PHYLOGEOGRAPHY AND CONSERVATION OF A NEWLY IDENTIFIED GALAXIID FROM THE JOUBERTINA AREA, SOUTH AFRICA

BY

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ABSTRACT

The dispersal of freshwater fishes in the Cape Floristic Region of South Africa has been attributed to river capture events and confluence of rivers during sea level regression. The role of low drainage divides and inter-basin water transfers have received less attention. A unique lineage of *Galaxias zebratus* (hereafter the Joubertina galaxias) occurs in two currently isolated river systems, the Gamtoos and Krom. The present study mapped the distribution of the Joubertina galaxias and used mitochondrial and nuclear DNA sequences to assess the processes that could have influenced its current distribution pattern. Analyses of both mitochondrial cytochrome *b* and nuclear (S7) sequences revealed that observed genetic differentiation cannot be explained by isolation between the Gamtoos and Krom River systems. No genetic differentiation was found between the Krom River System and the Twee River (a tributary of the Gamtoos River System). Shallow genetic differentiation (0.4% for cytochrome *b* and 0.3% for S7) was found between the Krom and the remaining populations in the Gamtoos River System. High levels of genetic structuring were observed within the Gamtoos River System with most tributaries having one or more unique alleles. Inter-basin dispersal during pluvial periods or recent human mediated translocation seems to be the most plausible explanations for presence of the Joubertina galaxias in the Krom River System. The present study also assessed the threats and habitat preferences of the Joubertina galaxias, and an evaluation of the conservation status of this lineage was done. The Joubertina galaxias is threatened mainly by alien fish invasion, habitat loss and loss of genetic diversity due to fragmentation of its populations. Due to its very restricted geographic range, small known population sizes and the intensity of threats to this lineage's survival, this lineage has been assessed as Endangered. The lineage has a restricted Area of Occupancy (AOO) and Extent of Occurrence (EOO). The extent of occurrence has declined in all tributaries and is continuing to decline in all except two tributaries that are secure. The lineage may have had natural fragmentation among its populations, but recent threats have completely isolated the populations. The threats affect the lineage's survival potential in the four tributaries which have small populations that are not presently viable. The densities observed for the Joubertina galaxias ranged from 0.16 - 1.3 fish /m^2 and the number of mature individuals for the whole population seems to be less than 2500 mature individuals. There is therefore a need for specific conservation actions to ensure the long-term survival of this unique lineage.

DECLARATION

I, the undersigned, hereby declare that 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 husband, Albert,

for all his unconditional love, encouragement and support throughout my studies,

my daughter Rutendo for understanding

and

my parents Otilia & Fanuel Mataruse

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CHAPTER 1

Thesis introduction

1.1. Diversity and distribution of the Galaxiidae

Freshwater fishes of the family Galaxiidae are restricted to temperate conditions throughout the Southern Hemisphere, with most species occurring in New Zealand and Australia (McDowall, 1970, 1971, 1973; Waters & Cambray, 1997; Burridge *et al*., 2012). The family also has representatives in southern South America, New Caledonia, Tasmania, the Falkland Islands and southern Africa (McDowall, 1969; 1973; Waters & Burridge, 1999; Waters *et al*., 2000). The family Galaxiidae has six genera and about 45 currently described species (Waters *et al*., 2000), although the discovery of deeply divergent lineages within many of the species (e.g. Allibone *et al*., 1993, 1996; Waters & Cambray, 1997; Waters *et al*., 2001a, b; Wishart *et al*., 2006; Swartz *et al*., unpublished; Chakona *et al*., unpublished) indicate that our current understanding of galaxiid fish diversity is incomplete. Most species are restricted to freshwater (e.g. *Galaxias zebratus*, *Galaxiella* spp. and *Brachygalaxias* spp.), but several are diadromous, with a regular marine juvenile phase in the life history (Waters *et al*., 2000; Wallis *et al*., 2001a). Members of the genus *Galaxias* are thought to be of Gondwanan origin (Barnard, 1943; McDowall, 1970; 1973; Rosen, 1974; but see also Burridge *et al*., 2012), but they are absent from Antarctica and India (McDowall, 1969; Gleeson *et al*., 1999). Only one species is currently recognised from the African continent, the Cape Galaxias (*Galaxias zebratus* (Castelnau, 1861)), which is an obligate freshwater species (Barnard, 1943).

The historical biogeography of most galaxiid fishes is uncertain. The distribution of the members of the galaxiid family has been attributed to Gondwanan fragmentation for non-diadromous species (Waters *et al*., 2000a; McDowall & Burridge, 2011) and marine dispersal for diadromous species (Waters *et al*., 2000a, b). McDowall (1973) considered marine dispersal as a possible explanation for the distribution of *G. zebratus*, but some authors argued in favour of the Gondwanan vicariance (Barnard, 1943 and Rosen, 1974; Waters *et al*., 2000b). The vicariance hypothesis was supported by the phylogenetic affinities of *G. zebratus* whose nearest relative was considered to be *Branchygalaxias bullocki* from Chile in South America (McDowall, 1973). A more comprehensive phylogeny by Burridge *et al*. (2012), however, suggest that *G. zebratus* is more closely related to *Neochanna* from Australia and New Zealand and *Galaxias platei* from South America. The Cape galaxias is considered one of the most distinctive and geographically isolated within the family Galaxiidae (Rosen, 1974) despite having a similar life history to other freshwater galaxiids (McDowall, 1973). The Cape galaxias differs morphologically from other *Galaxias* species, because it has a low number of vertebrae, reduced number of pelvic fin rays (fewer than seven which is typical in *Galaxias*) and lacks two latero-sensory pores beneath the lower jaw (McDowall, 1973, McDowall & Burridge, 2011).

1.2. Distribution, biology and habitat of *Galaxias zebratus*

Galaxias zebratus is restricted to the Cape Floristic Region (CFR) of South Africa (Figure 1.1). The CFR occupies a very small area (only 7%) of South Africa, but it is recognised globally as a hotspot for endemic plants (Goldblatt & Manning, 2000; Cowling *et al*., 2003;

Clark *et al*., 2009; Cowling *et al*., 2009) and animals (Wishart and Day, 2002; Kuhlmann, 2005; Kuhlmann, 2009; Linder *et al*., 2010). The diversity of the fish fauna associated with the CFR is considered to be one of the poorest in Africa (Skelton, 1986). The region hosts only one to ten primary freshwater fish species per river system and has only three widespread genera, namely *Galaxias, Pseudobarbus* and *Sandelia* (Linder *et al*., 2010). The genus *Galaxias* is currently represented by a single species, *Galaxias zebratus*. Until only recently, *G. zebratus* was considered to be one of the most widely distributed primary freshwater fishes in the CFR (Skelton, 1993, 2001; Cambray *et al*. 1995). The distribution range of this species spans across more than 10 currently isolated river systems, from the Olifants River system on the west coast to the Gamtoos River system in the eastern CFR (Cambray *et al*., 1995; Figure 1.1B).

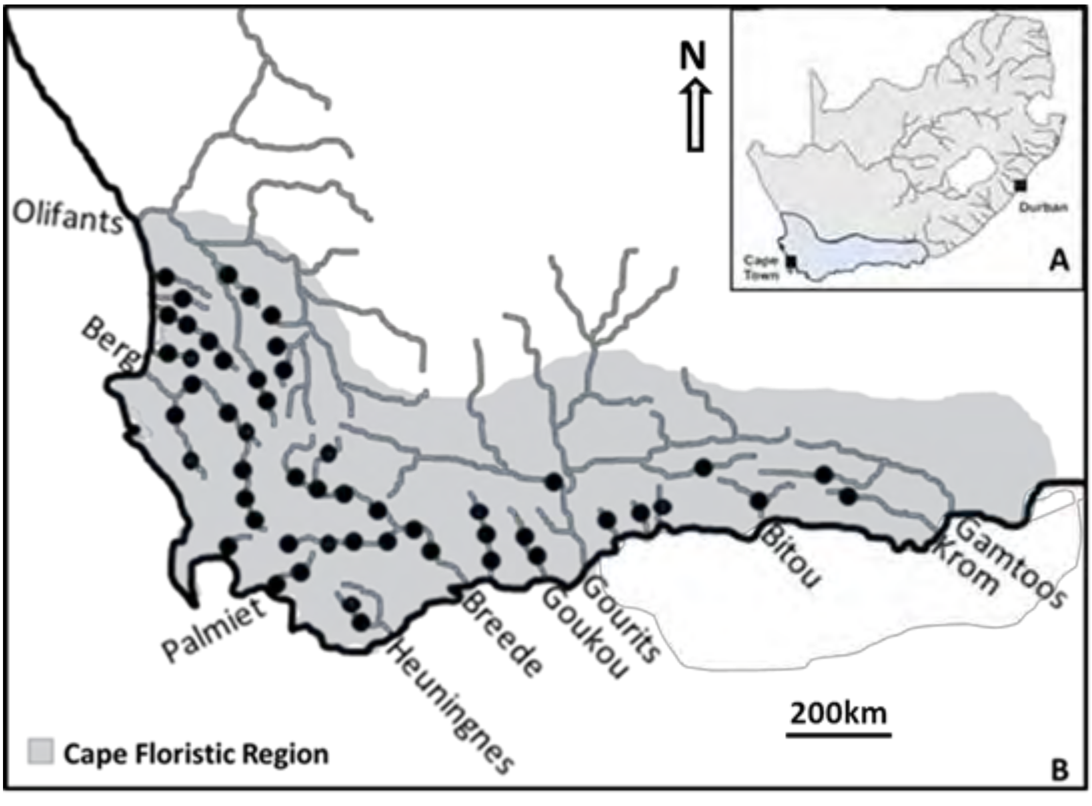

Figure 1.1: Map of South Africa showing the Cape Floristic Region (CFR) (A) and the localities where the Cape galaxias has been recorded (B; modified from Cambray *et al*., 1995).

Very little is known about the life history of *G. zebratus*. It is a small species, with a maximum recorded length of 75 mm (Jubb, 1967). Members of this species may appear almost translucent or may have a cryptic colouration depending on the nature of their habitat (Barnard, 1943). *Galaxias zebratus* occurs in a wide range of habitats from headwater mountain streams to lowland coastal streams and rivers, and is tolerant of both alkaline and acidic conditions (Barnard, 1943; McDowall, 1973; Waters & Cambray, 1995; Wishart, 2002; Chakona & Swartz, 2012). This species has been collected from fast flowing sections of rivers (riffles) but appears to prefer gentle currents, sheltered river reaches, and is usually found near the head of pools, but can also tolerate stagnant water (Barnard, 1943; Cambray *et al.*, 1995; Chakona & Swartz, 2012). *Galaxias zebratus* completes its entire life cycle in freshwater although brackish water may be tolerated during spawning (McDowall, 1973). The species is sexually mature at 38 - 40 mm in length and it has been suggested that it can breed throughout the year (Barnard, 1943).

1.3. Taxonomic history of *Galaxias zebratus*

Like other members of the family Galaxiidae, *G. zebratus* is characterized by a high degree of morphological variation. The Cape Galaxias has different forms and has been recognized as a species complex, but the conservative morphology that is common in galaxiid fishes has made it difficult for previous researchers to find characters with which to describe these forms as distinct species (Barnard, 1943; McDowall, 1973). Four species, namely *G. zebratus* (Castelnau, 1861), *G. punctifer* (Castelnau, 1861), *G. capensis* (Steindachner, 1894) and *G. dubius* (Gilchrist and Thompson, 1917) were described from South Africa. However, an extensive revision by Barnard (1943) failed to yield consistent characters to distinguish the species. Barnard (1943) placed *G. punctifer*, *G. capensis* and *G. dubius* in synonymy with Castelnau's (1861) species. Morphological and osteological data by McDowall (1973) could also not distinguish different species. Subsequent authors have therefore continued the use of the name *G. zebratus* for a single widespread and variable species (Jubb, 1965; Harrison, 1967; Skelton, 1993; Cambray *et al*., 1995).

1.4. Cryptic diversity within *Galaxias zebratus*

More recent molecular studies have revealed at least ten strongly diverged lineages within *G. zebratus* (Waters & Cambray, 1997; Van Niekerk, 2004; Wishart *et al*., 2006; Linder *et al*. 2010), confirming that the Cape galaxias is a species complex. Most of the lineages are likely to be described as separate species following taxonomic revision of this group (Swartz *et al*., unpublished). Only one of these lineages has a wide distribution range across the CFR (Van Niekerk, 2004; Chakona *et al*., 2011), while many of them have highly restricted geographic ranges (Swartz *et al*., unpublished; Chakona *et al*., unpublished). For example, the Joubertina galaxias is presently only known from upper Krom (Krom River System) and three tributaries of the Kouga catchment of the Gamtoos River System (Wabooms and Twee and Granaat) (Cambray, personal communication; Waters and Cambray, 1997; Cambray *et al*., 1995; Swartz *et al*., unpublished). This species prefers gentle currents within sheltered banks near the head of pools. They can tolerate stagnant water and wide temperature ranges (Cambray *et al*., 1995). Like other galaxiid fishes, individuals of the Joubertina galaxias have cryptic coloration, are transparent pale brown with light brown stripes, with the red gills, heart and vertebral column visible. Maximum size for individuals from the Krom and Gamtoos river systems is about 60 mm standard length (Cambray *et al*., 1995).

There is a lack of information regarding the biology and life history of this newly discovered *Galaxias* lineage. Of the known populations of the Joubertina galaxias, none occur with other indigenous fish species (*Sandelia capensis* or *Pseudobarbus afer*) (Cambray *et al.,* 1995). This may be due to sensitivity to competition and this species could have been excluded by fishes that are better adapted to mainstem habitats, resulting in preference for more upstream habitats where there is no competition from other fishes.

1.5. Research problem statement and rationale

The Joubertina galaxias is presently understood to occur in two isolated river systems and the sharing of the Krom and Kouga alleles may suggest relatively recent gene flow between populations of the two river systems (Waters & Cambray, 1997). The present study builds on this previous research by analysing the genetic structure of the Joubertina galaxias more comprehensively, since no population genetic information is available for the Joubertina galaxias. The Joubertina galaxias is confined to fresh water (Chapter 2). Both marine and terrestrial environments are therefore effective barriers to its dispersal. Species which inhabit either side of potential barriers are useful for assessing geological or climatic processes that could have shaped population genetic structure. The present study addresses the following questions: (i) how did the Joubertina galaxias acquire its present distribution in two currently isolated river systems, (ii) is there genetic differentiation within or between river systems and (iii) which processes allowed gene flow or caused isolation?

Previous explanations for the wide biogeographic range of galaxiids in general, inferred marine dispersal (McDowall, 1978; Berra *et al*., 1996; Waters *et al*., 2000) and vicariant hypothesis (Rosen, 1974, 1978; Craw, 1979) while more recent evidence by Burridge *et al*. (2012) suggests that both marine dispersal and vicariance played a crucial role in the distribution of galaxiids. Movement of freshwater fish between river systems is mainly facilitated by river capture events, river confluence during lower sea levels or through inland connections during wet periods (Durand *et al*., 1999). These processes have also been inferred to explain the diversification of freshwater fish in the CFR (Cambray *et al*., 1995; Waters & Cambray, 1997; Swartz *et al.,* 2007; 2009; Chakona *et al*., unpublished) and elsewhere (Waters *et al*., 1994; Waters & Wallis 2000; Burridge *et al*., 2007; Schönhuth *et al*., 2011). More recent anthropogenic processes have also been suggested to promote isolation (Meldgaard *et al*., 2002) or connectivity of freshwater fishes (Hughes *et al*., 2003; Therriault *et al*., 2005; Ramoejane, 2011).

The introduction of alien fishes, especially North-American black bass species (*Micropterus* spp.), has been noted as a major threat to the survival of indigenous species (Barnard, 1943). The level of impact that black bass, especially smallmouth bass (*Micropterus dolomieu*), is having on the small indigenous species has been severe, especially in the CFR where most native fish species are threatened (Harrison, 1961; Tweddle *et al*., 2009; Clark *et al*., 2009; Linder *et al*., 2010). Of the 16 endemic freshwater fish species in the CFR, 13 are threatened (Impson *et al*., 1999; Skelton, 2000). The effects of excessive water extraction and weirs have also posed a threat to indigenous freshwater fish in the CFR. The question therefore is: are these typical threats in the CFR affecting the survival potential of the Joubertina galaxias and what conservation management strategies can be recommended to secure the future survival of this lineage?

To answer these questions, information is required regarding the distribution, evolutionary history and conservation status of the Joubertina galaxias. To shed more light on the evolutionary history of the Joubertina galaxias, the following four factors that may have affected the distribution of fishes in the eastern CFR were investigated.

Geological history of the CFR: The most distinguishable geological features of the CFR are the Cape Fold Mountains, a series of mountain ranges that have a west to south-easterly orientation and run more or less parallel to the southern and south-western coastline of South Africa (Hattingh, 2008). Mountain uplift in southern Africa occurred in the late Jurassic (*ca.*140 million years ago (Ma)) and the region has been stable since the early Cenozoic (*ca.* 65 Ma) (Hendey, 1983). The Krom and Gamtoos River systems were similarly orientated to the Cape Fold mountains before the headwaters of the Krom were captured by the Gamtoos River system during the Cenozoic (*c.* 60 million years ago (Cambray *et al.*, 1995; Hattingh, 2008). Their form has remained stable since then (Hendey, 1983). As a result of the Kouga River capture, the Kouga became a major contributor of water to the Gamtoos River system, abandoning the Krom River system, leaving it as a much smaller drainage system. More recent tectonic events have occurred during the Miocene (*ca.* 22 Ma) and Pliocene (*c.* 5.3 – 2.6 Ma) (Partridge & Maud, 1987, 2000), but there is no evidence that these have altered the Krom and Gamtoos drainages.

Sea-level changes: Southern Africa has experienced repeated fluctuations in sea-levels throughout the Tertiary period (Hendey, 1983). During the mid-Miocene (*ca.* 15 Ma) to early Pliocene (*ca.* 5 Ma), sea levels in southern Africa raised to between 200 m and 300 m above the present sea level (Siesser & Dingle, 1981, Hendey, 1983). This was the major transgression experienced in southern Africa. Lowering of the sea level also experienced in southern Africa during the last glacial maximum (LGM) (*ca.*18 000 years ago) when sea levels ranged between 100 m and 200 m below the present coastline (Siesser & Dingle, 1981; Hendey, 1983). The Krom and Gamtoos River systems may have been connected and formed a common confluence during the low sea-levels of the LGM about 18 000 years ago (Hendey, 1983; Tankard, 1976; Rogers, 1985), but a phylogeography study revealed that isolation occurred between *Pseudobarbus afer* lineages from the Krom and Gamtoos prior to the LGM (Swartz *et al.*, 2009).

Climatic history of the CFR: During the Oligocene (*ca.* 23 Ma) to middle Miocene (*ca.* 10 Ma), southern Africa experienced periods of extreme wet conditions (Partridge *et al.*, 1999; Cowling *et al.*, 2009), which were followed by contemporary dry conditions by the end of the Pliocene (*ca.* 2.6 Ma) (Deacon *et al.*, 1992). This could have caused increased isolation of river systems and fragmentation of both aquatic and terrestrial habitats. The most recent wetter period was inferred to have occurred as recent as the Holocene Altithemal (*c.* 8 000 years ago) (Partridge *et al.*, 1999; Cowling *et al.*, 2009). Temporary periods of integration of the Krom and Gamtoos River systems may have occurred across low drainage divides during pluvial periods. Inland drainage connections through flooding during wet periods may facilitate the dispersal of freshwater fish (Jerry & Woodland, 1997; Craw *et al.*, 2007; Burridge *et al.*, 2008; Swartz *et al.*, 2009).

Inter-basin transfers: South Africa is experiencing serious water supply shortages as demand for freshwater is increasing for industry, agriculture and domestic use (DWAF, 1986). Several inter-basin water transfer schemes (IBT's) have been implemented throughout South Africa to increase fresh water supply in areas with inadequate water supply. Canals connect farm dams in the Krom and Upper Kouga catchments that could potentially allow migration of fish species between the Gamtoos and Krom River systems in either direction.

1.6. Conservation concerns

Joubertina is a small town in the Cacadu district municipality of the Eastern Cape Province of South Africa. It is located in the Langkloof valley, which is 160km long. The valley has been farmed since the 18th century and is a major fruit producing region. Farm dams, municipal dams, weirs and canals have been built in this area to provide irrigation for the intensive agriculture of particularly apple orchards. Streams of the upper Krom and upper Kouga are therefore extensively utilised for irrigation and domestic purposes. As a result, most of the streams in these catchments no longer have continuous flow throughout the year.

Alien invasive fish species such as the North American black bass species (*Micropterus* spp.) and the sharptooth catfish (*Clarias gariepinus*) have been recorded in several mainstem rivers and lower reaches of tributaries in the CFR, and are likely to occur in the study area. Invasive alien plant species, in particular *Pinus* spp. and *Acacia* spp are now the dominant vegetation in some catchments and riparian zones of the upper Krom and Gamtoos River systems (see Chapter 3). These exotic plants can alter stream characteristics in various ways, including accelerated riverbank erosion, increased sedimentation and reduced stream flow (Vertessy, 2000).

Conservation management should aim to allow evolutionary processes that could have shaped intraspecific diversity to continue into the future (Crandall *et al.*, 2000; Moritz, 1999; 2002) rather than to just conserve biological patterns. The focus should be mainly on protection of historically isolated lineages, because replacement of these cannot occur over a short period of time (Moritz, 1999; Moritz *et al.*, 2002). Molecular markers provide information regarding population history and the current distribution of genetic diversity is therefore a useful tool to define conservation units (Moritz, 1999).

1.7. Thesis aims

The first aim of the thesis was to comprehensively map the distribution of the Joubertina galaxias and to evaluate the genetic variation of this lineage, based on extensive field surveys and mitochondrial and nuclear DNA sequencing. Phylogeographic patterns of the lineage were assessed to determine underlying evolutionary processes and causal historical events (Chapter 2). Secondly, threats were assessed to evaluate the conservation status of the Joubertina galaxias using International Union for the Conservation of Nature (IUCN) criteria (Chapter 3).

CHAPTER 2

Historical abiotic events or human-aided dispersal: clarifying the evolutionary history of a newly identified galaxiid

*This chapter has been submitted to Hydrobiologia

ABSTRACT

The dispersal of freshwater fishes in the Cape Floristic Region of South Africa has been attributed to river capture events and confluence of rivers during sea level regression. The role of low drainage divides and inter-basin water transfers have received less attention. A recently discovered lineage of *Galaxias zebratus* (hereafter the Joubertina galaxias) occurs in two currently isolated river systems, the Gamtoos and Krom. The present study mapped the distribution of the Joubertina galaxias and used mitochondrial and nuclear DNA sequences to assess the processes that could have influenced its current distribution pattern. Analyses of both mitochondrial cytochrome *b* and nuclear (S7) sequences revealed that observed genetic differentiation cannot be explained by isolation between the Gamtoos and Krom River systems. No genetic differentiation was found between the Krom River system and the Twee River (a tributary of the Gamtoos River system). Shallow genetic differentiation (0.4% for cytochrome *b* and 0.3% for S7) was found between the Krom and the remaining populations in the Gamtoos River system. High levels of genetic structuring were observed within the Gamtoos River system with most tributaries having one or more unique alleles. River capture events and sea level changes does not explain present distribution of the Joubertina galaxias across the Krom and Gamtoos River systems, because it does not fit with the estimated time of genetic differentiation and the presence of in-stream natural barriers that would have prevented migration. Instead, inter-basin dispersal during pluvial periods or recent human mediated translocation seems to be the most plausible explanation for the shallow genetic divergence between populations from the two river systems.

2.1. Introduction

Freshwater taxa tend to display higher levels of genetic structuring among populations than marine taxa, because they generally have smaller effective population sizes and populations are often isolated by terrestrial or marine barriers to dispersal (Gyllensten, 1985; Ward *et al*., 1994; DeWoody & Avise, 2000). Strong genetic subdivisions are especially expected between river systems that are not connected (Meffe & Vrijnhoek, 1988; Waters & Cambray, 1997; Swartz *et al*., 2007, 2009; Chakona *et al*., unpublished). However, some freshwater taxa have a high salinity tolerance, and are therefore capable of marine dispersal. Such diadromous species are characterized by extensive geographic ranges, large population sizes and show low levels of genetic differentiation among populations even populations belonging to unconnected river systems (Waters & Burridge, 1999; Waters *et al*., 2000).

Species that are not tolerant of high salinities can naturally only disperse between isolated river systems through rare events such as river captures, confluence of rivers during lower sea levels, freshwater plumes in estuarine and marine environments or flooding of low drainage divides (Unmack, 2001; McGlashan & Hughes, 2001; Wong *et al*., 2004; Craw *et al*., 2007; Burridge *et al*., 2008; Sharma & Hughes, 2011). It may be possible that freshwater fish can be moved across geographical divides by waterspouts or can be dropped by aquatic birds. Such types of dispersal have been reported in Australia (Whitley, 1972; Glover, 1990) and West Africa (Ried, 1996), but remain poorly understood (Unmack, 2001). Unmack, (2001) suggested that there are other possibilities for random dispersal of freshwater fishes through accidental movement of eggs across geographical divides on birds' feet and/or feathers, while certain insects may be capable of moving eggs over short distances. These means of dispersal have, however, never been scientifically documented.

Several studies have inferred the role of river captures (Waters *et al*., 1994; Waters & Wallis 2000; Burridge *et al*., 2007; Swartz *et al*., 2007; Schönhuth *et al*., 2011), sea level changes (Bermingham & Avise, 1986; Swartz *et al*., 2007 & 2009) and inland drainage connections through flooding during wet periods (Jerry & Woodland, 1997; Craw *et al*., 2007; Burridge *et al*., 2008; Swartz *et al*., 2009) to explain the occurrence of closely related lineages in currently isolated river systems. Accumulating evidence also shows the role of anthropogenic influences in promoting isolation (e.g. Meldgaard *et al*., 2002; Gosset *et al*., 2006) or connectivity (Hughes *et al*., 2003; Therriault *et al*., 2005; Ramoejane, 2011) of freshwater restricted taxa.

Phylogeographic studies can be employed to understand the history of populations between and within drainages and can identify potential biogeographic processes that influence population genetic structure (Avise, 2000; Beheregaray, 2008; Sistrom *et al*., 2009). Species that occur on either side of potential barriers are useful for assessing processes that shaped population genetic structure. The Cape Floristic Region (CFR) of South Africa presents an ideal area to study processes that influence the evolutionary history of primary freshwater taxa due to the region's complex drainage patterns. Freshwater taxa in the CFR have been influenced by a potentially broad spectrum of events which include uplift of the Cape Fold Mountains (Hendey, 1983; Skelton, 1980; Maud, 1990; Hattingh, 2008; Cowling *et al*., 2009), sea-level changes (Tankard, 1976; Siesser & Dingle, 1981; Dingle *et al*., 1983) and climatic oscillations (Partridge *et al*., 1999; Cowling *et al*., 2009).

The impact of this complex history on the CFR's freshwater fauna has been studied with the aid of molecular methods (Waters & Cambray, 1997; Bloomer & Impson, 2000; Swartz *et al*., 2007; 2009; Chakona *et al.*, unpublished). Several unique genetic lineages of *G. zebratus* have been discovered across the CFR (Waters & Cambray, 1997, van Niekerk, 2004; Wishart *et al*., 2006, Chakona *et al*., unpublished, Swartz *et al*., unpublished), some of which are currently being described as new species (Swartz *et al*., unpublished). Only one of these lineages has a wide distribution range across the river systems of the CFR (Chakona *et al*., 2011), while most of them have restricted geographic ranges occurring in a single or two river systems (Swartz *et al*., unpublished; Chakona *et al*., unpublished). The present study builds on previous studies by analysing the genetic structure of one of these newly discovered lineages in the *Galaxias zebratus* species complex. This lineage, which occurs in two currently isolated river systems, the Krom and Gamtoos in the eastern CFR (Cambray *et al*., 1995; Waters & Cambray, 1997), will be referred to as the Joubertina galaxias in the present study, because the headwaters of the river systems drain upland areas adjacent to the town of Joubertina.

The Joubertina galaxias seems to be confined to headwater streams of the Krom and Gamtoos River systems and there has never been a report of this lineage near marine environments or even in main-stem habitats. Marine dispersal capabilities are therefore unlikely. During the Cenozoic (*c*. 60 million years ago), a tributary of the Krom River system (the present day Kouga catchment) was pirated by the Baviaanskloof River, a tributary of the Gamtoos River system (Cambray *et al.,* 1995; Hattingh, 2008; Figure 2.1A). If the Joubertina galaxias became isolated *c*. 60 million years ago, one would not only expect deep genetic divergence, but also clear morphological differences observable in the field between populations of the two river systems. Since Waters & Cambray (1997) found a low genetic divergence between the two systems and since no major morphological differences have been observed in the field, this “ancient river capture hypothesis” cannot explain the present distribution pattern of the Joubertina galaxias.

Changes in sea-levels during the Tertiary (Siesser & Dingle, 1981; Hendey, 1983; Tankard, 1976; Rogers, 1985) resulted in periods when connections between river systems in the same valley would have been possible (Swartz *et al.*, 2007; Swartz *et al.*, 2009). The Krom and Gamtoos River systems are proposed to have formed a common confluence during the low sea-levels of the last glacial maximum (LGM) about 18 000 years ago when the southern African coastline was about 130 m below present day levels. If the Joubertina galaxias exploited this connection to disperse between the two river systems, then relatively low levels of genetic differentiation would be expected between populations from the two river systems, with divergence time being consistent with the LGM (palaeoriver hypothesis; Figure 2.1B).

The CFR experienced periods of extreme wet and dry conditions, with the most recent wetter period being inferred to have occurred as recent as the Holocene Altithemal (*c.* 8 000 years ago) (Partridge *et al.*, 1999; Cowling *et al.*, 2009). Temporary periods of integration of the Krom and Gamtoos River systems during periods of heavy flooding could have occurred across the low drainage divide that separates the two river systems. This would be supported by finding shallow or lack of population structuring between the drainages (intermittent connections hypothesis; Figure 2.1C).

Waters & Cambray (1997) suggested a recent river capture event as a possible explanation for the occurrence of similar cytochrome *b* alleles in the Krom and Gamtoos River systems, but the small sample size (one sequence per river system) did not allow them to test this hypothesis. It is possible that the low drainage divide between the Twee and Krom may represent an abandoned river course due to river capture (recent river capture hypothesis; Fig. 2.1D).

There are also several man-made dams in the upper catchments of the two river systems (upper Krom and upper Kouga). Recent reports suggest that some of these dams are connected by a series of canals that divert water from the upper Krom to the Kouga catchment (Tweddle, 2009). When the dams spill, they discharge into the upper Kouga catchment, establishing a connection between the Krom and Gamtoos River systems. If little or no genetic structuring between populations of the two river systems were found, it is possible that human-mediated translocation occurred (inter-basin transfer hypothesis; Figure 2.1E).

The aims of the present study were to map the distribution, assess population genetic structuring and estimate divergence time between populations of the Joubertina galaxias to test the five hypotheses outlined above. By assessing past and present evolutionary patterns and processes, it may be possible to reconstruct population history that in turn could help to retain or reject the outlined hypotheses.

Figure 2.1: Five hypotheses could explain the present distribution of the Joubertina galaxias in the Krom and Gamtoos River systems. An ancient Krom-Kouga river capture (A), confluence of the Krom-Gamtoos river systems (B; Swartz *et al*., 2007; 2009), low drainage divide between Krom and Kouga Rivers (C), recent river capture (D) and man-made canals (E).

2.2. Materials and Methods

2.2.1. Study area

The upper Krom (Krom River system) and upper Kouga (Gamtoos River system) catchments are located in the approximately 160 km long Langkloof valley in the Eastern Cape Province of South Africa (Figure 2.2A). The valley has been farmed since the 18th century. The Joubertina galaxias is currently only known from the source of the Krom River (Krom River system) and the Wabooms and Twee tributaries of the Kouga catchment (Gamtoos River system) near the town of Joubertina (Cambray *et al.*, 1995; Waters & Cambray, 1997).

2.2.2. Sampling

A total of 76 localities (30 in the Krom and 46 in the Gamtoos) were sampled (Figure 2.2B) using a 3m seine net, snorkelling with a hand net, electric fishing or a combination of these methods. This was done to ensure that the entire range of the species was surveyed. Muscle tissues or whole fish samples were stored in 99% ethanol in the field, and upon returning to the laboratory, were transferred to a –70 °C freezer. The voucher specimens were fixed in 10% formalin and deposited in the National Fish Collection at South African Institute for Aquatic Biodiversity (SAIAB) as locality records and reference material for future studies.

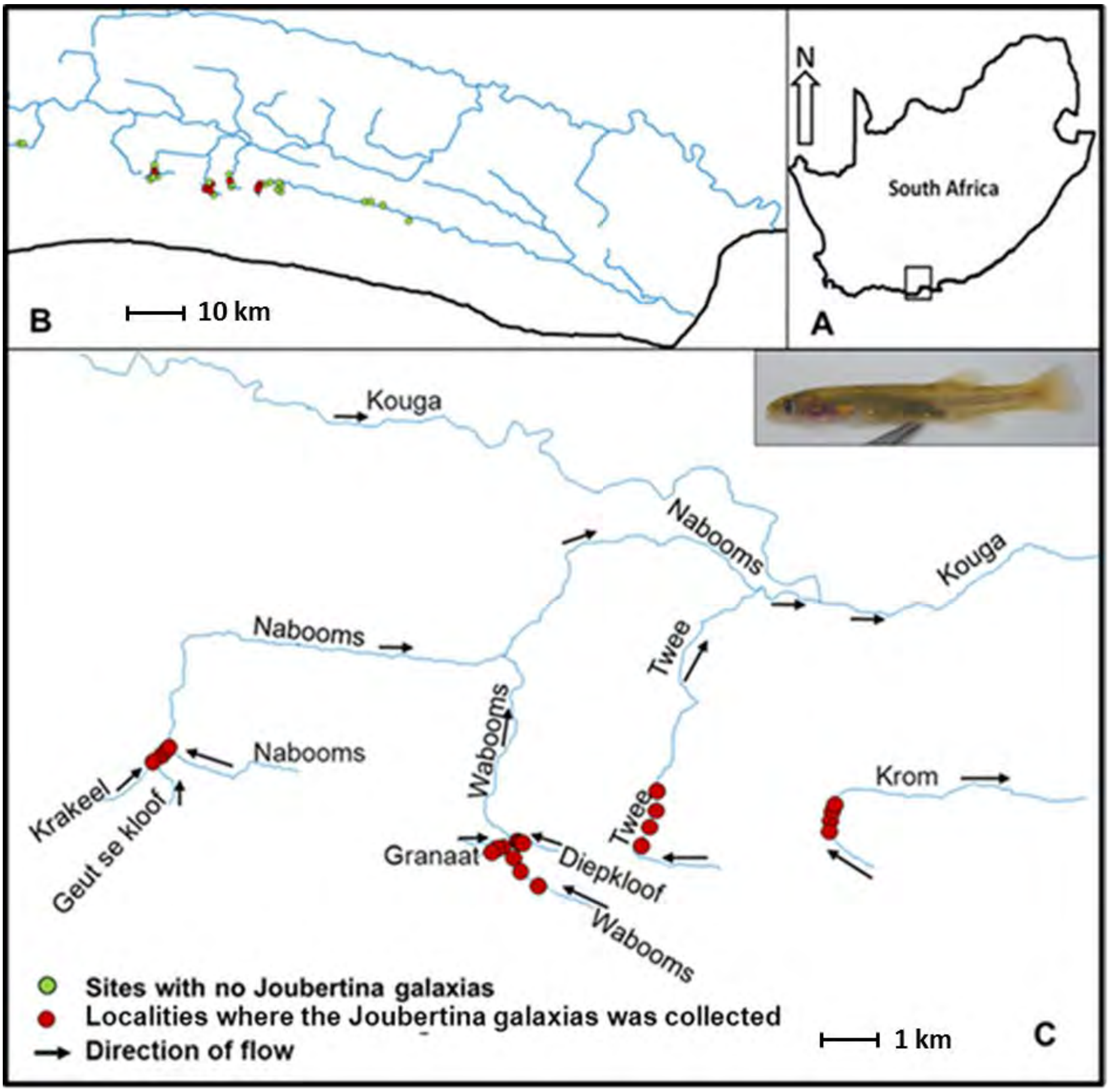

Figure 2.2 Map illustrating the study area (A), sampling sites that were visited to map the distribution of the Joubertina galaxias (B) and the present localities where the Joubertina galaxias was collected in the Krom and Gamtoos River systems (C). Arrows in map C indicate the direction of flow.

2.2.3. DNA extraction, amplification and sequencing

Total genomic DNA was isolated from muscle tissue using the Wizard® Genomic DNA purification kit (Promega, USA) following the manufacturer's protocol and was stored in a -70°C freezer. A section of the mitochondrial cytochrome *b* gene (*cyt b*) was amplified using the Polymerase Chain Reaction (PCR) with the primers Gcyt-Glu (5' – GAA AAA CCA CCG TTG TTA TTC A - 3') and Gcyt-Thr (5' CGA CTT CCG GAT TAC AAG ACC 3') (Waters & Wallis, 2001). Amplification was performed using the following thermal profile: initial denaturation at 94°C for 3 min and then 35 cycles of denaturing at 94 °C for 30 sec, primer annealing at 51 °C for 45 sec and product extension at 72 °C for 50 sec, followed by final extension at 72 °C for 7 min. Mitochondrial DNA was used because it has a rapid rate of evolution and a non-recombining mode of inheritance and is therefore a very useful tool for population level phylogeography. The mitochondrial sequence of the cytochrome *b* gene contains species-specific information concerning population history and evolutionary processes (Avise, 1994) and has been used in many phylogeographic studies on freshwater fish (e.g. Waters and Cambray, 1997; Swartz *et al*. 2007, 2009; Chakona et al., unpublished). However, due to its maternal inheritance, mitochondrial DNA can only provide information concerning female-mediated gene flow. Thus, it was essential to further characterize the genetic diversity of this species using nuclear DNA marker which captures both maternal and paternal gene flow. The nuclear S7 first intron was amplified using the primers S7RPEX1F (5' –TGG CCT CTT CCT TGG CCG TC 3') and S7RPEX3R (5' GCC TTC AGG TCA GAG TTC AT 3') (Chow & Hazama, 1998). Amplification of S7 gene was carried out with an initial denaturation at 95 °C for 1 min, followed by 30 cycles of amplification (denaturation at

95 °C for 30 sec, annealing at 60 °C for 1 min and extension at 72 °C for 2 min) followed with a final extension at 72 °C for 10 min. For both genes, amplification was performed in 25µl volumes containing 2.5µl of 10X polymerase reaction buffer, 2.5µl of 25mM $MgCl_2$, 2.5 µl of 8mM dNTP's, 0.5 µl of each primer (20pmol), 0.1 µl of 5U Super-Therm Taq Polymerase (Southern Cross Biotechnology, Cape Town) and 3-5 µl of DNA template (negative controls with no DNA template to test for contamination). Volumes were adjusted to a final volume of 25 µl with ddH_2O. PCR products were visualized using an UV transmitter after agarose gel electrophoresis and staining with ethidium bromide. All PCR products were purified and sequenced by Macrogen Inc. (South Korea).

2.2.4. Sequence editing, alignment and genetic analysis

A total of 121 *cyt b* sequences and 36 S7 sequences were generated for this study. Sequences were checked manually and edited using SeqMan (DNASTAR Lasergene 9 Core Suite). Alignment of corrected sequences was done using ClustalX (Thompson *et al*., 1997). The program DnaSP ver 5.10.01 (Rozas *et al*., 2003) was used to identify unique and shared alleles as well as the frequency of alleles (N) per population. Within-population variation, using haplotype diversity (H_D) (Nei 1987) and nucleotide diversity (π) (Nei & Tajima, 1981; Nei, 1987) and their standard errors were calculated using DnaSP version 5.10.01. A substitution model of sequence evolution that best fitted the data was selected for both genes using Modeltest ver 3.7 (Posada & Crandall, 1998). For both genes, genetic differentiation between populations was evaluated by calculating model corrected pairwise genetic distances between alleles in PAUP (Swofford, 2002). Divergence times between populations of the Krom and the Gamtoos were estimated using a variable molecular clock, which was recently calibrated by Craw *et al.* (2008). The authors used sequence divergences from New Zealand's galaxias populations with a known geological isolation history and proposed this as a potential dating tool for the late Quaternary evolutionary processes in the Southern Hemisphere. Divergence times (T_{div}) were computed using the formula from Craw *et al.* (2008):

$$\text{Divergence (\%)} = -2.2\mathrm{e}^{(-9T_{\mathrm{div}})} + 2.5T_{\mathrm{div}} + 2.2$$

Network analysis was performed to explore evolutionary relationships among alleles using TCS 1.21 (Clement *et al.*, 2000). TCS uses a statistical parsimony method, which links alleles with the smallest number of differences as defined by a 95% confidence criterion (Templeton *et al.*, 1992). Nested Clade Analysis (NCA; Templeton *et al.*, 1995; Templeton, 1998) was performed using GeoDis (Posada *et al.*, 2000) to evaluate the geographic association of alleles and to evaluate historical processes that could be responsible for the observed association. Hierarchical nesting was done according to Cunningham (2002). Alleles in the TCS network were grouped into hierarchical nesting levels from the tips to the interior of the cladogram. Interior clades were not nested until all tip clades were nested. A contingency test (10000 random permutations) was performed on each nested clade to test whether the null hypothesis of no association between clades and geographic location could be rejected. This was done by comparing the observed χ^2 values to distributions of random χ^2 values generated from 10000 random permutations using GeoDis. Geographic distances among populations were measured along the rivers using ArcMap 9.2 (ESRI software). Geographic clade distance (*D*c) (a measure of how geographically widespread individuals in a clade are); the nested clade distance (*D*n) (a measure of geographical distribution of individuals in a clade compared to all individuals in the nested clade), average interior versus tip clade distance (*ITc*) and average interior versus tip nested clade distances (*ITn*) and statistical inferences were calculated using the same program (Templeton *et al.*, 1995). Different evolutionary processes affect the above geographic distance statistics in different ways. It is therefore possible to infer which evolutionary processes could have played a role, using the inference key (2009) developed from Templeton (2004).

Analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) was done to assess population genetic structuring using the program Arlequin ver 2.0 (Schneider *et al.*, 2000). Four predefined hierarchical structures were tested for *cyt b* and S7 to assess which one explained most of the variation in the Joubertina galaxias: (i) two groups were defined to test the river systems and groups were therefore defined as the populations in the Krom and Gamtoos River systems; (ii) four groups were defined according to the catchments , namely the Krakeel, Wabooms (including Granaat and Diepkloof), Twee and Krom catchments; (iii) six groups were defined as the individual tributaries, namely the Krakeel, Wabooms, Granaat, Diepkloof, Twee and Krom and finally, (iv) two groups were defined differently to the first structure by combining the Twee and Krom populations, because they were sharing alleles, and compared to all the other populations in the Gamtoos. The significance of the variance components was determined with permutation tests of 1 000 replicates (Excoffier *et al.*, 1992).

Arlequin was also used to calculate pairwise population F_{ST} statistics for *cyt b* and S7 based on the most appropriate substitution model found in Modeltest. Tajima’s D (Tajima, 1989) and Fu's FS (Fu & Li, 1993; Fu, 1997) tests for neutrality were performed and tested for significance using 1000 permutations. These tests were performed to determine whether the *cyt b* and S7 genes were evolving according to neutral expectations (Tajima, 1989; Fu & Li, 1993). Significantly negative values for these tests may also suggest population expansion (Tajima, 1989).

2.3. Results

2.3.1. Distribution

Specimens of the Joubertina galaxias were collected from five different tributaries of the Gamtoos River system, namely the Granaat (N=30), Krakeel (N=25), Diepkloof (N=30) and Wabooms (N=30) (referred to in the present study as the western Kouga tributaries) and the Twee (N=30) in the eastern part of the Kouga catchment. The species was only found in one tributary of the Krom River system, namely the upper Krom (N=40). Two of these tributaries represent new distribution localities (Krakeel and Diepkloof) (Figure 2.2C), and the lineage therefore occurs in six tributaries and four broader catchments namely the Krom, Krakeel, Twee and Wabooms (grouping the three upper Wabooms tributaries, namely the Granaat, Diepkloof and Wabooms). Specimens were collected from three localities (N=10 per locality) each in the Granaat and Wabooms and four localities in the Krom (10 specimens per locality). Low numbers of individuals were found in the remaining tributaries. To reduce the impact on these populations and to increase the probability of capturing more of the population's genetic diversity, four localities each were sampled for the Twee (2, 8, 10 and 10 specimens per locality respectively), Krakeel (2, 5, 8 and 10 specimens per locality respectively) and Diepkloof (5, 5, 6 and 14 specimens per locality respectively) tributaries. Overall, the Joubertina galaxias was only found in 22 localities of the 72 localities that were surveyed (31%).

2.3.2. Mitochondrial DNA analysis

2.3.2.1. Cytochrome *b* diversity

Analysis of 121 individuals for 616 base pairs of cytochrome *b* resulted in seven alleles defined by nine variable sites (Table 2.1). No insertions or deletions were detected and no significant deviations from those expected under predictions of neutrality were observed when all alleles were analysed together (Tajima, 1989; D= 0.016, P>0.10; Fu and Li, 1993; F=0.816, P > 0.10).

Table 2.1: The geographic distribution of mitochondrial cytochrome *b* alleles. Sample sizes (*n*) used in this study and haplotype diversity (H_D) are given at the bottom of the table.

Haplotype	Krom	Twee	Diepkloof	Wabooms	Granaat	Krakeel
1	20	20				
2			20	11	8	1
3				8		
4					7	
5				1		
6					6	14
7						5
n	20	20	20	20	21	20
H_D	0.000	0.000	0.000	0.563	0.695	0.468

All the individuals from the Twee and Krom populations shared allele 1, but did not share this allele with any individuals from other populations. The Diepkloof also had only one allele (allele 2), but shared it with all the western Kouga tributaries (Diepkloof, Wabooms, Granaat and Krakeel). Allele 6 was restricted to the Granaat and Krakeel tributaries (Table 2.1). There were private alleles in the Wabooms (allele 3 and 5), Granaat (allele 4) and Krakeel (allele 7), which were not shared between any of the tributaries. Haplotype diversity (H_D) ranged from 0.468 to 0.695 for the Wabooms, Granaat and Krakeel, but was 0 for the Krom, Twee and Diepkloof populations.

The best substitution model selected for cytochrome *b* using ModelTest was the HKY model (Hasegawa *et al.*, 1985). The model allows for different rates of transitions and transversions as well as accommodating unequal base composition (unequal frequencies of the four nucleotides). Genetic distances between populations of Joubertina galaxias from the Krom and Gamtoos River systems based on the HKY model ranged from 0 - 0.65% (mean 0.37%). Genetic distances within and between populations of the Joubertina galaxias are shown in Table 2.2. Divergence between the Krom and Twee compared to the western Kouga populations ranged from 0.2 – 0.7%. The largest divergence was between the Wabooms and Granaat populations (0 - 1%) and the Wabooms and Krakeel populations (0 - 1%). Estimates of divergence times between the Krom and Gamtoos populations ranged between zero to 33 000 years ago.

Table 2.2: HKY genetic distances within and between Joubertina galaxias populations, based on mitochondrial cytochrome *b* sequences. The range is given as a percentage (%).

Population	Krom	Twee	Diepkloof	Wabooms	Granaat	Krakeel
Krom	0					
Twee	0	0				
Diepkloof	0.2	0.2	0			
Wabooms	0.2 - 0.7	0.2 - 0.7	0 - 0.5	0 - 0.7		
Granaat	0.2 - 0.7	0.2 - 0.7	0 - 0.5	0 - 1	0 - 0.7	
Krakeel	0.2 - 0.7	0.2 - 0.7	0 - 0.5	0 - 1	0 - 0.7	0 - 0.5

There were no significant differences in gene diversity between the Wabooms, Granaat and Krakeel populations, but the first two showed relatively higher values compared to the Krakeel population (Table 2.3). Nucleotide diversity was low overall ($\pi = 0.003$) within populations and did not differ significantly between the Wabooms, Granaat and Krakeel populations (Table 2.3).

Table 2.3: Mitochondrial cytochrome *b* pairwise F_{ST} values, nucleotide diversity (π) and gene diversity (δ) for Joubertina galaxias populations. All pairwise F_{ST} comparisons were statistically significant ($P < 0.05$), except for the comparison between the Krom and the Twee. All nucleotide diversity (π) and gene diversity (δ) values were not significantly different from each other.

Population	Krom	Twee	Diepkloof	Wabooms	Granaat	Krakeel	π	δ
Krom							0.000	0.000
Twee	0^{NS}						0.000	0.000
Diepkloof	1.000	1.000					0.000	0.000
Wabooms	0.641	0.641	0.354				0.003	0.563
Granaat	0.645	0.645	0.260	0.318			0.002	0.695
Krakeel	0.906	0.906	0.862	0.675	0.511		0.001	0.468

^{NS} = not significant.

2.3.2.2. Genetic structuring within and between populations

Allele 2 was the most widespread of the seven alleles, occurring in all the tributaries of the Gamtoos River system, except the Twee River (Figure 2.3). However, three of the Gamtoos tributaries, namely the Granaat, Krakeel and Wabooms, had unique alleles. Pairwise *F*-statistics and exact tests revealed significant levels of differentiation for all pairwise population comparisons, except between the Krom and Twee (Table 2.3), which suggest restricted gene flow among all the western Kouga populations. Apart from the lack of sharing of alleles between the Krom and Twee compared to all other populations, pairwise F_{ST} values revealed significant differentiation among the Krakeel, Granaat, Wabooms and Diepkloof populations ($0.250<F_{ST}<0.862$) (Table 2.3).

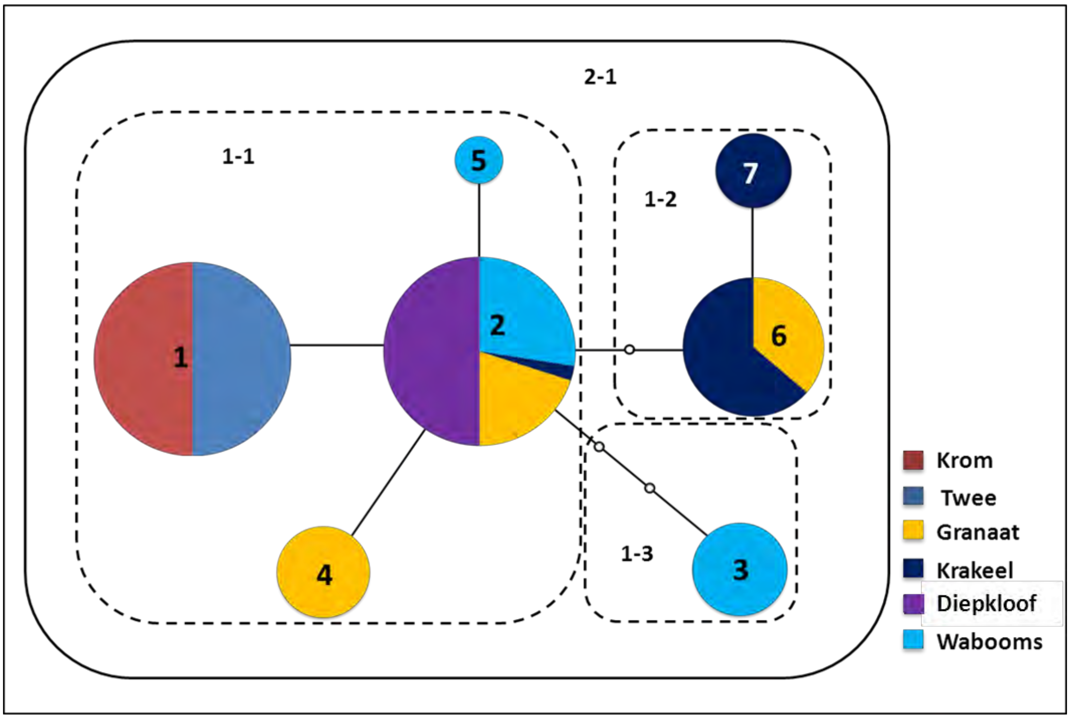

Figure 2.3 TCS allele network with 95% plausible set of mitochondrial cytochrome *b* allele connections showing nested clades for the Joubertina galaxias. The one-step clades are indicated by dashed lines and the two-step clade is indicated by a solid lined box.

AMOVA results indicated that much of the genetic variation was partitioned among tributaries (F_{CT}: 0.653 (65.3%) and F_{SC}: 0.689 (68.9%), $P < 0.001$) (Table 2.4). This evidence for genetic differentiation among populations of the Joubertina galaxias was also supported by pairwise F_{ST} and exact test values (Table 2.3). However, there was not significant mitochondrial DNA differentiation between the river systems and structuring between the systems explained less than 1% of the variation when the systems were used as groups (F_{CT}: -0.005 (-0.5%)). This result was due to the sharing of the same allele between individuals from the Twee and Krom populations. Variation within populations was also low; therefore most of the structuring was explained by variation among populations between tributaries. The overall φ_{ST} values were all significantly large for all four structures suggesting high levels of differentiation.

Table 2.4: AMOVA analysis of the distribution of mitochondrial cytochrome *b* genetic variation among Joubertina galaxias populations showing *F*-statistics and percentage variation in brackets.

Source of variation	Six tributaries	Four catchments	Two systems	Twee-Krom vs. W. Kouga
Among groups	0.653 (65.3%)	0.491 (49.1%)	-0.005 (-0.5%)	0.393 (39.3%)
Among populations within groups	0.347 (12.0%)	0.444 (22.6%)	0.686 (68.9%)	0.577 (35.1%)
Within populations	0.773 (22.7%)	0.717 (28.3%)	0.684 (31.6%)	0.743 (25.7%)
Overall φ_{ST}	0.947	1.060	0.951	1.170

2.3.2.3. Nested Clade Analysis (NCA)

NCA showed no significant associations between the distribution of alleles and geography for clade 1-3 ($P > 0.05$). The inferred pattern of geographic variation for clade 1-1 which comprised of alleles from the Krom and Gamtoos River systems was past gradual range expansion followed by fragmentation. For clade 1-2 which comprised of alleles from two tributaries of the Gamtoos River system, namely the Granaat and Krakeel, the inferred process was restricted gene flow with isolation by distance. The inferred process for the total cladogram was restricted gene flow with isolation by distance (Table 2.5).

Table 2.5: Results of Nested Clade Analysis showing clade (Dc), nested (Dn) and interior to tip clade (I-T) distances. Only clades with significant permutational X^2 probabilities for geographical structure have been included in the table.

Nesting level	Haplotype/ clade no.	Location	D*c*	D*n*	X^2-*P*	Inference chain	Inference key conclusion
1-1	1	Tip	184.8	301.0	150.7	1-2-3-5-6-13-21	Past gradual range expansion followed by fragmentation
	2	Interior	13.5	197.1			
	4	Tip	0.3	182.9			
	5	Tip	0	184.3			
		I-T	-140.5	-84.2			
1-2	6	Interior	101.7	93.5	3.3	1-2-11-17-4	Restricted gene flow with isolation by distance
	7	Tip	0.3	54.2			
		I-T	101.5	39.4			
2-1	1-1	Interior	244.3	247.7	131.0	1-2-3-4	Restricted gene flow with isolation by distance
	1-2	Tip	88.1	236.7			
	1-3	Tip	0.2	167.1			
		I-T	177.5	27.9			

2.3.3. Nuclear DNA

2.3.3.1. S7 diversity

Analysis of 36 individuals for 492 base pairs of S7 revealed 5 unique alleles defined by 4 variable sites. Only one heterozygotic position was found, therefore 72 sequences were used for analysis after separating the maternally and paternally derived sequences. No significant deviations from those expected under predictions of neutrality were observed when all S7 alleles were analysed together (Tajima, (1989); *D*= -0.630, P = 0.312; Fu and Li, (1993); *F*=-1.301, *P*= 0.223).

Allele 1 was found in all six tributaries, but allele 3 was restricted to the western Kouga tributaries and was absent in the Twee population. Alleles 2, 4 and 5 were private to the Krom, Twee and Diepkloof rivers respectively (Table 2.6). Haplotype diversity (H_D) ranged from 0.143 (Krom) to 0.750 (Diepkloof) and overall haplotype diversity was 0.512.

Gene diversity was high for the Granaat (0.546 ± 0.062) and Diepkloof (0.750 ± 0.097) populations compared to the other four populations (Table 2.6). Nucleotide diversity was not significantly different among any of the populations, ranging between 0 - 0.002. Overall nucleotide diversity was also low (π = 0.001) (Table 2.6).

Table 2.6: Distribution of nuclear DNA alleles, sample sizes (*n*) used in this study, haplotype diversity (H_D), nucleotide diversity (π) and gene diversity (δ) of the Joubertina galaxias. Nucleotide diversity (π) and gene diversity (δ) values were not significantly different between populations.

Haplotype	Krom	Twee	Diepkloof	Granaat	Wabooms	Krakeel
1	13	14	3	6	8	2
2	1					
3			3	6	2	10
4		2				
5			2			
n	14	16	8	12	10	12
H_D	0.143	0.233	0.750	0.545	0. 356	0.468
π	0.00	0.001	0.002	0.001	0.001	0.001
δ	0.143	0.233	0.750	0.546	0.356	0.303

2.3.3.2. Genetic structuring within and between populations

The substitution model that best fits the S7 data was found to be the F81 model (Felsenstein, 1981) using Modeltest. It assumes equal rates of change across sites and equal probabilities of transitions and transversions. This model was used to calculate genetic distances between S7 alleles within and between the Joubertina galaxias populations. Genetic distances between the Krom and Gamtoos River systems ranged between 0% and 0.4%. Distances between the Twee and western Kouga tributaries ranged between 0 to 0.3%. Results of the variable molecular clock revealed that the divergence between the Krom and Gamtoos alleles occurred relatively recently (about 0 – 19 800 years ago), coinciding with the very recent, Holocene or Pleistocene periods. Genetic distances within and between populations based on the F81 model were low (0% - 0.4%; Table 2.7).

Table 2.7: Genetic distance matrix within and between alleles of the Joubertina galaxias populations from six tributaries based on nuclear S7 sequences. The range is given as a percentage (%).

Population	Krom	Twee	Diepkloof	Wabooms	Granaat	Krakeel
Krom	0 - 0.2					
Twee	0 - 0.4	0 - 0.2				
Diepkloof	0 - 0.4	0 - 0.3	0 - 0.3			
Wabooms	0 - 0.2	0 - 0.2	0 - 0.3	0		
Granaat	0 - 0.2	0 - 0.2	0 - 0.3	0	0	
Krakeel	0 - 0.2	0 - 0.2	0 - 0.3	0	0	0

Results of pairwise *F*-statistics and exact test values (Table 2.8) revealed significant levels of differentiation between the Krom and the Granaat, Wabooms and Diepkloof tributaries. The highest F_{ST} value was observed between the Krom and Krakeel populations, while low F_{ST}

values were observed between the Krom and Granaat, Wabooms and Diepkloof populations. The Krom and Twee comparison did not show significant differentiation. Significantly different F_{ST} values were observed for comparisons between the Twee, Krakeel, Granaat, Wabooms and the Diepkloof populations with high values obtained for the Krakeel-Twee and Krakeel-Wabooms comparisons.

Table 2.8: Pairwise F_{ST} (S7) values of population differentiation among sites below the diagonal based on nucleotide content and haplotype frequencies (all other values were statistically significant; $P < 0.05$).

Population	Krom	Twee	Diepkloof	Wabooms	Granaat
Krom					
Twee	0.042^{NS}				
Diepkloof	0.416	0.401			
Wabooms	0.082	0.094	0.200		
Granaat	0.398	0.377	-0.006	0.099	
Krakeel	0.754	0.720	0.050	0.530	0.152

^{NS} = not significant.

AMOVA analyses indicated that much of the genetic variation was partitioned among tributaries, with no significant differentiation between the two river systems (Table 2.9). Variation among populations within groups ranged from 14 - 39.6%. The overall φST values were significantly low suggesting low levels of structuring. High levels of structuring were observed among the Gamtoos populations.

Table 2.9: Results of the AMOVA for populations of the Joubertina galaxias based on drainage structure (tributaries, catchments, river systems and Krom and Twee vs western Kouga tributaries), showing F-statistics and percentage variation in brackets. All values were statistically significant.

Source of variation	Six tributaries	Four catchments	Two systems	Twee -Krom vs. W. Kouga
Among groups	0.305 (30.5%)	0.280 (28.0%)	0.000 (0.01%)	0.362 (36.2%)
Among populations within groups	0.378 (26.3%)	0.194 (14.0%)	0.396 (39.6%)	0.486 (12.4%)
Within populations	0.568 (43.2%)	0.419 (58.1%)	0.396 (60.4%)	0.194 (51.4%)
Overall φ_{ST}	0.310	0.322	0.310	0.364

2.3.3.3. Phylogeographic structuring and Nested Clade Analysis (NCA)

Allele 1 that is central to the network is also the most common allele and was found in all six populations (Figure 2.4). It forms a single clade (clade1-1) with alleles 2 and 4, which are restricted to the Krom and Twee tributaries respectively. Clade 1-2 is restricted to the western Kouga tributaries and comprises of allele 3 (shared among all the western Kouga populations) and allele 5 (private to the Diepkloof tributary) (Figure 2.4).

NCA showed no significant associations between the distribution of alleles and geography for both clades 1-1 and 1-2 (*P* >0.05). A significant result was found for clade 2-1 (the total cladogram), with restricted gene flow with isolation by distance inferred as the underlying evolutionary process (Table 2.10).

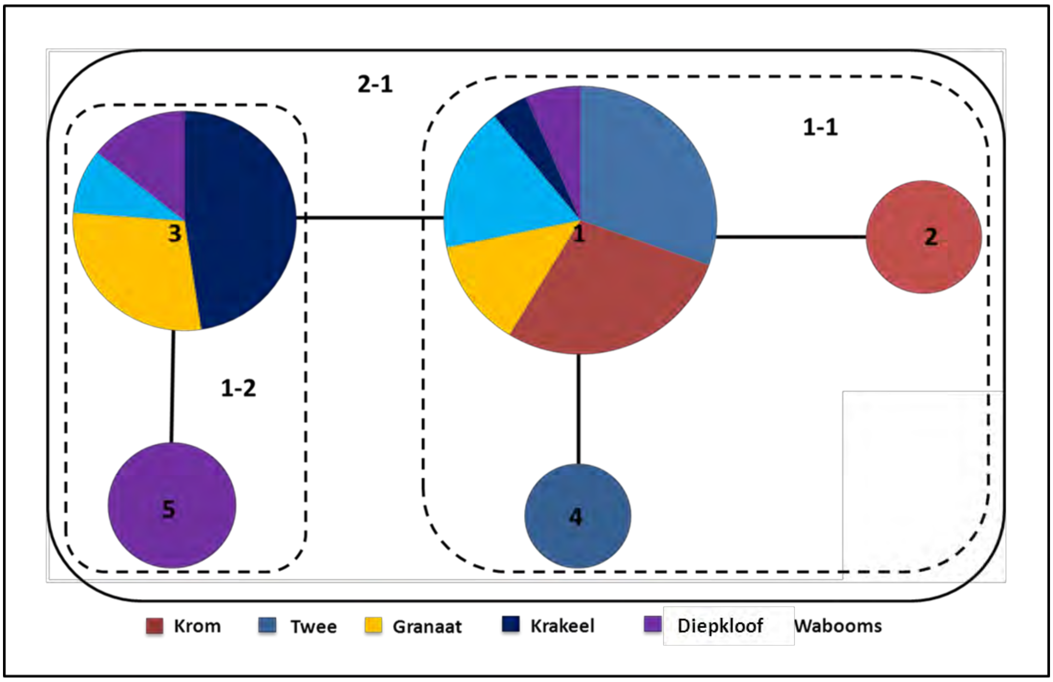

Figure 2.4 Network with 95% plausible set of S7 haplotype connections showing nested clades for the Joubertina galaxias. One-step clades are indicated by dashed lines and the two-step clade is indicated by a solid lined box.

Table 2.10: Results of the Nested Clade Analysis showing clade (Dc), nested (Dn) and interior to tip clade (I-T) distances. Only clade 2-1 had significant permutational X^2 probabilities for geographical structure.

Nesting level	Haplotype/ clade no.	Location	D*c*	D*n*	X^2-*P*	Inference Chain	Inference key conclusion
2-1	1-1	Interior	268.9	274.0	38.8	1-2-3-4	Restricted gene flow with isolation by distance
	1-2	Tip	117.2	234.4			
		I-T	151.6	39.6			

2.4. Discussion

It is generally expected that primary freshwater fish will display high levels of genetic differentiation among populations from historically isolated river systems, because dispersal opportunities are limited (Ward *et al.*, 1994; McGlashan & Hughes, 2001). The lack of genetic differentiation in the Joubertina galaxias between the Gamtoos and Krom River systems is not consistent with the expectation that primary freshwater fish generally display high levels of differentiation between populations from historically isolated rivers. Dispersal opportunities are usually limited between isolated systems (Ward *et al.*, 1994; McGlashan & Hughes, 2001), but populations from the Krom and Gamtoos River systems showed shallow genetic divergence as might be expected for diadromous species, which typically have large population sizes and high levels of gene flow (e.g. Waters *et al.*, 2000; Wong *et al.*, 2004). There is, however, no evidence at this stage to suggest that the Joubertina galaxias is diadromous. It is therefore likely that another mechanism allowed occurrence of this lineage in both systems.

The shallow genetic differentiation and recent divergence time estimate (about 0 – 33 000 years ago for cytochrome *b* and 0 – 19 800 years ago for S7) between the Krom and Gamtoos populations of the Joubertina galaxias is consistent with the proposed connection of the Krom and Gamtoos River systems during the LGM only about 18 000 years ago (Swartz *et al.*, 2007). The present Krom population, however, is isolated from the lower reaches of the Krom River system by two large waterfalls that would have prevented upstream migration. This could still have allowed unidirectional gene flow from the upper Krom to the Gamtoos tributaries if there were no barriers in the latter. In addition, Swartz *et al.* (2009) suggested that the historic isolation of the Krom and Gamtoos populations of *Pseudobarbus afer* was either due to isolation by a barrier during lower sea levels that is now flooded, or it is possible that the Krom was never part of the St. Francis palaeoriver system, forming a separate palaeoriver system during the LGM. It therefore seems likely that the Krom population of the Joubertina galaxias remained isolated during recent glacial periods and did not disperse through proposed palaeoriver systems. The absence of this lineage from middle and lower reach tributaries of both the Gamtoos and Krom River systems support this. If migration occurred between the river systems through downstream areas, individuals would have passed presently unoccupied tributaries. Even if this migration process never occurred, it is nonetheless surprising that the Joubertina galaxias has not recently colonised the lower tributaries of both the Krom and Gamtoos River systems from upstream sources, suggesting that lower altitude tributaries might not have suitable habitat. Given the instream barriers to dispersal and the absence of the Joubertina galaxias in lower altitude tributaries, dispersal through a confluence of the Gamtoos and Krom River systems during the LGM is unlikely and the palaeoriver hypothesis is therefore rejected.

Swartz *et al.* (2009) proposed inland-drainage dispersal during flooding events to explain the lack of divergence between *P. asper* populations from the Gourits and Gamtoos River systems. This may be the case for the genetic similarities between populations of the Joubertina galaxias from the Krom and the Gamtoos River systems. There is a low gradient area between the upper Krom and upper Kouga catchments, which could have facilitated temporary connection of these rivers and dispersal of this lineage during flooding in pluvial periods such as the Holocene Altithermal (*c.* 8000 years ago). Past gradual range expansion, followed by fragmentation was inferred from NCA (clade 1-1 of cytochrome *b*) and could be consistent with possible connections provided by low gradient areas. Dry conditions, which prevailed after the Holocene Altithermal, could have caused the dissociation of low gradient connections between the river systems, leaving too little time for loss of diversity or divergence of alleles. It is also possible that the low drainage divide was once the drainage line for the Twee flowing into the upper Krom catchment. Although possible, it seems unlikely that the Krom would have flowed into the Kouga catchment, because the upper Krom flows into a deeply incised gorge below the present distribution of the Joubertina galaxias. The genetic similarity between fish from the adjacent Twee and Krom compared to the western Kouga tributaries is consistent with both the "intermittent connections" and "recent river capture" hypotheses. Neither of these hypotheses can be rejected.

Finally, the lack of differentiation between the Krom and Twee populations could be an indication of ongoing exchange of individuals between the two populations across the drainage divide between the Gamtoos and Krom River systems due to the canals that connect the upper Krom catchment and upper Kouga catchment. The lower reaches of the Twee and the lower sections of the canals are not suitable for the permanent occurrence of the Joubertina galaxias, because of the occurrence of *Micropterus salmoides*, habitat destruction and unsustainable water abstraction. However, these canals could allow gene flow between the Krom and Twee populations during winter high flows or floods. Therefore the inter-basin transfer hypothesis cannot be rejected.

The direction of movement between the river systems is uncertain, but there are two possible scenarios that most likely explain the inference of gene flow between these two systems.

Firstly, alleles may have evolved from a common ancestral allele in the Gamtoos River system. The Twee River population would have become isolated relatively recently from the other Gamtoos populations and a mutation would have caused a unique mitochondrial cytochrome *b* allele to evolve in the Twee River. The nuclear data does not show clear isolation in the Twee (although there is a single private allele), but this could be due to insufficient time for mutations to accumulate. Connection of the rivers through the IBT (canal system) or natural low gradient areas, could have then allowed colonisation of the Krom River. Alternatively, the Krom was connected to some or all Gamtoos populations during wet periods and later became isolated during dry conditions. Assuming the Twee population went extinct or never existed in the first place; a unique allele could have become isolated in the Krom, which later established in the Twee through migration across the IBT or low gradient connections. The first scenario seems to be the most plausible explanation, because the Joubertina galaxias does not occur elsewhere in the Krom River system, but it occurs in five tributaries of the upper Kouga. It is therefore unlikely that the Twee tributary did not have the Joubertina galaxias when the Krom population became established. The most likely scenario is that, the occurrence of this lineage in the Krom is due to the recent IBT, but the present data cannot reject the possibility of intermittent dispersal across low gradients during wet periods, or a recent minor river capture event.

The Joubertina galaxias' low levels of genetic diversity within the Gamtoos River system was not surprising, given that the levels of genetic differentiation are usually low among fish populations within river systems compared to between systems (see Ward *et al*, 1994). Surprisingly, both nuclear and mitochondrial DNA revealed high levels of genetic structuring between Joubertina galaxias populations within the Gamtoos River system.

The geographic distribution of genetic diversity within river systems can be influenced by stream structure, physical barriers, isolation by distance and historical processes on population structure (Shaw *et al*., 1994; Lu *et al*., 1997; McGlashan, 2000; McGlashan *et al*., 2001). Isolation by distance is expected when the dispersal distance of an individual is less than the range of the species (Slatkin, 1993; McGlashan & Hughes, 2001; Sharma and Hughes, 2011). The Joubertina galaxias showed high levels of differentiation between the Twee and western Kouga populations and between the Krakeel and the three populations of the Waboom s catchment. This could be due to isolation by distance that was inferred with NCA between the Granaat and Krakeel (cytochrome *b*) and among all populations (cytchrome *b* and S7). In-stream barriers have been demonstrated to be effective in affecting population genetic structure (Currens *et al*., 1990; McGlashan & Hughes, 2000). High levels of differentiation between the Granaat and the other Gamtoos populations could have been influenced by a small waterfall in the Granaat River a few meters above the confluence with Wabooms River. This waterfall acts as a natural barrier preventing upstream migration of individuals from other populations to the upper Granaat. Stream fishes that are unhindered by natural barriers have highly connected populations and show low levels of genetic differentiation, which is probably the case for the Diepkloof and Wabooms populations. High levels of genetic structuring between Gamtoos populations could also suggest that the Joubertina galaxias has limited dispersal ability, a small home range and/or a preference for certain habitat types.

Recent impacts such as the spread of alien fishes, over abstraction of water and construction of weirs within the Gamtoos River system are causing population fragmentation which can cause loss of diversity, genetic drift and inbreeding in populations previously connected by gene-flow. This may appear like historical low gene flow when the fish were actually able to migrate historically. Loss of genetic diversity through fragmentation by weirs has been demonstrated (Meldgaard *et al.*, 2002) and this could also be the case for the present of private alleles in the Krakeel population which is fragmented by weirs. All populations of the western Kouga are extremely fragmented and isolated by long distances due to unsustainable agricultural activities and presence of alien fishes. The fragmented populations occur in small population sizes and it is possible that they recently became small and have not had the chance to lose their genetic diversity.

2.5. Conclusion

The fact that there is little or no differentiation between the Krom and Twee populations of the Joubertina galaxias is indicative of relatively recent or ongoing gene flow between currently isolated river systems. Recent human mediated IBT's from Twee to Krom is the most likely explanation for the observed distribution and genetic patterns, but relatively recent processes such as inter-basin dispersal due to intermittent freshwater connections through flooding of low drainage divides or a recent small scale river capture event, cannot be excluded as possible explanations. Migration due to the connections provided by palaeorivers during the LGM does not adequately explain the observed patterns of genetic differentiation and present distribution of the species. Ancient river capture can be rejected and any historical event older than about 33 000 years ago is unlikely to explain the observed patterns in the Joubertina galaxias. Genetic structuring within the Gamtoos River system was probably influenced by isolation by distance, physical barriers, a natural tendency to have a small home range facilitated by a preference for upper mountain stream habitats, a natural inability to disperse between catchments and/or recent alien fishes and human impacts. A more detailed study of the migration rates between the populations might help to reject more of these hypotheses, shed more light on the population history of the Joubertina galaxias and allow for clearer recommendations to be made on how the conserve the genetic diversity of this taxon.

CHAPTER 3

The status and distribution of the newly discovered and endemic Joubertina galaxias from the eastern Cape Floristic Region of South Africa

Abstract

The conservation status of most of the known endemic freshwater fish species in the Cape Floristic Region (South Africa) has been assessed, but recent discoveries have revealed several historically isolated lineages and undescribed species for which there is incomplete knowledge about taxonomic distinction, distribution and biology. One such lineage that form part of the *Galaxias zebratus* species complex and referred to as the Joubertina galaxias is threatened mainly by alien fish invasion, habitat loss and loss of genetic diversity due to fragmentation of its populations. Only six populations remain, one of which could be an extralimital population established through human activities. The lineage was assessed as Endangered according to IUCN categories, due to its very restricted geographic range, few remaining secure populations, small known population sizes and the intensity of threats to most of the populations. The lineage had a restricted area of occurrence and extent of occurrence. The extent of occurrence has declined in all tributaries and is continuing to decline in some. Only two populations are relatively secure from further decline. The lineage may have had natural fragmentation among its populations, but recent threats have completely isolated the populations. Survival potential is low in four of six populations and the number of mature individuals for the whole lineage seems to be less than 2500 mature individuals. It is therefore important for conservation agencies to inform local communities and landowners and to include this lineage in conservation management plans for the region.

3.1. Introduction

Globally, freshwater biodiversity is under severe threat due to widespread land use changes and associated habitat degradation, over-exploitation of water, pollution, flow modification and invasion by alien species (Revenga *et al*., 2005; Dudgeon *et al*., 2006; Magurran, 2009). These threats to freshwater biodiversity are interactive, but the greatest threat globally has been assessed as the introduction and spread of invasive alien fishes (Cowx, 2002). These impacts have collectively caused a sharp increase in the number of global freshwater fish extinctions (Harrison & Stiassny, 1999; Helfman, 2007; Hinton-Taylor *et al*., 2009). Some of the greatest extinctions due to alien fish introductions have been recorded in Africa, where Lake Victoria alone may have lost between 200 and 300 species of cichlids (Harrison & Stiassny, 1999).

The Cape Floristic Region (CFR), located at the southern tip of Africa, is a world renowned biodiversity hotspot (Myers *et al*., 2000; Wishart & Day, 2001; Goldblatt & Manning, 2002; Cowling *et al*., 2003; Kuhlmann, 2005; Kuhlmann, 2009; Linder *et al*., 2010). Diversity of primary freshwater fishes is low with one to ten primary freshwater fish species per river system and only three widespread genera, namely *Sandelia*, *Pseudobarbus* and *Galaxias* (Skelton, 1986; Linder *et al*., 2010). Despite this, the region has the highest number of endemic primary freshwater fishes in Africa south of the Zambezi River system (Thieme *et al*., 2005). Linder *et al*. (2010) estimated that there are 43 historically isolated lineages in the 18 nominal primary freshwater fishes in the CFR, a revelation that has important implications for conservation management, given that 11 of the 18 currently recognised primary freshwater fish species that occur in the CFR are already listed in threatened International

Union for Conservation of Nature (IUCN) categories (Critically Endangered, Endangered and Vulnerable) (Linder *et al.*, 2010). Some of the currently recognised species have broad geographical ranges, with some spanning several currently isolated river systems (e.g Chakona *et al.*, 2011), but many of the lineages in these species complexes have limited distribution ranges (e.g. Chapter 2; Bloomer & Impson, 2000; Swartz *et al.*, 2007, 2009).

Fishes of the CFR are mainly threatened by introduced piscivorous fishes and habitat modification through over-abstraction of water, channelization and bulldozing (Skelton *et al.*, 1995; Lombard *et al.*, 1997; Rouget *et al.*, 2003; Nel *et al.*, 2007; Tweddle *et al.*, 2009; Clark *et al.*, 2009). Tweddle *et al.* (2009) suggested that the introduction of alien fishes (especially *Micropterus* spp.) is the most serious of these threats. Alien fishes are causing the decline and local extinction of native fishes across the CFR (Tweddle *et al.*, 2009; Clark *et al.*, 2009).

Native freshwater fishes of the CFR typically survive in tributary streams in highly fragmented populations, mostly above barriers such as waterfalls or weirs that prevent upstream movement of alien fishes. Occurrence of potential cryptic species in these already threatened nominal species, presents a number of challenges. It firstly increases the number of species that require management plans and conservation actions. Secondly, lineages with more restricted distributions are more susceptible to alien fish impacts and more prone to extinction. It is also possible that different lineages may have different ecological requirements and may require different management strategies for conservation actions to be successful.

The discovery of extreme genetic diversity within the Cape galaxias (Waters & Cambray, 1997) provides an excellent example. Until recently, it was thought that Africa only had a single galaxiid species, namely *Galaxias zebratus*. This species was considered to be one of the most widespread in the CFR, occurring in more than 12 currently isolated river systems from the Olifants on the west coast to the Gamtoos in the eastern CFR (Cambray *et al*., 1995). Galaxiid fishes have relatively conservative morphology, which has made it difficult to find characters to describe the several morphologically variable forms of the Cape Galaxias as distinct species (Barnard, 1943; McDowall, 1973). Molecular research has, however, revealed that *G. zebratus* is a species complex with at least 10 deeply divergent lineages (Waters & Cambray, 1997; Van Niekerk, 2004; Wishart *et al*., 2006; Chakona *et al*., 2012; Swartz *et al*., unpublished). Some of these lineages are currently being described as distinct species (Swartz *et al*., unpublished).

Only one of the *Galaxias* lineages has a wide distribution range across the CFR (van Niekerk, 2004; Chakona *et al*., 2011). Most of the other proposed species, including the Joubertina galaxias (Chapter 2) which is subject of the present study, are currently known from only a few localities (Waters & Cambray, 1997; Chakona *et al*., unpublished; Swartz *et al*., unpublished). *Galaxias zebratus* is currently listed as Data Deficient because of the taxonomic problems that still exist (Darwall *et al*., 2009; IUCN 2011). The distribution ranges and conservation statuses of the newly discovered lineages of the *G. zebratus* complex therefore need to be evaluated. This information is needed for the implementation of effective conservation planning and management strategies.

Before 1995, the eastern limit of the distribution of *Galaxias zebratus* was thought to extend only to the Keurbooms River system on the border between the Western and Eastern Cape provinces of South Africa. Cambray *et al*. (1995) discovered an extension of this species' range when specimens were collected from two tributaries (the Twee (33º 53' 05"S and 23º 52' 57"E) and Wabooms (33º 52' 44"S and 23º 50' 32"E)) in the Gamtoos River system as well as the upper reaches of the Krom River system (33º 51' 54"S and 23º 56' 48"E). More specimens were also collected from the Granaat tributary (a suggested local name for the stream) of the Gamtoos River system in 1995. The GPS locality was wrongly indicated in Cambray *et al*. (1995) and is here corrected as 33º 52' 47.7" S and 23º 50' 23.9"E (Cambay, personal communication). Waters & Cambray (1997) revealed that populations of *G. zebratus* discovered by Cambray *et al*. (1995) from the Krom and Gamtoos River systems were genetically distinct (with sequence divergence ranging from 5.84 % -13.29 %) compared to other populations across the CFR. The taxonomic status of this lineage (hereafter referred to as the Joubertina galaxias) is currently being assessed (Swartz *et al*., unpublished). The Joubertina galaxias have a restricted geographic range, and until the present study, this lineage has only been collected from four tributaries, the source of the Krom River (Krom River system) and from three upper Kouga tributaries (Wabooms, Twee and Granaat in the Gamtoos River system) in the eastern CFR (Cambray *et al*., 1995; Cambray, personal communication).

The Joubertina galaxias populations are severely fragmented due to the occurrence of *Micropterus salmoides*, habitat destruction and unsustainable water abstraction within the Krom and Kouga catchments. Genetic data also revealed high levels of genetic structuring between Joubertina galaxias populations within the Gamtoos River system (Chapter 2). This could be due to isolation by distance, occurrence of natural barriers, limited dispersal ability within the Joubertina galaxias, a small home range and/or a preference for certain habitat types (Chapter 2).

The lack of differentiation between the Krom and Twee populations could be an indication of on-going exchange of individuals between the two populations across the drainage divide between the Gamtoos and Krom River systems. This could be made possible by the presence of canals that were constructed to connect the Krom and Twee rivers, and may be especially effective during the wet winter months. Recent processes such as inter-basin dispersal due to intermittent freshwater connections through flooding of low drainage divides or a recent small scale river capture event could also explain the lack of differentiation. The Krom is thought to have been colonised from the Twee stream through one of these processes and it could therefore be a newly established population (Chapter 2). Given these results and concern over the multiple threats to streams in the Joubertina area, the aims of the present study were to: (i) map the distribution of the Joubertina galaxias, (ii) document the major threats to this lineage and (iii) assess the conservation status of the lineage according to the latest IUCN criteria (IUCN, 2001).

3.2. Materials and methods

3.2.1. Sampling

Field surveys were done in January 2011, March 2011 and April 2012 during low flow conditions. A total of 22 tributaries (9 of the Krom and 13 of the Gamtoos River systems) and 72 localities (30 in the Krom and 42 in the Gamtoos) were sampled using a 3m seine net, snorkelling with a hand net, electric fishing or a combination of these methods. At least three sites were surveyed per tributary to establish the lower and upper limits of the lineage in each tributary. At each site, all habitats present were sampled, and all fishes captured were recorded. Geographic coordinates and site elevation were obtained in the field using a GPS for all sampling sites.

At each locality; temperature, pH, conductivity and total dissolved solids (TDS) were measured using portable electronic meters (Hanna Inc.). Water depth, stream width and the length of the stream segment were measured at localities where densities were measured using a graduated pole and tape measure. At each site surveyed, habitat types and flow conditions were visually assessed and classified as either pool or riffle depending on the depth and flow velocity. Deep areas with slow flow and smooth surface appearance were classified as pools while shallow areas with fast flow and uneven surface appearance were classified as riffles. Flow velocity was visually assessed and scored as static (0), slow (1), moderate (2) and fast (3). Static or slow velocity was regarded as a pool, whilst moderate to fast flow was classified as a riffle.

At each locality, dominant substratum was visually assessed and recorded according to particle sizes as mud/silt (< 0.063 mm); sand (0.063-2 mm); gravel (2-64 mm); cobbles (64-256 mm); boulders (256-330 mm) or bedrock following Quinn & Hickey (1990). Aquatic vegetation was scored as absent (0), scarce (< 30%), moderate (30-60%) and abundant (>60%). The extent of stream shading was visually assessed depending on the presence or absence of marginal vegetation and was scored as absent (0), scarce (< 30%), moderate (30-60%) and abundant (>60%). The presence or absence of riparian vegetation was also noted and classified into five categories defined in Table 3.1. Occurrence of non-native fishes was recorded as absent (0) or present (1).

Table 3.1 Classification of riparian vegetation

Riparian vegetation type	Description
Conifer dominated	Forested, more than 70% of trees are alien conifers
Hardwood dominated	Forested, more than 70% of trees are alien hardwood
Mixed vegetation	No dominance greater than 70%
Shrub	Dominated by woody vegetation less than 5m at maturity
Grass	More than 70% of the vegetation is grass

Classification trees (Breiman *et al*., 1984) were used to investigate habitat preferences of the Joubertina galaxias. The analysis was carried out in R version 2.15 (R Development Core Team, 2012) using R Libraries Tree (for classification trees) and Random Forest. Classification tree analysis explains variation of response variables based on a set of independent explanatory predictors, either numerical or categorical (Breiman *et al*., 1984; Ripley, 1996; De'ath, 2002). The analysis differentiates between pre-identified variables in the data as present or absent (De'ath & Fabricius, 2000). Classification trees were constructed using Joubertina galaxias presence/absence data for each site sampled. Physico-chemical variables (temperature, pH, TDS and conductivity), extent of shading, altitude, abundance of aquatic vegetation, type of riparian vegetation and bottom substrate were used as predictors in the analysis. The best predictive tree with the smallest predicted mean square error was selected using Cross-validation (De'ath & Fabricius, 2000). The accuracy of the predicted tree models was improved using Random Forests (a non-parametric bootstrapping procedure that constructs a set of trees by resampling the data and averaging the predictions from the bootstrap iterations) (Brieman, 2001). Heterogeneity between data sets within Random Forest trees was determined by the Gini index of variable importance (Breiman, 2002). A higher Gini index value indicates greater importance of the variable.

3.2.2. Assessment of population densities

Abundance and population densities of Galaxias were assessed at four localities, two in the upper Wabooms and two in the upper Granaat. The sites were chosen for this assessment because their catchments have been least impacted by humans (the riparian zones are still intact, the stream reaches have not been bulldozed, there are no alien fishes in the reaches and water flow has not been modified as there are no weirs, dams or water take off points above the localities) indicating that these are the remaining near natural populations of the lineage. Population densities of the fish were estimated as number per square meter using the three pass depletion method (Zippin, 1956; Lookwood & Schneider, 2000) using an electrofisher.

3.2.3. Assessment of threats

The assessment of threats was done following the guidelines provided by both the IUCN and the Global International Waters Assessment (GIWA, 2001). Major threats to the species were classified using the IUCN threat classification scheme version 3.0 (IUCN, 2001; see also Salafsky *et al*., 2008 and Kostoski *et al*., 2010). According to the IUCN, the threats could be in the past, ongoing or in the future, using a time frame of three generations or ten years, whichever is the longer (but not exceeding 100 years in the future) as is required by the Red List Categories and Criteria (IUCN, 2001). The environmental impacts were assessed using the scoring scheme provided by GIWA and four scoring ranks were used to quantify the severity of each key threat.

The distribution patterns of both indigenous and alien fishes in these streams were also assessed. The presence of invasive plants, in-stream or off-stream dams, physical in-stream impacts due to river channelization, Inter-Basin Transfers (IBTs), canalized rivers and weirs and groundwater abstraction were noted in the assessment criteria. To obtain an overview of the threats to Joubertina galaxias, the threats were identified and the levels of impacts were scored according to the degree of severity of the threats on the tributary and the lineage. The description of the level of impact was given in a scale ranging from low to severe (GIWA, 2001). If the estimated average impact score of all threats' score was < 0.5, then it was considered that there was no impact on the tributary; the level of impact was regarded to be slight if the average impact score was between 0.5 - 1; moderate if average impact score was between 1 - 1.5; high if average impact score was between 1.5 - 2 and severe if the average impact score was > 2 and/or if the maximum impact score of at least one threat was 3.

3.2.4. Assessment of the lineage's threatened status

The risk of extinction for the Joubertina galaxias was assessed according to the IUCN Red List Categories and Criteria Version 3.1 (IUCN, 2001). Table 3.2 presents a summarised description of the threatened categories of the IUCN. According to the IUCN Red List Categories and Criteria Version 3.1 (IUCN, 2001) and the Guidelines for Application of IUCN Red List Criteria at Regional Levels Version 3.0 (IUCN, 2003), all taxa listed as Critically Endangered, Endangered or Vulnerable are described as threatened. The IUCN Red List Categories and Criteria can be downloaded from www.iucnredlist.org.

Table 3.2 Description of the IUCN red list categories of threatened taxa that are relevant to the present study (www.iucnredlist.org).

IUCN threatened category	Description
Critically Endangered, CR	Species is considered to be facing an extremely high risk of extinction in the wild in 10 years or three generations
Endangered, EN	Species is considered to be facing a very high risk of extinction in the wild in 10 years or three generations
Vulnerable, VU	Species is considered to be facing a high risk of extinction in the wild in 10 years or three generations
Near Threatened, NT	Currently a species does not qualify for Critically Endangered, Endangered or Vulnerable, but is close to or is likely to qualify for a threatened category in the near future.
Least Concern, LC	Widespread and abundant taxa are included in this category. These cannot qualify for Critically Endangered, Endangered, Vulnerable or Near Threatened.

For each of these threat categories there is a set of five main criteria A-E, with a number of sub-criteria within A, B and C and an additional sub-criterion in D for the Vulnerable category (IUCN, 2001). The qualifying thresholds within the criteria A-E differ between the threat categories, but classification in any one of these criteria qualifies a taxon for listing (IUCN, 2001). The number of mature individuals was estimated using the equation $d*A*p$, where * is a multiplications sign, d is an estimate of population density, A is an estimate of area, and p is an estimate of the proportion of individuals that are mature (IUCN Red list Guidelines, 2011).

The estimation of Area Of Occupancy (AOO) for the Joubertina galaxias was not performed at the tetrad (2km x 2km) spatial scale, as recommended in the IUCN guidelines (IUCN, 2011), because rivers are linear habitats and freshwater fish are vulnerable to upstream impacts throughout a single population in a single tributary, making them more vulnerable than terrestrial organisms (ref). Instead, Area of Occupancy (AOO) was estimated by multiplying the stream length and average stream width for each tributary. Extend of Occurrence (EOO) was calculated by fitting a boundary to the species distribution, fitting hectads within the boundaries, and adding up the areas of the resulting polygons (IUCN guidelines; IUCN, 2011).

3.3. Results

3.3.1. Description and distribution

The Joubertina galaxias was found from only six mountain tributaries of the Krom and Gamtoos River systems (Chapter 2; Figure 3.1). The Krom River system has only one population that is restricted to the upper Krom, while five populations occur in tributaries of the Gamtoos River system, namely the Wabooms, Diepkloof, Granaat, Krakeel and Twee streams (Chapter 2). All known populations of the Joubertina galaxias do not occur with other native fishes, except at one locality in the Krakeel River where its distribution overlaps with *Sandelia capensis*.

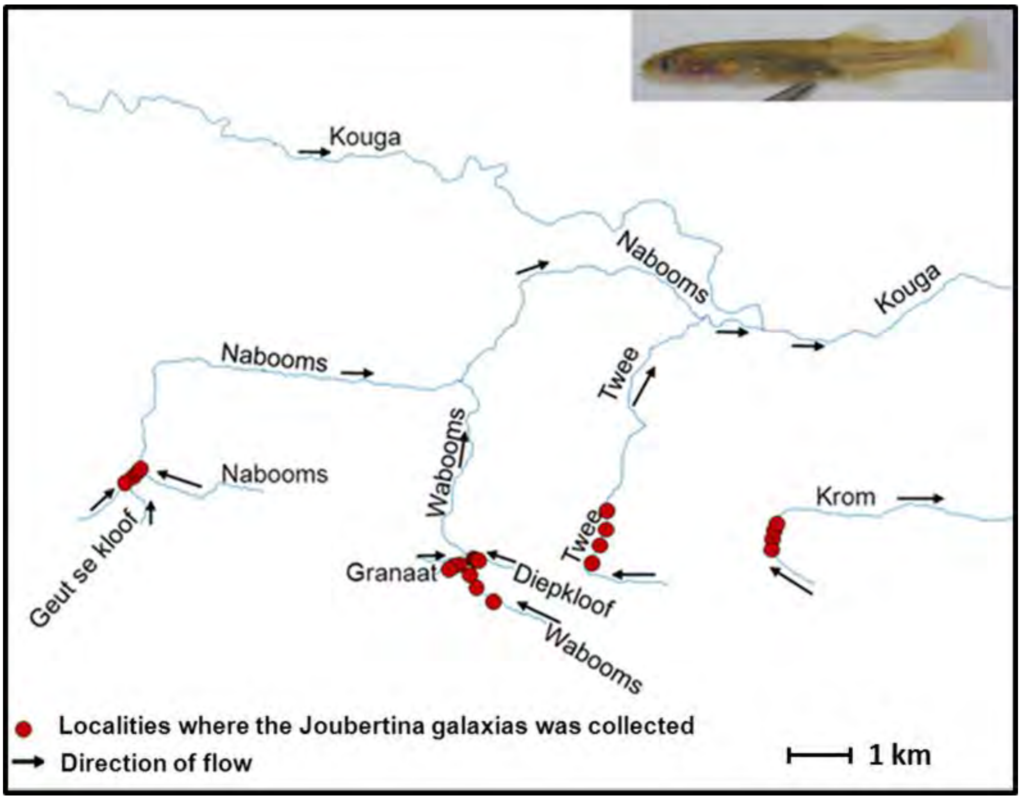

Figure 3.1 Map of the upper Krom River system and upper Kouga catchment in the Gamtoos River system showing sites where the Joubertina galaxias was collected. All sites sampled during this study are shown in Chapter 2 Figure 2.2.

The phylogeographic pattern of the Joubertina galaxias (Chapter 2) suggests low levels of gene flow among populations. It is therefore possible that there were no permanent populations in more mainstem habitats. Sharing of alleles between populations of the Joubertina galaxias from Gamtoos River system may suggest occasional migrations crossing the lower Twee, Nabooms and Wabooms possibly during floods, but competition from mainstem fishes (particularly *S. capensis*) may have prevented establishment of populations of any significant size in the mainstems (Figure 3.2 A). The remaining populations in the Gamtoos may therefore only represent a small proportion of the historical distribution range (Chapter 2; Figure 3.2 B). The Krom population is thought to have been introduced from the Twee stream through canals (Chapter 2). These populations now only occur in short headwater sections of the streams, many of them only 0.5 –1.2 km in length (Figures 3.1; 3.2 B).

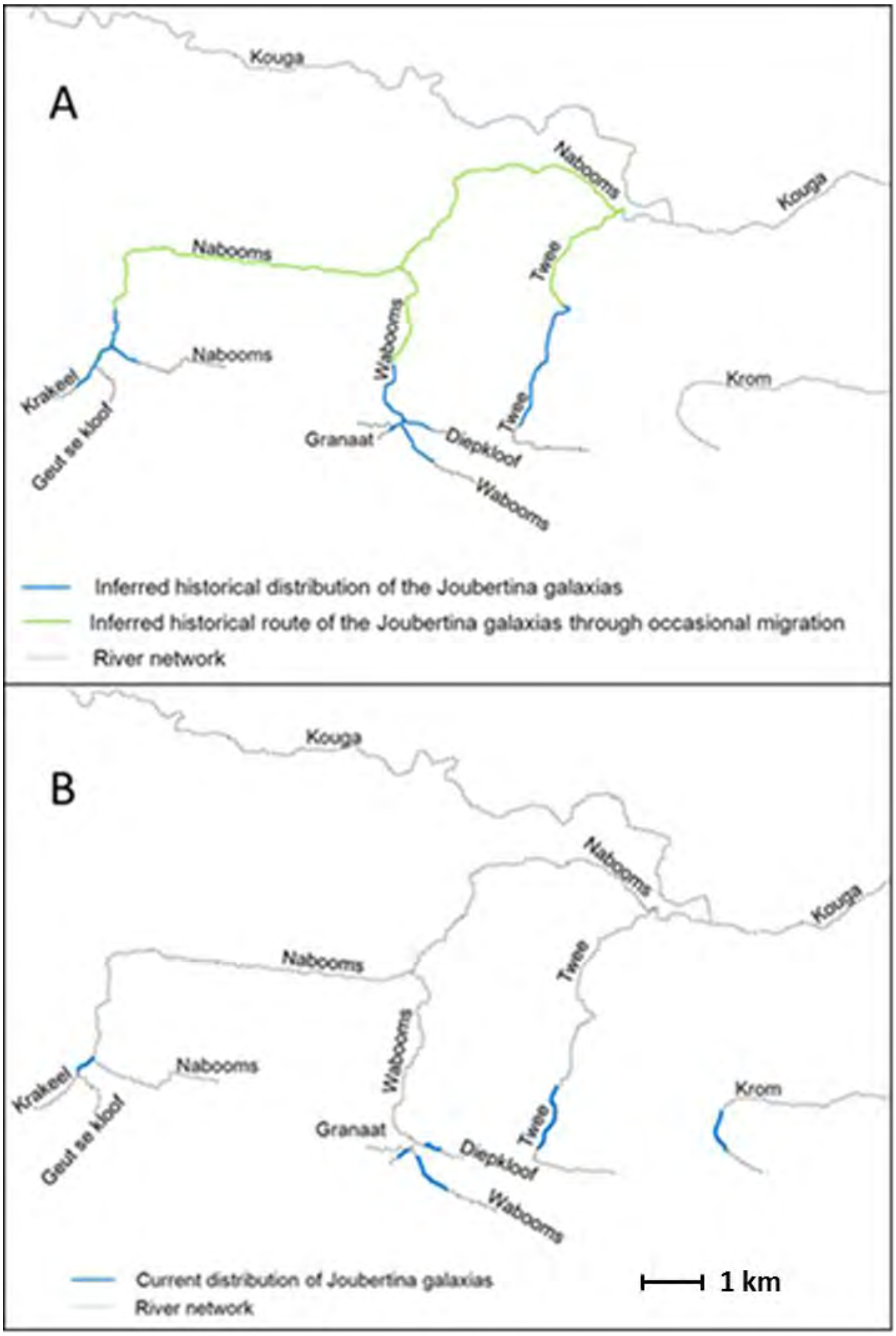

Figure 3.2 The inferred historical and current distribution of the Joubertina galaxias.

3.3.2. Habitat

The elevation of the surveyed sites ranged from 209 to 817 m above sea level, but the Joubertina galaxias only occurred in a narrow range of altitudes of 568 to 648 m above sea level. The surveyed streams ranged from <1 to 3.5 m in average width. The banks of the Gamtoos tributaries were sheltered, except for Twee stream that had previously been bulldozed. The dominant vegetation along these streams was riverine fynbos (mainly shrubs of the family Ericaceae and grasses) although submerged macrophyes (aquatic vegetation) were also common in some reaches (Figure 3.3). The upper Krom tributary is very narrow (±1m wide) and the banks are heavily vegetated with fynbos (mainly reeds and shrubs), and in some places the stream was completely shaded. Habitat descriptions of localities where the Joubertina galaxias were collected are summarised in Table 3.3. Conductivity (range 38-80μScm^{-1}) and total dissolved solids (range 19 - 40mg/l) were generally low, which is typical of mountain tributaries in the CFR. The pH values (range 4.97-6.28) suggested that the water was mostly acidic.

The Joubertina galaxias was collected in only two habitat types (pools and riffles). Although it appeared to prefer gentle currents within sheltered banks and was usually found near the head of pools, some fish were also collected in shallow waters and in riffles. The Joubertina galaxias can tolerate stagnant water (e.g. velocity for sites in the Krakeel and Twee was mostly static) and it was collected in temperatures ranging from 19 to 25^{o}C, but is expected to survive very cold water temperatures in winter with occasional snow on high-lying areas.

Figure 3.3 Typical habitats where the Joubertina galaxias were collected.

Table 3.3 Habitat description of six tributaries in the Krom and Gamtoos River systems where the Joubertina galaxias was collected.

Tributary	Habitat description				
	Channel flow conditions	Substratum	Riparian vegetation type	Aquatic vegetation	Stream shade
Twee	Single short pool up to 3m deep upstream with static and no flow downstream	Cobble with abundant boulders with occasional bedrock	Alien hardwood dominated	moderate	scarce
Krakeel	Small pools less than 1m deep with very slow flow upstream and downstream	Diverse, including boulders, cobbles, mud and silt	Mixed vegetation (grass & alien conifer)	abundant	scarce
Wabooms	Single short pool up to 1.5m deep bounded with riffles upstream and downstream with occasional static flow	Diverse, including bedrock, boulders, cobbles, gravel and silt	Shrub (Erica family)	scarce	moderate
Diepkloof	Mostly riffles, no pools observed	Mud and silt with occasional cobble, boulders and bedrock	Alien conifer dominated	scarce	abundant
Granaat	Riffles upstream and small pools downstream	Bedrock with occasional boulders, cobbles and sand	Mixed vegetation (grass & alien conifer)	scarce	moderate
Krom*	Small pools less than 1m deep bounded with riffles upstream and downstream	Bedrock with some boulders and large cobbles and mostly small sandy pools	Grassy fynbos and Ericaceae species	moderate	abundant

* = potential extralimital population

Classification tree analysis demonstrated that altitude, conductivity and the presence of aquatic vegetation are the habitat variables that strongly influence the distribution of the Joubertina galaxias (Figure 3.4). The Joubertina galaxias seems to be restricted to altitudes above 554 m and below 634 m above sea level. The ideal conductivity for the Joubertina galaxias seems to be below 80µScm^{-1}. It seems the lineage favours areas covered with aquatic vegetation, probably because these areas serve as refuge from predators, reduce energy expenditure and they may be preying on aquatic invertebrates that attach to plants. The mean decrease accuracy (MDA) for this analysis was very low (highest value was 0.02) meaning the model had excellent accuracy. The mean decrease in Gini index revealed that the five most important variables which produced noticeably higher mean decrease in Gini values were altitude, type of substrate, presence of aquatic vegetation, pH and conductivity (Figure 3.5).

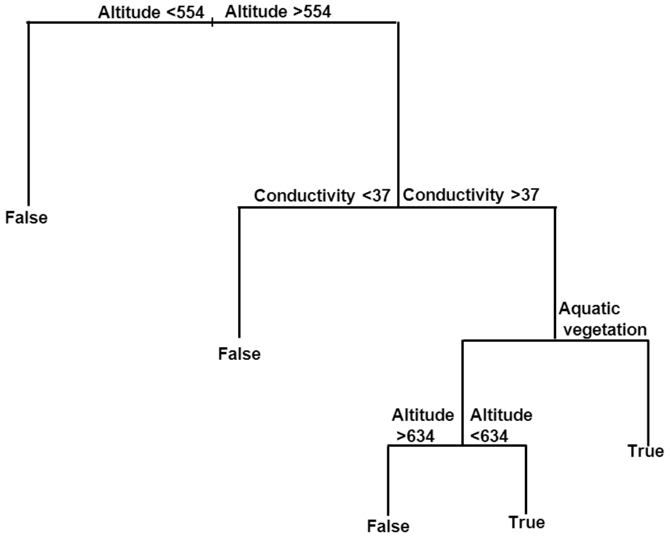

Figure 3.4 Pruned classification tree from analysis of the Joubertina galaxias present (true)/ absent (false) data. Misclassification error rate = 9%.

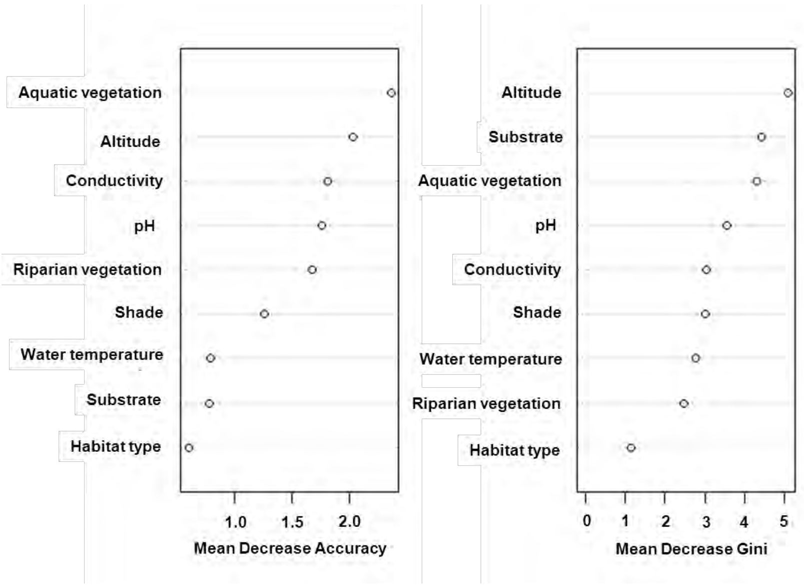

Figure 3.5 Mean importance of each environmental variable for the Joubertina galaxias. The values (1 to 5) indicate variable importance, with 1 indicating least important and 5 representing the most important variable in the model.

3.3.3. Abundance and density

The upper Granaat and upper Wabooms were the only sections where the Joubertina galaxias still occurs in near natural conditions. The Krakeel and Twee populations were severely affected by unsustainable agricultural activities, while the Diepkloof is highly affected by alien invasive fish species and sedimentation. The upper sections of the Krom seemed to have a relatively healthy population despite being affected by alien invasive fishes, but could not be used to assess habitat preference, because it may be a newly established population itself. The densities for Joubertina galaxias populations were therefore only calculated for the Granaat and Wabooms as the other four populations were viewed as severely impacted and not viable in the long term. The Joubertina galaxias densities ranged from 0.54 –1.3 fish/m^2 in the Wabooms tributary and 0.16–0.99 fish/m^2 in Granaat tributary (Table 3.4).

Table 3.4 Population densities of the Joubertina galaxias for the two sites sampled each in the Granaat and Wabooms tributaries. N is the number of fish captured per pass and T/N is the total number of fish captured in each pool.

Site	N pass 1	N pass 2	N pass 3	T/N	Pool length (m)	Pool width (m)	Pool area (m^2)	N.se	Density (N/m^2)
Wabooms 1	26	11	9	46	12.9	3.18	41.07	6.22	1.30
Wabooms 2	10	6	2	18	17.5	1.98	34.71	1.90	0.54
Granaat 1	5	3	2	10	15	4.47	67.00	2.15	0.16
Granaat 2	25	17	10	52	24.6	2.73	67.24	11.49	0.99

The estimated range of population sizes (N) were 1148-4420 and 120-1337 individuals in the Wabooms and Granaat, respectively (Table 3.5). The Joubertina galaxias was very uncommon in the other streams, particularly the Twee, Krakeel and Diepkloof. Densities were not calculated for th remaining steams, because they are likely to be unstable populations due to a variety of impacts.

Given that sampling localities covered the entire distribution range of this lineage within each tributary, it is possible that the abundances would not exceed 250 mature individuals in the Krom, Twee, Diepkloof and Krakeel. Only the Wabooms and Granaat tributaries had population sizes that possibly have more than 250 mature individuals per stream. Very few mature individuals per population were caught during sampling surveys (an average of 30%), with sub-adults being the most common. The estimated number of mature individuals for the Granaat and Wabooms tributaries ranged from 36 to 401 and 344 to 1326 individuals respectively (Table 3.5), therefore suggesting that the number of mature individuals may be fewer than 2500.

Table 3.5 Estimated ranges of population sizes and the number mature individuals of the Joubertina galaxias in the Granaat and Wabooms tributaries calculated from the densities obtained from the four sampled sites in Table 3.4.

Tributary	Length (m)	Width (m)	Total area of distribution (m^2)	Density (N/m^2)	Population size (N)	Mature individuals (N)
Wabooms	850	2.5-4	2125-3400	0.54-1.3	1148-4420	344-1326
Granaat	600	1.25-2.25	750-1350	0.16-0.99	120-1337	36-401

3.3.4. Decline

The data suggest that Joubertina galaxias may have experienced a decline from the inferred historical distribution and abundance in relation to its current distribution (Figure 3.2). Downstream sections of the lineage's range may have been lost in the Krakeel, especially in sections where the tributaries become wider with a lower gradient above where *Sandelia* would have been abundant. Range losses also occurred in the Twee due to unsustainable agricultural activities and to a lesser extend in Diepkloof, due to alien invasive fishes and water abstraction. Slight range loss has also been experienced in the Wabooms and Diepkloof due to the presence of alien invasive fishes in the Joubertina dam. All remaining populations are now extremely fragmented and isolated by dams, habitat destruction and alien fishes. The Joubertina dam has contributed to habitat loss of the Joubertina galaxias in the Granaat (below the waterfall), Wabooms and Diepkloof streams by changing stream habitat to lacustrine habitat.

3.3.5. Threats

Key threats to the upper Gamtoos and upper Krom River systems were assessed as defined by IUCN threats classification scheme version 3.0 (Table 3.6). Only four classes of threats were assessed for the Joubertina galaxias (Table 3.7). Climate change and severe weather were not considered in this study due to incomplete knowledge about the severity of these threats although their impact is predicted to increase in future (Hulme *et al*., 2001; Coetzee *et al*., 2009). Of the six streams in which the Joubertina galaxias currently occur, only the catchment of upper Granaat and Wabooms have not been directly impacted by human activities (Table 3.7).

Table 3.6: Scoring table for the Krom and Gamtoos River systems showing key threats as defined by IUCN threats classification scheme version 3.0, current impact and expected future impact changes (shown using arrows). Four scores which are: 0-no known impact; 1-slight impact; 2-moderate impact and 3-severe impact, were used for the assessment of the current impacts affecting the two river systems following the scoring scheme provided by GIWA (2001). For each threat class, the average scores were calculated and maximum values were also given. The level of knowledge regarding these threats was subjectively estimated.

Threat class	Key threat	Knowledge of threat	Impact	Average impact	Maximum impact
1 Residential & commercial development	1.1 Housing & urban areas	-	0	0	0
	1.2 Commercial & industrial areas	-	0		
	1.3 Tourism & recreation areas	-	0		
2 Agriculture & aquaculture	2.1 Annual & perennial non-timber crops	Well known	2 ↑	1	2
	2.2 Wood & pulp plantations	Less known	1↑		
	2.3 Livestock farming & ranching	Less known	1 ↑		
	2.4 Marine & freshwater aquaculture	-	0		
3 Energy production & mining	3.1 Oil & gas drilling	-	0	0	0
	3.2 Mining & quarrying	-	0		
	3.3 Renewable energy	-	0		
4 Transportation & service Corridors	4.1 Roads & railroads	-	0	0	0
	4.2 Utility & service lines	-	0		
	4.3 Shipping lanes	-	0		
	4.4 Flight paths	-	0		
5 Biological resource use	5.1 Hunting & trapping terrestrial animals	-	0	0	0
	5.2 Gathering terrestrial plants	-	0		
	5.3 Logging & wood harvesting	-	0		
	5.4 Fishing & harvesting aquatic resources	-	0		
6 Human intrusions & Disturbance	6.1 Recreational activities	-	0	0	0
	6.2 War, civil unrest & military exercises	-	0		
	6.3 Work & other activities	-	0		
7 Natural system Modifications	7.1 Fire & fire suppression	Moderately known	1↑	2.3	3
	7.2 Dams & water management/use	Well known	3↑		
	7.3 Other ecosystem modifications	Less known	3↑		
8 Invasive & other problematic species & genes	8.1 Invasive non-native/alien species	Well known	3↑	1	3
	8.2 Problematic native species	-	0		
	8.3 Introduced genetic material	-	0		
9 Pollution	9.1 Domestic & urban waste water	-	0	0.5	2
	9.2 Industrial & military effluents	-	0		
	9.3 Agricultural & forestry effluents	Moderately known	2↑		
	9.4 Garbage & solid waste	-	0		
	9.5 Air-borne pollutants	Less known	1↑		
	9.6 Excess energy	-	0		
10 Geological events	10.1 Volcanoes	-	0	0	0
	10.2 Earthquakes/tsunamis	-	0		
	10.3 Avalanches/landslides	-	0		
11 Climate change & severe weather	11.1 Habitat shifting & alteration	-	0	0	0
	11.2 Droughts	-	0		
	11.3 Temperature extremes	-	0		
	11.4 Storms & flooding	-	0		
	11.5 Other impacts	-	0		

The arrow indicates the likely direction of future changes: ↑- impact increases; →- no change; ↓- impact decreases

Table 3.7: Criteria used for the assessment of threats to the Joubertina galaxias using four scores which are: 0-no know impact; 1-slight impact; 2-moderate impact and 3-severe impact. *-Potentially extralimital

Tributary	Impact of threats				Average impact	Maximum impact	Level of impact
	Agriculture & aquaculture	Natural system modifications	Invasive & other problematic species & genes	Pollution			
Wabooms	1	1	1	0	0.75	1	Slight
Krakeel	3	3	1	1	2	3	Severe
Granaat	0	0	0	0	0	0	None
Twee	3	3	2	1	2.25	3	Severe
Diepkloof	3	3	2	1	2.25	3	Severe
Krom*	2	2	3	1	2	3	Severe

3.3.5.1. Alien fish invasion

The most serious threat to the Joubertina galaxias is predation by alien invasive largemouth bass (*Micropterus salmoides*) (see Tables 3.6 and 3.7). The Joubertina galaxias has been eliminated from the sections of the Wabooms, Diepkloof and Krom streams that have been invaded by *M. salmoides*. The Granaat population is protected from invasion by a waterfall which prevents upstream movement of alien fishes. Restriction of the Joubertina galaxias to stream habitats that have not been invaded by alien fishes is indicative of the high susceptibility of this species to predation. The presence of *M. salmoides* is a major cause of population decline and also prevents recolonisation and recovery of the Joubertina galaxias. Even though Krom may be an alien range for Joubertina galaxias, no specimens were collected in the canals to the farm "Opening" dam in the Krom tributary where *M. salmoides* occur.

The future survival of the Joubertina galaxias is uncertain, because the remaining populations that persist in the predator-free headwaters are at a high risk of being invaded, particularly during periods of heavy rainfall, since largemouth bass is present in these streams immediately below the Wabooms, Diepkloof and Krom populations. The Granaat population is relatively safe, because it has a natural waterfall that prevents the upstream invasion of *M. salmoides*. The Wabooms is protected by a small man-made barrier associated with the gravel road across it just above the Joubertina dam, but it could eventually break, especially during floods. It is therefore important to assess and monitor the strength of this structure. Direct introduction by anglers remains a threat in these tributaries, but is considered a relatively low threat due to the low potential for angling in such small streams.

3.3.5.2. Degradation and loss of habitat

Mountain tributaries of the Gamtoos and Krom River systems naturally have perennial flow, but excessive water extraction have transformed downstream sections of many streams into seasonal dry-beds during the dry season. Water is extracted from the surface through weirs (Krakeel) and canals (Krom) and from underground with pumps (Twee and Krakeel) and used for agricultural and domestic purposes (see Figure 3.6). The Diepkloof, Krakeel, Twee and Krom populations have been severely affected by habitat loss due to over-abstraction of water for agriculture. Major portions of the streams run dry, especially the Twee and Krakeel streams where most of the flow of the stream has been diverted for agriculture. Population size in these streams has been severely reduced. Stream structure and critical habitats for fish have also been destroyed directly through bulldozing to channelize flow, especially in the Twee and Krakeel streams. Physical damage of habitats, destruction of riparian vegetation, unnatural levels of stream bank erosion and in-stream sedimentation were observed in all tributaries (Figure 3.6).

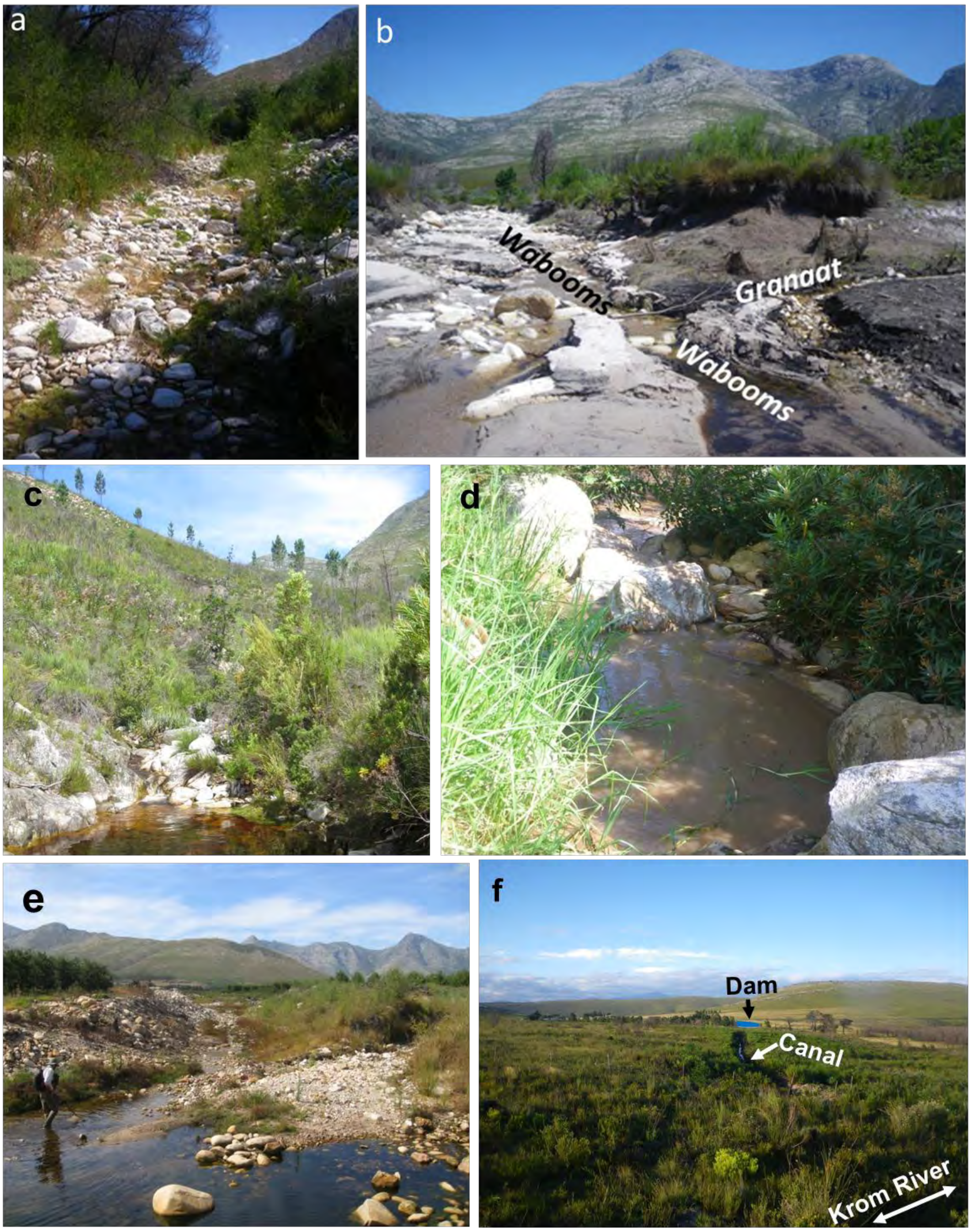

Figure 3.6 Some of the major threats to the Joubertina galaxias (a) over-abstraction of water and bulldozing in the Twee River; (b) sedimentation, erosion and siltation of the Waboomš and Granaat streams due to the Joubertina dam; (c) invasion of riparian zone by *Pinus* trees along Waboomš River; (d) increased water turbidity in the Diepkloof River due to irrigation back-flow; (e) habitat homogenisation due to bulldozing in the Krakeel River and (f) canal (in which bass was collected) diverting water from the Krom River into a farm dam.

3.3.5.3. Dams and inter-basin water transfers

There are several dams that have been built in catchments of the Gamtoos and Krom River systems to store water for agricultural and domestic use. Alien fishes were subsequently introduced for angling. These dams, in particular the Joubertina dam are therefore acting as reservoirs for the spread of invasive alien fishes (especially in the Wabooms (below the low water bridge (33º 52' 50.1" S and 23º 50' 26.2"E)), Diepkloof (33º 52' 42.6" S and 23º 50' 37.3"E) and Granaat (below waterfall (33º 52' 47.2" S and 23º 50' 23.9"E)), potentially repopulating in the Diepkloof and therefore preventing recolonisation of native fishes in this stream. Canals that connect the Krom and Twee streams could have and may still be facilitating the spread of bass and the Joubertina galaxias between the Gamtoos and Krom River systems.

3.3.5.4. Genetic isolation

All Gamtoos populations of the Joubertina galaxias had significantly high pairwise F_{ST} values (Chapter 2). This suggests that there may historically have been little or no migration between Gamtoos populations, with no permanent populations in more mainstem habitats. Fragmentation may therefore be a relatively natural pattern for this lineage. Occasional migration in the more mainstem habitats could have been possible during flooding as indicated by the sharing of alleles between populations from the Gamtoos River system. However, all known populations of the Joubertina seem to have become completely isolated (Figure 3.2B). They are isolated by alien invasive fishes, habitat destruction and the man-made structures (e.g. weirs in the Krakeel stream). It is therefore very unlikely that contemporary gene flow is occurring and may lead to negative effects of inbreeding.

3.3.6. IUCN conservation status assessment.

Only the Critically Endangered, Endangered and Vulnerable IUCN categories were relevant to assess the extinction risk of the Joubertina galaxias. According to the nature of the available data of the Joubertina galaxias, the assessment of this lineage was carried out quantitatively against just two IUCN criteria (B and C; Table 3.8) dealing with geographic range, population size and demographic changes. Criterion A was not assessed despite the possibility that *M. salmoides* could have caused a rapid decline of more than 50% of the population, because this probably happened more than 10 years ago, just after they were first introduced. More recently (the last 10 years) the decline of habitat quality due to agricultural activities and continued impacts by bass may have caused a further 5-20% decline in population size. These threats have not ceased, but it is possible that they are causing a slower decline than in the past. Criterion D was not assessed because the population is not very small and although the AOO is very small, the Joubertina galaxias was collected from more than five locations (five if the potentially extralimital Krom population is excluded). Also, criterion E was not assessed because no quantitative analysis was carried out to estimate the extinction probability of the Joubertina galaxias based on known life history, habitat requirements and threats.

The available data and assessment for the Joubertina galaxias are shown in Table 3.8. The assessment shows that the Joubertina galaxias is Endangered. The lineage may have had natural fragmentation among its populations due to isolation by distance and competition from native fishes, but recent threats have completely isolated populations. The severity of the threats on most of the populations also affects the lineage's survival potential, with only the Granaat and Wabooms that are secure. The extent of occurrence has declined and is continuing to decline, except in the Granaat and Wabooms tributaries. The densities observed for the Joubertina galaxias ranged from 0.16 - 1.3 fish /m^2 and the number of mature individuals for the whole population seems to be less than 2500 mature individuals. The Joubertina galaxias had a restricted AOO and EOO and this can continue to decline if threats persist. This lineage qualifies under Endangered due to continuous decline of the Krakeel, Krom, Twee and Diepkloof populations. The lineage could be assessed as Critically Endangered due to its restricted geographic range, but the occurrence in more than one location, presence of more than 250 mature individuals and the presence of relatively secure Wabooms and Granaat populations, make the Endangered classification more realistic.

Table 3.8: Summary of the thresholds for the IUCN Criteria (IUCN, 2001) and results for the assessment of the Joubertina galaxias in South Africa.

Criterion	Thresholds			Criteria for qualifying	Result
	Critically Endangered	*Endangered*	*Vulnerable*		
B. Small geographic range + fragmented, declining or fluctuating	Extent of occurrence <100 km² OR area of occupancy <10 km² + two of the following: (i) severely fragmented or only a single location (ii) continuing decline (iii) extreme fluctuations	Extent of occurrence <5000 km² OR area of occupancy <500 km² + two of the following: (i) severely fragmented or no more than 5 locations (ii) continuing decline (iii) extreme fluctuations	Extent of occurrence 20000 km² OR area of occupancy <2000 km² + two of the following: (i) severely fragmented or no more than 10 locations (ii) continuing decline (iii) extreme fluctuations	CR: B1(a, b)	EOO has been reduced to 31.3 km^2 and is continuing to decline. Populations are severely fragmented and at least four of the five natural populations have become isolated. However, two of the populations remain secure and is likely to survive and maintain its genetic diversity and the species still survives in 5 natural locations.
				CR:B2(a, b)	AOO has been reduced to 8 km^2 and is continuing to decline. Populations are severely fragmented and at least four of the five natural populations have become isolated. However, two of the populations remain secure and is likely to survive and maintain its genetic diversity and the species still survives in 5 natural locations.
C. Small population and declining	<250 mature individuals, population declining	<2500 mature individuals, population declining	<10000 mature individuals, population declining	EN: C1	Population size is estimated to be 380-1727 mature individuals and only the Wabooms and Granaat likely have a number of mature individuals greater than 250 each. The other four subpopulations probably have <250 mature individuals) is probably continuing to decline and is vulnerable to extirpation at any time.

3.4. Discussion

The range and abundance of almost all endemic riverine fishes of the Cape Floristic Region has declined due to a combination of factors, including degradation of habitat, hydrological alterations, increased sedimentation, pollution, loss of stream connectivity and the introduction and spread of non-native species (Skelton, 2002; Cambray, 2003; Woodford & Impson, 2004; van Rensberg et al., 2009; Tweddle *et al*, 2009). As with previous studies that revealed that most of the remnant populations of freshwater fishes in the CFR are highly fragmented (e.g. Clark *et al*., 2009; Chakona & Swartz, 2012), the Joubertina galaxias populations are isolated and confined to mountain tributaries. Priority should be given to the Gamtoos populations because the Krom is probably an extralimital population.

3.4.1. Current status and threats

The present classification of the Joubertina galaxias as Endangered indicates that the lineage faces “a very high risk of extinction in the wild in 10 years or three generations” (IUCN, 2001). There is therefore need for urgent conservation actions and management plans that will be effective within this time frame. Similar to most other threatened fishes (Cowx, 2002; Skelton, 2002; Cambray, 2003; Woodford & Impson, 2004; van Rensberg *et al*., 2009; Tweddle *et al*, 2009), the Joubertina galaxias is threatened by multiple inter-dependent impacts. There are several potential risks associated with the isolated nature of remnant populations of this lineage in fragmented habitats. Small, isolated populations are vulnerable to long-term stochastic processes and potential loss of genetic heterogeneity (Frankham *et al*., 2002; Frankham, 2005). Small headwater streams are characterised by extreme variability in environmental conditions and are susceptible to stochastic events such as dewatering of stream channels and forest fires that could result in loss of fish, particularly from fragmented habitats (Schlosser & Angermeier 1995; Rieman & Clayton 1997).

Adequate levels of gene flow are necessary to prevent the deleterious effects of inbreeding (Frankham *et al*., 2002; Frankham, 2005). While gene flow levels among all the Joubertina galaxias populations could have been naturally low, the current situation where all known populations of this lineage are highly fragmented and the presence of alien predators in intervening waters makes it unlikely that migration could occur between remnant populations. This could affect the lineage's long term viability and evolutionary potential due to higher chances of inbreeding, which in turn could cause loss of genetic variability and increase the risk of extinction (Frankham *et al*., 2002; Frankham, 2005). Although baseline data on densities is unavailable, population sizes are likely to have been larger in the past, such that even though migration levels could have been low, higher numbers would have increased the effective population size and reduced the general effects of inbreeding. Future studies should determine the actual population sizes required to maintain long-term viability of the remnant populations of the Joubertina galaxias, at the same time estimating the size of habitat required to support those populations.

The near natural stream reaches where the Joubertina galaxias occur are usually composed of heterogeneous substrates (a mix of cobbles, pebbles, some boulders, gravel and sand) with the banks covered by marginal and bank trailing vegetation. This habitat complexity is important as it may provide cover to the fishes, and also provide important habitat for aquatic invertebrates that could be the main food source for this lineage. Catchment disturbance, particularly through bulldozing (which is common in the Gamtoos and other river systems in the CFR), smothers and homogenises the substrates due to increased input of sediments, while disturbance of the river banks may lead to loss of bank-trailing and marginal vegetation. Bulldozing can cause increase in water turbidity and changes in water chemistry such as lowering levels of dissolved oxygen particularly after sediment pulses, while loss of bank cover may increase water temperature. Loss of riparian vegetation could also increase the possibility that pesticides and fertilisers could be washed into the streams with runoff from irrigation and storm events. Poor water quality and pesticides can potentially affect sensitive early life stages (eggs, larvae and young juveniles) and could lead to fish mortality.

Another potential threat to the Joubertina galaxias populations is the presence of non-native fishes in the catchment. During surveys for the present study, native fishes were seldom collected in areas invaded by non-native piscivorous fishes. *Micropterus salmoides*, *M. dolomieu* and *Clarias salmoides* were collected in the lower reaches and mainstem areas during surveys for the present study. Although the impact of non-native fishes in the Gamtoos River system has not been empirically studied, they have the potential to be detrimental to a small species such as Joubertina galaxias. Previously Woodford and Imposon (2004) found that *Galaxias* was particularly susceptible to predation by alien piscivores (*Micropterus* spp. and *Oncorhynchus mykiss*). Because alien fishes currently occur in the lower reaches of the tributaries that currently harbor remnant Joubertina galaxias populations, there are concerns that, if not managed, they may invade the upper reaches.

3.4.2. Recovery planning and management issues

As an immediate measure, there is need for conservation authorities to implement appropriate mechanisms that would ensure protection of critical Joubertina galaxias habitat to secure the remaining populations. Long-term conservation programmes should not focus on conserving biological patterns only, but should also seek to maintain evolutionary processes (Moritz, 1994; 1999). Rehabilitation of habitats and establishment of connectivity among remaining populations of Joubertina galaxias will allow expansion and migration of individuals. This will allow larger and more stable populations (e.g. Wabooms and Granaat) to "rescue" smaller populations (e.g. Diepkloof), thereby reducing potential deleterious effects of inbreeding. There is also need to assess the minimum stream flow that is required to sustain all life history stages of the Joubertina galaxias. Care should be taken in designing rehabilitation projects, because restoration may allow the spread and establishment of unwanted non-native species.

Given that a large proportion of the Joubertina galaxias habitat occurs within private owned land, there is need for liaising with the relevant land owners/stakeholders regarding management options. While involvement of people in local communities has long been identified as one of the important factors to successful conservation projects (Cambray & Pister, 2002), the greatest challenge currently facing conservation authorities in the study area is poor awareness of the issues of water and freshwater biodiversity conservation. There is therefore a need for environmental education, explaining ecological impacts of complete water abstraction and the spread of alien fishes and plants. Lower reaches of almost all of the rivers where the Joubertina galaxias occur have been severely degraded by a number of activities, particularly bulldozing and channelization, which caused severe degradation of fish habitat. A key action is to provide local farmers/land owners with appropriate knowledge and expertise to develop low-impact flood management practices that minimise destruction of instream habitats.

3.4.3. Assessing effectiveness of recovery actions

The primary measure of success for the conservation and recovery measures will be the down-listing of the current “Endangered” status of the Joubertina galaxias. A more effective way to achieve the down-listing would be to secure the remaining populations and putting measures that would enable more populations to recover to more than 250 mature individuals. Recovery after rehabilitation should be monitored to assess any changes in the status of the remaining populations.

CHAPTER 4

Synthesis and general discussion

4.1. Thesis conclusions

The primary aim of the present thesis was to investigate the genetic diversity and geographic distribution of the Joubertina galaxias and to evaluate its conservation status. The research expands on the work done by Cambray *et al*. (1995), Waters & Cambray (1997), van Niekerk (2004) and Swartz *et al*. (unpublished). The previous work showed that the Joubertina galaxias is a unique lineage, possibly endemic to the upper Krom (Krom River System) and Kouga (Gamtoos River Sytem) catchments, with very little differentiation between the two river systems. Only four populations were known from previous research. In the present study, five populations of the Joubertina galaxias were identified in the Gamtoos River System (adding the Krakeel and Diepkloof as new populations) and the continued presence of this lineage was confirmed in the upper Krom River in the Krom River System. Individuals from the same tributary were regarded as a single population and appear to be relatively isolated due to alien fish invasion and the construction of dams and weirs. Mitochondrial and nuclear DNA analyses revealed high levels of genetic differentiation in the Gamtoos River System (Chapter 2), suggesting limited historical gene flow between populations. Consistent with Waters & Cambray (1997), low genetic differentiation was found between populations from the Gamtoos and Krom River systems, suggesting recent dispersal.

In the present study, the Krom population was genetically more closely related to the Twee population in the Gamtoos River System compared to other Gamtoos populations (Chapter

2). This suggests recent connectivity or possibly even ongoing dispersal between these two populations of supposedly historically isolated river systems. More recent processes such as inter-basin dispersal due to intermittent freshwater connections through flooding of low drainage divides, recent river capture or inter-basin water transfers (IBT's) were the most plausible explanations for the observed patterns. Genetic structuring within the Gamtoos river system could have been influenced by isolation by distance and physical barriers such as waterfalls (Chapter 2). These results suggest that migration may be limited between tributary streams of the Gamtoos River System.

In Chapter 3, the Joubertina galaxias was classified as Endangered according to IUCN criteria and is therefore facing a risk of extinction in the wild over the next 10 years or three generations. The Twee, Diepkloof and Krakeel populations are most at risk of extinction. The Krom population is also at risk of extinction, but is possibly an introduced population. The risk of extinction is mainly as a result of habitat modification and destruction through alteration of flow regimes (in particular over-abstraction, inter-basin water transfers, damming and weirs) (e.g. Twee, Diepkloof, Krakeel and Krom), invasion of remaining populations by alien fish species (e.g. Diepkloof and Krom) and potential loss of genetic diversity through general effects of inbreeding.

The extremely fragmented range and geographic isolation of the populations of the Joubertina galaxias in headwater streams makes this lineage highly vulnerable to local extinctions due to limited opportunities for range expansion genetic "rescue" from other areas.

4.2. Conservation recommendations

The Granaat population is protected from alien fish invasion by a waterfall and may therefore not require conservation management actions at this stage. In contrast, the Wabooms populations should be secured against invasion, since it is the largest and therefore most viable population, and it is only protected by small man-made structures associated with the road bridge. Ideally, a larger and more permanent weir structure should be built at the site of the lower bridge just above the Joubertina Dam to ensure that upstream migration of bass to the upper Wabooms during flooding is impossible. Alternatively, bass should be removed from the entire catchment above the Joubertina Dam, which would then act as a barrier to invasion. Although weirs have helped to secure indigenous fish populations against invasion by alien fishes, their position in the streams need to be carefully selected to prevent potential adverse impacts such as reducing population range. More immediate measures should prevent further loss of range of the remaining populations by active removal of alien fishes or use of an approved piscicide such as rotenone (e.g. Lintermans & Raadik, 2003) in the Diepkloof tributary, lower Wabooms, lower Granaat and possibly also Joubertina dam. It is possible that the Joubertina galaxias could establish in the dam, but there is also a high risk that bass would be reintroduced in the dam if such a measure is not understood and supported by the local municipality, communities, landowners and anglers. Education will be the most effective way to control the spread of alien invasive fishes, because once alien fishes are established in streams, effective measures to eradicate them are very difficult to employ and it is very expensive to completely remove them from a system. Eradication of alien fishes may facilitate range expansion and increase the size of the existing Joubertina galaxias populations as this species would be able to spread downstream to increase its distribution. This has been successfully implemented for the barred galaxias in Australia, leading to its recovery (Saddlier & Raadik, 1995; Lintermans & Raadik, 2003; Raadik *et al*., 2010).

Rehabilitation programs in the Twee and Krakeel tributaries through restoration of environmental flows may increase the range and size of the populations, and would in turn reduce the risk of inbreeding. These populations then need to be secured from alien fishes by building weirs lower down in the tributaries. Ideally, rehabilitated stream sections should encompass critical habitats of other native fishes, such as *Sandelia*. Chakona and Swartz (2012) found that this genus is particularly vulnerable to local extinction in the Breede River System, due to their preference for lower reaches of tributary streams where typical CFR impacts have an interactive and accumulated effect. This will have the added advantage from a scientific perspective that exclusion of the Joubertina galaxias by *Sandelia* through competition proposed in the present study can be tested. Lastly, it is important that conservation agencies liaise with stakeholders and include information from the present study in management plans for this lineage. The Joubertina galaxias is a critical flagship species in the eastern CFR, since their presence indicates high quality water (Cambray *et al*., 1995), the absence of alien invasive fishes and generally well-functioning aquatic ecosystems. Without immediate intervention from conservation authorities, the number of locations and the number of mature individuals of the Joubertina galaxias will likely continue to decline.

4.3. Future research requirements for conservation management

Additional surveys are required to assess if the Joubertina galaxias does not occur in some of the western tributaries of the Kouga catchment. Wider geographic sampling should also include all the farm dams in the area, to assess whether additional alien fish species can invade and identify areas that are vulnerable to invasion from existing alien species. This will also help to assess how water, and therefore potentially also fish, is transferred between or within catchments. More variable molecular markers such as microsatellites need to be utilised to more accurately assess gene flow patterns in this lineage. Better knowledge of the migration and diversity patterns will help to more accurately assess the risk of genetic inbreeding. In addition, ecological and behavioural studies, as well as better population size estimates will help to understand future demographic changes in the Joubertina galaxias. Such studies will improve conservation management plans, but the existing information provides an adequate framework for the prioritisation of initial conservation actions that will ensure the continued survival of the Joubertina galaxias.

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