founded on sound scientific principles supported by knowledge at all levels of biological diversity i.e. gene, species and ecosystem levels. The threats include among others the following:

- Modification and destruction of natural habitat.
- Environmental pollution (e.g. eutrophication, toxification, salination, etc.).
- Direct exploitation of aquatic fish species.
- Climate change.

In South Africa, which is a developing country, these threats are compounded by the scarcity of the water resources and the uneven distribution of water resources. The scarcity of the water resources limits the developing countries' capacity to meet the millennium development goal (MDG) of ensuring that water and proper sanitation are provided to all citizens. The sluggish or even negative economic growth of the year 2009 brought about by the global economic crisis will exacerbate the negative impact that the scarce water resources have on meeting this MDG. The water resource of the country is regarded as one of the catalysts for economic development. The water use sectors such as agriculture, mining, and industries require water for production and this often brings about over-abstraction and pollution. Flow dependent fish species are therefore affected by both the deterioration of water quality (modified physico-chemical conditions) and low flows of the river systems. One such a group of flow dependent species is in the genus *Chiloglanis*.

The genus *Chiloglanis* includes 45 species of which eight are described from southern Africa (Skelton, 2001; Friel and Vigliotta, 2008 and Vigliotta, 2008). The genus is characterized by jaws and lips that are modified into a sucker or oral disc used for attachment to a variety of substrates and feeding in lotic systems (habitats formed in running water). It is for this reason that the species that belong to this genus are commonly known as suckermouth catfishes. The suckermouths are typically found in fast flowing waters but over varied substrates and water depths. This project focuses on three species, namely *Chiloglanis pretoriae* van der Horst 1931, *C. swierstrai* van der Horst 1931 and *C. paratus* Crass 1960, all of which occur in the Limpopo River System (Jubb and Le Roux, 1969 and Skelton, 2001). The genus' taxonomy has undergone major

revision over the years, as some species thought to be different were found to be synonyms (Table 1). Although the study is not intended to resolve these taxonomic puzzles in *Chiloglanis* species, the results will serve as one of the milestones for such a future study.

Table 1. List of type localities for *Chiloglanis* species focused on in the present study.

SPECIES	**AUTHORS**	**TYPE LOCALITY**	**TYPE MATERIAL**
C. pretoriae	van der Horst 1931	Crocodile River, Pretoria District, Transvaal, South Africa	Holotype
C. pumilus Synonym *C. pretoriae*	van der Horst 1931	Apies River and Crocodile River, Pretoria District, Transvaal, South Africa	Syntypes
C. paratus	Crass 1960	Concrete wall of Pongola River barrage $31^0$30'E & 27^0 23'S	Holotype, Paratypes
C. swierstrai	van der Horst 1931	Crocodile River, Pretoria District, Transvaal, South Africa	Holotype
C. engiops Synonym *C. swierstrai*	Crass 1960	Lower Pivaan River, Pongola River, 31^0 11'E & 27^0 25'S	Holotype, Paratypes

Chiloglanis species display habitat specificity and this might contribute to the genus' vulnerability to habitat destruction, as their mode of feeding is highly specialized on substrate surfaces. In the high mountainous streams with rocks in flowing water *C. pretoriae* is most commonly found (Jubb and Le Roux, 1969). *Chiloglanis swierstrai* is a downstream dweller that lives over sandbanks, burying itself in the loose sand, whereas *C. paratus* prefers rocky riffles with a higher water column (greater depth) relative to *C.*

pretoriae (Skelton, 2001). All three species may overlap in their distribution when the habitat provides their required ecological niches.

Socio-economically, the Limpopo River System has contributed significantly to the Gross Domestic Product (GDP) of South Africa, especially in the mining sector, which is more prevalent in the Upper Olifants catchment. Currently, there seems to be a shift of focus from the east (Olifants catchment) to the west (Mokolo and Lephalala catchments) with the view to develop South Africa's coal reserves to meet the current energy needs. The development of these coal reserves will increase competition for the scarce water resources with other water users such as national parks, agriculture and aquatic ecosystems. The stakeholders in the Mokolo and Lephalala catchments are putting pressure on the South African government to move rapidly to ensure that the lessons learnt from the Upper Olifants catchment's environmental pollution are not repeated in the Mokolo and Lephalala catchments (Business Report, 2009, 11 September). It is for these reasons that the Limpopo River System has to be well understood to strike a balance between socio-economic and environmental components and the suckermouth fishes provide a group of model species for such a study.

The suckermouth catfishes have been extensively used in aquatic surveys as indicators of impacts from anthropogenic activities and the health of the river systems (James and Barber, 1991; Kleynhans et al., 1992 and Pollard, 2000). The genus is currently used as part of the fish assemblage assessment during River Health Programmes (RHP) (Kleynhans, 1999), which is one of the eleven national monitoring programmes run by the Department of Water Affairs (DWA) in South Africa. The genus is also used in the determination of environmental flows during reserve determination, as part of implementation of chapter 3 of the South African National Water Act of 1998 (NWA). The reserve in this context relates to the water required to protect the aquatic ecosystem. Jubb and Le Roux (1969) reported that it was impossible to collect *C. pretoriae* and *C. swierstrai* from the type locality in the upper reaches of the Crocodile River due to pollution. A related species to the Limpopo suckermouth catfishes, *C. bifurcus* Jubb & le Roux 1969, which is endemic to the Inkomati River System in Mpumalanga Province, occurred at a lower altitude than expected as a result of the shading effect of plantations of exotic vegetation in the Nels River catchment (Schulz, 1996). Kleynhans et al. (1992) also reported that *C. bifurcus* suffered serious mortality from the impacts of a paper mill effluent spill in the Elands and Crocodile Rivers. The negative impacts of these anthropogenic activities differ in scope and magnitude. There is often a dichotomy in terms of how the suckermouth catfishes respond to such negative impacts. On the one hand suckermouth catfishes may recover and recolonise after a few sporadic spills, especially when there are refugia within the natural range (Kleynhans, 1992). On the

other hand the catfishes may completely disappear if multiple impacts, such as extreme fluctuations of water flow, high levels of contaminants, concrete canalization of some urban systems, impoundments and so forth, occur system-wide.

Genetic diversity should be an integral component of conservation strategies (Wilson, 1985; Hughes and Noss, 1992; Angermeier and Karr, 1994; Moritz, 1999; Templeton et al., 2001 and Moritz, 2002). In some instances genetic considerations have been used as a prerequisite for management of biodiversity, especially on issues regarding translocation of populations and interbasin water transfers (Meador, 1992; Spellerberg, 1996 and Grobler and Matlala, 2002). Genetic diversity brings about variety in shape, colour, behaviour, resistance to disease and tolerance to adverse conditions and it is therefore of utmost importance if natural populations are to survive changes in the environment, pathogens and parasites (Gray, 1996 and Gaines et al., 1997). Conversely, a loss of microevolutionary potential of species will limit the capacity of populations to adapt to changing environmental conditions (Ehrlich and Pringle, 2008).

The emergence of landscape genetics has added an impetus to the application of genetic diversity in conservation biology, as it provides information about the interaction between landscape features (e.g. waterfalls, matrix of poor habitat, impoundments, etc.) and microevolutionary processes, such as gene flow, genetic drift and selection (Manel et al., 2003). Genes interacting at some development level control morphological characters and there is often a relationship between geographic patterns of morphological and genetic variation (Sugg et al., 1997). The inclusion of a molecular phylogenetic approach provided a new perspective to the systematics of *Noturus* catfishes compared with previous analyses based on a morphological dataset alone (Hardman, 2004). The investigation of both molecular and morphological variation in the suckermouth fishes is therefore important to provide a robust assessment of intra-and interspecific patterns of diversity.

The main aims of this study were as follows:

- Updating records on the distribution of the three *Chiloglanis* species in the Limpopo and associated river systems.
- Assessing within and between *Chiloglanis* species variation based on morphological and genetic analyses.
- Formulating management recommendations to water resource managers based on our results and analyses.

2. MATERIALS AND METHODS

2.1. The Study Area and Sampling

The Limpopo River System is one of the three east-flowing river systems that drain major parts of the Gauteng, North West, Limpopo and Mpumalanga Provinces in South Africa. This international river system forms the border between South Africa and Botswana. Lower in the system it forms the border between Zimbabwe and South Africa before it flows into the Indian Ocean in Mozambique. In South Africa, management of this international river system takes place within Water Management Areas (WMAs) (DWAF, 1999) and there are four WMAs, which are situated within this river system, namely, the Crocodile West Marico, Limpopo, Luvuvhu-Letaba and Olifants catchments (Figure 1).

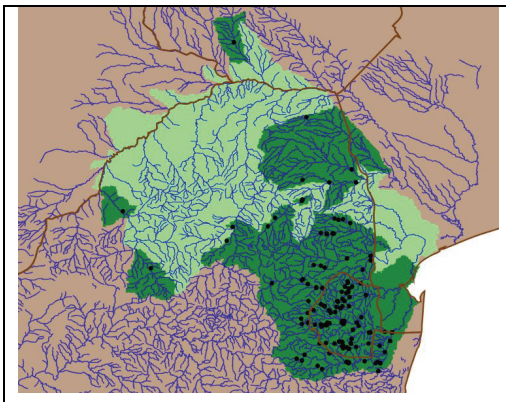
Figure 1. Water Management Areas in South Africa (DWAF, 1999) with four WMAs surveyed highlighted in red.

A total of eighty eight (88) sites in the Limpopo River System were surveyed from November 2006 through November 2008. The *Chiloglanis* species were collected at thirty nine (39) of the sites surveyed. Although the Limpopo River System constitutes the study area, we have included two sites in the adjacent Sabie River, which is part of the Inkomati River System for comparison between the two systems. The site selection for collection of *Chiloglanis* was guided by the historical distribution based on collection records from the South African Institute for Aquatic Biodiversity (SAIAB) and other museums (Figure 2). In some instances new sites were surveyed guided by factors such as suitable habitat for the three species, accessibility and proximity to RHP sites.

The sampling mainly followed the electrofishing method, which involves the creation of an electric field in water to which fish respond by some form of forced swimming and immobilization, with the ultimate purpose of rendering the fish easy to catch (Cowx and Harvey, 1993). The gear used was a Samus 725G and fishes were collected both in a net and a down-stream set seine-stop net.

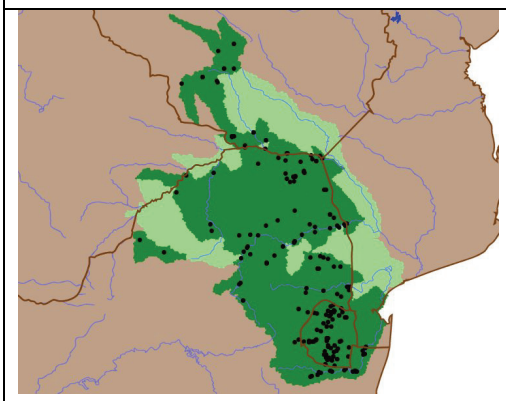
Fifteen (15) adult fish were collected per site for morphological analysis. A muscle tissue sample was taken from 10 fish per site for DNA analysis. Fish were photographed prior to preservation to record different colour morphs. The whole fish and those, from which muscle tissue was dissected, were fixed in 10% formalin solution and transferred to the SAIAB collection facility as voucher specimens. After fixation in formalin all specimens were transferred through a series of ethanol solutions (10-50%), up to and stored in 70% ethanol.

We also recorded water use activities in the vicinity. A rapid habitat assessment method was used to collect relevant habitat information for ecological water resource monitoring in the Olifants catchment (DWA, 2009).



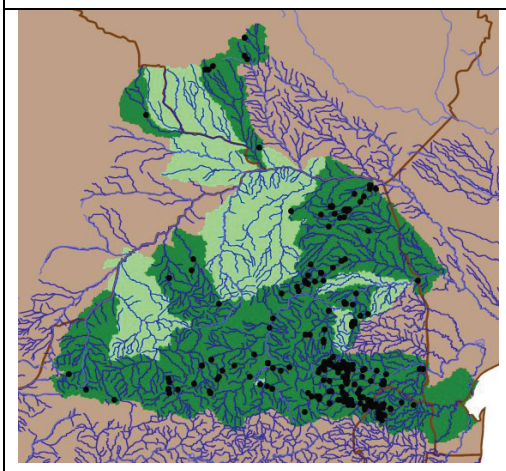
(A)

C. swierstrai van der Horst 1931



(B)

C. paratus Crass 1960



(C)

C. pretoriae van der Horst 1931

Figure 2. Historical distribution of three *Chiloglanis* species in southern Africa based on collection records from SAIAB and other museums; a photograph of specimens of each species is shown in the right hand panel. *C. swierstrai* recorded mostly in the Lowveld and Swaziland rivers (A). *C. paratus* distribution covered a wider area although not in high abundance (B). *C. pretoriae* is most widely distributed and abundant of the three species (C).

2.2. Morphological Methods

Quantitative characters employed in this study included both linear measurements and meristic characters. Measurements were taken with digital calipers and recorded to the nearest 0.1 mm. Specimens were, as far as possible, of adult proportions i.e. larger than 50 mm standard length. This was done to minimize bias due to growth allometry. Fifteen adult specimens for each *Chiloglanis* species from each locality were selected for both measurements and counts. However, there are sites where the required number of fifteen fish could not be successfully collected (these sites are specifically addressed in section 3 Results and Discussion). Museum specimens of the same *Chiloglanis* species collected from previous surveys from Swaziland, Botswana and Zimbabwe were included in the morphological analysis for comparison.

Linear measurements were taken with the following clarifications:

- Measurements on the head (e.g. orbit diameter) were taken from the bony margins of the elements concerned.
- Predorsal and postdorsal lengths were taken along the horizontal line to the point of intersection of the vertical through the base of the leading dorsal ray.
- Measurements were taken on the left side except where damaged.

Approximately thirty four (34) measurements and counts were made on groups of the three species. Linear measurements taken in this study included:

- Total length (TL)
- Standard length (SL)
- Head length (HL)
- Head width (HW)
- Body depth (BD)
- Predorsal length (PDL)
- Prepelvic length (PPL)
- Preanal length (PAL)
- Snout length (SnL)
- Inter-orbital width (IOW)
- Pectoral girdle width (PGW)
- Orbit length (OL)
- Snout to anterior nostril length (S-NL)
- Posterior nostril to orbit length (N-OL)
- Nostril width (NW)
- Maxillary barbel length (MBL)
- Mandibular barbel length (MnBL)

- Sucker disc width (SDW)
- Sucker disc length (SDL)
- Mouth width (MW)
- Mandibular tooth pad width (MnTW)
- Maxillary tooth patch width (MTW)
- Maxillary tooth patch length (MTL)
- Pectoral spine length (PSL)
- Pectoral fin length (PFL)
- Dorsal spine length (DSL)
- Dorsal fin length (DFL)
- Dorsal base length (DBL)
- Caudal fin upper lobe (CFU)
- Caudal fin lower lobe (CFL)
- Caudal fin fork (CFF)
- Caudal peduncle length (CPL)
- Caudal peduncle depth (CPD)
- Anal fin base length (ABL)

Morphological data were analyzed using Principal Component Analysis (PCA) using the programme Statistica version 6 (Installshield Software Corporation). Principal Component Analysis involved the use of variables to generate a plot of Eigenvalues so as to identify factors that show a significant proportion of variation. This was followed by plotting of identified factors to show the contribution of variables to the factors under consideration and all variables outside negative 0.5 and positive 0.5 quadrants were considered to contribute significantly to the factor. Each variable was separately plotted on the factor-planes to analyse interspecific and intraspecific variation among and within the populations of *Chiloglanis*.

2.3. Molecular Methods

Muscle tissues used for molecular analysis were dissected from the same specimens that were used for morphological analysis. However, in some instances where adult specimens were not sufficient, juveniles were included for this molecular analysis. The DNA extraction followed the Chelex protocol (Estoup et al., 1996). Two freshwater catfish specific primers (ACB1F and ACB2R) were used to polymerase chain reaction (PCR) amplify the 5' end of the cytochrome *b* gene of the mitochondrial DNA (mtDNA). This gene is widely used in vertebrate phylogenetic and phylogeographic studies (Meyer 1993); the mtDNA protein coding genes evolve at a faster rate than similar genes in the nuclear genome and are therefore sensitive to historical changes in population size and

can be used for the inference of patterns of colonization and gene flow. MtDNA is however not without limitations; the genome represents a single linked genetic locus and is maternally inherited, free of recombination, thus it only reflects historical patterns of variation along the female generations (Zhang and Hewitt, 2003).

Polymerase chain reaction was performed in 25 µl volumes containing 1 x buffer, 2 mM $MgCl_2$, 0.2 mM of each of the four nucleotides, 25 pmol of each primer, 0.75 U of Super-Therm DNA polymerase (Southern Cross Biotechnologies) and 100-200 ng template DNA. Conditions for PCR cycling involved an initial denaturation of 4 min at 94^{o}C, then 35 cycles of 30 sec at 94^{o}C, 30 sec at 50^{o}C and 45 sec at 72^{o}C, finishing with a final extension of 5 min at 72^{o}C. The PCR products were ethanol precipitated, followed by elution in ddH_2O. Cycle sequencing was performed in 10 µl volumes, containing 100 ng of purified DNA as a template, 3.2 pmol primer (either ACB1F or ACB2R) and 2 µl of ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Cycle sequencing products were precipitated using the EDTA/salt method (Applied Biosystems). Nucleotide sequences were determined through ABI 3100/3130 automated sequencers. Consensus sequences were obtained from forward and reverse sequences in Sequence Navigator 1.01 (Applied Biosystems) and were aligned using Clustal X (Thompson et al., 1997) and checked manually.

Unique alleles were determined using PAUP version 4 (Swofford 2003). DnaSP version 4 (Rozas et al., 2003) was used to calculate haplotype (H_D) and nucleotide diversity (π). Haplotype diversity reflects the probability that two randomly drawn mtDNA sequences from the sample are different (Nei and Tajima, 1981), while nucleotide diversity represents the average number of nucleotide differences per site between two sequences (Nei, 1987). Genealogies based on 95% confidence of connections among alleles (Templeton et al., 1992) were determined using statistical parsimony as implemented in TCS (Clement et al., 2000). Mismatch distributions of pairwise nucleotide differences between individuals were evaluated against predicted growth and decline or constant population size models in DnaSP. Changes in population size over time leave specific signatures on the patterns of polymorphism among samples (Slatkin and Hudson, 1991 and Rogers and Harpending, 2002).

2.4. Aquatic Habitat Assessment Methods

The site selection for rapid habitat assessment followed standard methods described in the Rapid Habitat Assessment Model Manual (DWA, 2009). The Ecological Water Requirements (EWR) sites were selected at localities where *Chiloglanis* species were collected both during the current and historical surveys in the Olifants catchment. The River Health Programme (RHP) sites in close vicinity were also recorded. A transparent

head Velocity Rod (VR) (Fonstad, et al., 2005) was used at the cross-section transects to measure depth and velocity. Substrate types, submerged vegetation and cover were recorded and measured as described in the Rapid Habitat Assessment Model Manual (DWA, 2009). Physico-chemical parameters such as Total Dissolved Solids (TDS, ppm), pH, Temperature (T, ^{o}C), Dissolved Oxygen (DO, % saturation, mg/l) and Electrical Conductivity (EC, mS/m) were measured on site using a multiparameter meter HI 9828. Rapid habitat assessment data were analysed using the Comp_RHAM, which is a model developed by Dr Drew Birkhead of Streamflow Solutions cc.

3. RESULTS AND DISCUSSION

3.1. Updated Records on the Distribution of *Chiloglanis*

The current distribution trend in *C. pretoriae*, which is the most widely distributed of the three species in the four catchments, corresponds with the historical distribution (Figure 2), although there were several historical sites where the species could not be successfully sampled currently due to loss of suitable habitat (Figure 3). It was not possible to collect *C. swierstrai* at four sites where the species was previously recorded in the four primary catchments outside the Kruger National Park (KNP). It does appear that *C. swierstrai* is mainly restricted in the KNP in these four primary catchments (Figure 4). *Chiloglanis paratus* showed a decline with regard to distribution pattern as manifested from the current records. This is more pronounced in the Limpopo and Olifants primary catchments upstream of the Kruger National Park. There are signs of disappearance from the Mogalakwena, Sand and Middle Olifants catchments in the recent survey (Figure 5).

The disappearance of *Chiloglanis* species from areas previously recorded may be attributed among others to factors such as flow modifications, changes in system connectivity, physico-chemical conditions and lack of vegetation cover. However, the lack of capture might not necessarily mean that the suckermouth catfishes have disappeared. There is a possibility that the current surveys coincided with seasonal or even long term environmental fluctuations which resulted in the species' temporary absence from certain areas within the system. It is therefore necessary to consider some further studies regarding distribution of these species.

Populations of *C. swierstrai* were collected at six sites in the Olifants and Luvuvhu-Letaba primary catchments. Populations of *C. pretoriae* and *C. paratus* were collected at twenty six (26) and nineteen (19) sites from the four primary catchments, respectively.

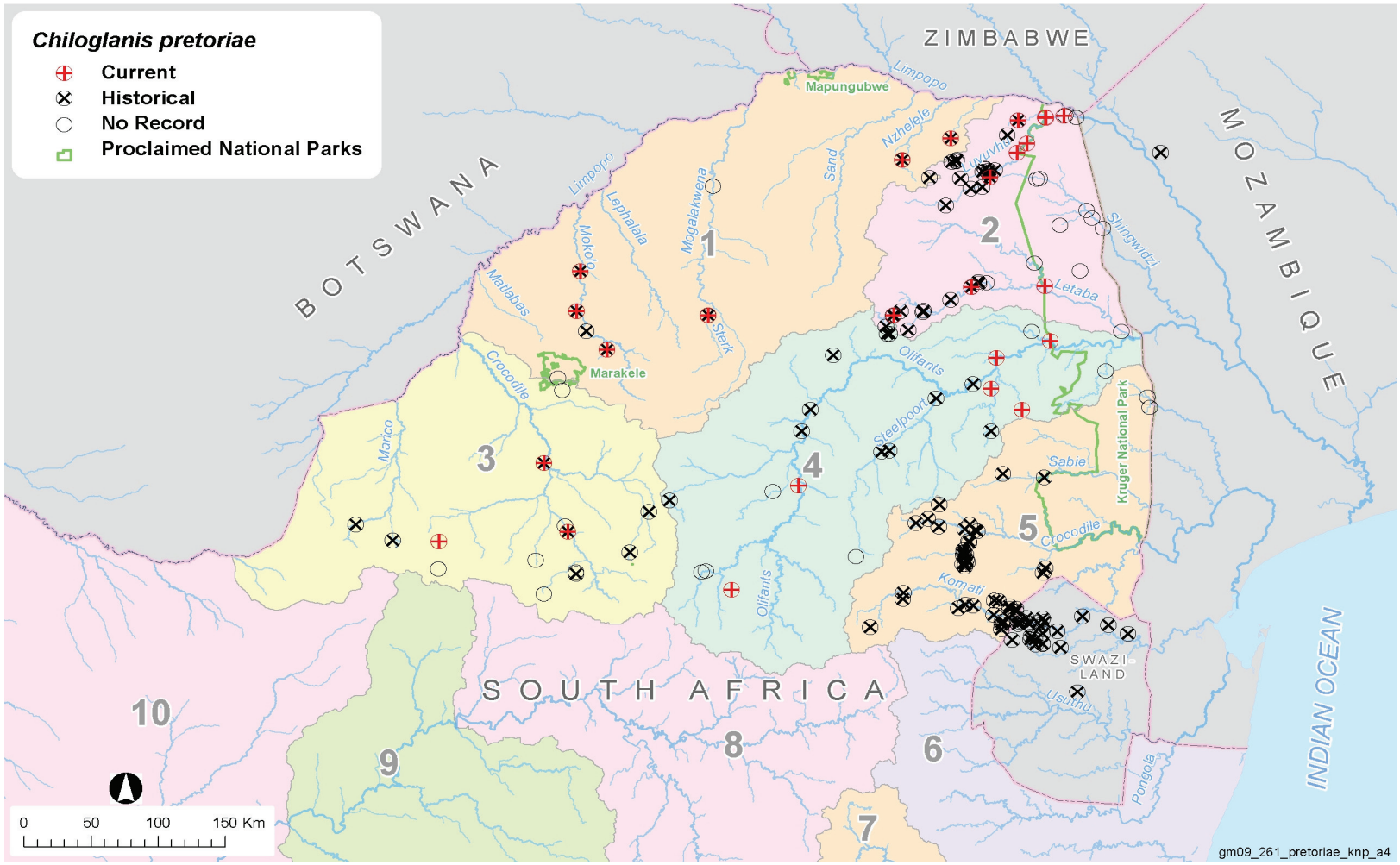


Figure 3. Distribution of *Chiloglanis pretoriae* showing historical records in southern Africa and updated records in the four WMAs.

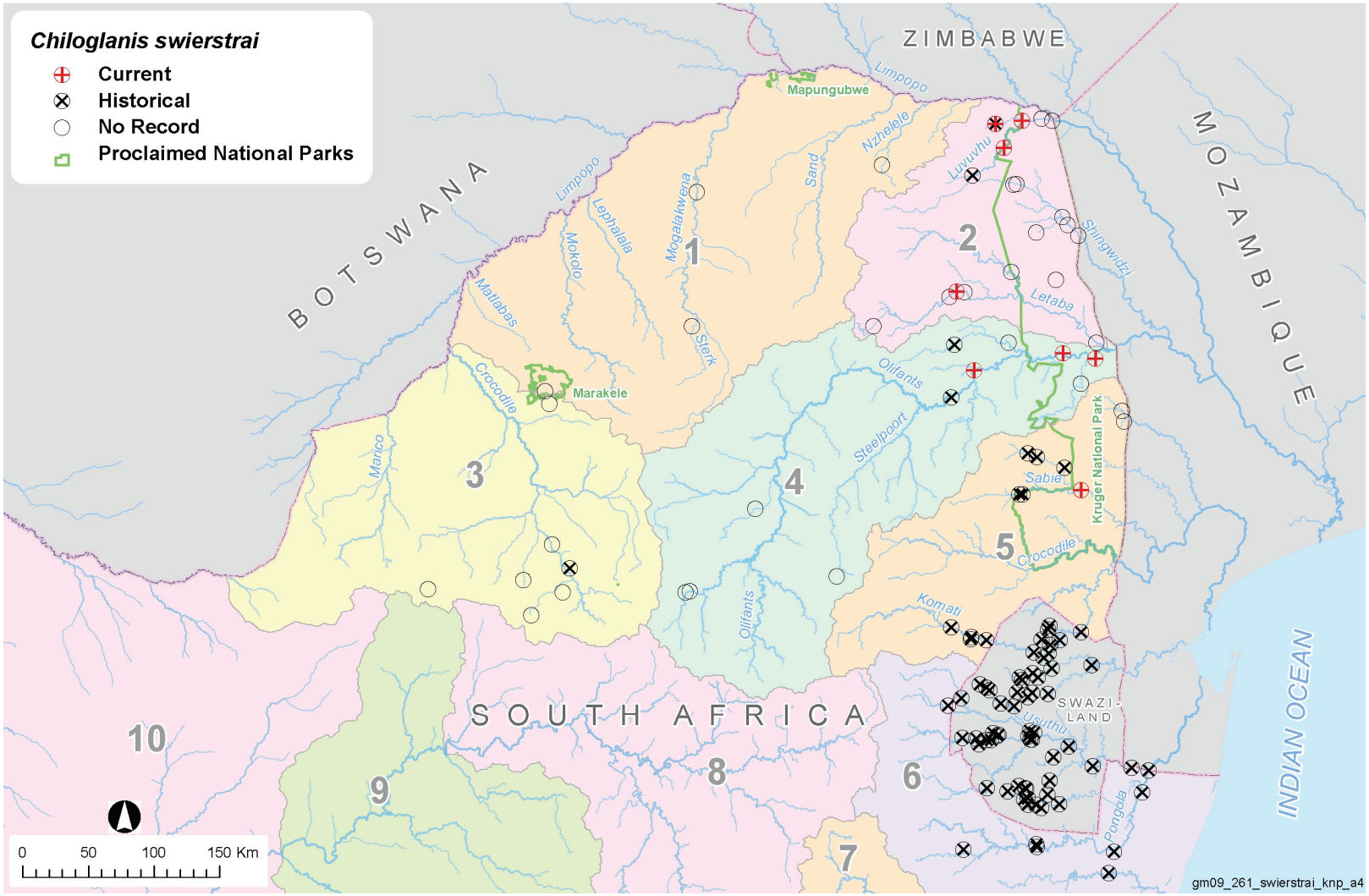


Figure 4. Distribution of *Chiloglanis swierstrai* showing historical records in southern Africa and updated records in the four WMAs.

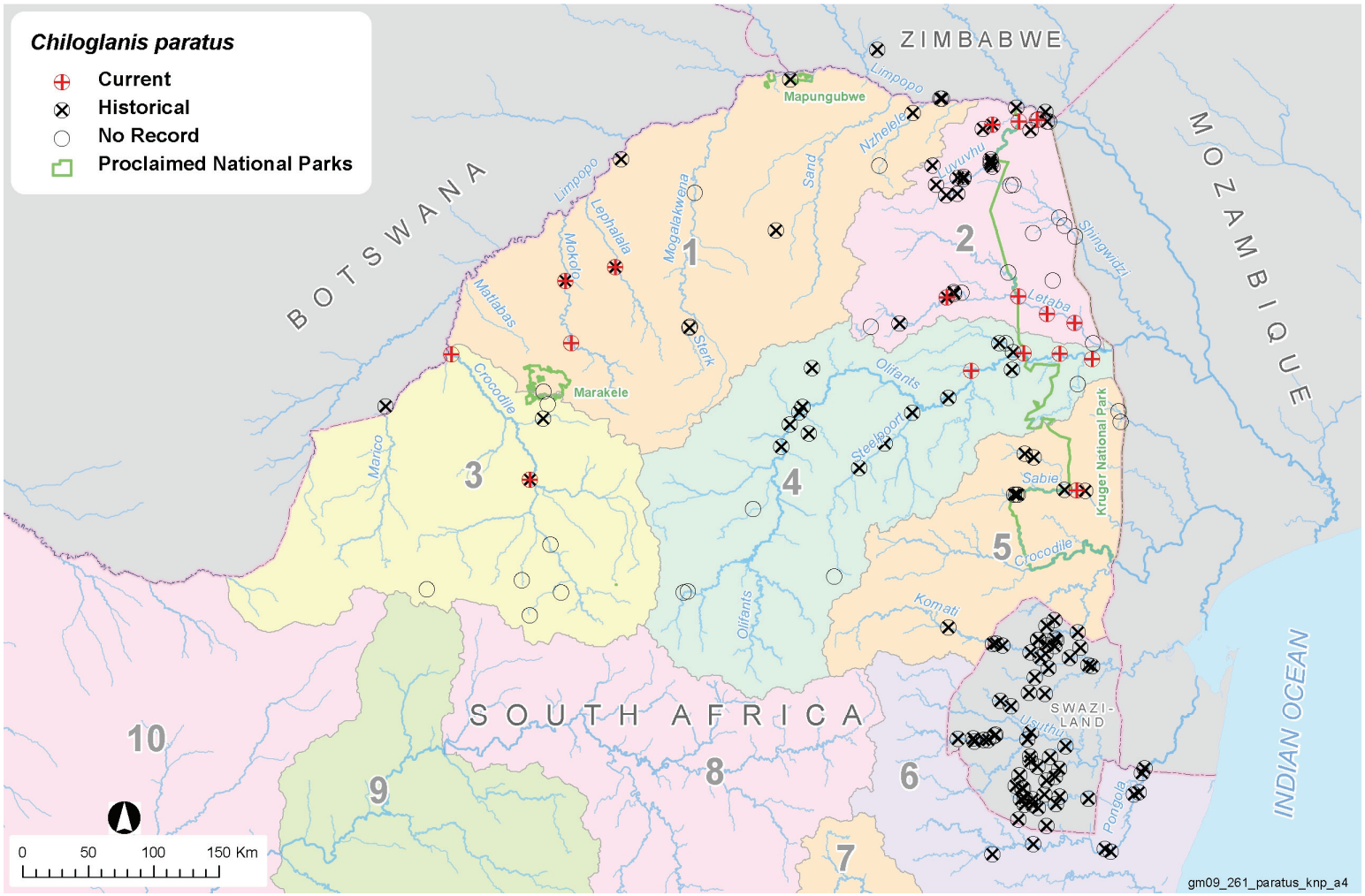


Figure 5. Distribution of *Chiloglanis paratus* showing historical records in southern Africa and updated records in the four WMAs.

3.2. Morphological Analysis

A PCA plot of all specimens for factors 2 x 3 showed separation between the three species of *Chiloglanis* (Figure 6). The separation is even more pronounced when an interspecific analysis is performed using factors 1 x 2 (Figure 7). *Chiloglanis swierstrai* is clearly distinct from *C. paratus* and *C. pretoriae* based on morphology.

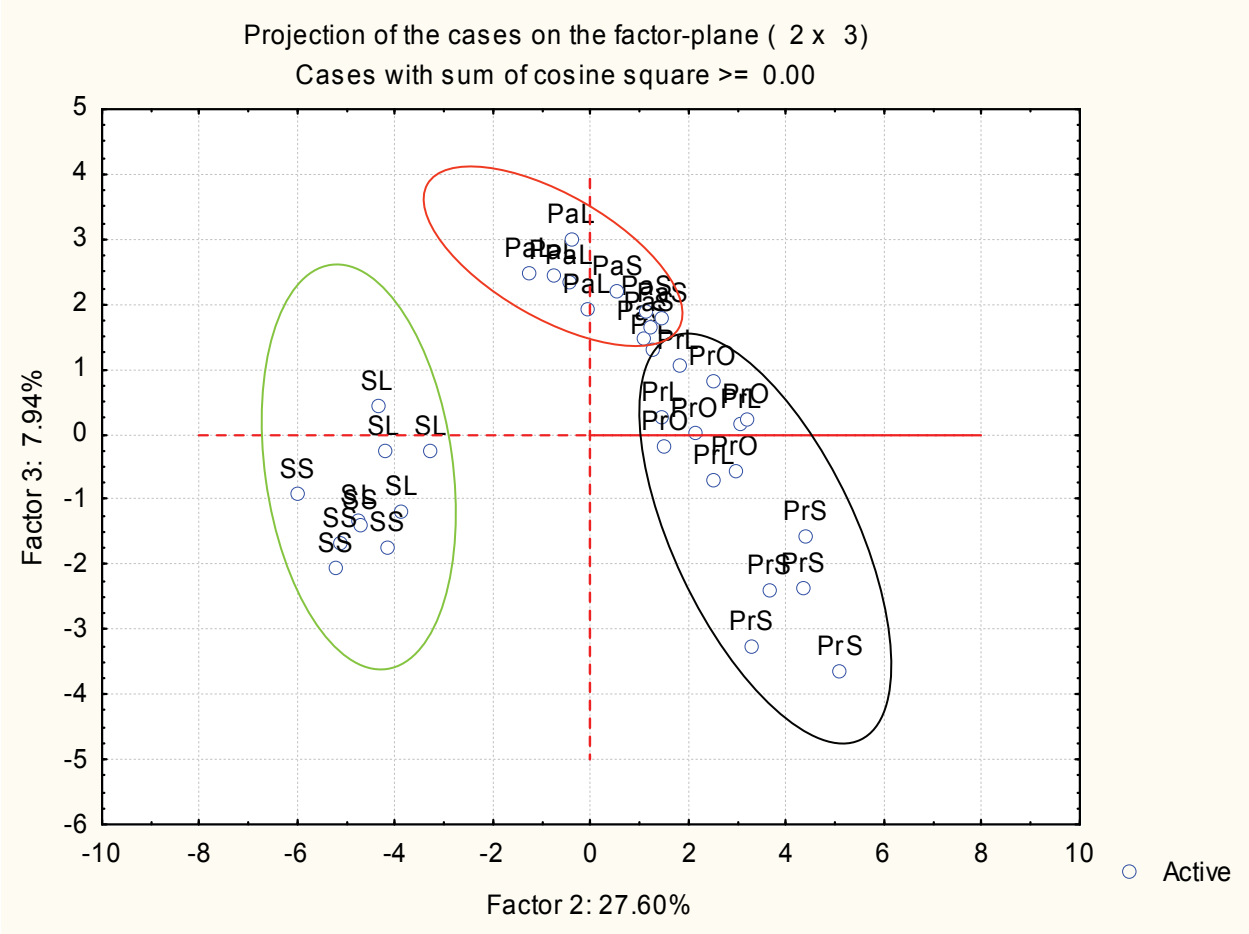


Figure 6. PCA plot of factors 2 x 3 for three *Chiloglanis* species: *C. swierstrai* Swaziland (SS) and Limpopo (SL) are clearly separated from *C. paratus* Swaziland (PaS) and Limpopo (PaL) and *C. pretoriae* Swaziland (PrS, Olifants (PrO) and Limpopo (PrL).

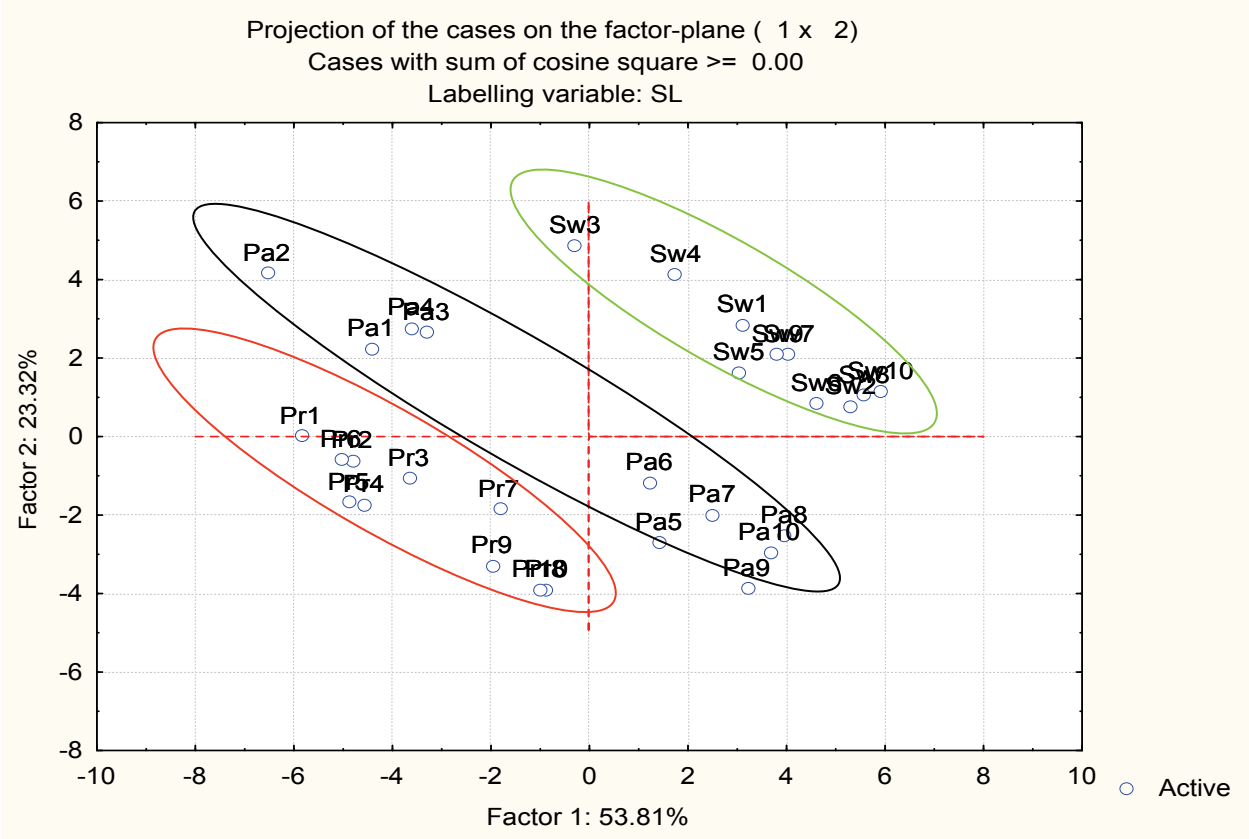


Figure 7. PCA plot of factors 1 x 2 for three *Chiloglanis* species: *C. swierstrai* (Sw) is clearly separated from *C. paratus* (Pa) and *C. pretoriae* (Pr).

An intraspecific analysis of populations of *C. pretoriae* from different localities suggests morphological variation across the catchments. The population of *C. pretoriae* (Cr1 to Cr20) from the Crocodile River, which forms part of the Inkomati River System seems to separate from the populations of *C. pretoriae* from the Limpopo River System (Figure 8). A population of *C. pretoriae* from the Matopos River, a tributary of Limpopo in Zimbabwe, did not group with populations from the Limpopo River and Inkomati River in South Africa. The separation of the populations of *C. pretoriae* seems to confirm the distinction of the different colour morphs displayed in the species across the different geographical areas (Figure 9).

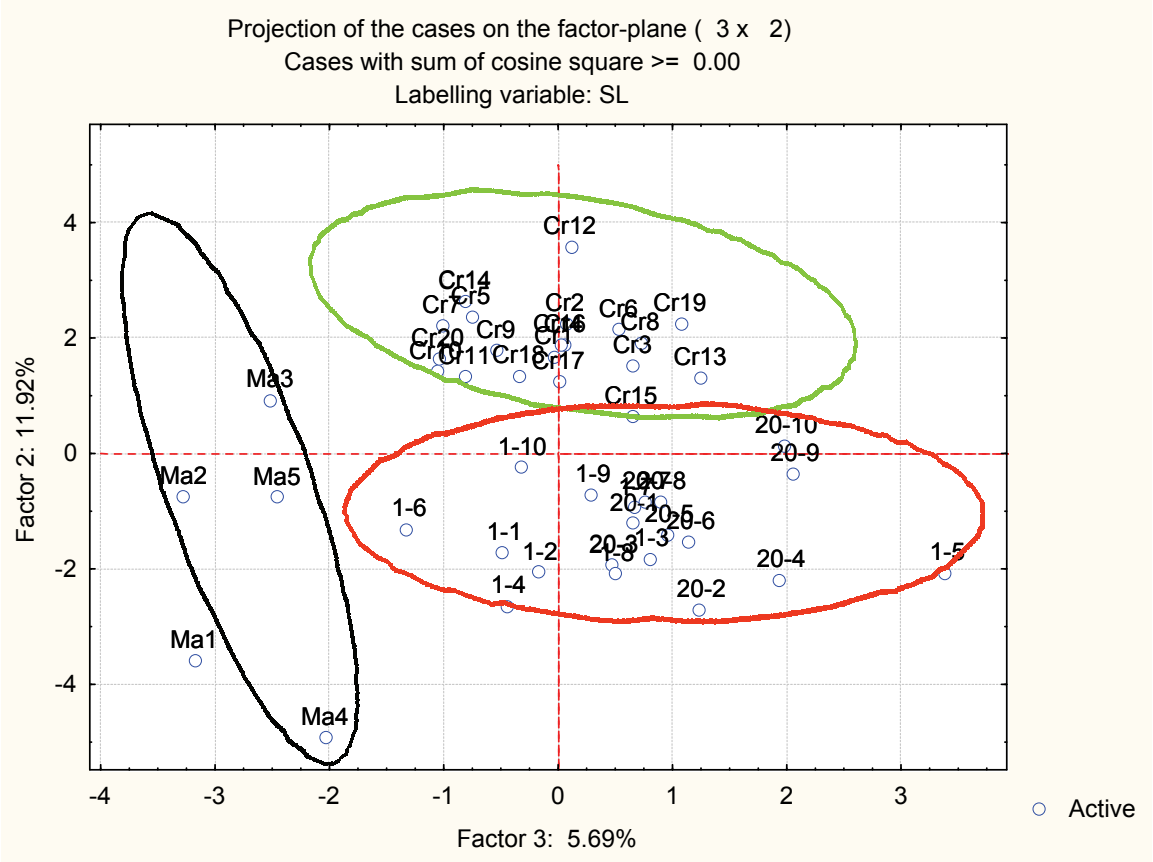


Figure 8. PCA plot of factors 2 x 3 for *C. pretoriae* from two different river systems: *C. pretoriae* Inkomati Swaziland (Cr1 to Cr20) are clearly separated from *C. pretoriae* Limpopo Zimbabwe (Ma1 to Ma5) and Limpopo South Africa (1-1 to 1-10 and 20-1 to 20-10).

Figure 9. Colour morphs of *C. pretoriae* from Crocodile-West River (top) South Africa, Lubuyane River part of the Inkomati System (middle) in Swaziland and Mokolo River (bottom) South Africa.

There are similar trends evident from the intraspecific analysis of populations of *C. swierstrai* (Figure 10) and *C. paratus* (Figure 11) from different geographical areas across the species' distribution range. The populations of *C. swierstrai* (20-1 to 20-10) from the Limpopo River System separate clearly from the populations of *C. swierstrai* from the Inkomati (N1 to N5) and Phongolo (S1 to S5) River Systems.

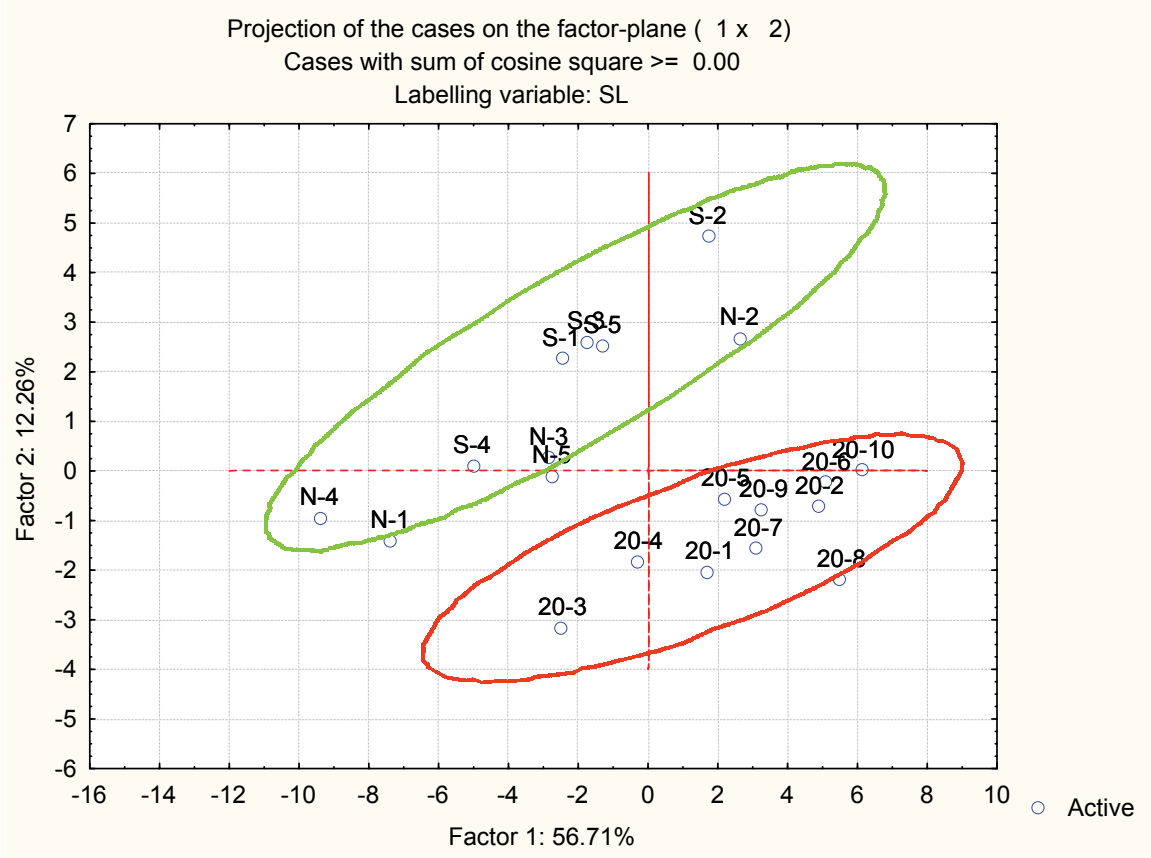


Figure 10. PCA plot of factors 1 x 2 for *C. swierstrai* from different river systems: *C swierstrai* Limpopo South Africa (20-1 to 20-10) clearly separated from *C. swierstrai* Phongolo Swaziland (N-1 to N5) and *C. swierstrai* Sabie South Africa (S-1 to S-5).

The population of *C. paratus* (Sw1 to Sw5) from the Phongolo River System in Swaziland separated from populations of *C. paratus* (L1 to L20 and 5-1 to 5-10) from the Limpopo River system in South Africa (Figure 11).

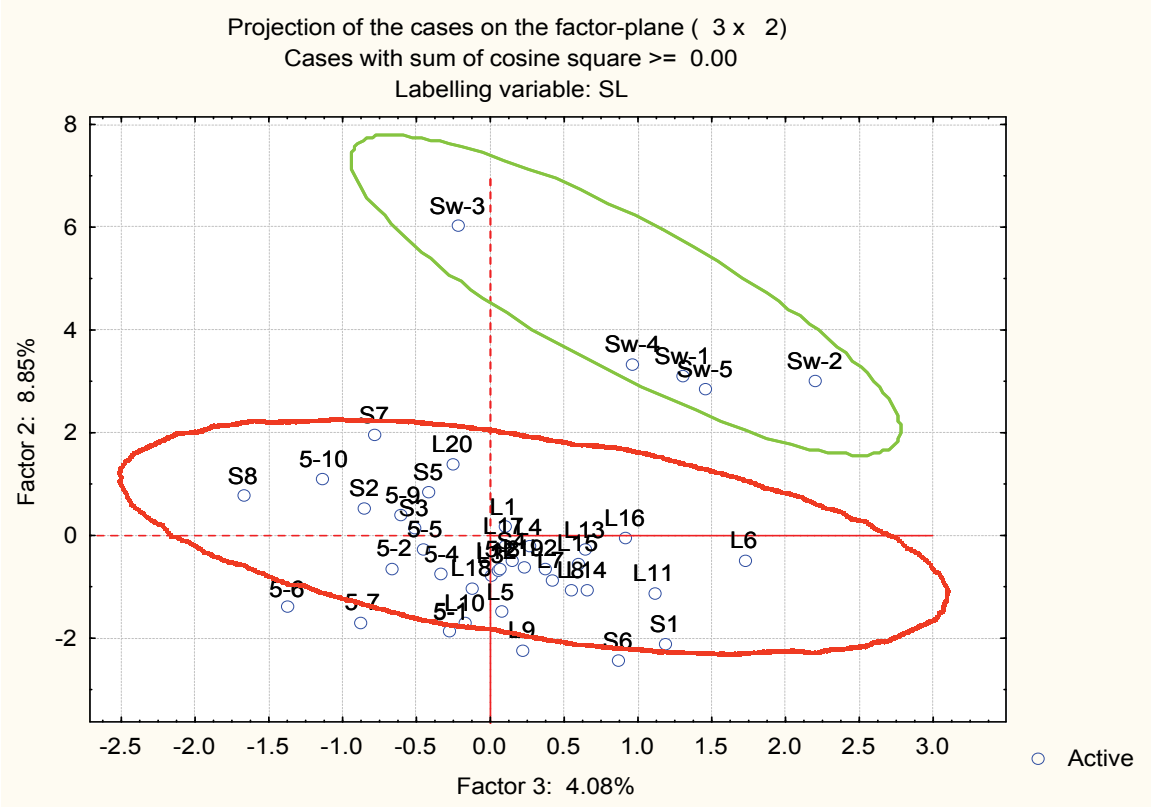


Figure 11. PCA plot of factors 2 x 3 for *C. paratus* from different river systems: *C. paratus* Phongolo Swaziland (Sw) clearly separated from *C. paratus* Limpopo South Africa (L1 to L20) and Shashe Botswana (S1 to S8).

This separation supports different colour morphs in the two river systems (Figure 12). The population of *C. paratus* (S1 to S8) from the Shashe River (a tributary of the Limpopo River in Botswana) did not separate clearly from the South African populations of *C. paratus*.

Figure 12. Different colour morphs of *C. paratus* from Shashe (Botswana) (Top), Limpopo (South Africa) (Middle) and Phongolo (Swaziland) (Bottom).

3.3. Phylogeographic Analysis

Basic statistics for the individuals analysed thus far, based on ~ 600 bases of the mtDNA cytochrome *b* gene, are summarized in Table 2. All three species showed considerable genetic diversity. *Chiloglanis paratus* displayed the most unique alleles (highest haplotype diversity).

Table 2. Summary statistics for variation observed within three *Chiloglanis* species based on a portion of the mtDNA cytochrome *b* gene.

Species	Samples (N)	Variable sites (S)	Number of haplotypes	Nucleotide diversity (π, SD)	Haplotype diversity (H_D, SD)
C. swierstrai	36	25	12	0.62%, 0.1%	0.56, 0.1
C. paratus	28	35	18	0.62%, 0.15%	0.9, 0.05
C. pretoriae	93	32	27	0.53%, 0.03%	0.89, 0.02

The Limpopo River System populations of *C. swierstrai* have similar alleles represented in all the tributaries, especially the inferred ancestral allele, A01 (Figure 13). The narrow distribution of *C. swierstrai* in the Limpopo River System might contribute to this condition, as the species could not be collected at four sites where it was previously recorded. Although allele A01 was also represented in the Olifants River, some unique alleles not sampled elsewhere in the system, were recorded here. The most significant result is that the Limpopo River System alleles were found at very low frequency in the Sabie River, which had several unique and genetically very distinct alleles (A07, A08, A09, A10 and A12); only Sabie River allele A08 was recorded from one Olifants River fish.

The *Chiloglanis paratus* allele network (Figure 14) shows several divergent alleles (e.g. A13-17), recorded from the Limpopo and Olifants Rivers. Allele A02 has a wide geographic distribution, especially throughout the Limpopo River while the other alleles are only recorded from single localities.

Chiloglanis pretoriae proportionately showed the most alleles and the allele network (Figure 15A) is further characterized by several high frequency alleles e.g. A02, A14, A17.

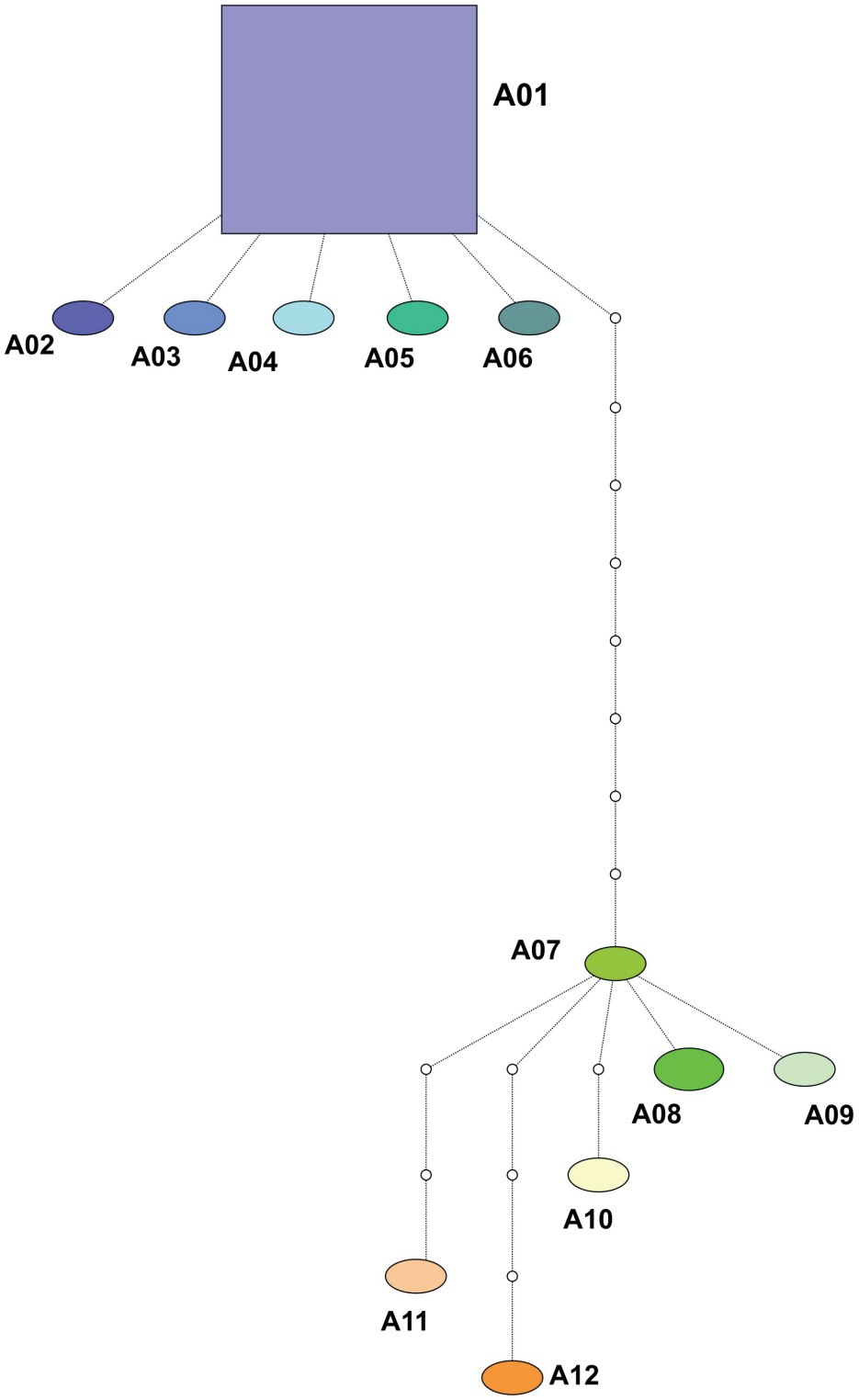


Figure 13(A). Allele network of 12 unique cyt *b* sequences with a large number of mutational changes between the Limpopo (A1-6) and Sabie (A7-12) Rivers.

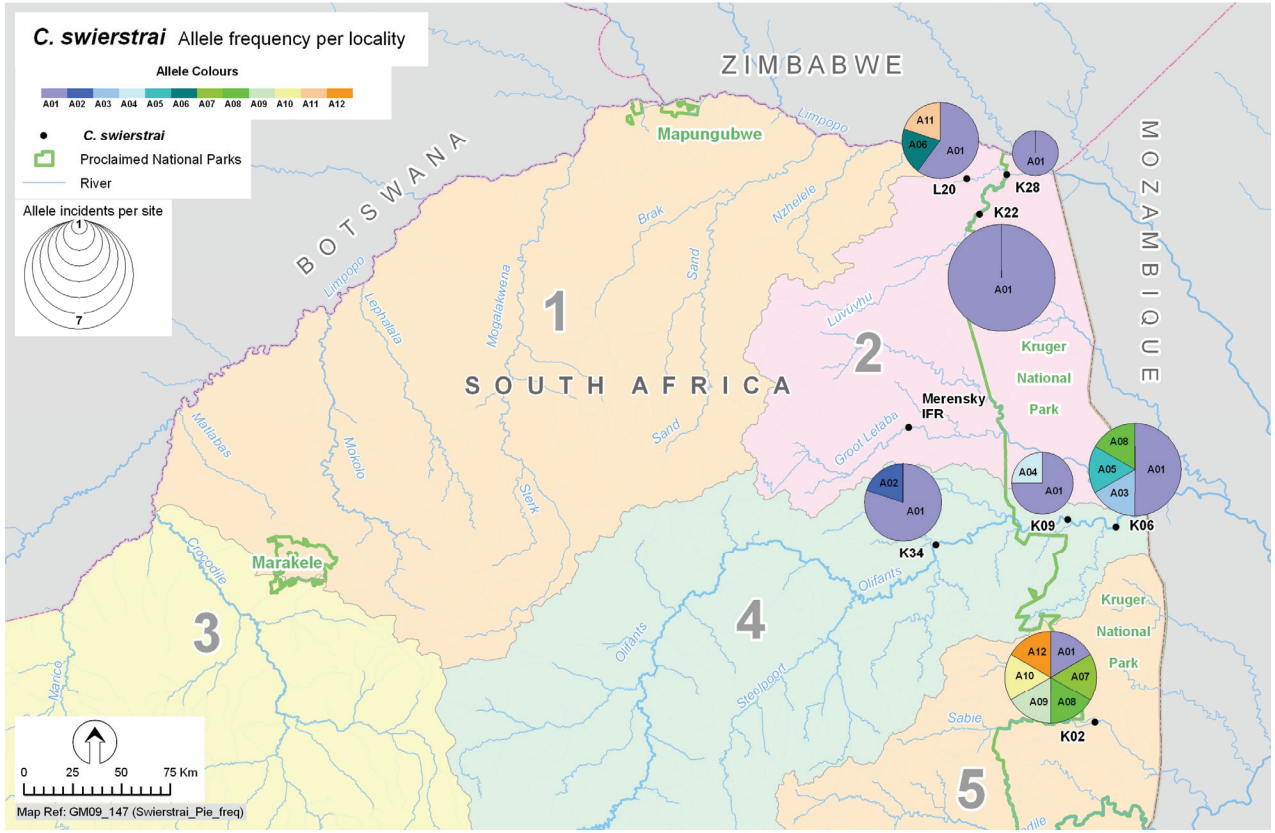
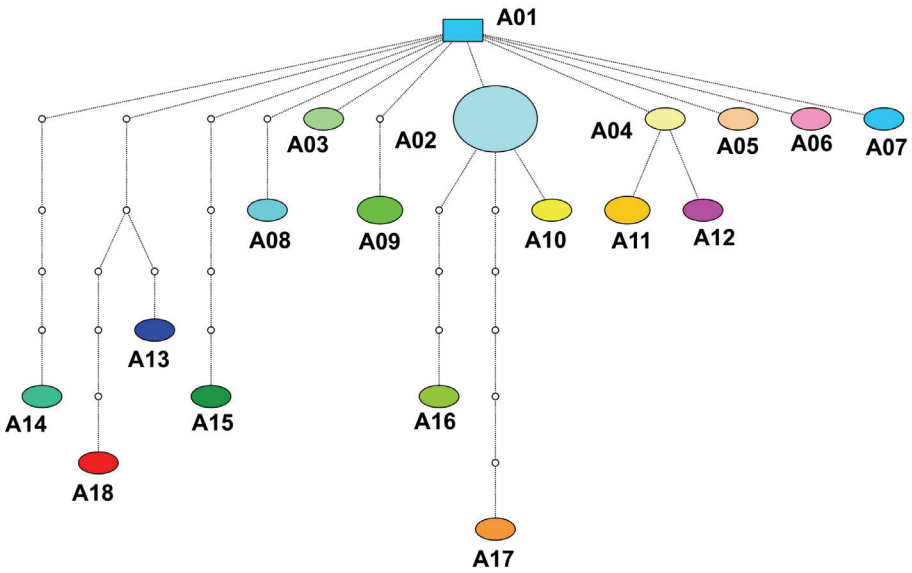
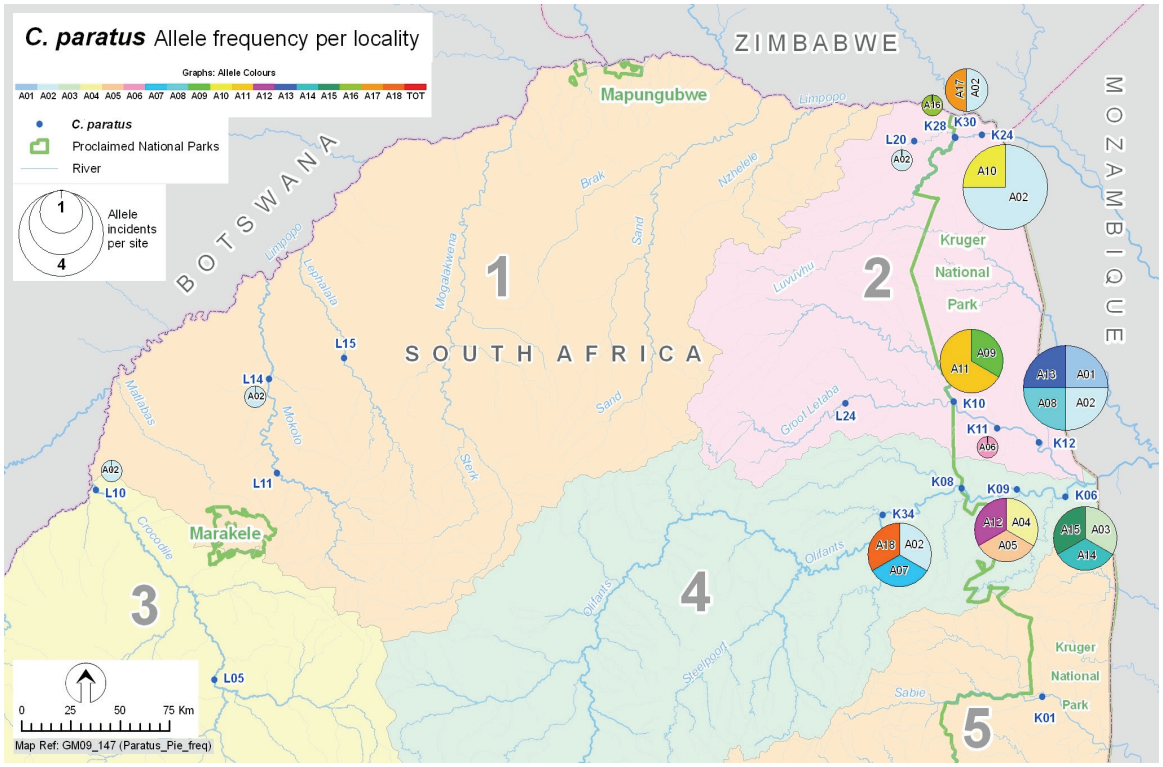


Figure 13(B). Phylogeographic analysis of *C. swierstrai* from the Limpopo and neighbouring river systems in South Africa. Distribution and frequency of 12 alleles in the Limpopo System and Sabie River; only A01 that was sampled at high frequency in the Limpopo System was found in one fish from the Sabie indicating historical isolation between these rivers.

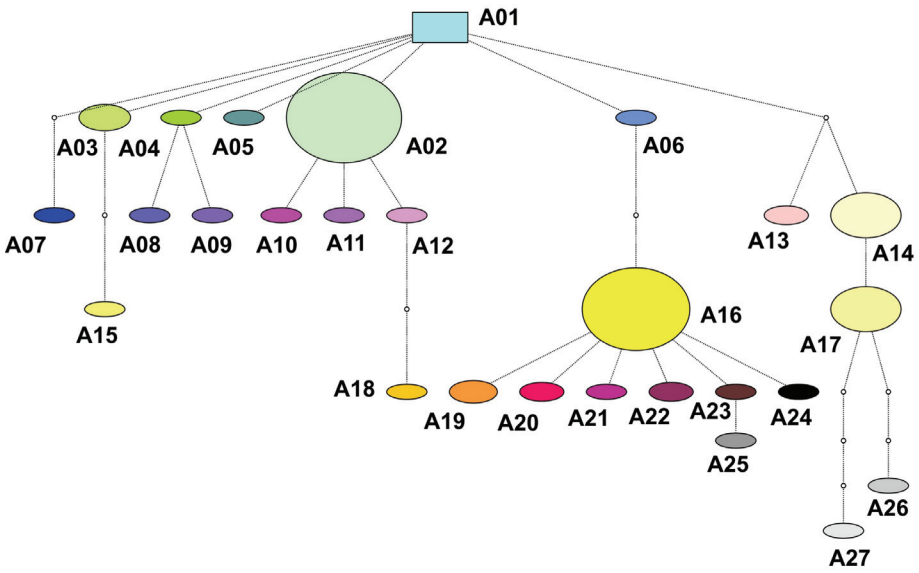


(A)

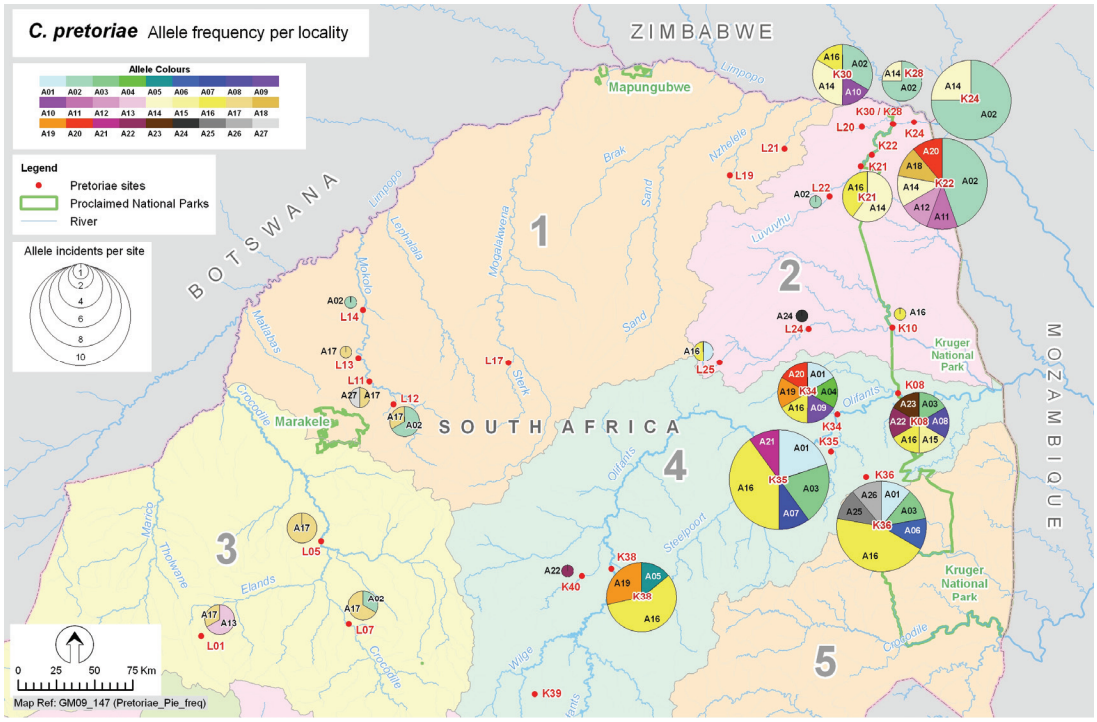


(B)

Figure 14. Phylogeographic analysis of *C. paratus* from the Limpopo River System in South Africa. (A) Allele network of 18 unique cyt *b* sequences containing several divergent alleles; (B) Distribution and frequency of 18 alleles in the Limpopo System; only A02 was widely recorded while the other alleles were locality specific.



(A)



(B)

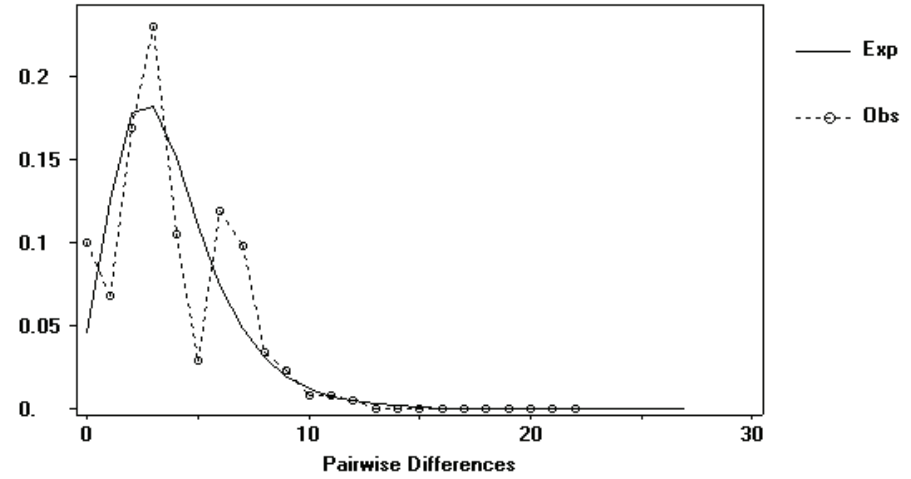
Figure 15. Phylogeographic analysis of *C. pretoriae* from the Limpopo River System in South Africa. (A) Allele network of 27 unique cyt *b* sequences, mostly connecting with few mutational changes between them; (B) Distribution and frequency of 27 alleles in the Limpopo System; a few alleles are widely distributed and the Olifants River appears to be historically isolated from the remainder of the catchments.

Despite the sharing of some alleles between the different rivers, there appears to be clear differences in the geographic distribution of the alleles in *C. pretoriae*. For example, allele A02 was only recorded from the Limpopo, Luvuvhu, Mokolo and Crocodile-West Marico and not from the Olifants System. Similarly alleles A01 and A03, despite a close genetic relationship with allele A02, were only recorded from the Olifants System and not from the northern and western parts of the species' distribution range. Table 2 summarizes the inferred historical gene flow rates among four regions: Olifants (1), Limpopo-Luvuvhu (2), Mokolo (3) and Crocodile-West Marico (4). In all instances there is asymmetrical gene flow, e.g. only from Olifants to Limpopo-Luvuvhu and not in the opposite direction. The most significant connections are between Limpopo-Luvuvhu and Mokolo as well as between Mokolo and Crocodile-West Marico. The history of these proposed connections has to be explored further and the historical female gene flow should be contrasted with overall historical and current gene flow rates.

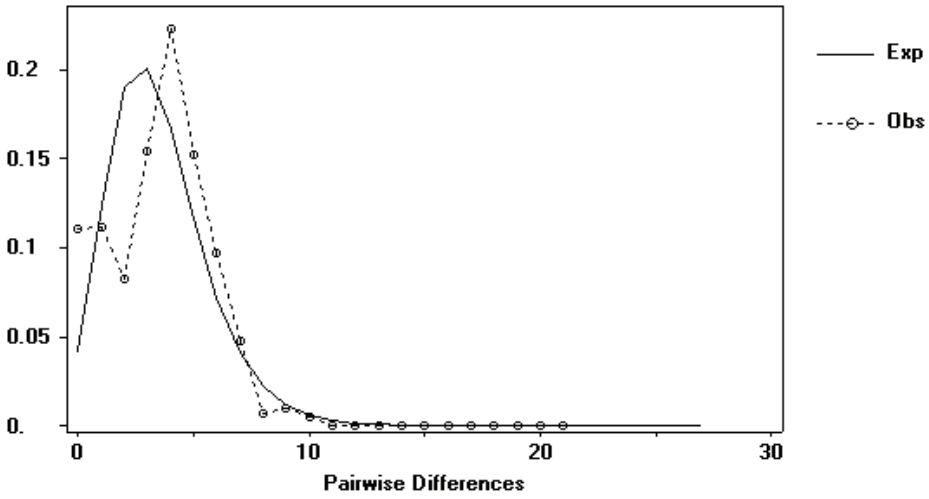
Table 3. Historical female gene flow patterns (i.e. number of effective female migrants per generation) between four regions of *C. pretoriae* distribution; '+' indicates the receiving population

		FROM			
		1, +	2, x	3, x	4, x
TO	1	-	0	0	0
	2	1.49	-	0	0.51
	3	0	2.2	-	0
	4	0	0	7.96	-

The distribution of genetic differences between alleles within a species can be used to reveal aspects of its history. Data for *C. paratus* (Figure 16A) and *C. pretoriae* (Figure 16B) showed a significant fit to a model of population growth and decline. In future we will date these expansions, but from the current analysis it appears that the period of population growth may coincide between these two species, indicating that they could be affected similarly by environmental changes. The pattern in *C. swierstrai* was more complex. Overall, the species appeared to display a long-term stable population size (Figure 17A). However, when the two groups within the species (see Figure 13A) were analysed independently (Group 1: Alleles A01-A06; Group 2 Alleles A07-A12), group 1 fitted a stable population model (Figure 17B) whereas group 2, mainly recorded from the Sabie River, showed population growth and decline (Figure 17C).

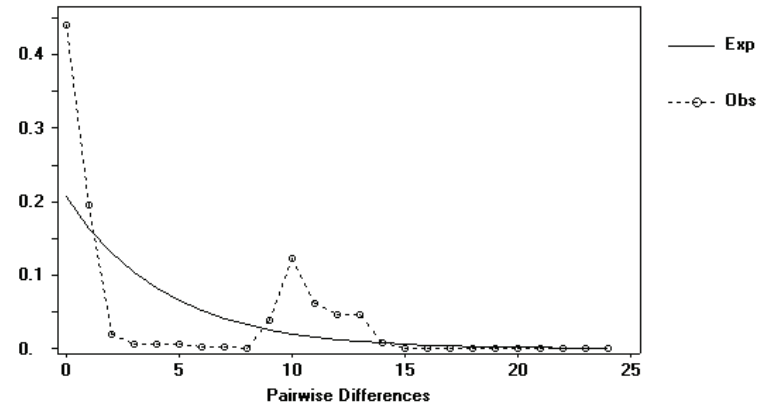


(A)

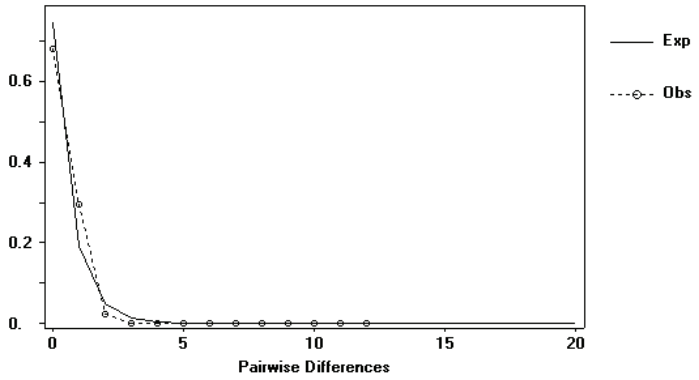


(B)

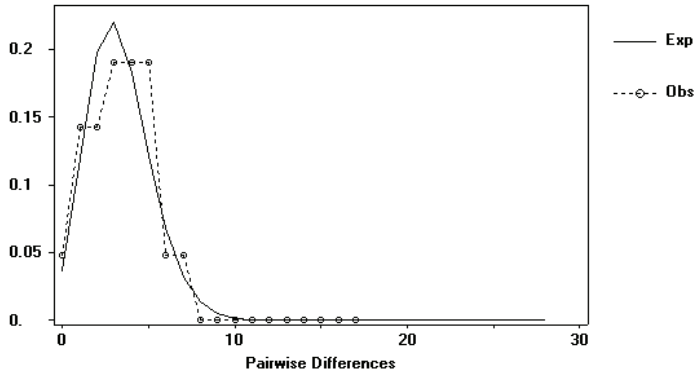
Figure 16. *C. paratus* (A) and *C. pretoriae* (B) showed a significant fit to a model of population growth and decline.



(A)



(B)



(C)

Figure 17. The *C. swierstrai* displayed a long-term stable population size (A), Group 1 fitted a stable population model (B) and group 2 showed population growth and decline (C)

3.4. Aquatic Habitat Assessment Analysis

An updated record on the distribution of three *Chiloglanis* species in the Limpopo and associated river systems and a synopsis of activities that are likely to devastate the natural habitat of *Chiloglanis* species suggest a decline in distribution of the three species. The results from the aquatic habitat assessment carried out in this study suggest that balancing of components of the system such as velocity, depth, substrate and physico-chemical parameters is of overriding importance. A rapid habitat assessment carried out in the Olifants River suggests that suitable habitat for *Chiloglanis* is a function of velocity, depth, substrate and physico- chemical conditions of the system. Site B3Mo of the Moses River (a tributary of the Olifants River) was described as a run riffle with a discharge value of 1.09 m^3/sec, oxygen saturation of 118.5% and TDS of 145 ppm and *C. pretoriae* was collected. Site B3Ela of the Elands River was described as a run riffle with a discharge value 0.19 m^3/sec, oxygen saturation of 114.5% and TDS of 664 ppm and no *Chiloglanis* was collected; only *Barbus* spp, *Labeo* spp, *Oreochromis mossambicus* and *Amphilius* were collected. There might be other factors that, when combined with high TDS, contributed to the absence of *Chiloglanis* at this site. Constant releases from the dam upstream increase the water column at site B3Ela. However, such a condition might be tolerable to the less sensitive fish species. Angermeier and Karr (1994) pointed out that when a river is dammed it results in homogenization of habitat types, thus reducing habitat integrity and/or diversity and leading to declines of fish populations adapted to the natural hydrological regime.

4. CONCLUSIONS AND RECOMMENDATIONS

The study has provided a finer scale assessment of habitat specificity for each of the three species. As expected the distribution of the three species reflected patterns typical of flow dependent primary freshwater fish species. *Chiloglanis pretoriae,* the most abundant of the three species, was found to inhabit shallow rapids, cascades and waterfalls of the headwater streams conforming to the previous findings on the distribution of the species (Gaigher and Pott, 1972 and Skelton 2001). *Chiloglanis paratus* appeared in the deeper rapids and runs habitat of the streams, although not as abundant as *C. pretoriae*. We recorded *C. swierstrai* in sandy substrate runs or sand pockets within deeper rocky runs. Clear loose sand seemed to have a vital role to facilitate burrowing. Although a rapid habitat assessment was only carried out in the Olifants catchment, there are trends emerging to suggest that suitable habitat for *Chiloglanis* is a function of velocity, depth, substrate and physico-chemical conditions of the system. However, factors such as vegetation cover, predation, seasonality, historical events of hydrological extremes (drought and floods) and so forth, should also be considered.

The genetic and morphological results point towards isolation and divergence of tributary populations, which suggests little or no connection between these populations. Isolation seems to be dictated by many factors including ecological and physical barriers, although the extent to which such factors isolate the populations differ from river system to river system. The dispersal of fishes depends on direct connections between river systems and the history of basin inter-connections (Lundberg, 1993 and Bermingham and Martin, 1998). Our analyses suggest that there must have been historical inter-connections that existed between the Limpopo River System and adjacent river systems that might have influenced the current distribution of the *Chiloglanis* species. The genetic and morphological variation within and between populations from different systems is significant, suggesting that the genus *Chiloglanis* is a good surrogate for studying fragmentation and impacts on aquatic systems. Geographical variation in fish colour patterns is supported by the results of the morphological and molecular analyses.

Chiloglanis swierstrai is morphologically distinct from the other two species and also shows significant intraspecific morphological diversity. The latter is confirmed by the DNA analysis, with two distinct clusters evident in the Limpopo System and the Sabie River. In both *C. pretoriae* and *C. paratus*, fish from Swaziland are morphologically distinct from those of the Limpopo River System. More extensive collecting is required for *C. pretoriae* as there is considerable variation among fish from Zimbabwe and also

clear distinction from the Limpopo System. With the exception of one shared allele, each site analysed in *C. paratus* harbored unique mtDNA alleles suggesting isolation between the rivers. *C. pretoriae* was extensively sampled and 27 alleles were identified. The geographic distribution of these alleles suggests limited exchange between the Olifants and the Limpopo/Luvuvhu. The extent of inter-connection of the Limpopo, Mokolo and Crocodile West Marico needs further investigation but some isolation is also apparent.

We make the following recommendations:

- The Limpopo and Inkomati River Systems must continue to be managed separately avoiding Inter-basin Transfers (IBTs) where possible until the two genetically distinct populations of *C. swierstrai* are well understood and such patterns are confirmed using other aquatic species that inhabit both systems. The WRC Consultancy K8/677 on *Labeobarbus polylepsis*, may support this conclusion.
- There are specific sites that should be given immediate special attention with the view to curb further fragmentation of the isolated populations. These sites are in the upper catchments of the Crocodile West Marico and the headwaters of the upper Olifants catchment.
- A monitoring programme must be established at selected sites to determine if the current records indeed represent long term absence from sites where the species have been recorded in the past. Such a programme could be incorporated into the on-going RHP of DWA.
- In tandem with the proposed monitoring programme, biological studies (both *in situ* and laboratory-based) should be carried out to determine factors responsible for the declines. Such factors could include water flow preferences, temperature and toxicity to certain pollutants (sedimentation, nitrates and certain metals). This could follow a research model including postgraduate studies.
- A detailed mapping of environmental impacts across the Limpopo River System that pose threats to *Chiloglanis* species should be undertaken. This could be linked with catchment studies of DWA and environmental science postgraduate studies might be another option.
- Ways should be explored for the inclusion of genetic diversity indices in the assessment of the ecological integrity of South Africa's rivers.
- A follow-up study should include all outlying populations of these three species as well as comprehensive analyses of other *Chiloglanis* species and should again be based on a combination of genetic and morphological approaches. Such a study will require more financial resources, amongst others from the Water Research Commission.

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APPENDIX 1

Table 4. Sites surveyed for the Limpopo River System suckermouth catfish project.

Site #	Location	Coordinates						
		South				East		
		°	'	"		°	'	"
k1	Sabie River below Skukuza	24	57	56		31	40	38
k2	Sabie River below Skukuza	24	57	50		31	41	09
k3	Sweni River near bird hide	24	28	25		31	58	31
k4	Nwanetsi River on Gudzani Road	24	24	04		31	57	11
k5	Timbavati River below Piet Grobler Dam	24	13	49		31	38	01
k6	Olifants River at Balule low level bridge	24	03	10		31	43	48
k7	Letaba River at low level bridge near Olifants	23	56	37		31	43	53
k8	Olifants River near Mamba weir	24	02	29		31	12	39
k9	Olifants River halfway between Mamba and Olifants camp	24	01	55		31	29	07
k10	Letaba River at Mahlangeni low level bridge	23	39	02		31	08	54
k11	Letaba River at Mopani road crossing	23	45	33		31	22	12
k12	Letaba River at road bridge near Letaba camp	23	48	41		31	34	55
k13	Pioneer Dam – below wall	23	31	35		31	23	52
k14	Shingwedzi River at Dipeni	23	12	49		31	32	40
k15	Shingwedzi River at Kanniedood Dam	23	08	39		31	27	47
k16	Klein Letaba at Sawutini	23	29	28		31	03	37
k17	Shingwedzi River below Silwervis Dam	23	12	39		31	13	58
k18	Shingwedzi River at Shingwedzi camp high bridge	23	05	37		31	25	07
k19	Mphongolo at Shibauwene waterhole	22	53	03		31	03	50
k20	Malahlapanga hot springs	22	53	22		31	02	22

Table 4 continues

k21	Levuvhu at Dongadziva	22	42	35		30	53	19
k22	Levuvhu at Shidzivane	22	38	15		30	57	08
k23	Levuvhu bridge near Pafuri	22	25	22		31	13	09
k24	Bobomene bridge	22	25	40		31	12	34
k25	1km upstream of Crooks Corner	22	25	55		31	18	00
k26	1km above site 25	22	25	46		31	15	18
k27	Mutale weir	22	26	16		31	04	40
k28	Mutale old weir	22	26	44		31	04	32
k29	Mutale at M-Levuvhu confluence	22	26	52		31	04	46
k30	Levuvhu at M-Levuvhu confluence	22	26	52		31	04	46
k31	Letaba River, 1st weir below Hans Merensky NR	23	38	48		30	43	08
k32	Letaba River, 2nd weir below Hans Merensky NR	23	41	18		30	52	05
k33	Selati River above Phalaborwa	23	58	41		31	04	25
k34	Olifants River at Mica	24	11	03		30	49	34
k35	Blyde River on R527	24	24	19		30	47	53
k36	Klaserie River on R531	24	32	33		31	02	07
k37	Steelpoort near Stoffberg R579	25	25	56		29	51	24
k38	Olifants below Loskop dam on R33	25	09	45		29	24	56
k39	Moses River on R33	25	12	40		29	13	30
k40	Groot River on R25	25	38	58		29	52	02
k41	Bronkhorstspruit on R25 near Bronkhorstspruit town	25	47	44		28	45	22
k42	Bronkhorstspruit on R104 in Bronkhorstspruit	25	48	21		28	43	20

Table 4 continues

L1	Groot Marico River at the road crossing just south of Groot Marico town	25	38	34		26	45	21
L2	Skeerpoort Stream on Hartebeeshoek Farm (plot 6)	25	49	55		27	47	1
L3	Magalies River at a road bridge in town	25	59	45		27	33	2
L4	Sterkspruit on main tar Rustenburg-Pretoria Road	25	45	26		27	28	59
L5	Crocodile River at David Brink Bridge on Northam Road	25	3	45		27	31	11
L6	Tributary of Roodekopjes Dam – north of Brits	25	30	24		27	41	37
L7	Tributary of Roodekopjes Dam – north of Brits at Rice Flour Technology center	25	32	59		27	42	58
L8	Sundays River on upper Buffelshoek Farm, weir site	24	32	16		27	38	27
L9	Sundays/Sand River at concrete bridge south-east of Thabazimbi	24	27	11		27	36	25
L10	Middle Crocodile west of Thabazimbi	24	34	57		26	24	49
L11	Mokolo River near Hermanusdorings town	24	6	45		27	48	8
L12	Sterkstroom Stream – tributary of Mokolo River on main road	24	14	29		27	57	41
L13	Mokolo River at weir below the Mokolo Dam	23	58	15		27	43	32
L14	Mokolo River at main road bridge just south of Lephalale Town	23	41	11		27	44	47
L15	Lapalala River at main road bridge (R518)	23	34	46		28	6	56
L16	Palala River at bridge on Vaalwater Road	23	59	5		28	24	18
L17	Sterk River on Marken road near Mokamole village	23	58	13		28	41	39
L18	Mogalakwena River below Glen Alpine Dam	23	9	18		28	40	55
L19	Nzhelele stream above Nzhelele dam	22	48	8		30	3	31
L20	Mutale River at bridge between Masisi and Thohoyandou	22	28	31		30	52	45

Table 4 continues

L21	Nwanedi River below Nwanedi dam	22	37	50		30	23	58
L22	Levuvhu River at bridge on the Thohoyandou-Punda Maria road	22	53	47		30	41	54
L23	Channel below Nsami Dam wall	23	15	33		30	46	19
L24	Groot Letaba River at bridge near Letsitele	23	41	9		30	36	41
L25	Groot Letaba on the road south west out of Tzaneen	23	54	50		30	3	13