Comparative biogeography and ecology of freshwater fishes in the Breede and associated river systems, South Africa

by

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is, to the best of my knowledge, original. This thesis has not been previously submitted, either in part or in its entirety, for the award of any other degree at any other university.

Signature: …………………………

Date: ………………………

Thesis summary

Distribution patterns and levels of genetic diversity in extant taxa are a product of complex palaeogeographic processes and climatic oscillations as well as the species' intrinsic ecological adaptations. The Cape Floristic Region of South Africa presents a unique system for studying the processes that promote species diversification and distribution patterns. This region has a high degree of endemism of both terrestrial and aquatic biota and is clearly isolated from neighbouring areas by the Cape Fold Mountains and the Great Escarpment. The objective of this study was to firstly examine the ecology of freshwater fishes belonging to the genera *Galaxias*, *Pseudobarbus* and *Sandelia* in the south-western CFR. This was followed by an assessment of the genetic diversity of these taxa. Unique lineages were identified and their distribution was mapped. The work aimed to explore the role of the region's complex palaeogeographic and climatic history as well as the role of the species' ecological adaptations in driving lineage diversification and shaping contemporary distribution patterns. The four main components of the study can be summarised as follows:

1. Habitat associations of three widely distributed lineages of *Galaxias zebratus Pseudobarbus burchelli* and *Sandelia capensis* were evaluated at multiple localities in minimally disturbed mountain tributaries of the Breede, Duiwenhoks and Goukou River systems. The lineages have distinct habitat associations which were related to differences in their morphological traits. The slender-bodied *Galaxias* 'nebula' and the fusiform-shaped *Pseudobarbus* 'Breede' are capable of exploiting upper reaches with faster water

velocity. By contrast, the laterally compressed *Sandelia* ‘eastern’ is restricted to lower reaches, making this lineage more susceptible to a wide array of impacts.

2. A recently discovered lineage of *Galaxias zebratus*, (*Galaxias* ‘nebula’), was found to be capable of tolerating emersion for a prolonged period of time. This is the first time that such capabilities have been documented in an African galaxiid. These adaptations have implications for the interpretation of *Galaxias* ‘nebula’s wide distribution range.

3. The phylogeography of *Galaxias* ‘nebula’ across its entire distribution range was investigated using two mitochondrial genes (cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*)). This lineage has a complex evolutionary history that was influenced by both intrinsic and extrinsic factors. Rare events such as episodic drainage connections during Pleistocene and Holocene pluvial periods, possibly augmented by river confluences during periods of lower sea-levels and river capture events seem to be the most credible explanation for the extensive contemporary distribution and the relatively shallow genetic divergence between different river systems.

4. Mitochondrial cyt *b* sequences were used (i) to assess genetic diversity in *G. zebratus*, *P. burchelli* and *S. capensis* from the south-western CFR and (ii) to determine the roles of intrinsic ecological adaptations and extrinsic landscape and climatic changes in promoting genetic diversification and shaping present day distribution patterns of lineages in the three taxa. Marine incursions during periods of major sea-level transgressions are proposed to have isolated populations in upland refugia, thereby

driving allopatric divergence in these species. Subsequent connections of rivers during wetter periods and lower sea-levels are proposed to have facilitated post-speciation dispersal of lineages to attain present day distribution patterns.

While detailed morphological studies and further genetic analysis are needed to substantiate the taxonomic status of the newly discovered lineages of *Galaxias zebratus*, *Pseudobarbus burchelli* and *Sandelia capensis*, results of the present study indicate that the south-western CFR represents a previously unrecognised centre of freshwater fish diversity and microendemism in the broader Cape Floristic Region. Accurate identification of lineages and comprehensive mapping of their distribution is a fundamental pre-requisite for ecological studies, assessing conservation status and implementation of appropriate conservation measures.

Dedication

To my daughter Rutendo,

my wife and friend Gamuchirai

my parents Assumpta and Runesu Chakona

… and to all those dedicated to the conservation of the CFR's unique freshwater fishes …

Thesis acknowledgements

I gratefully acknowledge my supervisors, Ernst Swartz and Gavin Gouws from the South African Institute for Aquatic Biodiversity and Paulette Bloomer from the University of Pretoria, for their support, guidance, mentorship, intellectual input and great working alliance from the beginning of this study until the end. Ernst conceived the research idea, administered the project and helped define its direction. Gavin’s unrelenting patience, assistance and guidance in the early days of my lab work and data analysis is greatly appreciated.

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Research outputs

Peer reviewed articles

Chakona, A., Swartz, E. & Magellan, K. (2011). Aerial exposure tolerance of a newly discovered galaxiid fish. *Journal of Fish Biology* **78**, 912–922.

Conference presentations

Chakona, A., Gouws, G., Swartz, E. & Bloomer, P. (2011). Vicariance or dispersal: does the distribution pattern of *Galaxias* ‘nebula’ conform to previous biogeographic hypotheses? (Abstract and oral presentation). 9th SASSB Conference (19-21 January 2011, Rhodes University, Grahamstown.

Chakona, A., Swartz, E., Gouws, G. & Bloomer, P. (2009). Genetic differentiation of a widespread Galaxias lineage across currently isolated river systems (Abstract and oral presentation). 10th Anniversary Conference, Southern Africa Society for Systematic Biology, 25-27 July 2009, Natalia, Illovo Beach, KwaZulu-Natal, South Africa.

Popular articles and newsletters

Chakona, A. (2010). Biodiversity field work in the Cape Floristic Region: Experiences and lessons learned from the Breede River expedition of 2008 to 2009 (Newsletter article). South African Biosystematics Initiative students’ newsletter, March 2010.

Chakona, A. (2010). Genetic diversity and habitat requirements of native fish in the Cape Floristic Region. South African Institute for Aquatic Biodiversity (SAIAB) research nugget for 2010/11.

Chakona, A. (2009). Comparative phylogeography, ecology and conservation of *Pseudobarbus*, *Galaxias* and *Sandelia* in the Breede and associated river systems (Popular article). South African Institute for Aquatic Biodiversity Annual Highlights Report, 2008-2009.

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Chapter 1

Thesis introduction

1.1. Overview

The Cape Floristic Region (CFR) located at the southern tip of Africa (Fig. 1.1) is renowned for its high plant diversity and endemism that is unrivalled by other Mediterranean-type ecosystems in the world (Goldblatt & Manning, 2000; Linder, 2003; Kreft & Jetz, 2007). Although the CFR's vertebrate diversity is much lower, the region is a hotspot of high endemism for several animal groups, including primary freshwater fish, reptiles, mammals and insects (Wright & Samways, 1998; Wishart & Day, 2002; Thieme *et al*., 2005; Linder *et al*., 2010).

Despite a long history of research, (the first description of a South African freshwater fish species was done by Burchell in 1822 (reviewed by Skelton 1996)), knowledge of the processes that promoted diversification and shaped distribution patterns of the CFR's biota is still incomplete. Earlier studies assessed taxonomic diversity and the distribution of taxa based on phenotypic (morphological) characters (e.g. Barnard, 1943; Bond & Goldblatt, 1984; Skelton, 1980a, b, 1986, 1988, 1996). More recently, molecular markers have been used to assess genetic diversity of the CFR's biota (Waters & Cambray, 1997; Bloomer & Impson, 2000; Wishart & Hughes, 2001; Daniels, 2003; Linder & Hardy, 2004; Roos, 2004; van Niekerk, 2004; Swartz *et al*., 2004, 2007, 2009; Swartz, 2005; Wishart *et al*., 2006; Daniels *et al*., 2006; Smit, *et al*., 2007; Price *et al*., 2007, 2010;

Tolly *et al*., 2006, 2009; Swart *et al*., 2009; Verboom *et al*., 2003, 2009; Gouws *et al*., 2010; Schnitzler *et al*., 2011).

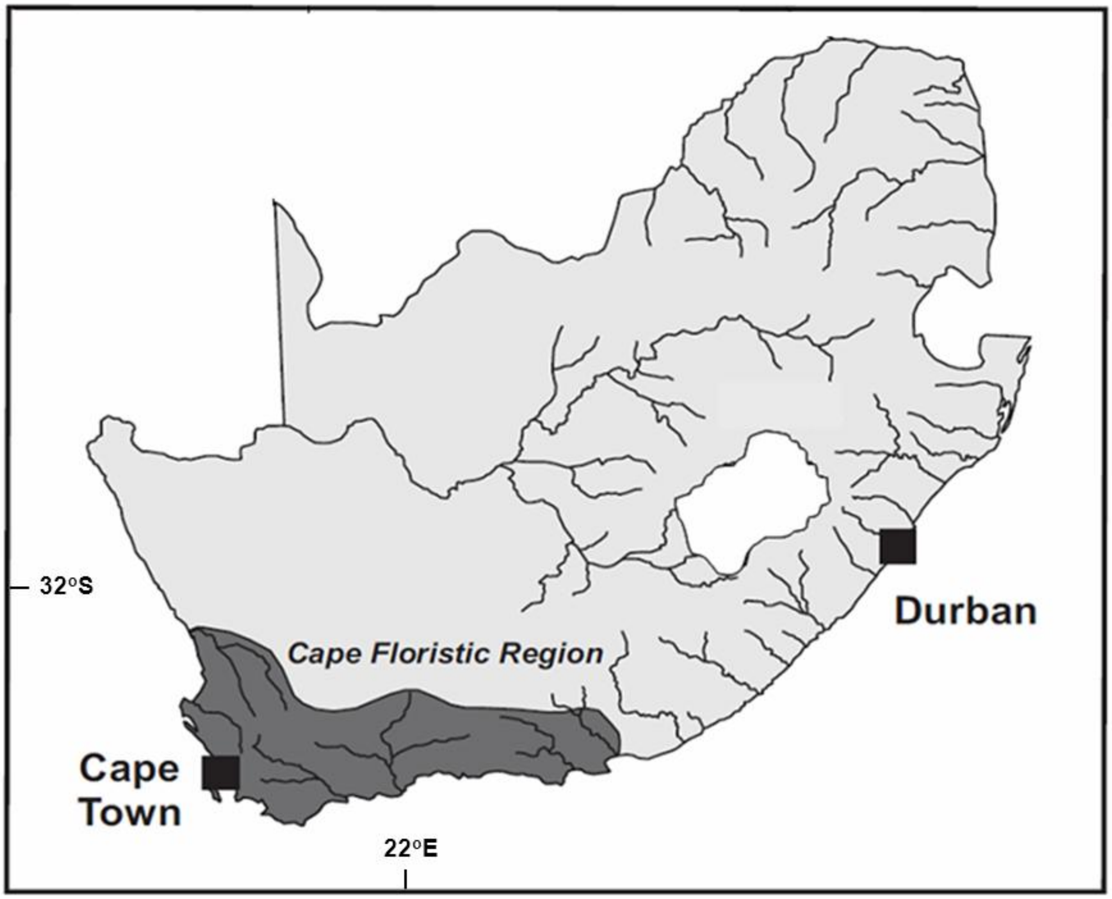

Fig. 1.1: Map of South Africa showing the location of the Cape Floristic Region (CFR).

Many of these studies have uncovered differentiation within traditionally recognised species, indicating that hidden or cryptic diversity may be a common phenomenon. Thus, genetic approaches are revealing that earlier studies underestimated biodiversity in the CFR. These studies are also providing valuable information about the effects of geological and climatic events on the evolution and distribution of regional biotas (e.g. Verboom *et al*., 2003; Linder, 2003; Tolley *et al*., 2006; Swartz *et al*., 2007, 2009; Price *et al*., 2007, 2010; Gouws *et al*., 2010). Southern Africa experienced a complex evolutionary history marked by mountain uplift, sea-level fluctuations and climatic oscillations that impacted both the landscape and the biota.

1.2. Geological history of the Cape Floristic Region

The most prominent geological feature of the CFR is the Cape Fold Mountains (Fig. 1.2). The southern ranges of the Cape Fold Mountains run parallel to the south coast and the western ranges run parallel to the west coast of South Africa. These two ranges meet in the south-western CFR, providing an interesting arena for the evolution of complex drainage systems. Major orogenic events in southern Africa began in the late Jurassic (*ca.* 140 million years ago (Ma)) (Hendey, 1983). All major present day landform features and drainage basins were probably established by the early Cenozoic (*ca.* 65 Ma), and their form is thought to have been relatively unaltered since then (Hendey, 1983). Thus, the CFR landscape can be considered to be very old and stable. However, evidence of more recent tectonic events also exists (Partridge & Maud, 1987, 2000). The first of two major events, called the Post-African I event, is thought to have occurred in the Miocene (*ca.* 22

Mya), while the second, called the Post-African II event, occurred in the Pliocene (*ca*. 5.3 – 2.6 Ma) (Partridge & Maud, 1987, 2000). This renewed tectonic activity is thought to have rejuvenated mountain uplift and erosion, increasing possibilities for drainage rearrangements. This suggests that even though the major features are very old, the fine-scale geomorphology of the CFR has been undergoing some changes since as late as the Pliocene. Tectonism also affected sea-levels (see section 1.4).

1.3. Climatic oscillations

Southern Africa experienced repeated climatic oscillations marked by extremes of wet and dry conditions since the Oligocene through to the Holocene epoch (Partridge *et al*., 1999; Marlow *et al*., 2000; Zachos *et al*., 2001; Cowling *et al*., 2009). Wetter periods are thought to have prevailed during the late Oligocene (*ca*. 25 Mya) to middle Miocene (*ca*. 15 Mya) (Marlow *et al*., 2000; Zachos *et al*., 2001; Cowling *et al*., 2009). The present dry conditions of the region were probably established by the end of the Pliocene (*ca*. 2.6 Mya) (Deacon *et al*., 1992; Tyson & Partridge, 2000). Some parts of the region, for example coastal areas of the southern CFR experienced wetter climatic conditions during the Holocene Altithermal (*ca*. 8 000 - 6 000 years ago) (Partridge *et al*., 1999). It is possible that adjacent river systems could have experienced intermittent connections through the inundation of low drainage divides, while wetlands on drainage divides could have sporadically discharged to different catchments during pluvial periods. Progressive aridification in more recent periods could have forced increased isolation of river systems and fragmentation of aquatic habitats.

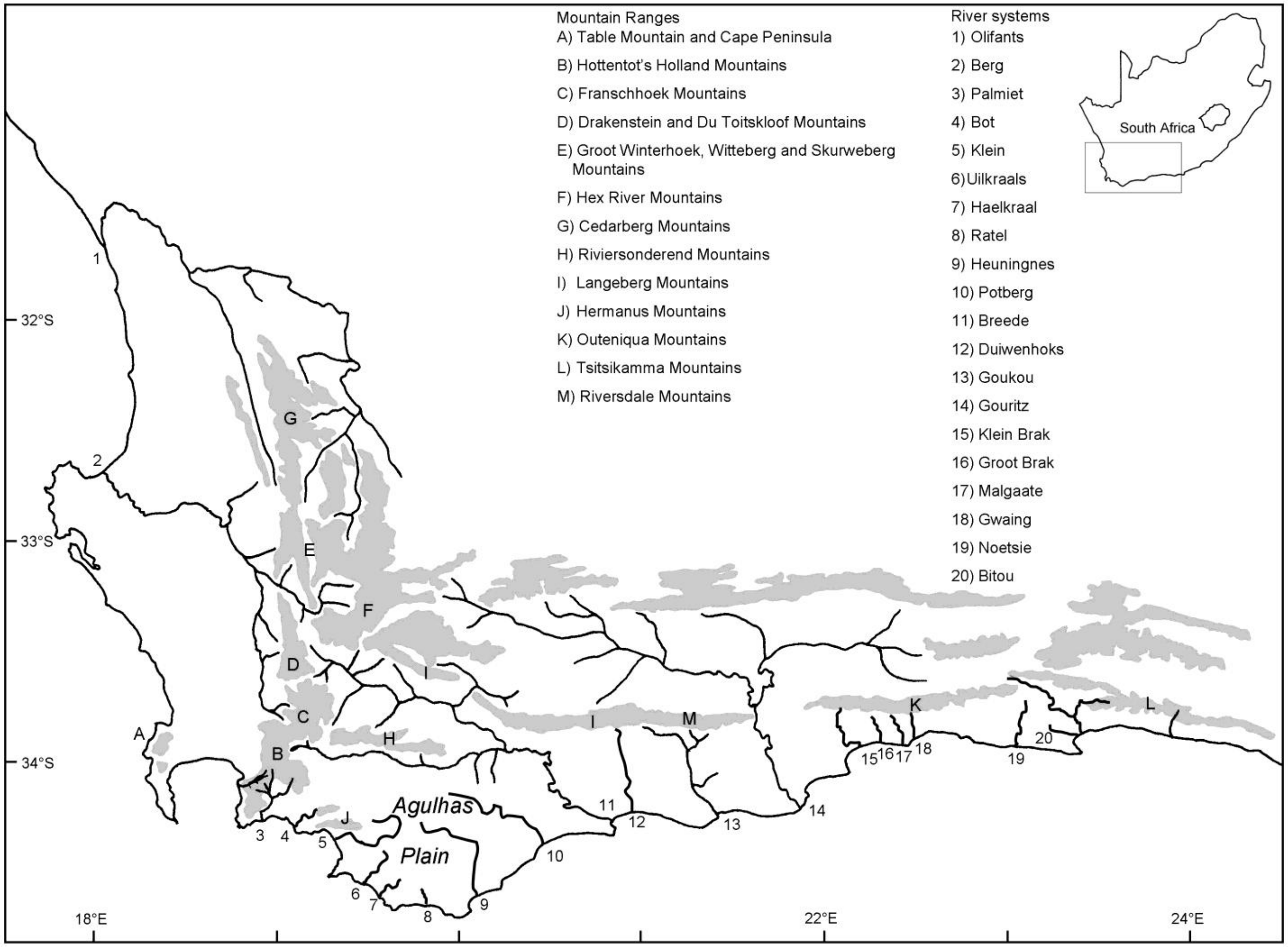

Fig. 1.2: Map of the Cape Floristic Region showing the Cape Fold Mountains, the Agulhas Plain and river systems mentioned in the text.

1.4. Sea-level fluctuations

Geological changes (section 1.2) and climatic oscillations (section 1.3) can affect sea-levels that in turn can affect drainage patterns. Global sea-level records show that the coastlines of continents have been in a continual state of flux due to changes caused by tectonism, and glacial and interglacial cycles (e.g. Matthews & Poore, 1980; Hendey, 1983; Siddall, *et al*., 2003). Repeated fluctuations in sea-levels around southern Africa occurred throughout the Tertiary period (Siesser & Dingle, 1981; Hendey, 1983). The last major transgression in southern Africa occurred during the mid-Miocene (*ca.* 15 Ma) to early Pliocene (*ca.* 5 Ma) when sea-levels ranged between 200 m and 300 m above the current coastline (Siesser & Dingle, 1981; Hendey, 1983). Subsequent transgressions were less dramatic, rising no more than 30 m above present levels (Rogers, 1985). The last major regression in southern Africa was experienced during the last glacial maximum (LGM) (*ca*. 22 000 – 18 000 years ago) when sea-levels ranged between 100 m and 200 m below the current coastline (Tankard, 1976; Siesser & Dingle, 1981; Hendey, 1983; Rogers, 1985; Ramsay & Cooper, 2002). These fluctuations could have produced recurrent expansion and contraction of terrestrial habitats, as well as repeated connection and separation of adjacent river systems (Hendey, 1983). Several low-altitude river systems could have also been drowned during periods of major transgressions (Siesser & Dingle, 1981; Hendey, 1983).

1.5. Linking past processes with current biodiversity patterns

The present geographical distribution of living organisms is a result of intrinsic biological and extrinsic geological and climatic factors. DNA sequencing has provided researchers with a powerful tool to investigate geographical genetic variation and to assess intraspecific phylogeographic structuring for a wide variety of taxa (Avise, 2000, 2004; Beheregaray, 2008). Molecular studies have facilitated the discovery of cryptic diversity within several traditionally recognised species (e.g. Gouws *et al*., 2004; Bickford *et al*., 2007; Brown *et al*., 2007; Elmer *et al*., 2007; Oliver *et al*., 2009). This has profound implications for evolutionary and biogeographic theories, taxonomy, diversity estimates, ecological studies and biodiversity conservation (Bickford *et al*., 2007). The ability of phylogeographic studies to approximate the timing of diversification provides researchers with a tool for describing the chronology for the cessation of gene flow among populations, and hence provides insights into the processes that were most influential in shaping present biodiversity patterns (Avise, 2000). Phylogeographic studies have identified Pleistocene climatic oscillations, sea-level fluctuations and tectonism as important drivers of diversification and biogeographic patterns of taxa worldwide (e.g., Hewitt, 2000; Montoya-Burgos, 2003; Near *et al*., 2003; Near & Benard, 2004; De Bruyn & Mather, 2007; Castoe *et al*., 2009; McCulloch *et al*., 2010).

In southern Africa, topographic uplift during the Miocene-Pliocene epochs has been linked to the present day spatially-heterogeneous environment of the CFR. Topographic and soil heterogeneity, associated with extreme climatic conditions, has been identified as an important driver of floral diversification and endemism in this region (Cowling *et al*., 2009; Schnitzler *et al*., 2011). Fragmentation of once widely-distributed populations caused by Plio-Pleistocene environmental fluctuations is considered to have been responsible for the diversification of mammal and reptile species in the CFR (Smit *et al*., 2007; Swart *et al*., 2009; Tolley *et al*, 2009). Drainage alterations, vegetation changes, climatic and sea-level fluctuations have been linked to the evolution of terrestrial insects in the CFR (e.g. Price *et al*., 2007). Mountain uplift, river capture events and sea-level regressions have been suggested to have had a strong influence on the diversification and biogeographic patterns of the CFR's freshwater fishes (Swartz, 2005; Swartz, *et al*., 2007, 2009).

The evolutionary histories of fishes that are confined to freshwater habitats are usually indicative of drainage history, because their dispersal is constrained by the intervening terrestrial and marine barriers. They can therefore only disperse following direct connections between different freshwater systems via relatively rare events such as drainage re-arrangements, coalescence of adjacent river systems during periods of lowered sea-levels, episodic connections of low drainage divides during periods of flooding or through freshwater plumes into oceans (Burridge, *et al*., 2006; Craw *et al*., 2007; Thacker, *et al*., 2007). Ecological adaptations (e.g. habitat preferences and environmental tolerances) influence the ability of a species to utilise these dispersal mechanisms (e.g. Burridge *et al*., 2008a). The distribution patterns of primary freshwater fishes may therefore reflect geological and climatic changes of a region as well as their ecological adaptations. Concordant geographical distributions of lineages or species could indicate the influence of a common historical event (geological or climatic history), whereas different patterns would reflect differences in ecological adaptations (Bermingham & Martin, 1998).

The present study contributes to the understanding of the influence of intrinsic ecological adaptations and the geological and climatic history of southern Africa in shaping the diversity and distribution of regional biota by comparing the genetic diversity of primary freshwater fishes endemic to the CFR belonging to three genera, namely *Galaxias*, *Pseudobarbus* and *Sandelia*.

1.6. Diversity and distribution of freshwater fishes in the Cape Floristic Region

The CFR has a low diversity of primary freshwater fishes, with only four families (Cyprinidae, Galaxiidae, Anabantidae and Austroglanidae) and only 19 formally described species at present (Skelton, 2001). The taxonomic richness of river systems is low, ranging from zero to ten species per system, with the majority of the systems that contain fish having only two to four species. Based on recognised habitat discontinuities as well as the distribution of endemic primary freshwater fishes, Skelton (1980a) recognised eight main catchments or biogeographic zones in the CFR, namely (1) Olifants, (2) Berg, (3) western Agulhas, (4) Breede and adjacent rivers, (5) Gourits, (6) south coastal rivers, (7) Gamtoos and adjacent rivers, and (8) Swartkops, Sundays and adjacent rivers (Fig. 1.3). The present study focused on the Western Agulhas and the Breede and adjacent rivers, hereafter called the south-western CFR.

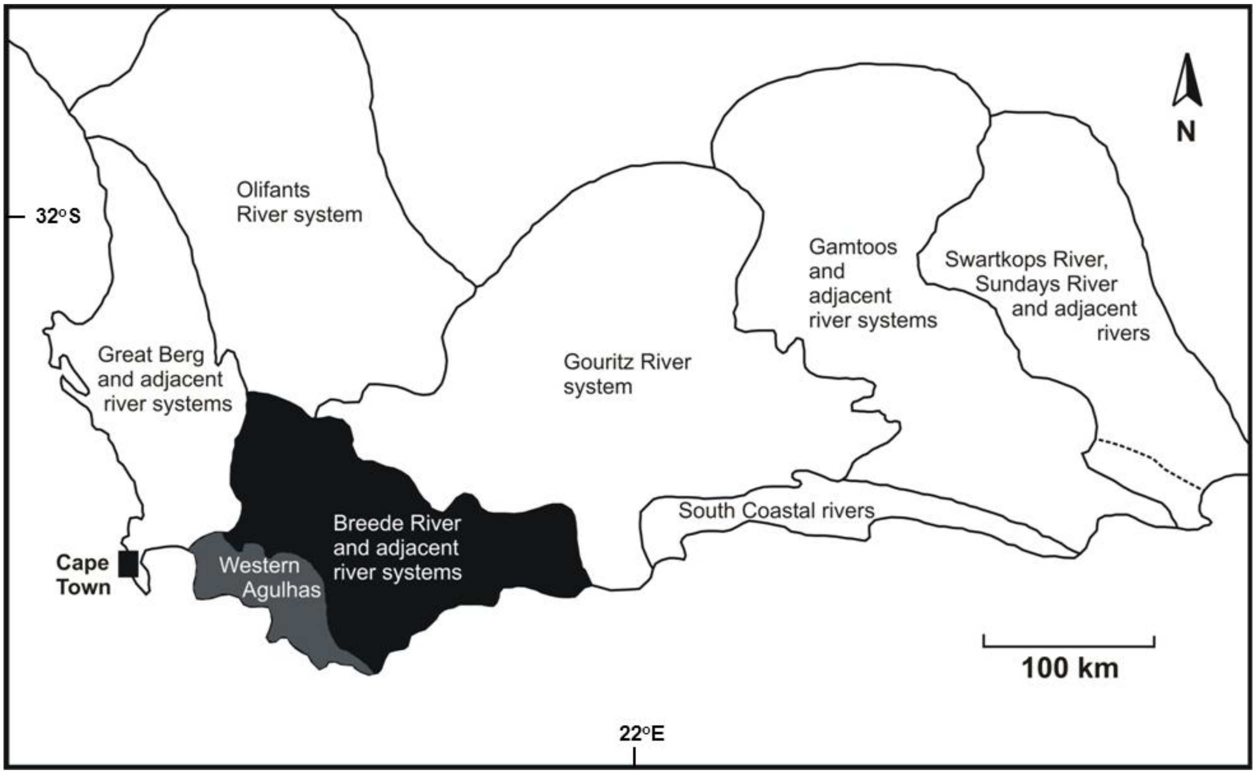

Fig. 1.3: Major biogeographic zones proposed for freshwater fish of the Cape Floristic Region (CFR) following Skelton (1980a). The study area for this research is indicated by black and grey shading.

Galaxias, *Pseudobarbus* and *Sandelia* are the most widely distributed of the CFR's seven primary freshwater fish genera. *Pseudobarbus* is the most speciose of these with six endemic species in the CFR, namely *Pseudobarbus burchelli* Smith, 1841, *Pseudobarbus burgi* (Boulenger, 1911), *Pseudobarbus afer* (Peters, 1864), *Pseudobarbus asper* (Boulenger, 1911), *Pseudobarbus phlegethon* (Barnard, 1938) and *Pseudobarbus tenuis* (Barnard, 1938) (reviewed by Skelton, 1988). Only *Pseudobarbus quathlambae* (Barnard, 1938) found in the Lesotho highlands occurs completely outside the CFR. *Sandelia* is represented by *Sandelia bainsii* Castelnau, 1861 and *Sandelia capensis* (Cuvier, 1831), but only the latter is found in the CFR (Barnard, 1943; Skelton, 2001). *Galaxias zebratus* Castelnau, 1861, a CFR endemic, is the only galaxiid currently recognised from Africa (Barnard, 1943; McDowall, 1973; Skelton, 2001). Molecular research has uncovered unexpected genetic diversity in all these species, indicating the existence of cryptic species complexes (Waters & Cambray, 1997; Bloomer & Impson, 2000; Roos, 2004; van Niekerk, 2004; Swartz, 2005; Swartz *et al*., 2004, 2007, 2009; Wishart *et al*., 2006). Whereas the traditionally recognised species had broad geographical ranges, many of the newly discovered lineages have more restricted distributions.

The occurrence of *Galaxias*, *Pseudobarbus* and *Sandelia* species across currently isolated river systems suggests that some mechanism has facilitated the movement of these fishes from one drainage basin to another. Swartz (2005) and Swartz *et al*. (2007, 2009) examined the phylogenetic relationships of the *Pseudobarbus* species and the influence of sea-level changes and river capture events on the distribution of genetic lineages across the CFR. River confluences during the lower sea-levels of the LGM seem to explain the common occurrence of closely related genetic lineages of *Pseudobarbus burchelli* and *P. afer* in currently isolated river systems (Swartz, 2005; Swartz *et al*., 2007, 2009). These authors also identified a number of closely related lineages occurring in river systems that did not coalesce during the LGM. River capture events and dispersal via low drainage divides during pluvial periods were invoked as alternative mechanisms to explain the occurrence of *P. asper* in both the Gouritz and Gamtoos River systems and *P. tenuis* in the Gouritz and Keurbooms/Bitou River systems. River capture also seems to be the most plausible explanation for the close relationship between genetic lineages of *P. phlegethon* in the Olifants River system on the west coast and a lineage of *P. afer* on the south coast (Swartz *et al*., 2007, 2009). The occurrence of the Breede lineage of *P. burchelli* in the Goukou River system that was part of the Gouritz palaeoriver system during the LGM was also attributed to a possible river capture event between the Duiwenhoks and Goukou River systems (Swartz *et al*., 2009). Comparative phylogeographic studies of other freshwater restricted taxa, such as the co-distributed *G. zebratus* and *S. capensis* provides a more powerful approach for investigating the processes that influenced the evolutionary history of stream fishes in the CFR, incorporating the role of ecological adaptations.

1.7. Ecology of freshwater fishes of the Cape Floristic Region

Ecological and biological knowledge for many of the CFR's freshwater fishes is scant. Some research has been done on the reproductive biology of *Pseudobarbus afer* and *P. asper* (Cambray, 1994), reproductive and feeding biology of *P. burchelli* (De Wet, 1990; Whitehead *et al*., 2007) and habitat preferences of *Labeobarbus capensis* (Paxton, 2008).

However, much of this research will have to be reassessed with the discovery of new lineages that could be different species. Knowledge of the ecological adaptations of species has diverse applications. These include providing a better understanding of the intrinsic and extrinsic determinants of biogeographic and phylogeographic patterns (e.g. Graham *et al*., 2004; Burridge *et al*., 2008a), prediction of past distribution patterns (e.g. Hugall *et al*., 2002; Nogués-Bravo, 2009), assessing conservation status (IUCN, 2003) and formulation of effective conservation strategies for threatened species (Ferrier, 2002; Filipe *et al*., 2004; Brooks, 2010).

1.8. Conservation of the CFR's freshwater fishes

Many of the freshwater fishes of the CFR are at risk of extinction due to widespread invasion by alien fishes, excessive water abstraction and habitat degradation (Skelton, 1990; Skelton *et al*., 1995; Cambray, 2003; Tweddle *et al*., 2009; Clark *et al*., 2009; Swartz & Tweddle, 2011). During the last century, the endemic fish fauna of the CFR has undergone severe attrition, and almost all main-stem populations have been extirpated (Clark *et al*., 2009; Chakona, unpublished). The remaining native fish populations persist in isolated headwater tributaries with little potential for gene flow due to fragmentation. The paucity of information on the biology, ecology, genetic diversity and evolutionary history of freshwater fishes of the CFR is a major impediment to effective conservation of the region's aquatic biodiversity. Effective conservation strategies should consider species-level diversity, genetic diversity within species, as well as maintaining the evolutionary processes responsible for promoting such diversity (Moritz & Faith, 1998).

Genetic diversity allows species to adapt to environmental changes, and also promotes long-term evolutionary potential (Moritz & Faith, 1998). The current management approach for indigenous fishes of the CFR may be considered to be ineffective, because it does not consider all the different levels of diversity.

1.9. Study objectives and description of study area

The first objective of this study was to contribute towards a better understanding of the ecology of freshwater fishes in three genera (*Galaxias*, *Pseudobarbus* and *Sandelia*) by comprehensively sampling river systems in the south-western CFR. The second objective was to identify unique genetic lineages within these genera and map their distribution. Thirdly, the study explicitly investigated the influence of the CFR's geological and climatic history, as well as species ecology, in driving lineage diversification and shaping present distribution patterns. It is hoped that the insights gained from this study will contribute to effective conservation of the highly-threatened, endemic fishes of the study area by helping conservation authorities and non-governmental organisations to prioritise their management actions.

The south-western CFR can be viewed as an excellent microcosm for studying biogeographical patterns and processes that influenced the evolutionary history of the region's freshwater biota. This is because the south-western CFR is clearly demarcated from surrounding areas by the Hottentot's Holland, Franschhoek, Drakenstein and Du Toitskloof Mountain ranges to the west, the Hex River and Langeberg Mountain ranges to the north and the Gouritz basin to the east (Fig. 1.2). This region is also of interest because it has several river systems of different sizes with diverse habitats for aquatic organisms. The Breede drains inland areas beyond the coastal mountain ranges, while the Duiwenhoks and Goukou originate in the mountain ranges that run parallel to the south coast (Fig. 1.2). Most of the tributaries of the Breede, Duiwenhoks and Goukou River systems are oligotrophic with low conductivity, while the coastal-draining rivers on the Agulhas Plain have high conductivity due to high salt content (Deacon, *et al*., 1992). Some of the rivers of the south-western CFR were fragmented, significantly reduced or inundated during the Miocene-Pliocene marine transgression (Siesser & Dingle, 1981; Hendey, 1983; Fig. 1.4). Many of the rivers also coalesced during the most recent marine regression during the LGM (Hendey, 1983; Fig. 1.4).

The study area has only four currently recognised indigenous primary freshwater fish species: three smaller species (*Galaxias zebratus*, *Pseudobarbus burchelli* and *Sandelia capensis*) and a large cyprinid barb, *Barbus andrewi* (Skelton, 2001). Van Niekerk (2004) discovered three genetically distinct lineages of *Galaxias zebratus* in the south-western CFR based on mitochondrial DNA (mtDNA) control region and cytochrome *b* (cyt *b*) sequences. Another study by Roos (2004) revealed that individuals of *Sandelia capensis* from the Breede/Goukou and Heuningnes River systems were genetically distinct based on control region and cyt *b* sequences. However, small sample sizes and large geographic sampling gaps prevented comprehensive mapping of the distribution ranges of these lineages.

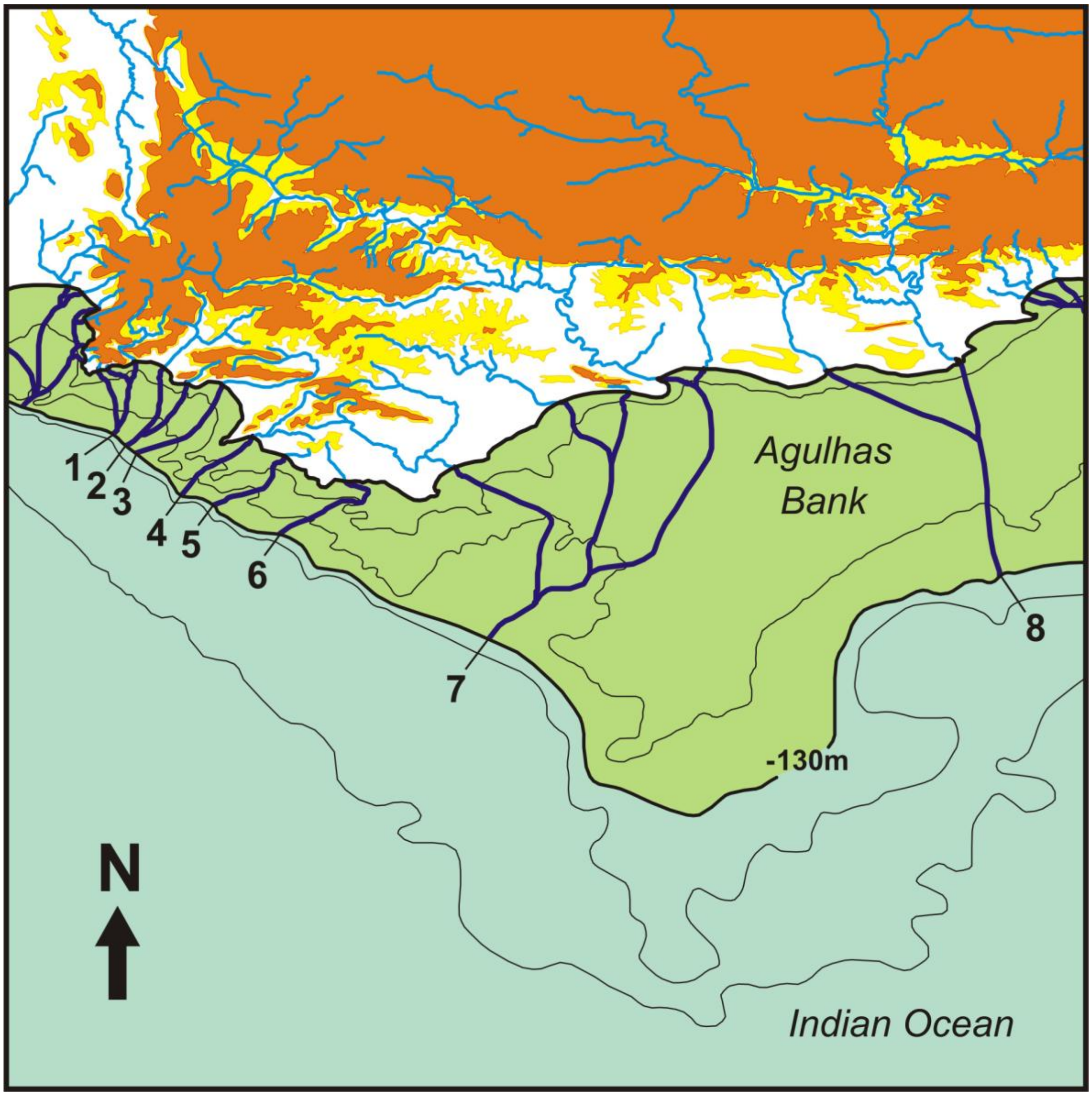

Fig. 1.4: Map showing potential fragmentation of river systems during the Miocene-Pliocene sea-level transgression and the proposed palaeoriver systems (1-8) of the Last Glacial Maximum (LGM) in the south-western Cape Floristic Region (CFR). The potential range of the maximum transgression level is indicated by the area in yellow (represented by the present day 200-300m contour lines bordering the yellow area). The white area would therefore have been vulnerable to marine incursion, whereas the area in orange was possibly never affected by the Miocene-Pliocene transgression. The approximate LGM sea-level is represented by the -130m contour line. The palaeorivers are 1) Palmiet, 2) Bot-Onrus, 3) Klein, 4) Uilkraals, 5) Haelkraal, 6) Ratel, 7) Breede-Heuningnes-Duiwenhoks and 8) Gourits-Goukou.

A more comprehensive study by Swartz (2005) uncovered three historically-isolated genetic lineages within *Pseudobarbus burchelli*. These comprise of a widespread Breede lineage occurring in the Breede, Duiwenhoks and Goukou River systems, a second lineage confined to the Tradou River (a tributary of the Breede River system) and another from a single locality in the Heuningnes River system. Swartz (2005) hypothesised that the common occurrence of the Breede lineage of *Pseudobarbus burchelli* across the currently isolated Breede and Duiwenhoks River systems in the south-western CFR could be explained by the confluence of these rivers during low sea-levels of the LGM, while river capture events were proposed to explain the occurrence of this lineage in the Goukou River system which belonged to a different palaeoriver system (Fig 1.4). The present study extends previous research with fine-scale geographic sampling, an assessment of ecological factors and by comparing the genetic diversity of *Galaxias*, *Pseudobarbus* and *Sandelia* to assess the factors that influenced the evolutionary history of freshwater fishes in the south-western CFR.

The questions arising from the research objectives were:

- What factors influenced the distribution of freshwater fishes of the south-western CFR?
- Do the fishes differ in their habitat associations, and what are the biogeographic and conservation implications of such variations?
- Are there more unidentified genetic lineages within currently recognised fish species of the south-western CFR, and what are their distribution ranges?

- What effects did mountain uplift, climatic oscillations and sea-level fluctuations have on the diversification and distribution of lineages in *Galaxias*, *Pseudobarbus* and *Sandelia* in the south-western CFR?
- Is there evidence that temporal patterns of divergence and geographical distribution patterns are shared among lineages of these co-distributed genera?
- Based on the temporal and geographic patterns of divergence, is it possible to formulate a hypothesis of lineage diversification and distribution for freshwater fishes of the south-western CFR?
- What were the past distributions of the lineages prior to human impacts?
- What are the major threats to the fishes of the south-western CFR, and what are appropriate measures to prevent further loss of biodiversity?

1.10. Structure and format of the thesis

Different components, a published paper and three other planned publications are structured as separate chapters. The background to this research is given in the General Introduction. Secondly, due to lack of quantitative data on the ecological attributes of freshwater fishes within the study area, habitat preferences of three widely-distributed, sympatric lineages of *Galaxias*, *Pseudobarbus* and *Sandelia* were investigated in least-impacted, mountain tributaries of the Breede, Duiwenhoks and Goukou River systems (Chapter 2). Thirdly, the study documents, for the first time, emersion tolerance capabilities in an African galaxiid fish (Chapter 3). In Chapter 4, the phylogeography of this species, the most widespread lineage of *Galaxias*, was investigated and the role of intrinsic and extrinsic factors in shaping the present genetic structure and distribution pattern of this lineage across the CFR was evaluated. In Chapter 5, a comparative study of *G. zebratus*, *P. burchelli* and *S. capensis* was done to investigate the influence of geological and climatic events in promoting lineage diversification and in shaping the distribution patterns of lineages within these species. A synthesis and discussion of the findings of this research, and suggestions for future research, is presented in the final chapter.

Chapter 2

Comparative ecomorphology and habitat use of three indigenous fishes in undisturbed mountain tributaries of the Breede, Duiwenhoks and Goukou River systems

Abstract

Habitat associations of three recently discovered fish lineages (*Galaxias* ‘nebula’, *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’) in 51 undisturbed mountain tributaries of three river systems in the south-western Cape Floristic Region were assessed using presence and absence data. For each species, Manly’s indices were used to determine habitat preferences, and logistic regression was used to relate species occurrence to simple habitat measures (mean wetted width, maximum depth, elevation and slope). The prediction that habitat preferences would be related to body shape was supported. The slender-bodied *Galaxias* ‘nebula’ and the fusiform-shaped *Pseudobarbus* ‘Breede’ were found to be capable of exploiting upper reaches with faster water velocity, while *Sandelia* ‘eastern’, which has a laterally compressed body and comparatively larger pectoral fins, is a habitat specialist preferring lower reaches with slower current velocities. Logistic regression modelling showed that the probability of detecting the larger-bodied lineages (*Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’) increased with increase in stream size and water depth, while the smaller bodied *Galaxias* ‘nebula’ exploited a range of water depths and stream sizes. This is the first comprehensive and quantitative information on habitat preferences of these lineages, which has implications for explaining contemporary biogeographic and phylogeographic patterns, inferring past distribution patterns and informing effective conservation management of these fishes.

2.1. Introduction

The Cape Floristic Region (CFR) of South Africa is a hotspot of high endemism for freshwater fishes. However, basic biological and ecological knowledge for many of the species remain largely unknown. It is currently difficult to determine the historical distributions of native freshwater fishes of the CFR due to hydrological modifications, degradation of habitat and widespread invasion of the region’s riverine environments by non-native piscivorous fishes. The most dominant of these are the sharptooth catfish *Clarias gariepinus* (Burchell, 1822), largemouth bass *Micropterus salmoides* (Lacepède, 1802), smallmouth bass *M. dolomieu* (Lacepède, 1802), spotted bass *M. punctulatus* (Rafinesque, 1819), bluegill sunfish *Lepomis macrochirus* Rafinesque, 1819, rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) and brown trout *Salmo trutta* Linnaeus, 1758. These impacts have collectively resulted in local extinctions in some mountain tributaries and extirpation of almost all main-stem populations of native freshwater fishes (Clark *et al*., 2009; Chakona & Swartz, unpublished). The remaining populations persist in isolated headwater tributaries, often above physical barriers that prevent upstream migration of alien predators (Clark *et al*., 2009; Chakona & Swartz, unpublished).

A better understanding of the factors that affect fish occurrence in these undisturbed catchments has diverse applications. These include the prediction of past distribution patterns (e.g. Hugall *et al*., 2002; Nogués-Bravo, 2009), providing a better understanding of the ecological and evolutionary determinants of contemporary biogeographic and phylogeographic patterns (e.g. Graham *et al*., 2004; Burridge *et al*., 2008a), projecting potential impacts of environmental changes (e.g. Chu *et al*., 2005; Xenopoulos & Lodge, 2006) and informing effective conservation planning and management (e.g. Ferrier, 2002).

A pattern of distinct habitat preferences and longitudinal zonation has been observed repeatedly across many studies of riverine fishes (e.g. Bhat, 2004; Morán-López *et al*., 2005; Santoul *et al*., 2005). However, these studies were conducted in watersheds with higher fish diversity, where competitive exclusion and predation may interact with abiotic factors to produce distinct spatial patterns in stream fishes (e.g. Gilliam *et al*., 1993). It is unclear, however, whether fishes in species-poor regions such as the CFR would also exhibit non-random distribution patterns and specific habitat preferences.

The near-natural condition of upland tributaries of the Breede, Duiwenhoks and Goukou River systems in the south-western CFR (Fig. 1.2) offered a unique opportunity to study the factors that influence the distribution of native fish assemblages in the absence of major confounding impacts such as pollution, sedimentation and alien fishes. River systems of this study area were previously thought to have only four indigenous primary freshwater fishes, currently *Galaxias zebratus*, *Pseudobarbus burchelli*, *Sandelia capensis* and *Barbus andrewi* (see Skelton, 2001). Recent molecular studies have however discovered several deeply divergent genetic lineages within *G. zebratus*, *P. burchelli* and *S. capensis* (Roos, 2004; van Niekerk, 2004; Swartz, 2005; see also Chapter 5). Taxonomic revision of these groups is underway and some of the lineages will be described as new species. Many of the lineages in the south-western CFR have highly restricted geographic ranges (see Chapter 5), except three (hereafter *Galaxias* 'nebula', *Pseudobarbus* 'Breede' and *Sandelia* 'eastern'), which are subject of the present study. *Galaxias* 'nebula', *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' are broadly co-distributed across the Breede, Duiwenhoks and Goukou River systems (see Chapter 5). Apart from the recently discovered emersion tolerance capabilities of *Galaxias* 'nebula' (Chapter 3 published as Chakona *et al*., 2011), almost nothing is known about the ecology of these fishes.

The purpose of the present study was to explore and quantitatively describe the habitat features that most strongly influence the distribution of *Galaxias* 'nebula', *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' in undisturbed mountain tributaries of the Breede, Duiwenhoks and Goukou River systems. Specific objectives were: 1) to identify and describe habitat preferences of *Galaxias* 'nebula', *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' and (2) to develop statistical models for these lineages in relation to environmental variables that could be used to predict their occurrence in the river systems.

It was hypothesised that habitat preferences would be related to the species' morphological traits because many studies have found that morphological characters of freshwater fishes are directly linked to their ecology (Wikramanayake, 1990; Douglas & Matthews, 1992; Motta *et al*. 1995, Chan, 2001; Brinsmead & Fox, 2002; Bhat, 2005; Leal *et al*., 2011). It was predicted that the slender bodied *Galaxias* 'nebula' (Fig. 2.1) would be capable of exploiting upper stream reaches with higher current velocities because a slender body shape reduces drag, thereby reducing the energetic expenditure necessary to maintain position in flowing water (Webb, 1984; McLaughlin & Grant, 1994). By contrast, the laterally-compressed *Sandelia* ‘eastern’ (Fig. 2.1) was predicted to be mainly restricted to lower river reaches with slow current velocities because deeper-bodied fishes are generally associated with poorer swimming performance (Bronmark & Miner, 1992; Webb, 1998). The medium-bodied *Pseudobarbus* ‘Breede’ (Fig. 2.1) was predicted to penetrate to intermediate elevations. The larger-bodied species (*Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’) were predicted to show preference for deeper habitats which are known to reduce predation risk from terrestrial predators (Harvey & Stewart, 1991).

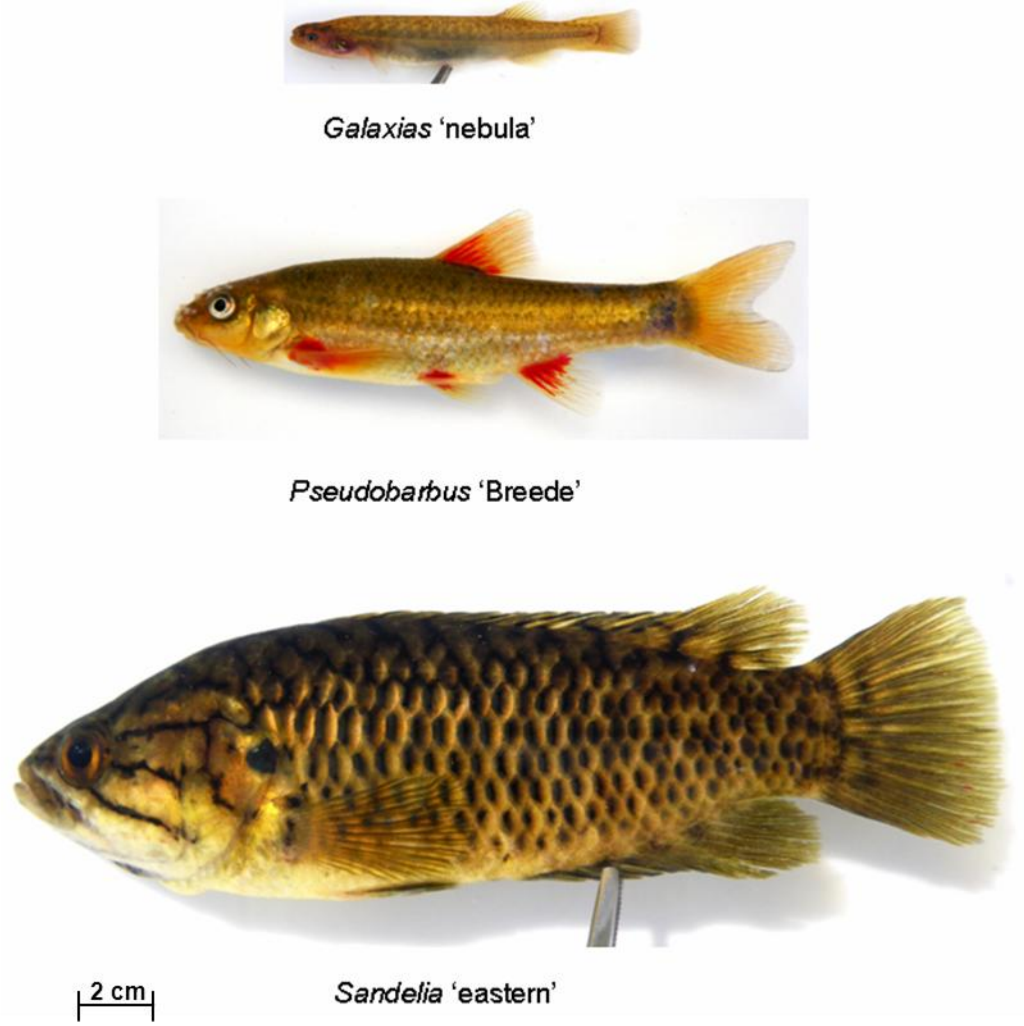

Fig. 2.1: Body forms of *Galaxias* 'nebula', *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' scaled to relative adult size.

2.2. Materials and methods

Presence-absence sampling

Intensive sampling of river systems within the south-western CFR was conducted during low-flow conditions between November 2008 and December 2009. A total of 318 sites from 154 rivers were sampled during this period. The sampled localities encompassed a suite of habitats ranging from steep mountain tributaries to low-gradient main-stem reaches. For purposes of assessing habitat use, only sites from undisturbed sections of the rivers were used. This was done to ensure that the distribution of individuals was based on intrinsic habitat preference and not influenced by anthropogenic disturbance. Sites were classified as undisturbed and included in the present study if they were: (i) located in river reaches where water flow and in-stream habitat were not impacted by man-made structures such as weirs or water diversion canals and pipes, (ii) located upstream of agricultural or residential areas, (iii) not invaded by alien fishes, and (iv) not isolated by natural barriers to dispersal (e.g. waterfalls). Following these criteria, data from 116 sites from 51 undisturbed mountain tributaries of the Breede, Duiwenhoks and Goukou River systems (Fig. 2.2) could be used to assess the factors influencing the distribution of *Galaxias* ‘nebula’, *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’.

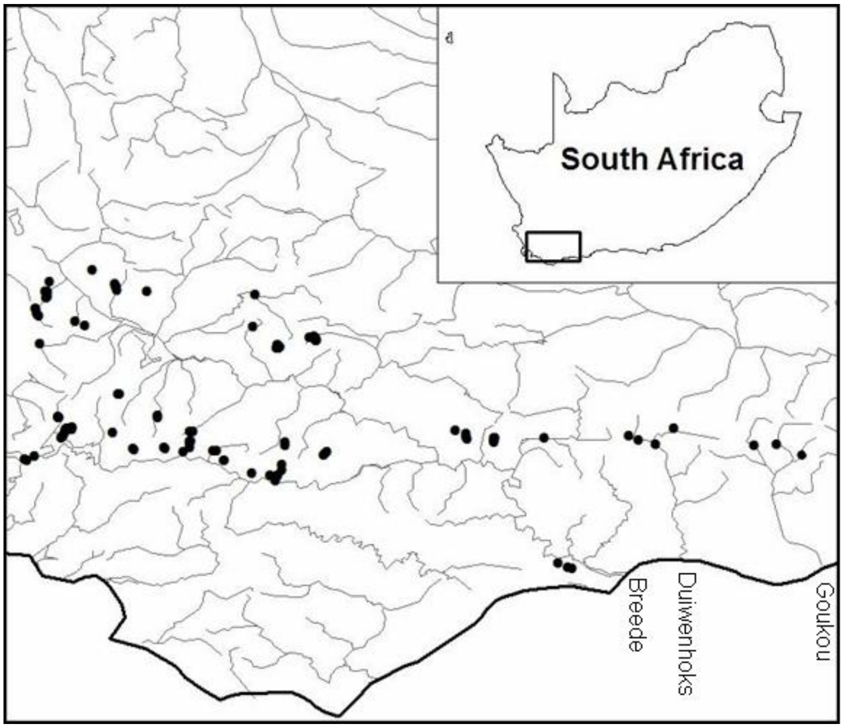

Fig. 2.2: Map showing the localities sampled for this study. The insert shows the location of the south-western Cape Floristic Region (CFR).

Sampling techniques varied depending on the size of the stream, depth and water clarity. Electrofishing (with a SAMUS-725MP electrofisher) was used for sampling in shallow riffle stream sections with cobble-boulder substratum, while the occurrence of fish in pools with clear water was determined by snorkelling and visual census. Deep tannin-stained pools were sampled with a seine net (3 m length, 3 mm mesh size). While there may be advantages in considering fish densities at each station rather than their presence or absence alone, the present study encompassed a wide geographic region. Time and resource constraints precluded assessment of fish densities at many of the localities sampled. Therefore, the presence-absence approach was used for the present study. *Galaxias* ‘nebula’, *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ at each site were either observed or captured using the methods described above. Captured fish were identified and quickly returned to the water alive, but at some of the localities up to ten individuals per species per tributary were retained for genetic analyses (see Chapters 4 & 5). The location of each sampling site was recorded with a hand-held Global Positioning System (GPS) receiver with accuracy within 10 m.

Morphological traits of the fish

To compare the morphological traits of *Galaxias* ‘nebula’, *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’, twenty adults of each taxon were measured for standard length (SL), maximum body depth (BD), maximum body width (BW) and pectoral fin length (PL). Aleev’s Y-distance (Aleev, 1969 cited by Chan, 2001 and Chuang *et al*., 2006) was measured as the distance from the anterior point of the snout to an imaginary vertical line at the point of the maximum depth. The height of the caudal fin was measured using vernier calipers and area of the caudal fin was determined by tracing the caudal fin on graph paper. These measurements were then used to calculate various morphological traits: compression index (CI), relative body depth (RBD), relative pectoral fin length (RPL), index of trunk shape (AleevY) and caudal fin aspect ratio (CFAR)). The sources, descriptions, formulae and interpretation of each of these metrics are given in Table 2.1. These traits were selected because they can predict fish adaptations to particular habitat conditions (Aleev, 1969 cited by Chan, 2001 and Chaung *et al*., 2006; Gatz, 1979a, b; Chan, 2001; Chuang *et al*., 2006; Leal *et al*., 2011).

Table 2.1: Calculations and interpretations of morphological traits examined in the present study. The morphological traits represent: compression index (CI), relative body depth (RBD), relative pectoral fin length (RPL), index of trunk shape (AleevY), and caudal fin aspect ratio (CFAR). The measurements: maximum body width (BW), maximum body depth (BD), standard length (SL), Y-distance, caudal fin height (CFH) and caudal fin area (CFA) are described in the text.

Morphological trait	Formula	Description and interpretation	References
CI	BW/BD	A low CI value indicates a comparatively laterally compressed body and is expected in fishes inhabiting lentic habitats or river reaches with slow-flowing waters.	Gatz (1979a)
RBD	BD/SL	Fishes with high RBD values have increased capacity to make vertical movements. High RBD values are expected in fish preferring habitats with slow current velocities.	Gatz (1979a)
RPL	PL/SL	Fishes with high RPL values are characterised by increased capabilities of maneuvering at low speeds, and inhabit slow-flowing waters.	Gatz (1979b)
AleevY	Y-distance/SL	The AleevY index is roughly a measure of the fish's hydrodynamic abilities. A high AleevY value indicates a comparatively more streamlined body shape and is characteristic of fishes with better swimming performances. High AleevY values are therefore expected in fishes capable of exploiting faster current velocities.	Aleev (1969) cited by Chan (2001) and Chuang *et al*., (2006); Videler & Wardle (1991)
CFAR	CFH^2/CFA	CFAR is a measure of caudal fin shape. Fishes with high CFARs have comparatively more lunate or forked caudal fins which generally increase the amount of thrust and reduce drag while in motion. This results in better swimming performance. Thus high CFAR values are expected in fishes that are capable of exploiting reaches with faster flowing waters. Low values of CFAR on the other hand indicate more square shaped tails. Fishes with low CFAR values generally suffer high drag penalties, and thus have poorer swimming performances. Low CFAR values are therefore expected in fishes inhabiting slow-flowing waters.	Gatz (1979b); Chan (2001); Videler (1993)

Potential predictors

At each sampling locality, habitat was characterised by quantitative and qualitative measurements of nine environmental variables (Table 2.2). Portable electronic meters were used to measure temperature and conductivity (Hanna EC/TDS/Temperature Tester, HI98311 (DiST 5)) and pH (Hanna pH/Temperature tester HI98128). Local habitat features were characterised by measuring channel width and depth, and by assessing bottom substratum and aquatic vegetation. At each locality, 4 to 8 transects were set up to measure these physical habitat variables. Depth was measured with a graduated pole at three equally spaced intervals for each transect. Maximum depth was the greatest water depth measured among transects. Transect widths were used to calculate mean wetted width (used here as proxy for stream size) for each sampling locality. Dominant substratum was visually estimated and characterised as silt-sand (< 2 mm), gravel (10 – 64 mm), cobble (64 - 256 mm), boulders (> 256 mm) and bedrock (solid rock surfaces) following Bain *et al*. (1985) and Gibson *et al*. (1998). Dominant substratum was categorized into 6 classes (Table 2.2). Aquatic vegetation was also visually assessed and characterised as none (0), scarce (< 30%), moderate (30 – 60%) and abundant (> 60%). Elevation and channel slope for each site were calculated from longitude and latitude data using GIS Spatial Analyst. Following Oberdorff *et al*. (2001), water velocity at each site was estimated using the formula:

$$V = \log WID + \log DEP + \log SLO - \log(WID + 2DEP)$$

where V = velocity, WID = mean wetted width, DEP = maximum depth and SLO = slope.

Data analysis

Indices such as forage ratios, selectivity or preference indices that compare the resources or habitats used with those available are crucial for evaluating the non-randomness of a species' distribution or resource selection (e.g. Manly *et al*., 1993). In the present study, Manly *et al*.'s (1993) index, β_i, was used to calculate habitat preferences for each species. The index is given by:

$$\beta_i = \frac{\alpha_i}{\sum_{j=1}^{K} \alpha_j}$$

where β_i is the preference for habitat type *i*, α_i is the proportion of habitat type *i* used for category *j*, and the denominator represents the sum of all proportions of the *K* categories of habitat type *i*. Manly's index ranges from 0 to 1, with $\beta_i > \frac{1}{K}$ representing positive selection, $\beta_i < \frac{1}{K}$ indicating avoidance and $\beta_i = \frac{1}{K}$ signifying random selection of habitat type *i* (Yeates & Barmuta, 1999; Manly *et al*., 2002). The variables were categorised prior to analyses (Table 2.2).

Table 2.2: The categories and frequencies of the environmental variables measured in minimally disturbed headwater tributaries of the Breede, Duiwenhoks and Goukou River systems.

Variables	Categories	Frequency	Variables	Categories	Frequency
Maximum depth (m)	< 0.2	4	Conductivity (µS/cm)	< 50	36
	0.2 – 0.5	26		50 - 100	46
	0.5 - 1	51		100 - 200	14
	> 1	35		> 200	9
Mean wetted width (m)	< 2	27	Temperature (^{o}C)	< 20	43
	2 - 5	65		20 - 25	52
	> 5	24		> 25	5
Gradient (m/km)	<5	35	Aquatic vegetation	No (NO)	95
	5 - 10	30		Scarce (SC)	13
	10 - 20	33		Moderate (MO)	5
	> 20	18		Abundant (AB)	3
Elevation (m.a.s.l.)	< 100	3	Dominant substratum	Silt-Mud (SM)	5
	100 - 200	16		Sand (SA)	8
	200 - 400	61		Cobble (CO)	18
	> 400	36		Cobble-Gravel (CG)	15
				Cobble-Boulder (CB)	28
pH	< 5	16		Boulder-Bedrock (BB)	42
	5 - 7	81			
	> 7	8			

Logistic regression models were used to describe the relationships between species occurrence and habitat variables. Logistic regression has been used to describe fish-habitat relationships at a variety of scales (e.g. Rich *et al*., 2003; Morán-López *et al*., 2005; Amadio *et al*., 2005). It was appropriate for modelling species responses in the present study because of the binomial nature of the response variables (presence/absence) (Manly *et al*., 1993; Boyce *et al*., 2002). Advantages of logistic regression are that it requires limited assumptions regarding normality or homocedasticity (Hosmer & Lemeshow, 1989; Trexler & Travis, 1993). These assumptions are rarely met for ecological data due to the patchy distribution of organisms (Trexler & Travis, 1993).

Furthermore, because logistic regression analysis gives the probability of occurrence of a species as a function of an environmental variable(s), it is ideal for modelling the presence of a species in unsampled areas (Manly *et al.*, 1993). Logistic regression modelling may therefore be used to predict geographic patterns of occurrence of species from regions where survey data are limited. This approach may therefore provide information to predict past distributions of species in systems where distribution patterns may have been altered. The form of the logistic regression is given by:

$$P(x) = \frac{e^{\text{logit}}}{1 + e^{\text{logit}}}$$

where $P\ (x)$ is the probability of occurrence of the species as a function of a measured environmental variable x, logit is the linear regression model: Logit = $\beta_0 + \beta_1 X_1 + \beta_2 X2 + \dots \beta_p X_p$, β_0 is the regression constant or intercept, β_i are regression coefficients and X_p are the measured environmental variables (Manly *et al.*, 2002).

Logistic regression models were computed separately for each species with the presence (1) or absence (0) of individual species as dependent variables and the environmental variables as the independent variables using SPSS version 16.0 (SPSS Inc.). Inclusion of environmental variables in logistic models was determined by first comparing the significance of differences of the values of each variable between localities where fish were present and those from localities where fish were absent using Mann-Whitney tests (for continuous variables) or a chi-square tests (for categorical variables). Non-significant variables (pH, conductivity, temperature, aquatic vegetation and dominant substratum) were excluded from further analyses.

Separate single analyses were performed to relate the presence or absence of each species to each environmental variable that was found to differ significantly between localities with and without fish (i.e. mean wetted width, maximum depth, gradient and elevation). Likelihood ratio tests were used to assess the significance of regression parameters (β_i) in explaining the variation in the dependent variables. Chi-square (χ^2) goodness-of-fit tests were to assess the fit of the models to the data (Hosmer & Lemeshow, 1989; Trexler & Travis, 1993). Significance of the environmental variables was assessed using the Wald test (Tabachnik & Fidell, 1996). Odds ratios were used to evaluate the relative importance of the variables. Ratios with confidence intervals that did not include 1 were considered to be statistically significant (Hosmer & Lameshow, 1989). Model predictive success was evaluated using the percent reclassification success of fish presence or absence using the original data. Probability plots were developed for each species and each of the four environmental variables separately to illustrate species responses to the environmental variables.

Next, multiple stepwise logistic regressions were performed to determine the most influential variables for predicting the probability of occurrence of each species. Multiple stepwise logistic regressions were performed using significant variables for each species from the single regressions. Each variable had a probability of entry of 0.05 and removal of 0.10. Because multiple logistic regression is sensitive to multicollinearity (Trexler &

Travis, 1993), Pearson's correlation coefficients were computed to examine correlations among the habitat descriptors. Leathwick *et al*. (2005) recommended that highly-correlated variables ($r > 0.7$) should be excluded from analyses to reduce redundancy. The same tests and procedures used for the single regressions were used for assessing goodness-of-fit of the models to the data, and testing the significance of the regression parameters and significance of the variables. Multivariate probability plots were developed using the significant variables detected from multiple logistic regression analysis to allow for a simultaneous view of a species' responses along more than one dimension.

Principal components analysis (PCA) was performed on the morphological traits of the species to observe their distribution in morphological space. One-way analysis of variance (ANOVA) was used to test whether there were significant differences in morphological traits among the species. If significant differences were detected, Tukey post-hoc tests were performed to test for pairwise differences among the species. All analyses were performed using SPSS version 16.0 (SPSS Inc.) and PAST (Hammer *et al*., 2001).

2.3. Results

Galaxias 'nebula' was the most widespread of the three lineages, occurring in 68 of the 116 sites and 39 of the 51 undisturbed streams. *Pseudobarbus* 'Breede' was more restricted, but was encountered in 75 of the 116 sites and 33 of the 51 undisturbed streams. *Sandelia* ‘eastern’ only occurred in 40 of the 116 sites and 24 of the 51 streams (Fig. 2.3).

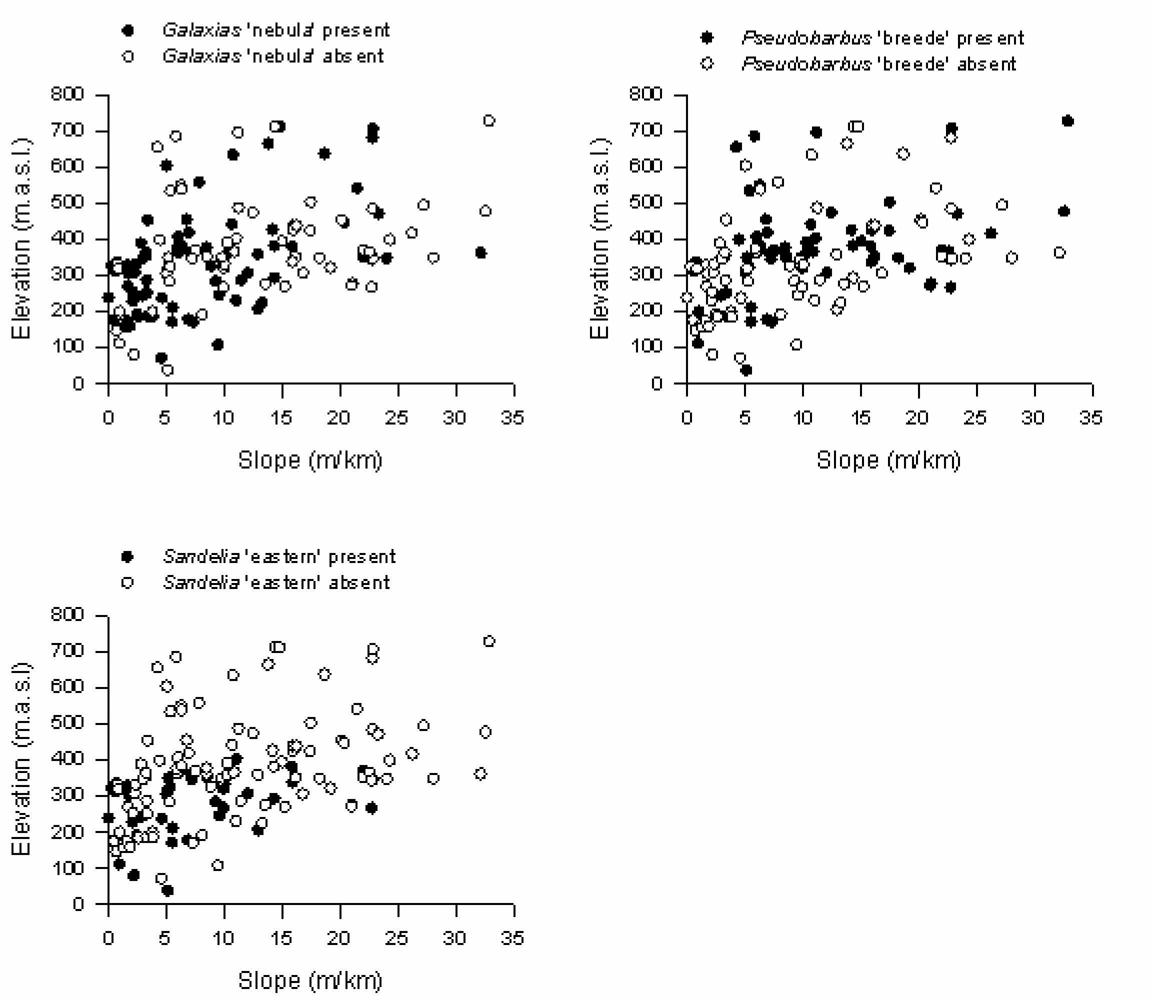

Fig. 2.3: Plots of the longitudinal distribution patterns of *Galaxias* ‘nebula’, *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ across upland tributaries of the Breede, Duiwenhoks and Goukou River systems.

Scatter plots depicting the distribution patterns of the individual lineages in relation to elevation and stream gradient revealed substantial variation among lineages (Fig. 2.3). The plots showed a wider dispersion of points for *Galaxias* ‘nebula’ and *Pseudobarbus* ‘Breede’, indicating that these two lineages occur at diverse elevations and a wider range of slopes compared to *Sandelia* ‘eastern’. *Sandelia* ‘eastern’ was restricted to reaches with low elevation (< 425 metres above sea-level (m.a.s.l.)) and low gradient (< 25 m/km) (Fig. 2.3). Cumulative percentage trajectories of the lineages confirmed these differences in longitudinal distribution patterns of the lineages, with *Galaxias* ‘nebula’ and *Pseudobarbus* ‘Breede’ penetrating further upstream than *Sandelia* ‘eastern’ (Fig. 2.4).

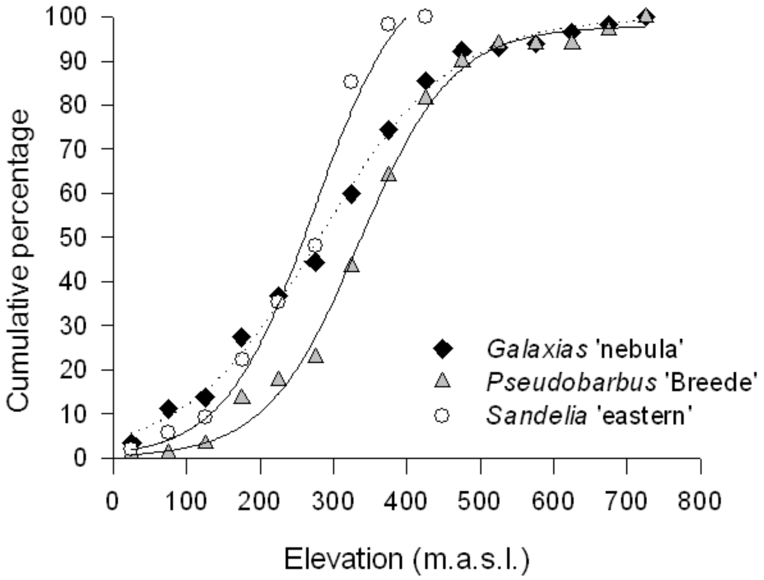

Fig. 2.4: Cumulative percentage frequencies of sites with *Galaxias* ‘nebula’, *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ along the longitudinal gradients of upland streams in the south-western Cape Floristic Region (CFR).

Habitat preference indices revealed clear differences in microhabitat use among the lineages (Fig. 2.5 – 2.7). *Galaxias* ‘nebula’ exhibited random use of habitats, inhabiting a range of water depths, gradient, elevation, pH, conductivity, temperature, bottom substratum and occupying sites with no aquatic vegetation, as well as those with dense macrophytes (Fig. 2.5). This lineage only showed moderate avoidance of wider streams (> 5m).

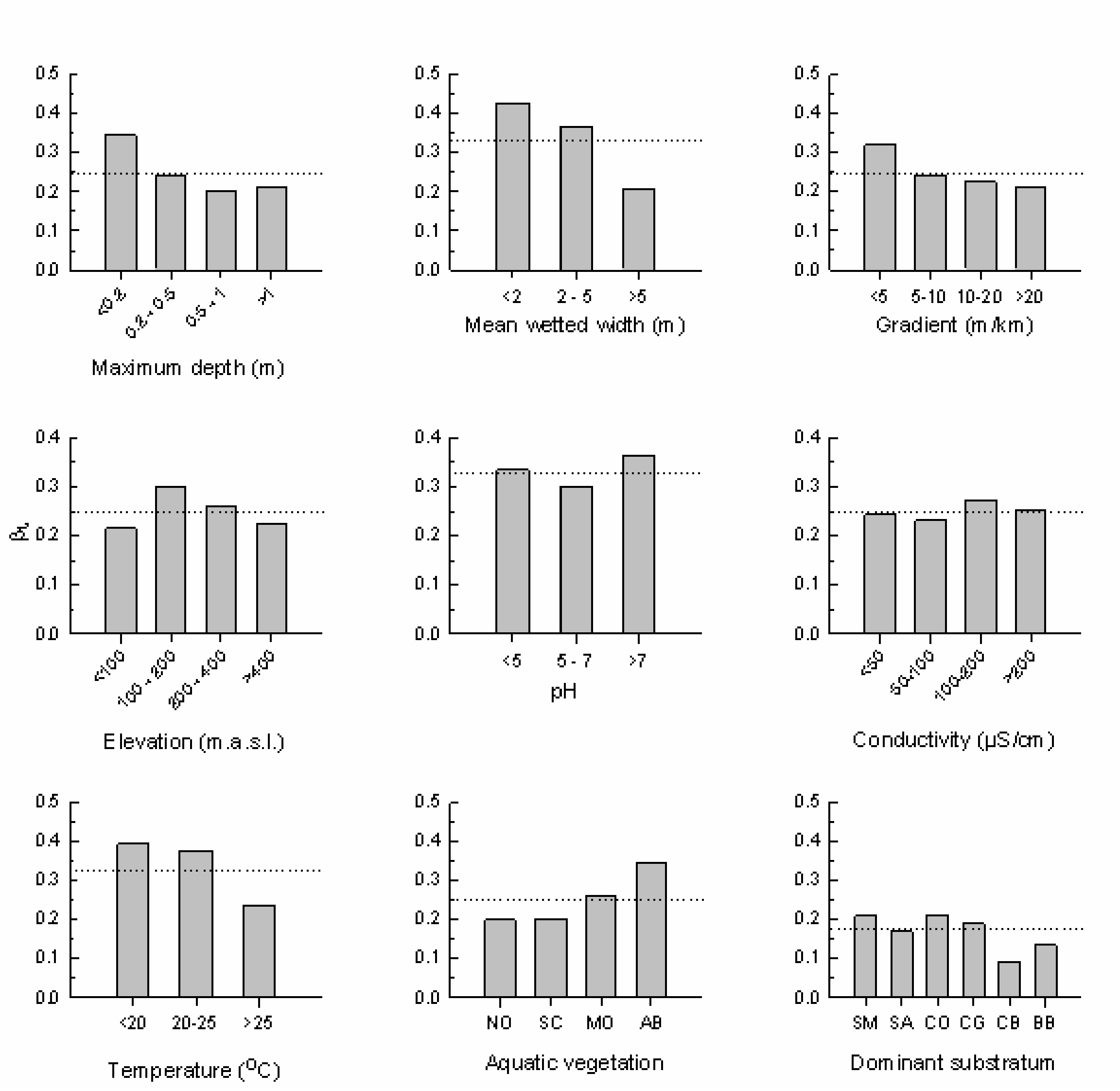

Fig. 2.5: Manly’s indices (*βi*) for *Galaxias* ‘nebula’ for nine environmental variables considered in the present study. The dotted horizontal line represents random use (no preference/avoidance).

By contrast, *Pseudobarbus* ‘Breede’ showed strong avoidance of shallow habitats (< 0.2 m maximum depth) and smaller streams (< 2 m mean wetted width) (Fig. 2.6). This lineage was absent from the eastern catchments of the Duiwenhoks and Goukou River systems that have dense macrophytes and fine substratum. *Pseudobarbus* ‘Breede’ was slightly under-represented in sites at lower elevations (< 100 m.a.s.l), sites with low pH (< 5) and higher water conductivities (> 200 µS/cm) (Fig. 2.6).

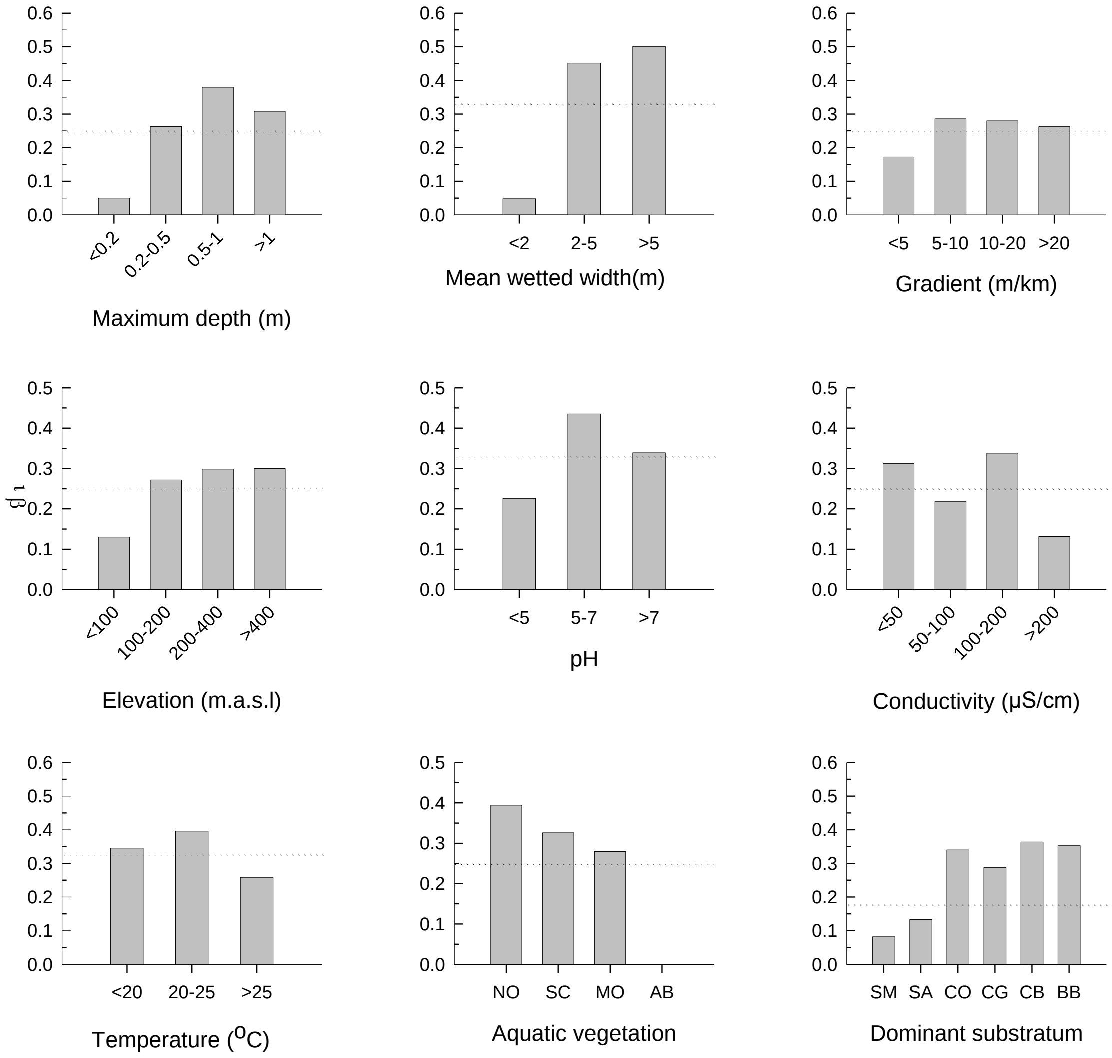

Fig. 2.6: Manly’s indices (*βi*) for *Pseudobarbus* ‘Breede’ for nine environmental variables considered in the present study. The dotted horizontal line represents random use (no preference/avoidance).

Similar to *Pseudobarbus* ‘Breede’, *Sandelia* ‘eastern’ also showed strong avoidance of shallow habitats and smaller streams (Fig. 2.7). However, *Sandelia* ‘eastern’ differed from both *Galaxias* ‘nebula’ and *Pseudobarbus* ‘Breede’ in its strong avoidance of higher elevations (> 425 m.a.s.l) and steeper gradients (> 20 m/km) (Fig. 2.7).

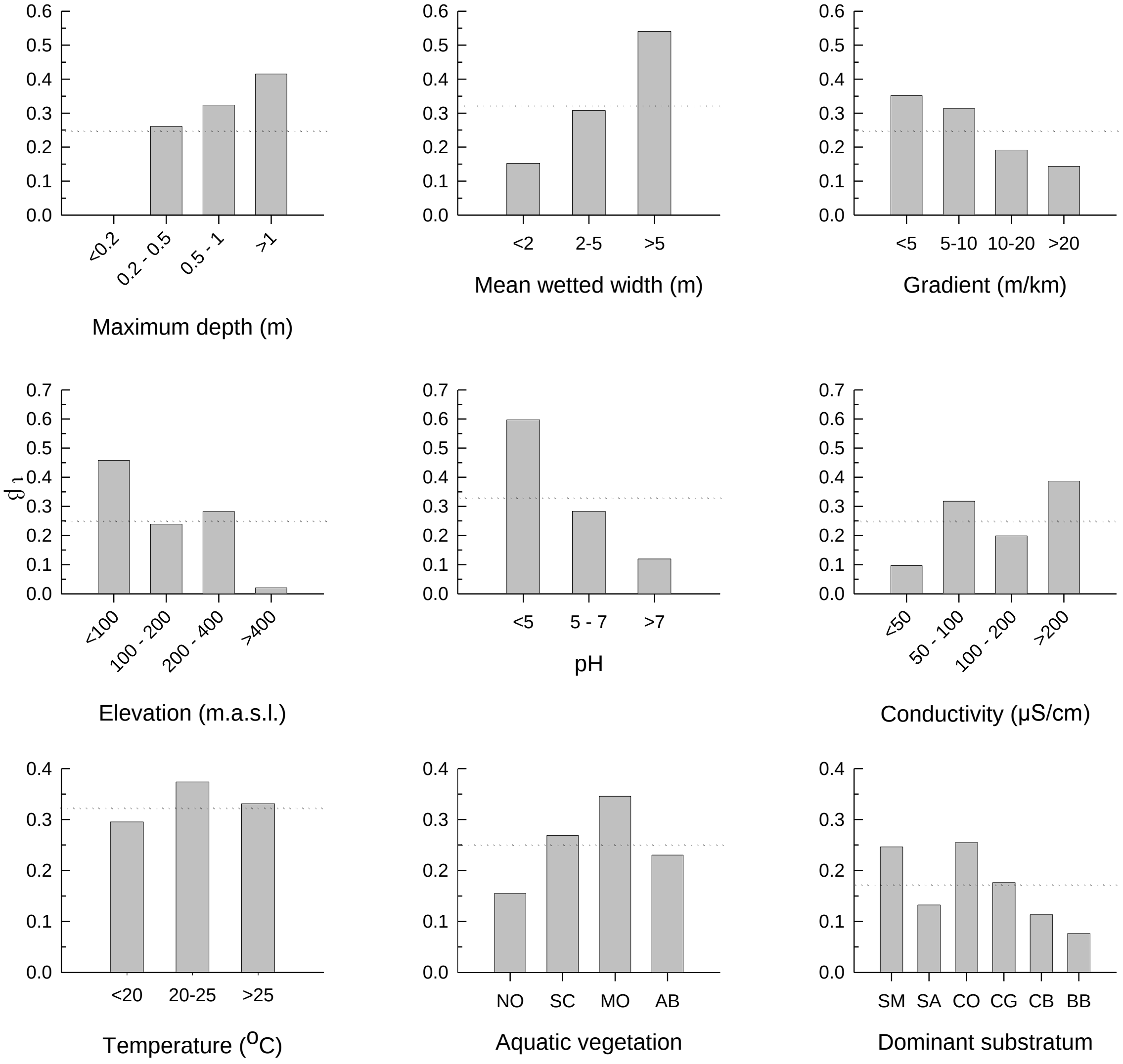

Fig. 2.7: Manly’s indices (βi) for *Sandelia* ‘eastern’ for nine environmental variables considered in the present study. The dotted horizontal line represents random use (no preference/avoidance).

Table 2.3: Results of simple logistic regression analyses of habitat variables influencing the presence and absence of *Galaxias* ‘nebula’, *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ from the south-western Cape Floristic Region (CFR). * Indicates statistically significant variables.

	Parameter estimate	Standard error	Wald χ^2	*P*	Odds ratio	95% CI for odds ratio	Goodness-of-fit test				Resubmission	
							χ^2	DF	*P*	R^2	+ve	-ve
Galaxias ‘nebula’												
Mean wetted width	-0.410	0.112	13.401	0.000*	0.663	(0.533, 0.826)	17.573	1	0.000*	0.189	84%	40%
Depth	-0.006	0.004	2.095	0.148	0.994	(0.987, 1.002)	2.126	1	0.145	0.024	88%	13%
Elevation	-0.001	0.001	0.936	0.333	0.999	(0.996, 1.001)	0.944	1	0.331	0.011	94%	8%
Gradient	-0.042	0.025	2.938	0.087	0.958	(0.913, 1.006)	3.012	1	0.083	0.035	85%	21%
Pseudobarbus ‘Breede’												
Mean wetted width	1.154	0.249	21.488	0.000*	3.171	(1.947, 5.166)	43.491	1	0.000*	0.445	94%	70%
Depth	0.025	0.007	12.962	0.000*	1.025	(1.011, 1.039)	18.618	1	0.000*	0.212	92%	43%
Elevation	-0.002	0.001	1.283	0.257	0.998	(0.996, 1.001)	1.290	1	0.256	0.016	100%	0%
Gradient	-0.005	0.026	0.042	0.837	0.995	(0.946, 1.046)	0.042	1	0.837	0.001	100%	0%
Sandelia ‘eastern’												
Mean wetted width	0.304	0.101	9.144	0.002*	1.355	(1.113, 1.650)	10.673	1	0.001*	0.123	29%	90%
Depth	0.012	0.004	7.201	0.007*	1.012	(1.003, 1.021)	7.765	1	0.005*	0.091	24%	92%
Elevation	-0.010	0.002	19.555	0.000*	0.990	(0.985, 0.994)	31.845	1	0.000*	0.335	43%	83%
Gradient	-0.089	0.031	8.346	0.004*	0.915	(0.861, 0.972)	9.981	1	0.002*	0.115	31%	80%

The results of simple logistic regression analyses for *Galaxias* ‘nebula’, *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ are presented in Table 2.3. Mean wetted width was the only statistically significant predictor of *Galaxias* ‘nebula’ occurrence (χ^2-goodness of fit = 17.573, d.f. = 1, P = 0.00, Nagelkerke’s R^2 = 0.189, 66% overall correct classification).

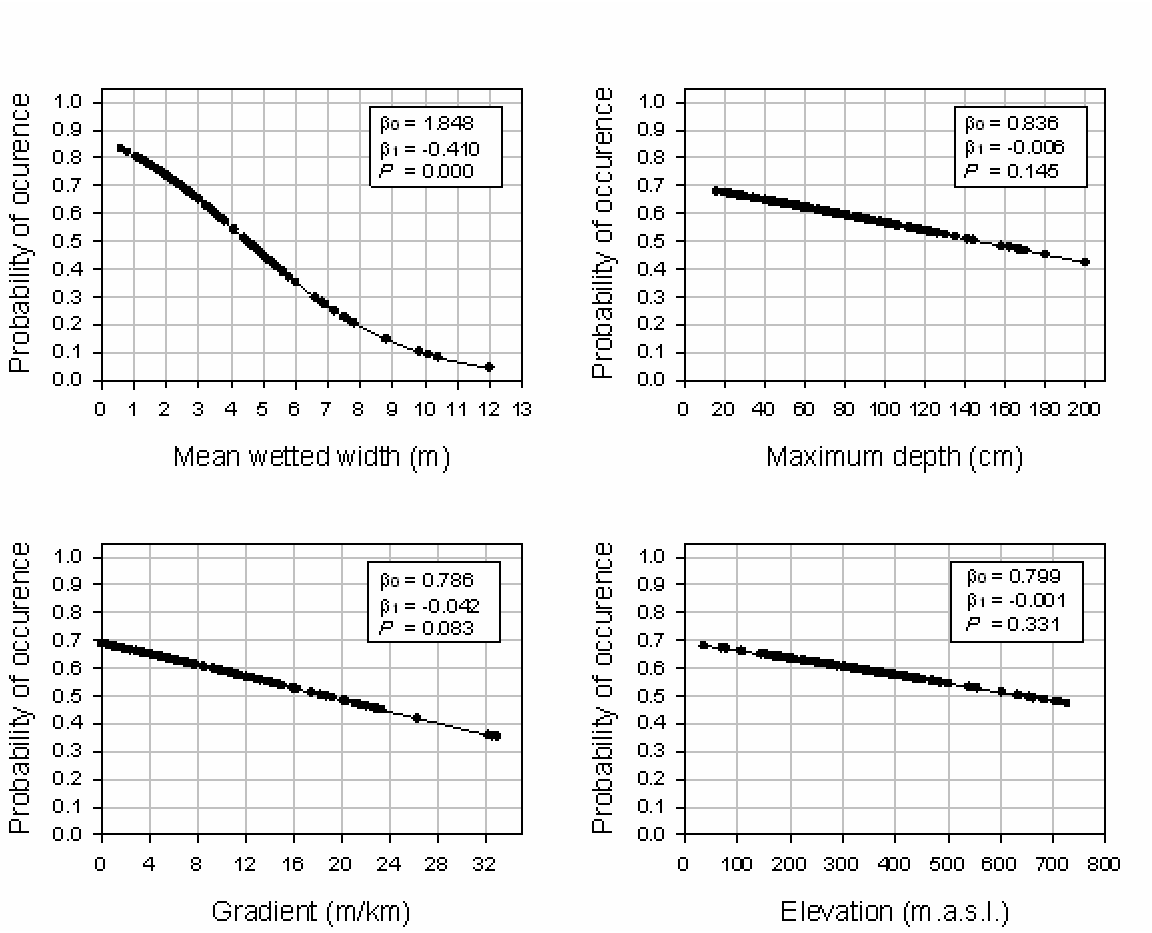

Fig. 2.8: Predicted probabilities of occurrence of *Galaxias* ‘nebula’ as a function of mean wetted width, depth, gradient and elevation determined using logistic regression.

The probability of occurrence of *Galaxias* ‘nebula’ was predicted to decrease as stream size increases (Fig. 2.8). The other three models showed poor fit to the data: maximum depth (χ^2-goodness of fit = 2.126, d.f. = 1, P = 0.145, Nagelkerke’s R^2 = 0.024, 57% overall correct classification), elevation (χ^2-goodness of fit = 0.944, d.f. = 1, P = 0.331, Nagelkerke's R^2 = 0.011, 59% correct classification) and gradient (χ^2-goodness of fit = 3.012, d.f. = 1, P = 0.083, Nagelkerke's R^2 = 0.035, 59% correct classification) (Table 2.3).

Only two of the predictors (mean wetted width and maximum depth) had significant odds ratios (i.e. their confidence intervals did not include 1) for *Pseudobarbus* 'Breede' (Table 2.3). Increasing probability of occurrence of *Pseudobarbus* 'Breede' was predicted by increasing stream size (indexed by mean wetted width) (χ^2-goodness of fit = 43.491, d.f. = 1, P = 0.000, Nagelkerke's R^2 = 0.445, 86% overall correct classification) and increasing depth (χ^2-goodness of fit = 18.618, d.f. = 1, P = 0.000, Nagelkerke's R^2 = 0.212, 76% overall correct classification) (Table 2.3; Fig. 2.9). Logistic regression showed that elevation and gradient were poor predictors of *Pseudobarbus* 'Breede' occurrence: elevation (χ^2-goodness of fit = 1.290, d.f. = 1, P = 0.256, Nagelkerke's R^2 = 0.016, 67% overall correct classification) and gradient (χ^2-goodness of fit = 0.042, d.f. = 1, P = 0.837, Nagelkerke's R^2 = 0.001, 67% overall correct classification) (Table 2.3; Fig. 2.9).

For *Sandelia* 'eastern', all four variables (mean wetted width, maximum depth, elevation and gradient) had statistically significant odds ratios (Table 2.3), indicating that they were all good predictors of *Sandelia* 'eastern' occurrence.

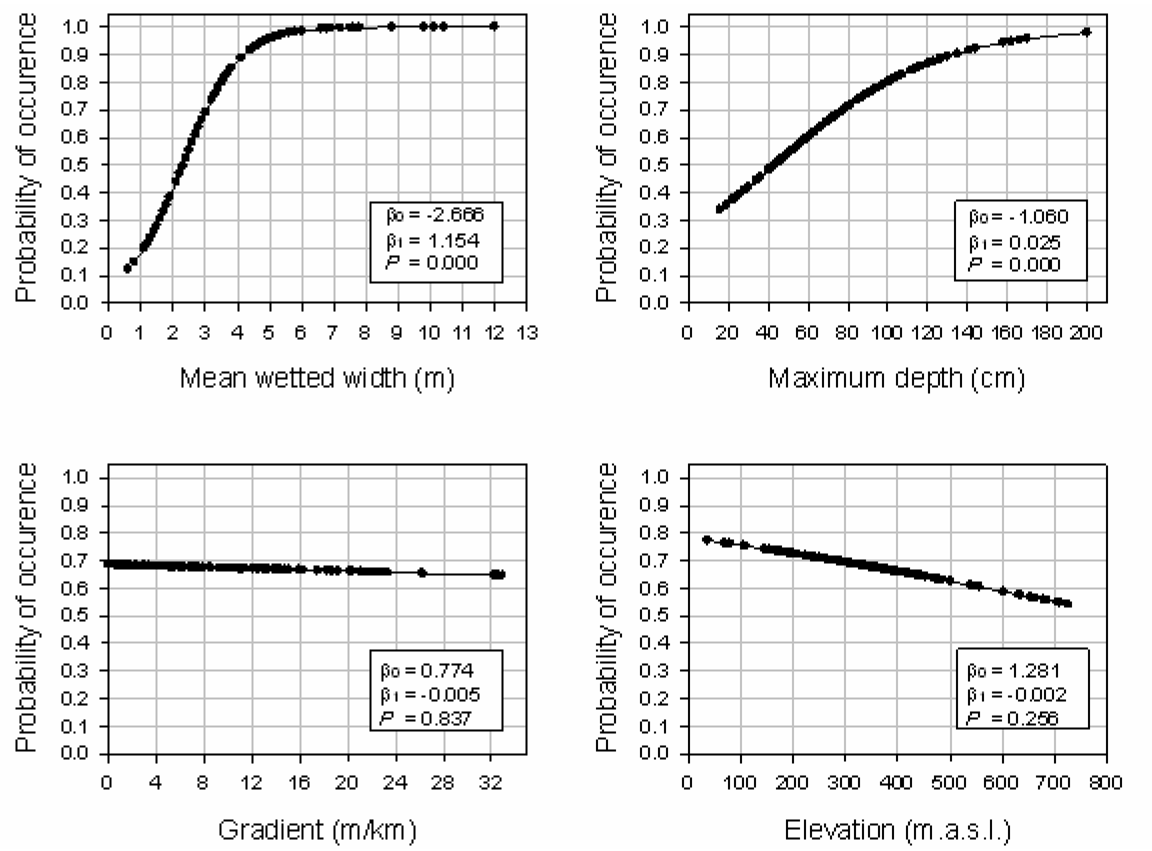

Fig. 2.9: Predicted probabilities of occurrence of *Pseudobarbus* 'Breede' as a function of mean wetted width, depth, gradient and elevation determined using logistic regression.

Increasing probability of occurrence of *Sandelia* 'eastern' was associated with increasing stream size (χ^2-goodness of fit = 10.673, d.f. = 1, P = 0.000, Nagelkerke's R^2 = 0.123, 67% overall correct classification) and increasing depth (χ^2-goodness of fit = 7.765, d.f. = 1, P = 0.005, Nagelkerke's R^2 = 0.091, 66% overall correct classification), whereas the probability of occurrence of this lineage was predicted to decrease with increasing elevation (χ^2-goodness of fit = 31.845, d.f. = 1, P = 0.000, Nagelkerke's R^2 = 0.335, 68% overall correct classification) and increasing gradient (χ^2-goodness of fit = 9.981, d.f. = 1, P = 0.002, Nagelkerke's R^2 = 0.115, 62% overall correct classification) (Table 2.3, Fig. 2.10).

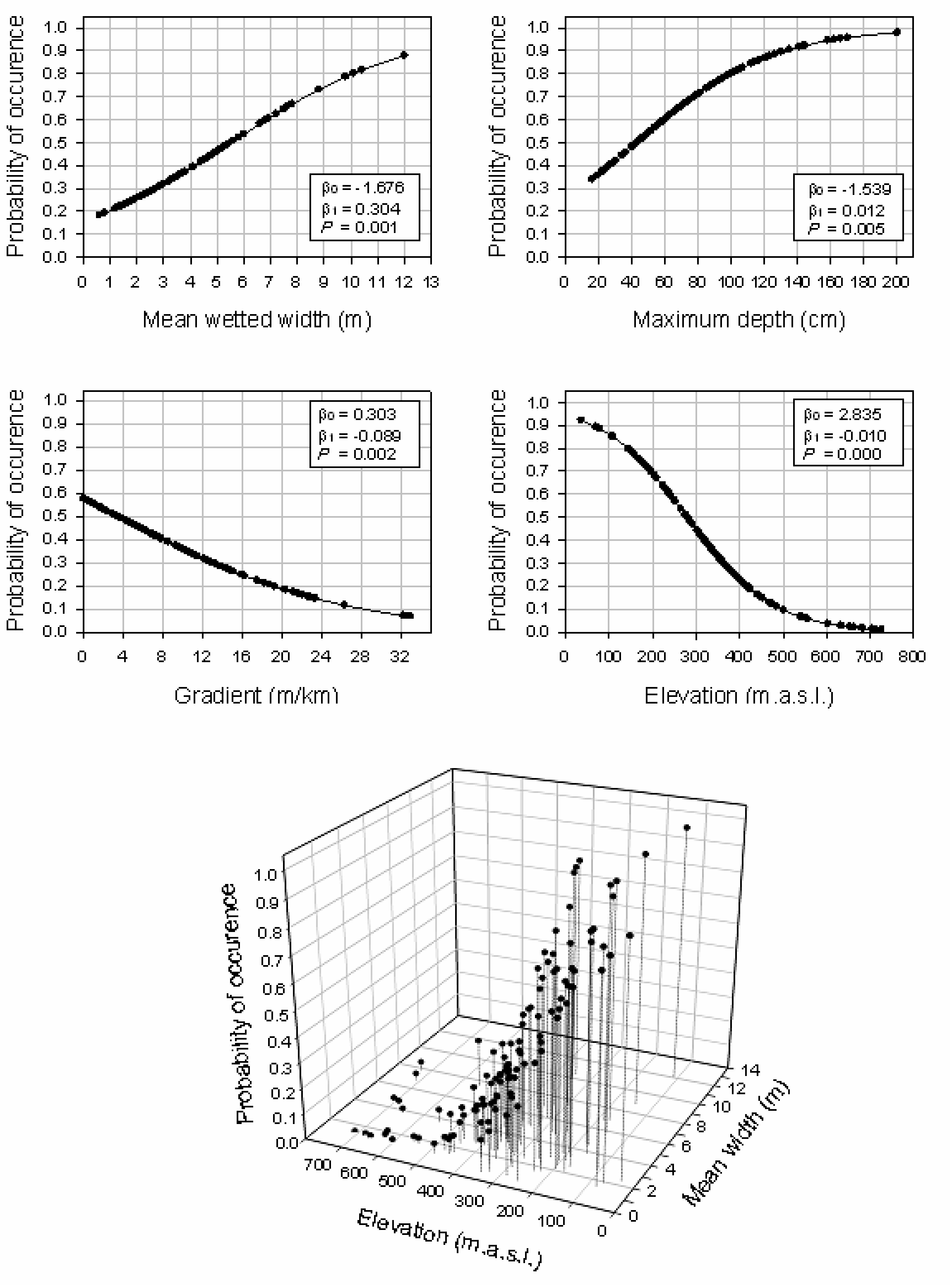

Fig. 2.10: Predicted probabilities of occurrence of *Sandelia* 'eastern' as a function of mean wetted width, depth, gradient and elevation determined using logistic regression.

Significant variables from the simple logistic regressions were used to construct multiple stepwise logistic regressions to determine the minimal adequate set of variables for predicting each lineage's probability of occurrence. Multiple logistic regression analysis was not done for *Galaxias* 'nebula' because only one variable (mean wetted width) was found to be statistically significant from the simple logistic regressions (Table 2.3).

For *Pseudobarbus* 'Breede', the multiple logistic regression model contained only two significant variables (mean wetted width and maximum depth), whereas for *Sandelia* 'eastern' the model contained all four variables (mean wetted width, maximum depth, elevation and gradient). Pearson's correlation coefficients for these four variables were less than 0.7 (Table 2.4). As a result no variables were excluded from analysis.

Table 2.4: Pearson's correlation coefficients for the four variables that differed significantly between localities with fish and those without fish.

	Variable	1	2	3
1	Mean wetted width			
2	Maximum depth	0.457		
3	Elevation	-0.074	-0.044	
4	Gradient	-0.055	0.109	0.464

Mean wetted width was found to be the most important predictor of *Pseudobarbus* 'Breede' occurrence in the multiple logistic regression model (Table 2.5). Odds ratios of maximum depth were not statistically significant, despite this variable being a significant predictor in the simple logistic regression analysis. Inclusion of maximum depth in a model containing mean wetted width did not improve prediction of *Pseudobarbus* 'Breede' presence (Tables 2.5).

For *Sandelia* 'eastern', the logistic regression model containing mean wetted width, maximum depth, elevation and gradient had a 62% correct classification rate for sites with this lineage, with mean wetted width and elevation being the significant predictors of *Sandelia* 'eastern' presence (Table 2.5). Inclusion of gradient and maximum depth in a model containing mean wetted width and elevation did not improve classification success for *Sandelia* 'eastern'. A combined plot of these two variables, giving a simultaneous view of their influence on *Sandelia* 'eastern' occurrence (Fig. 2.10), shows that the probability of occurrence increases with decreasing elevation and increasing stream size (mean wetted width) (χ^2-goodness of fit = 42.983, d.f. = 2, $P = 0.000$, Nagelkerke's R^2 = 0.432, 74% overall correct classification).

Table 2.5: Results of multiple logistic regression analyses of habitat variables influencing the presence and absence of *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ from the south-western Cape Floristic Region (CFR). * Indicates statistically significant variables.

	Intercept	Mean wetted width	Maximum depth	Elevation	Gradient	Resubmission	
						Positive	Negative
Pseudobarbus ‘Breede’	overall goodness-of-fit test: χ^2 = 44.857; d.f. = 2; P = 0.000; R^2 = 0.457; 86% overall correct classification						
Parameter estimate	-2.925	1.032	0.008			92%	73%
Standard error	0.739	0.263	0.007				
Wald χ^2	15.676	15.447	1.291				
P	0.000	0.000*	0.256				
Odds ratio	-	2.808	1.008				
95% CI for odds ratio	-	(1.678,4.699)	(0.994,1.022)				
Sandelia ‘eastern’	overall goodness-of-fit test: χ^2 = 45.483; d.f. = 4; P = 0.000; R^2 = 0.452; 75% overall correct classification						
Parameter estimate	1.180	0.303	0.008	-0.010	-0.031	62%	83%
Standard error	0.892	0.142	0.006	0.003	0.038		
Wald	1.751	4.575	2.111	14.130	0.662		
P	0.186	0.032*	0.146	0.000*	0.416		
Odds ratio	-	1.354	1.009	0.990	0.969		
95% CI for odds ratio	-	(1.026, 1.787)	(0.997, 1.020)	(0.985, 0.995)	(0.900, 1.045)		

Variation in morphological traits

The mean values for morphological traits of *Galaxias* ‘nebula’ *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ measured in this study are presented in Table 2.6. ANOVA results indicated that the three lineages differ significantly in all five morphological traits measured (Table 2.6). *Galaxias* ‘nebula’ has significantly higher compression index (CI) and AleevY values than both *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’, while *Sandelia* ‘eastern’ has significantly higher relative body depth (RBD) and relative pectoral fin length (RPL) than both *Galaxias* ‘nebula’ and *Pseudobarbus* ‘Breede’ (Table 2.6). With the exception of the caudal fin aspect ratio (CFAR) which is significantly higher in *Pseudobarbus* ‘Breede’ than *Galaxias* ‘nebula’ and *Sandelia* ‘eastern’, results of the other four metrics indicate that *Pseudobarbus* ‘Breede’ has a body form that is intermediate to that of *Galaxias* ‘nebula’ and *Sandelia* ‘eastern’ (Table 2.6).

Table 2.6: Comparison of morphological traits (compression index (CI), relative body depth (RBD), relative pectoral fin length (RPL), AleevY and caudal fin aspect ratio (CFAR)) (mean ± SD) between *Galaxias* ‘nebula’, *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ from the Breede, Duiwenhoks and Goukou River systems (n = 20 for each species). * All post-hoc pairwise comparisons using Tukey’s test were statistically significant (P < 0.001).

Morphological trait	*Galaxias* ‘nebula’	*Pseudobarbus* ‘Breede’	*Sandelia* ‘eastern’	*F*-value	*P*-value
CI	0.805 ± 0.091	0.682 ± 0.042	0.557 ± 0.053	71.309	0.000*
RBD	0.119 ± 0.014	0.230 ± 0.017	0.320 ± 0.028	471.967	0.000*
RPL	0.123 ± 0.015	0.189 ± 0.022	0.233 ± 0.033	103.551	0.000*
AleevY	0.652 ± 0.021	0.476 ± 0.029	0.441 ± 0.026	391.214	0.000*
CFAR	0.827 ± 0.223	1.596 ± 0.186	1.041 ± 0.205	74.752	0.000*

The PCA analysis reduced the five morphological measures to two components explaining 91.8% of the total variance in the considered aspects of morphology of the three species (Table 2.7). The first principal component axis (PCA axis 1) explained almost three-quarters of the variance (eigen value = 3.636; variance = 72.7%) and was found to be determined by relative body depth (RBD), AleevY, relative pectoral fin length (RPL) and compression index (CI) (Table 2.7). Caudal fin aspect ratio (CFAR) was found to load heavily on the second principal component axis (eigen value = 0.952, variance = 19.044) (Table 2.7).

Table 2.7: Factor loadings, eigen values and variances from the principal components analysis for morphological traits of *Galaxias* 'nebula', *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' from the Breede, Duiwenhoks and Goukou River systems (n=20 for each species).

Morphological trait	Principal component	
	1	2
Compression index (CI)	-0.464	0.313
Relative body depth (RBD)	0.501	-0.192
Relative pectoral fin length (RPL)	0.488	-0.096
AleevY	-0.492	-0.160
Caudal fin aspect ratio (CFAR)	0.230	0.911

PCA axis	Eigen value	% Total variance	Cumulative eigen value	Cumulative eigen value %
1	3.636	72.711	3.636	72.711
2	0.952	19.044	4.585	91.755

The differences in morphological traits resulted in three distinct clusters segregated on the first two PCA axes (Fig. 2.11). The cluster of the species with the highest AleevY and CI values (*Galaxias* ‘nebula’) had negative values along the first PCA axis, while the species with the largest RBD and RPL (*Sandelia* ‘eastern’) had positive values along this axis. The cluster of the species with high CFAR (*Pseudobarbus* ‘Breede’) occurred in the region of higher scores on the second PCA axis.

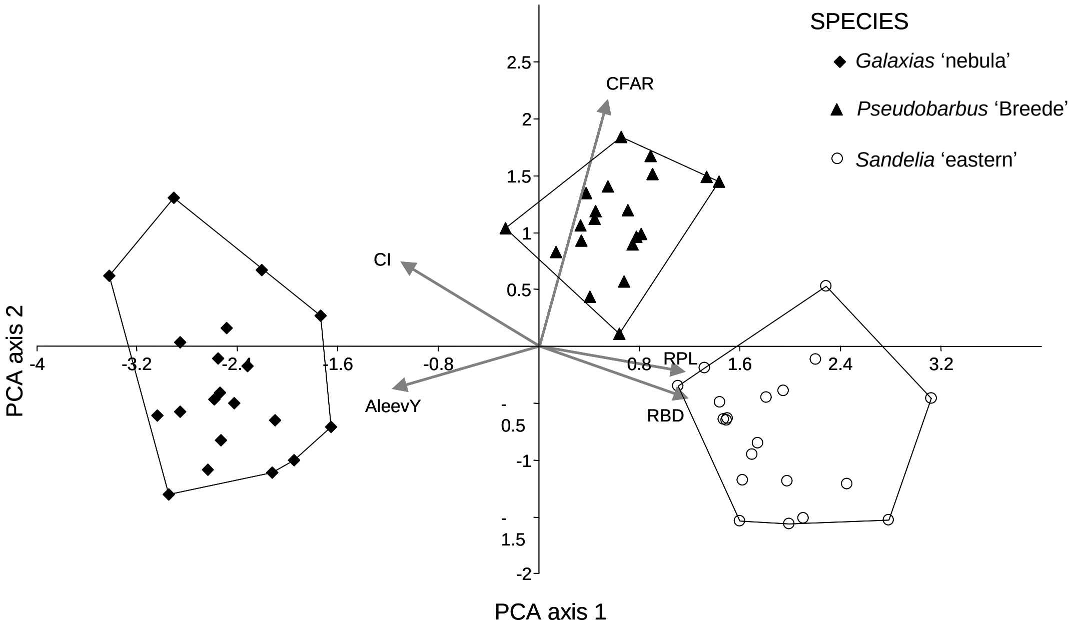

Fig. 2.11: PCA scatter plot of *Galaxias* ‘nebula’, *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ based on five morphological traits namely AleevY, compression index (CI), caudal fin aspect ratio (CFAR), relative pectoral fin length (RPL) and relative body depth (RBD). The arrows indicate the direction and strength of the factor loadings of the variables.

2.4. Discussion

Comparative habitat use and past distributions

Results of the present study revealed clear differences in habitat use among the three species. *Galaxias* 'nebula' seemed to be less particular about its habitat use as individuals of this species were collected from almost all habitat types sampled in the present study. This species was capable of exploiting smaller and shallower streams and penetrated to higher elevations than both *Pseudobarbus* 'Breede' and *Sandelia* 'eastern'. Both *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' preferred deeper habitats in wider streams, and both avoided smaller streams and shallow habitats. The two species, however, differed in that *Pseudobarbus* 'Breede' occurred at localities with a wider range of gradients (slopes) and penetrated to higher elevations than *Sandelia* 'eastern'. The latter was restricted to sites with low gradient and elevations below 425 m. *Pseudobarbus* 'Breede' was absent from reaches with abundant aquatic vegetation and preferred reaches with coarser substrates, while *Sandelia* 'eastern' was not influenced by the presence or absence of aquatic vegetation. *Sandelia* 'eastern' tended to avoid reaches that were dominated by boulders and bedrock.

Out of the nine environmental variables measured in the present study, only stream size had a major influence on the distribution of *Galaxias* 'nebula'. Results of this study predicted that *Galaxias* 'nebula' was less likely to occur in larger streams. This was surprising for two reasons. Firstly, individuals of this species occurred in large numbers at one locality in the main-stem Breede River and this species was also collected in wider reaches of the lower Smalblaar and Slanghoek Rivers (these sites were excluded from the present analysis because they were invaded by alien fishes and impacted by bulldozing of the river beds). Secondly, genetic studies show little differentiation among tributary populations of this species within each of the three river systems (see Chapter 4). This indicates current (or recent) gene flow connecting tributary populations in each river system through the main-stems. This suggests that *Galaxias* 'nebula' is capable of at least using the wider and deeper main-stem habitats as conduits of gene flow, had these habitats not been invaded by alien predators and competitors.

The negative relationship between *Galaxias* 'nebula' occurrence and stream size may reflect the influence other factors (e.g. biotic interactions) that were not considered in the present study. Field observations suggest that habitat use of *Galaxias* 'nebula' may be affected by the presence of *Pseudobarbus* 'Breede'. *Galaxias* 'nebula' was consistently absent from sites in larger rivers such as the Wit and the Upper Riviersonderend where *Pseudobarbus* 'Breede' occurred in large numbers. Yet, as discussed above, *Galaxias* 'nebula' is capable of exploiting larger stream habitats when *Pseudobarbus* 'Breede' is absent (e.g. in the lower Smalblaar and Slanghoek). Underwater observations in several tributaries of the Breede River system also indicate a substantial increase in the numbers of *Galaxias* 'nebula' immediately above the upper limits of *Pseudobarbus* 'Breede' and at localities where the latter occurs in lower numbers. *Sandelia* 'eastern' coexists with the other native species and does not seem to be affected by their presence. Whether or not the negative relationship between *Galaxias* 'nebula' and stream size is a result of competitive interactions with *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' (whose probability of occurrence increases with increase in stream size) is currently unknown and merits further investigation.

Pseudobarbus ‘Breede’ and *Sandelia* ‘eastern’ were predicted to be capable of exploiting wider and deeper habitats beyond their present distribution ranges. Indeed, both species occur in the Sanddrifs Dam, a large reservoir built on a tributary of the Hex River in the upper Breede River catchment. It is, however, unclear at this stage whether these taxa can exploit the deeper sections of the dam, but it seems that wave action against the rocky shoreline of this dam probably creates favourable conditions that support the existence of these taxa in the littoral sections of the dam. The dam also has clear water that allows light penetration to support the growth of periphyton and the occurrence of invertebrates. The existence of *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ in the dam suggests that they both could have been capable of at least dispersing through the wider main-stem habitats prior to invasion of these habitats by non-native predators and competitors. This inference is supported by results from molecular studies, which show shallow differentiation among tributary populations of each species within the three river systems (Swartz, 2005; Chapter 5). This indicates that tributary populations in the same river system were historically connected through the main-stems prior to fragmentation caused by predation or competition from alien fishes that now dominate in the main-stems.

Interspecific morphological differences and habitat use

Habitat preference of the three taxa seems to be related to their body forms. All five morphological traits measured in the present study differed significantly among the three lineages. The lack of overlap on the Principal Components Axes suggests that the three lineages fall into discrete morphological categories. *Galaxias* 'nebula' has a more slender, cylindrically-shaped body and smaller pectoral fins than *Pseudobarbus* 'Breede' and *Sandelia* 'eastern'. These features reduce hydraulic stress on stream fishes and thereby reduce the energetic demands of maintaining position in flowing water (Webb, 1984; Videler, 1993). Species with these traits usually have better swimming performance and are capable of exploiting river reaches with faster current velocities (Videler and Wardle, 1991). Results of the present study are consistent with these expectations because *Galaxias* 'nebula' was found to be capable of penetrating further into upper reaches than both *Pseudobarbus* 'Breede' and *Sandelia* 'eastern'. Estimates of velocity from the present study showed that upper reaches have faster current than lower reaches (Fig. 2.12).

Pseudobarbus 'Breede' has a fusiform body shape and higher caudal fin aspect ratio (forked tails), two traits that are known to reduce drag and increase swimming ability (thrust) in faster-flowing water (Webb, 1984; Videler, 1993). In this study, *Pseudobarbus* 'Breede' was found to be capable of utilizing habitats at higher elevations (and hence faster current velocities; Fig. 2.12) than *Sandelia* 'eastern', but not as high as *Galaxias* 'nebula'. The body shape of *Pseudobarbus* 'Breede' was found to be intermediate to that of *Galaxias* ‘nebula’ and *Sandelia* ‘eastern’. This may possibly explain why the upper limit of *Pseudobarbus* ‘Breede’ falls between the upper limits of *Galaxias* ‘nebula’ and *Sandelia* ‘eastern’.

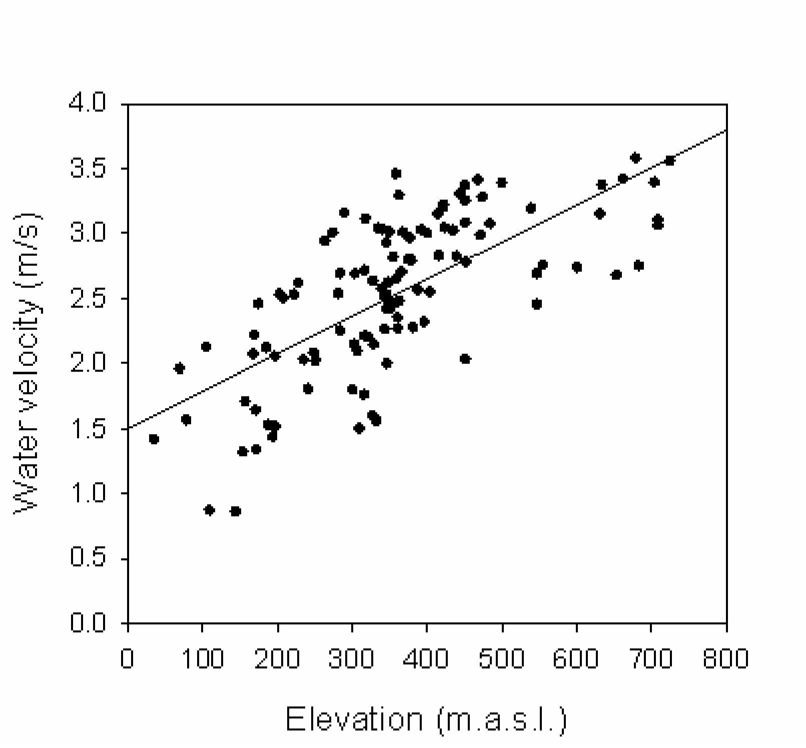

Fig. 2.12: Longitudinal changes in velocity along mountain streams of the Breede, Duiwenhoks and Goukou River systems. Velocity was approximated using the equation: Velocity = log Width + log Depth + log Slope – log (Width + 2Depth).

Sandelia ‘eastern’ has a laterally compressed body, more squarely-shaped tail and larger pectoral fins, and was found to be more restricted in its habitat use, preferring lower elevation reaches with moderate gradients and slower current velocities (Fig. 2.12). This is consistent with findings from other morphological studies that found that deeper bodied fishes with laterally compressed bodies, larger pectoral fins and lower caudal fin aspect ratios (more square shaped tails) have poor swimming performances due to high drag penalties (Webb, 1998; Chan, 2001). It is likely, therefore, that *Sandelia* ‘eastern’ may not be capable of maintaining position under greater turbulence due to increased energetic demands. This may explain why this species avoided localities with steeper gradient in the upper reaches. Steep gradients and the dominance of boulder-bedrock substratum at higher elevations are associated with high turbulence which in turn exerts substantial hydraulic stress on stream biota (Robertson *et al*., 1995).

The prediction that body size would influence habitat selection was supported. The larger sized species (*Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’) showed stronger selection for wider streams and deeper habitats. Smaller streams are characterised by high flow variability and are unpredictable compared to larger streams and rivers, and they are likely to experience periods of low or even no flow (Poff & Ward, 1989). Smaller streams in other Mediterranean regions are known to experience flash floods and extreme conditions of high temperature and low oxygen concentrations during periods of low or no flow when the streams recede into isolated pools (Gasith & Resh, 1999; Dean & Richardson, 1999), conditions that *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ may not tolerate. Occurrence of *Galaxias* ‘nebula’ in these highly variable small streams where other species could not survive may be related to its intrinsic ecological attributes, in particular emersion tolerance (Chakona *et al*., 2011). Preferential use of deeper pool habitats by fish is usually attributed to greater vulnerability to wading/diving predators (Harvey & Stewart, 1991). Given the relatively larger body sizes of *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’, deeper water is likely to provide protection from predators such as otters and avian piscivores. This is in contrast to *Galaxias* ‘nebula’ which is a small cryptic species that can easily find concealment from avian or terrestrial predators in shallow habitats.

The absence of *Pseudobarbus* 'Breede' from the eastern-draining tributaries of both the Duiwenhoks and Goukou Rivers was unexpected because these tributaries were both wide and contained deep pool habitats. There are a number of possible explanations for this. Both tributaries were characterised by dense macrophytes (predominantly *Prionium serratum*), fine substrate and slow to static flow. It is possible that the absence of *Pseudobarbus* 'Breede' from these tributaries may be linked to potential hypoxic conditions due to increased amounts of decomposing organic matter and reduced mixing of water, as found for overgrown rivers and swamps elsewhere (Melnychuka & Chapman, 2002). Diet has also been shown to influence species distribution in other studies (Morán-López *et al*., 2005). The sub-terminal mouth of *Pseudobarbus* 'Breede' suggests an adaptation to feeding on insects and periphyton scraped from rock surfaces. This was observed in the field. Limited light penetration due to shading by macrophytes as well as the dominance of fine substrate could have limited the growth of periphyton and reduced colonisation by insects associated with rock surfaces in the eastern sub-catchments of the Duiwenhoks and Goukou Rivers. Thus, absence of this species from the two tributaries may be linked to trophic adaptations. Alternatively, low water velocity could also be linked to this pattern. The Hjulström sediment deposition curve predicts that fine sediments (< 0.2 mm), which were the dominant substrate type in the two sub-catchments, settle out of the water column when water velocities are less than 0.3 m/s (Gordon *et al*., 1992). This suggests that *Pseudobarbus* 'Breede' may be intolerant of low water velocity or static conditions. Indeed, *Pseudobarbus* 'Breede' were absent from deep static side pools in the Wit and Klip Rivers, but were abundant in the lotic sections of the rivers and predominantly concentrated at the pool-riffle interphase. This suggests a strong affinity for lotic (flowing) conditions. This may seem to contradict the occurrence of this taxon in Sanddrifs Dam discussed earlier, but wave action provides constant movement and mixing of water in the dam. Conditions along the dam's littoral zone may therefore mimic 'lotic' conditions. All these explanations are plausible, but a better understanding of such ecological mechanisms requires more directed field work and/or experiments.

Results from the present study are consistent with findings of research on stream fishes from elsewhere that suggests a strong relationships between fish morphology and ecology (Wikramanayake, 1990; Douglas & Matthews, 1992; Motta *et al*. 1995, Chan, 2001; Brinsmead & Fox, 2002; Bhat, 2005; Leal *et al*., 2011). Ecomorphological analyses may therefore provide additional information to help predict habitat preference of other freshwater fishes in the Cape Floristic Region and elsewhere. There is, however, a need for swimming performance studies on *Galaxias* 'nebula', *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' to substantiate the predictions about their swimming abilities discussed in the present study.

Other factors such as competition, predation, resource availability and temporal variation in habitat use can also influence the distribution patterns and habitat selection in stream fishes (e.g. Angermeier & Karr, 1983; Gasith & Resh, 1999). These factors were not incorporated in the present study. Therefore, the results need to be interpreted with caution. Nevertheless, the results indicate that ecomorphological characters, such as body shape and fin size, may be useful indicators of habitat preference for stream fishes in the CFR.

Biogeographic and phylogeographic implications

The habitat preference information from this study is essential for making predictions and explaining contemporary biogeographic and phylogeographic patterns of freshwater fishes in the study area (see Chapters 4 & 5). For example, the wide geographic range of *Galaxias* ‘nebula’ across the CFR may be attributed to its ability to utilise different dispersal mechanisms (Chakona *et al*., 2011; Chapters 3 & 4), which may include interdrainage dispersal via connections of mountain tributaries during wetter periods, river captures (which are mainly characteristic of upland catchments) and river confluences during periods of low sea-levels. Making use of this broad range of dispersal mechanisms is possible given its utilisation of a wide variety of habitat types. By contrast, *Pseudobarbus* ‘Breede’ and *Sandelia* ‘eastern’ have more restricted ranges, probably due to less dispersal options in lower river reaches compared to *Galaxias* ‘nebula’.

Implications for conservation

The invasion of CFR streams by non-native species and habitat loss are considered to be the greatest threats to the freshwater biodiversity of this region (Tweddle *et al*., 2009). The development of agricultural activities and irrigation schemes during the early and mid-1900s was predominantly in the low-gradient valleys. Consequently, downstream reaches of almost all mountain tributaries are severely degraded. Therefore, the restriction of the remaining populations of *Sandelia* 'eastern' to the lower reaches of the mountain streams exposes them to multiple impacts and stressors which include increased susceptibility to invasion by alien predators from the main-stems, hydrological alteration and habitat loss due to the building of water abstraction structures in upper reaches, sedimentation and increased water turbidity, and pollution and pesticides from intensive agricultural activities.

Building of weirs to prevent upstream migration of exotic species has been considered to be one of the best conservation strategies to secure the remaining native populations in the CFR. The species' cumulative frequencies in relation to elevation along the streams considered in the present study (Fig. 2.4) could be useful for making initial assessments of appropriate locations for such barriers, so that protected stream reaches will include preferred habitat features for all species. These trajectories are also useful for assessing the potential impacts of developments, for example the building of water abstraction structures at a nominated altitude within a given stream. Field surveys indicate that *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' have been extirpated from streams where weirs or water abstraction structures have been built above the upper limits of these species. In some instances, the remaining populations only occur in a very short stretch of river above the weirs, indicating that these weirs have been built just below the species' upper limit. These populations have high extinction risk because the remaining habitat may not be optimal. There is also lack of migration from other areas, as the weirs form effective barriers. In most cases the reaches below the weirs either dry completely during the dry season or are invaded by aliens. There is a need for many water take-off points that are located in the upper river reaches to be moved lower down to enable the native fishes to recover and extend their distribution ranges.

Translocation has also been considered to be a useful strategy in the recovery of threatened species (Maitland, 1995). Besides being a controversial suggestion (particularly for streams of the CFR where the upper fishless reaches harbour unique aquatic invertebrate taxa), the data presented here indicate that such measures may not help certain species. For example, moving *Sandelia* 'eastern' into upper reaches above their natural distribution ranges may not be a viable long-term conservation measure since they appear to be adapted to lower river reaches with moderate channel slopes and slower-flowing waters. Appropriate conservation management strategies for *Sandelia* 'eastern' and other species with similar ecological preferences would rather be the rehabilitation and protection of their preferred habitats (slow-flowing pools) in lower river reaches of the tributary catchments.

Chapter 3

Aerial exposure tolerance of a newly discovered galaxiid fish

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Abstract

Laboratory experiments were conducted to investigate tolerance and physiological responses of *Galaxias* ‘nebula’, a newly discovered and widespread African galaxiid, to aerial exposure. This species can tolerate emersion for at least 36 hrs. Changes in water level and dewatering did not induce the fish to burrow into substrate or find refugia, nor was there detectable mucus production following aerial exposure. However, opercular movement, a proxy for gill ventilation rate, did vary with changes in water level. The initial steady ventilation rate increased significantly when the fish were partially emersed and ventilation ceased immediately upon total air exposure. When fish were re-immersed, there was first a period of hyperactivity with a corresponding inflated gill ventilation rate which was restored to pre-treatment levels within two hours. This is the first documented case of amphibious capabilities in an African galaxiid which has implications for the interpretation of its widespread distribution pattern.

3.1. Introduction

Many fishes have the ability to extract oxygen from both air and water (Daxboeck & Heming, 1982; Martin *et al*., 1993, 2004; Graham & Lee, 2004; Ip *et al*., 2004; Sloman *et al*., 2008). These fish can be divided into two classes, amphibious and aquatic air-breathers (Graham, 1997). Amphibious air-breathers utilize atmospheric oxygen during periods of exposure to air (emersion) and many of them are capable of surviving periods without surface water (Martin *et al*., 1993; Sayer, 2005; Taylor *et al*., 2008). In contrast, aquatic air-breathers never leave water, but periodically break the water surface to gulp air (Gonzales *et al*., 2006). These capabilities confer several advantages which include providing alternatives to escape or evade predators and competitors (Sayer & Davenport, 1991), avoidance of hypoxic conditions and survival in temporary habitats (Eldon, 1979*a*; Graham, 1997; McPhail, 1999; Sayer, 2005). Studies of air-breathing fish are crucial to our understanding of the evolution of vertebrate air-breathing and the fish-tetrapod transition (Brauner *et al*., 2004; Graham & Lee, 2004). Knowledge of the patterns of air-breathing capabilities is also important to our understanding of the factors that shape the biogeography and evolutionary history of freshwater fishes (Main, 1989; Fraser *et al*., 1995).

Air-breathing fishes use a combination of behavioural, structural and physiological adaptations to survive exposure to air (Sayer, 2005). Behavioural adaptations may involve burrowing into subterranean refugia or piles of vegetative debris and logs, where cool damp conditions may allow fishes to survive temporary cessation of surface flow, as observed in the mangrove killifish *Kryptolebias marmoratus* (Poey) (Taylor *et al*., 2008) and the Australian salamanderfish *Lepidogalaxias salamandroides* Mees (Berra & Allen, 1989). Breathing out of water may be facilitated by possession of accessory respiratory structures which include specialized lungs in the African lung fish *Protopterus* species (Maina & Maloiy, 1985), pharyngeal organs, such as the heavily vascularised buccopharyngeal cavity in the Gobiidae (Gee & Gee, 1995) and cutaneous respiration in the Canterbury mudfish *Neochanna burrowsius* (Phillipps) (Eldon, 1979*b*; Meredith *et al*., 1982). Physiological adaptations to emersion include modification of gill function (Graham *et al*., 1985; Ong *et al*., 2007), metabolic adjustments (McPhail, 1999; Richards, 2010) and mechanisms to reduce toxic waste products in air (Ip *et al*., 2004; Randall *et al*., 2004). All of this research examined fish species known to be capable of air-breathing. It is much more difficult to establish, via non-lethal means, whether a newly discovered species has this capacity. The present study focuses on the amphibious survival capacity of a newly discovered galaxiid fish by assessing some of its behavioural and physiological adaptations for emersion survival.

The Galaxiidae consist of generally small osmeriform fishes confined to cool temperate regions in the Southern Hemisphere (McDowall, 1970, 1973). Several galaxiid species are known to be capable of air-breathing (Cadwallader & Backhouse, 1983; Koehan & Raadik, 1991; Thompson & Withers, 1999; O'Brien & Dunn, 2007) and research has addressed the ecological and evolutionary implications of this. For example, the amphibious air-breathing capabilities of *N. burrowsius* and its propensity to move over land could have resulted in its wide geographic distribution and the shallow genetic structuring of this species across the Canterbury Plains in New Zealand (Davey *et al*., 2003). Paraphyletic relationships between isolated populations of the roundhead galaxias *Galaxias gollumoides* McDowall & Chadderton in New Zealand have been attributed to the hypoxia tolerance of this facultative swamp dweller, which could have allowed it to utilise intermittent wet connections to disperse between catchments (Burridge *et al*., 2008a). However, while knowledge of the ecology and air-breathing capabilities of galaxiid fishes in New Zealand (Eldon, 1979*a, b*; McDowall, 1999; Main, 1989; McPhail, 1999; O'Brien & Dunn, 2007), Australia (Cadwallader & Backhouse 1983; Koehan & Raadik, 1991; Thompson & Withers, 1999) and South America (Hickford *et al*., 2010) is reasonably extensive, the current study is the first to address this potential in African galaxiid fishes.

Africa was thought to be represented by a single Galaxiidae species, the Cape Galaxias *Galaxias zebratus* (Castelnau). However, recent phylogeographic studies have revealed that *G. zebratus* is a species complex consisting of several unique lineages that are endemic to the Cape Floristic Region of South Africa (Waters & Cambray, 1997; Wishart *et al*., 2006). In continuing research, many more lineages are being discovered and all of them are highly restricted geographically, except for one widespread lineage (hereafter *Galaxias* 'nebula') which is the subject of this study. African galaxiid fishes appear to be confined to freshwater (McDowall, 1973). *G.* 'nebula' occurs in a range of habitats from fast-flowing mountain tributaries to coastal streams and swamps in river systems that are currently isolated by marine and terrestrial barriers.

In this study, a series of experiments were used to assess whether *G*. ‘nebula’ individuals can adjust their behaviour and physiology in response to emersion, and more importantly, reverse to a normal state after re-immersion. Specific predictions are that *G.* nebula 1) is tolerant of emersion 2) can respond to declining water levels by actively seeking refugia and 3) has respiratory physiological responses to emersion. These intrinsic ecological and physiological adaptations may explain its wide distribution and utilization of diverse habitats.

3.2. Materials and Methods

The research was conducted under permit from CapeNature (permit number: AAA-004-000205-0035) issued only after approval of methods by a review panel. Specimens of *G.* ‘nebula’ were collected from the Hex River (33º 31' 44.2" S; 19º 32' 24.3"E) on 25 April 2009 for the emersion tolerance and refugia use experiment (n = 60) and on 29 April 2009 for assessing physiological responses to emersion (n=25). The fish were transported to a nearby field laboratory and were kept overnight in aerated holding tanks. All the water used was collected from the same site and kept at ambient temperature. Sand for substrate and rocks and vegetation (*Juncus* sp.) for refugia were also collected at the same site.

To determine the tolerance of *Galaxias* ‘nebula’ to aerial exposure, 18 plastic trays (20 × 14 × 8 cm) were filled with 1.5 cm deep sand, one rock (7 – 10 cm length and 4 - 6 cm width), a clump of vegetation covering about 40 cm^2 and 0.5 l of water. Two medium [3

– 4.5 cm total length (L_T)] and one large (5 – 6.5 cm L_T) fish were placed in each tray and allowed to acclimate for 6 h. The trays were covered with a net to reduce external disturbances. The experiment consisted of five phases: 1) pre-treatment (P); 2) recession (R); 3) partial emersion (Pe); 4) emersion (E) and 5) re-immersion (Ri). Phases two (R), three (Pe) and four (E) were de-watering phases. With the exception of the emersion phase (E) (described below) the duration of each phase was 6 h. Each tray was observed at the start, mid-point and end of each phase, recording the number of fish in refugia (*i.e.* under the rock or vegetation) or visible in the open areas of the tray and noting any movement. The emersion (E) phase had four exposure periods (6, 12, 24 and 36 h) and observations were carried out at the beginning of this phase and after 3, 6, 12, 18, 24, 30 and 36 h of emersion (depending on the duration of the phase). Three randomly selected trays were designated as controls and left with water throughout the experiment. The remaining 15 trays were randomly assigned to an emersion exposure period such that there were three replicates for the 6 h exposure period, three replicates for the 12 h exposure period, three replicates for the 24 h exposure period and six replicates for 36 h. The ambient temperature ranged between 18 and 22 °C throughout the experiment.

For the pre-treatment phase (P), the trays were simply monitored to record the behaviour of the fish before experimental manipulations. For the recession phase (R), 0.2 l of water was drained from each of the treatment trays, such that the fish remained completely immersed in water. It took an average of 2 min to drain this volume of water from each tray. Another 0.2 l of water were drained from all treatment trays during the partial-emersion phase (Pe) leaving about 1 mm depth of water so that fish were partially exposed to air. For the emersion phase (E), all the surface water was drained from the treatment trays, leaving only the sand, vegetation and underside of rocks moist and fish completely exposed to air. Finally, for the re-immersion phase (Ri), 0.5 l of water was added to the respective trays at the end of each emersion period (*i.e.* after 6, 12, 24 and 36 h). The survival of the fish was confirmed and the time that the fish took to recover following re-immersion was noted. The fish were monitored for at least another 12 h to assess post-treatment behaviour and survival. Observations were conducted throughout these phases as set out above. Any dead fish encountered during treatment observations were immediately removed from the trays. Given that a combination of factors may influence the subsequent survival of fish in laboratory experiments, the experiment was terminated after 36 h of emersion when two fish died between 30 and 36 h of exposure. Only four of the 54 fish used in this experiment died. In addition to the two above, one died during acclimation and another died after 24 h of emersion. At the end of the experiment, the fish were released alive at the site of collection.

In the second experiment, the respiratory physiological response of *Galaxias* ‘nebula’ to treatments (*i.e.* recession, partial-emersion, emersion and re-immersion) was determined by visually quantifying opercular movement and calculating gill ventilation rate (GVR) as the number of opercular movements per minute. Twenty trays (10 controls and 10 treatments) were set up with 1.5 cm deep sand and 0.5 l of water. One medium sized fish (3 – 4.5 cm L_T) was added to each tray and left to acclimate for 6 h. The same five phases as above (pre-treatment, recession, partial emersion, emersion and re-immersion), each lasting 3 h, were carried out on all treatment trays. The control trays remained with water throughout the experiment. During each phase, three 1 min observations (*i.e.* immediately after the treatment, at mid point of treatment and finally at the end of the treatment) were carried out to record the frequency of opercular movements (a measure of gill ventilation rate) for both control and treatment fish. No fish died during this experiment and these fish were also released at their exact collection site.

For experiment one, data were converted into proportions of fish in refugia, then, as sample sizes and variances were small, means of all the measurements per phase for each tray were taken and used as the dependent variable in analyses. As all treatment fish established their positions (either cover or open) by the end of partial-emersion and remained in these positions throughout the emersion phase, only the data for the first 6 h of the emersion phase were used for all analyses to account for the varying duration of emersion. First, to establish whether there were any diurnal differences in use of refugia, the control data was subjected to a Friedman test with observations over time as the independent variable. Then, to assess whether exposure period affected refugia use, separate Kruskal-Wallis tests were carried out on the data from the emersion and re-immersion phases (four and five, the two phases likely to be affected) with exposure period as the independent variable. Finally, a Friedman test on the treatment trays with phase as the independent variable was used to assess differences in refuge use in different phases.

For experiment two, as data sets for operculum movement were normally distributed (assessed using Kolmogorov-Smirnov tests) parametric analyses were used. Levene's test was used to assess homogeneity of variance (MANOVA) and where data was not spherical (repeated measures ANOVAs) a Greenhouse-Geisser correction was applied. Any diurnal effect on GVR was assessed using repeated measures ANOVA on the control data over all phases with time as the repeated measure and operculum movement as the dependent variable. To assess differences in GVR with phase another repeated measures ANOVA was carried out with the three repeated measurements per phase nested within the repeated measure of phase. However, in the fourth phase (emersion) only zeros were recorded (see results) so this data set was omitted from this analysis. Then, as the interaction term was not significant (see results), simple analyses were carried out. First, the data per phase for each tray was converted into means and used in a Friedman test with the five phases as the independent (repeated) factor to assess differences in GVR between phases, and post-hoc Wilcoxon tests were used to find where these differences occurred. Finally, to assess differences over time within each phase, a repeated measures MANOVA was used with the three observations per phase as independent variables and operculum movements in each phase as dependent variables. For any phase in which significant differences were found, post-hoc Tukey’s tests were used to determine at what stage these differences occurred.

3.3. Results

In the first experiment, *Galaxias* ‘nebula’ was able to withstand emersion for up to 36 hours. There was no discernible pattern in the use of refugia among control fish (Friedman test, d.f. = 4, $P > 0.05$; Fig. 3.1). For the treatment fish, exposure period did not affect refugia use in either the emersion (Kruskal-Wallis test, d.f. = 4, $P > 0.05$; Fig. 3.1) or re-immersion (Kruskal-Wallis test, d.f. = 4, $P > 0.05$; Fig. 3.1) phases, nor did refuge use change between phases (Friedman test, d.f. = 4, $P > 0.05$; Fig. 3.1).

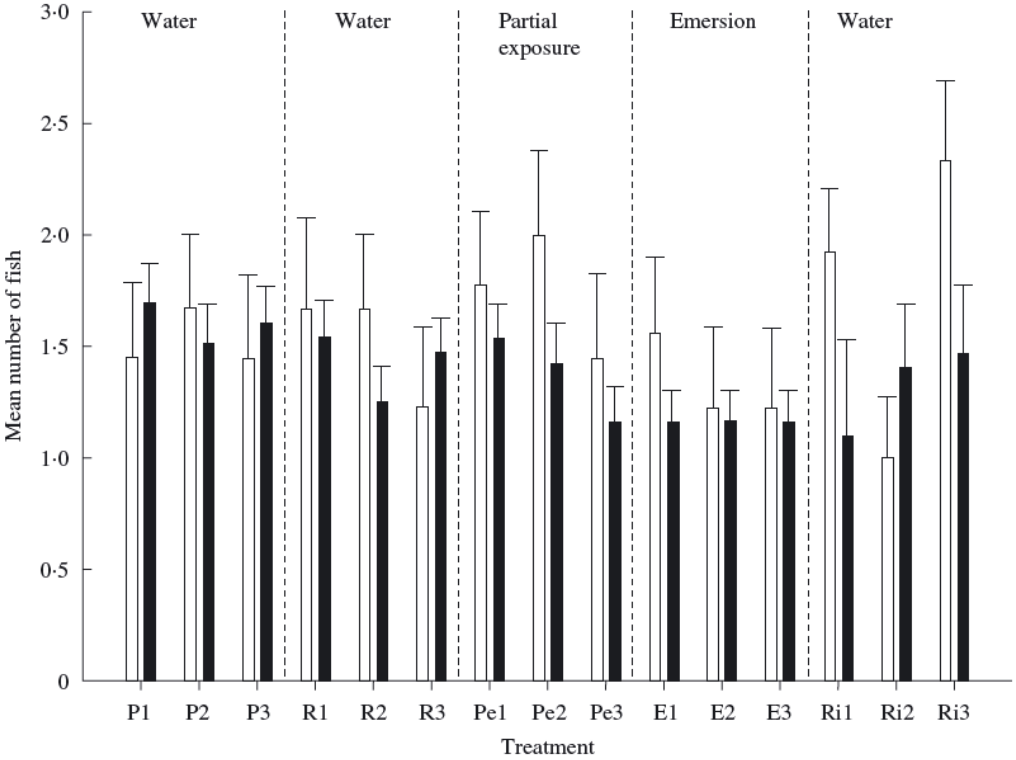


Fig. 3.1: Response of Galaxias 'nebula' to air exposure showing the mean number of fish in cover for control (□) and experimental (■) trays. The five phases are denoted by: P = pre-treatment, R = recession, Pe = partial emersion, E = emersion and Ri = re-immersion. 1 = start (0 h), 2 = midpoint (3 h) and 3 = end (6 h) of phase. Only data for the first 6 h of the emersion phase are presented. Values are mean ± s.e. n = 3 for control and 15 for experimental trays. The figure was reproduced from Chakona *et al*., (2011).

All fish immediately responded to partial-exposure (Pe) by moving over the wet sand using serpentine movements. Some fish made excavations in the sand while moving, mainly around rocks and clumps of vegetation, but none of the fish burrowed into the substratum. Most fish became inactive while maintaining a dorso-ventral position within the first 2 hrs of emersion, but a few individuals occasionally moved laterally or rolled onto their dorsal side. No opercular movements or other visible respiratory behaviour were observed, nor was there any visible mucus production. All fish responded rapidly to re-immersion. Approximately 25% of the fish were positively buoyant, suggesting that their gas bladders were inflated or they had taken an air bubble into the mouth and gill chamber. All fish expelled an air bubble through the mouth immediately following re-immersion. Many of the fish immediately started active swimming, about 50% even swimming hyperactively, but all fish had calmed within the first 2 hrs of re-immersion.

In the second experiment, there was no diurnal difference in opercular movement in the control fish (Repeated Measures ANOVA, d.f. = 3, 29, $P > 0.05$). The treatment fish did significantly differ in GVR over both phase (Repeated Measures ANOVA, d.f. = 2, 16, $P < 0.05$) and time within phases (Repeated Measures ANOVA, d.f. = 2, 16, $P < 0.05$). However, the interaction term was not significant (Repeated Measures ANOVA, d.f. = 4, 32, $P > 0.05$). These factors were thus analysed separately. Mean opercular movement significantly differed between the five phases (Friedman test, d.f. = 4, $P < 0.001$) with post-hoc pairwise comparisons showing significant differences between the partial exposure and emersion phases (Wilcoxon, $n = 10$, $P < 0.05$) and between these phases and all others (Wilcoxon, partial emersion, $n = 10$, $P < 0.05$; emersion (all comparisons), $n = 10$, $P < 0.05$). Partial-exposure of fish to air resulted in a sharp increase in gill ventilation rate from an average of 67.1 to 93.5 beats min^{-1} and was maintained around this rate throughout this phase, while emersion caused an instant cessation of opercular movement (Fig. 3.2).

A significant difference in opercular movement was found within the re-immersion phase, but no significant differences were detected within the other three phases (Table 3.1). All fish immediately switched back to gill breathing when re-immersed, exhibiting an initial phase of hyperventilation (90 beats min^{-1}) with GVR declining and approaching pre-treatment and control values within the first 2 hrs of re-immersion (Fig. 3.2).

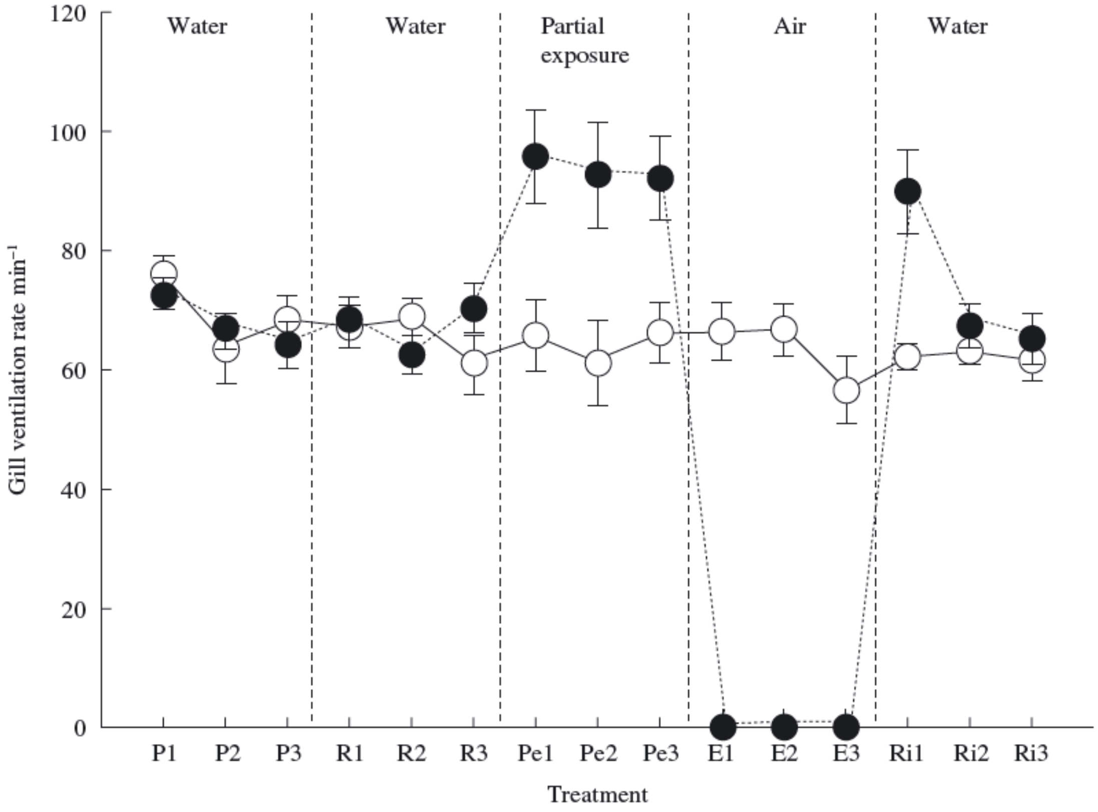


Fig. 3.2: Gill ventilation rates (mean ± s.e.) of Galaxias ‘nebula’ for control (○) and experimental (●) trays during pre-treatment (P), recession (R), partial emersion (Pe), emersion (E) and re-immersion (Ri) phases. 1 = start (0 h), 2 = midpoint (1·5 h) and 3 = end (3 h) of phase. For both control and experimental trays, n = 10 for each observation time for each phase. The initial pre-treatment values were determined after 6 h of acclimation in river water. The figure was reproduced from Chakona *et al*., (2011).

Table 3.1: Results from the repeated measures Multivariate Analysis of Variance (MANOVA) comparing significance of differences in gill ventilation rates for experimental fish within pre-treatment, recession, partial-emersion and re-immersion phases.

Phase	d.f.	*F*	*P*
Pre-treatment	2,18	1.621	0.225
Recession	2,18	0.861	0.440
Partial-emersion	2,18	0.141	0.870
Re-immersion	2,18	15.452	<**0.001**

3.4. Discussion

The ability of *Galaxias* 'nebula' to tolerate emersion for a prolonged period of time suggests that this species has amphibious survival capabilities. They exhibited both behavioural and physiological responses consistent with known amphibious adaptations in other fishes (Meredith, 1985; Sayer, 2005). The limited activity in *G.* 'nebula' when emersed probably indicates suppression of metabolic rate corresponding to reduced energy and oxygen demand when exposed to an environment where respiration is constrained (Crocker & Cech, 1997; Richards, 2010). Maintenance of a dorso-ventral posture, facilitated by a low anguiliform body shape, is likely to enhance emersion tolerance by reducing pressure on the opercular region, which in turn may enable the fish to inflate their buccal cavities (Meredith, 1985). Species that are unable to maintain this position when removed from water, such as *G. maculatus*, have low emersion tolerance (Meredith, 1985). The occasional rolling observed in *G.* 'nebula' may function to rehydrate the skin and expose different body surfaces, which may facilitate gas exchange, enhance waste excretion and prevent desiccation of gills through rehydration from residual water within the opercular cavity (Meredith, 1985). The rapid cessation of opercular ventilation in response to emersion is probably a crucial reflex to prevent desiccation of gills. The initial period of hyperventilation after re-immersion most likely functions to actively remove waste products accumulated during emersion from gill and body surfaces (Daxboeck & Heming, 1982; McPhail, 1999). Active swimming may also function to separate gill lamellae and force oxygenated water over the gill surfaces to enhance gaseous exchange.

Secretion of substantial amounts of mucus is considered to be a critical mechanism that enhances emersion tolerance, because it keeps the gills and skin surfaces moist for efficient gaseous exchange (Eldon, 1978; Meredith, 1985) and also facilitates retention of air bubbles in buccal and opercular cavities (Meredith, 1985). However, *Galaxias* 'nebula' did not produce detectable mucus which could have affected their ability to retain air-bubbles in the buccal cavity as none of the fish were observed to exhibit distinctly distended jaws or inflated opercular regions. However, some amphibious fishes, such as *Lepidogalaxias salamandroides*, do not produce large quantities of mucus when emersed, but can still respire aerially (Martin *et al*., 1993). In the case of *G.* 'nebula', it is likely that they cannot withstand considerable dehydration through complete drying of their habitat. Nevertheless, cutaneous respiration cannot be ruled out in this species, especially under very humid conditions or if the skin is kept moist for efficient gas exchange.

There are several alternative mechanisms by which *G.* 'nebula' may tolerate emersion. Gas bladders may function as accessory air-breathing organs (Brauner *et al*., 2004). In this study, fish were positively buoyant when re-immersed, suggesting that their gas bladders may have been inflated. Galaxiid fishes have been reported to survive loss of surface water by burrowing into subterranean refugia (Eldon, 1979*b*; O'Brien, 2005; Davey *et al*., 2006; Dunn & O'Brien, 2006; O'Brien & Dunn, 2007). Although the fish in this study did not attempt to burrow or actively seek refugia with decreasing water levels, it may be that the choice of substrate or the duration of water loss was influential. Both *G. cobitinis* McDowall & Waters (Dunn & O'Brien, 2006) and the river kokopu *G. vulgaris* Stokell, 1949 (Davey *et al*., 2006) burrow faster in coarser substrates. The small pore size of the sand used in this study may have inhibited subterranean burrowing. Alternatively, water decline may be more gradual under natural conditions. Here, the necessarily rapid water draw down may not stimulate a burrowing response. Finally, fish may switch to anaerobic respiration (Sloman *et al*., 2008), a possibility yet to be tested for *G*. 'nebula'.

Intrinsic ecological attributes such as emersion and hypoxia tolerance may facilitate interdrainage dispersal via intermittent wet connections in freshwater restricted taxa (Hurwood & Hughes, 2001; Thacker *et al*., 2007; Burridge *et al*., 2008a). *G*. 'nebula' has a relatively widespread distribution across several currently isolated river systems across the Cape Floristic Region. Many of these systems had common confluences during the lower sea-levels of the last glacial maximum, forming palaeoriver systems that are now submerged. These connections along with river capture events played an important role in allowing wide distributions of some redfin *Pseudobarbus* (Cyprinidae) lineages (Swartz *et al*., 2007, 2009). However, *G*. 'nebula' occurs across several of these palaeoriver systems indicating that historical river connections do not adequately explain the current distribution pattern of this lineage, especially since conventional river capture is a relatively rare event in the evolution of drainage networks (Bishop, 1995). The propensity to move over land is common in galaxiid fishes and is well documented for mudfishes (*Neochanna* species) (Eldon, 1978). Field observations suggest that *G*. 'nebula' can move over damp soil. It is possible, therefore, that the amphibious adaptations exhibited by *G*. 'nebula' contributed to its wide distribution pattern.

While this is the first demonstration of emersion tolerance in an African galaxiid, the occurrence of air-breathing adaptations in galaxiid fishes is well documented. For example, the blackstriped dwarf galaxias *Galaxiella nigrostriata* (Shipway) (Thompson & Withers, 1999) and the Australian mudfish *Neochanna cleaveri* (Scott) (Cadwallader & Backhouse, 1983; Koehan & Raadik, 1991) from Australia and *Neochanna* species from New Zealand (O'Brien & Dunn, 2007) all have amphibious adaptations similar to those described here, and one widespread species, the common galaxias *G. maculatus* (Jenyns), from South America, New Zealand and Australia (Berra *et al*., 1996) has terrestrial egg development (Hickford *et al*., 2010). The incidence of amphibious traits in galaxiid fish from all Gondwanan continents in the Southern Hemisphere suggests that it could be an ancestral phenomenon or that it evolved independently several times. It could also occur in many more galaxiids than presently known, but further research is required to assess whether these traits are homologous. Knowledge of intrinsic ecological attributes of freshwater limited taxa is crucial in explaining large scale biogeographical and phylogeographical patterns (Hurwood & Hughes, 2001; Thacker *et al*., 2007; Burridge *et al*., 2008a).

Chapter 4

Extrinsic and intrinsic influences on the phylogeography of the Cape Floristic Region's most widespread freshwater fish species

Abstract

Cenozoic tectonic events and Pleistocene climatic fluctuations have been proposed as the principle factors that influenced distribution patterns of flora and fauna in the Cape Floristic Region (CFR) of South Africa. The present study explicitly evaluated these hypotheses by comparing divergence times among populations of a widely distributed freshwater fish (*Galaxias* 'nebula') to known geological and climatic events. The extent to which genetic variation in *Galaxias* 'nebula' was affected by uplift of the Cape Fold Mountains, terrestrial barriers between contemporary river systems, coalescence of rivers during low sea-levels and episodic freshwater connections was tested by analysing mitochondrial COI and cyt *b* sequence data from across the species' entire distribution range. Phylogeographic patterns of this species reflect more recent (Pleistocene-Holocene) connectivity of populations across the CFR. The present study rejects the hypotheses that Cenozoic uplift of the Cape Fold Mountains and river capture events played a dominant role in influencing the contemporary phylogeographic pattern of *Galaxias* 'nebula'. Palaeoriver systems also do not adequately explain the evolutionary history of this species. Inter-basin dispersal via episodic freshwater connections during Pleistocene and Holocene pluvial periods, possibly augmented by rare events such as river capture and sea-level regression, seems to be the most credible explanation for the extensive contemporary distribution and relatively recent inter-basinal divergence of *Galaxias* 'nebula' across the CFR.

4.1. Introduction

Spatial and temporal patterns of genetic diversity are a result of both contemporary and historical processes. Phylogeographic studies have been used extensively to decipher evolutionary processes that shaped the temporal and spatial distribution patterns of gene genealogies for a wide variety of taxa (Avise, 2000; Beheregaray, 2008), including plants (e.g. Dick, 2010), birds (e.g. Drovetski *et al*., 2010), freshwater and marine fishes (e.g. Faulks *et al*., 2010; Gaither *et al*., 2010), amphibians (e.g. Lemmon *et al*., 2007) and invertebrates (e.g. Gouws *et al*., 2010; McCulloch *et al*., 2010). However, there are apparent disparities in the global distribution of such studies, with much of the previous research efforts having focused on taxa from developed countries in the Northern Hemisphere compared to considerably fewer studies from the Southern Hemisphere (Beheregaray, 2008).

The Cape Floristic Region (CFR) located at the southern tip of Africa contains a high diversity of endemic freshwater biota (Barnard, 1943; Skelton, 1980a, 1986; Wishart & Day, 2002). Phylogeographic studies are contributing to our understanding of within-species genetic diversity and the evolutionary forces that influenced diversification of the region's freshwater taxa (e.g. Waters & Cambray, 1997; Bloomer & Impson, 2000; Wishart & Hughes, 2001; Wishart *et al*., 2006; Daniels *et al*., 2003, 2006; Swartz, 2005; Swartz *et al*., 2007, 2009; Gouws *et al*., 2010; see also Chapter 5). Formation of the Cape Fold Mountains, river capture events and river confluences during lower sea-levels are thought to be the most important factors that strongly influenced the biogeography of obligate freshwater taxa in the CFR (Barnard, 1943; Skelton, 1980a, 1986; Wishart & Hughes, 2001; Daniels, 2003; Daniels *et al*., 2006; Swartz *et al*., 2007, 2009). This region experienced a complex geological and climatic history marked by extensive uplifting and mountain building processes (Hendey, 1983; Cowling *et al*., 2009), periodic sea-level regressions and transgressions (Tankard, 1976; Siesser & Dingle, 1981; Dingle *et al*., 1983; Rogers, 1985; Ramsay & Cooper, 2002), as well as periods of severe and extreme wet and dry conditions (Partridge *et al*., 1999; Marlow *et al*., 2000; Zachos *et al*., 2001; Cowling *et al*., 2009).

The formation of the Cape Fold Mountains had a significant influence on the evolution and development of contemporary drainage systems in the CFR. In the early Cenozoic (*ca*. 65 million years ago (Mya)), the CFR landscape experienced strong uplift, but the region is thought to have been remarkably stable since then (Hendey, 1983). Present-day drainage systems of the CFR are therefore thought to have remained unaltered since the early Cenozoic (Hendey, 1983). However, Partridge & Maud (1987, 2000) suggested that two further uplift events occurred after the early Cenozoic. The first event, called the Post-African I uplift event, occurred in the Miocene (*ca*. 22 Ma). The second event, called the Post-African II uplift event, occurred as late as the Pliocene (*ca*. 5.3 – 2.6 Ma). This suggests that the contemporary landscape features of the CFR were established as late as the end of the Pliocene (reviewed by Cowling *et al*., 2009).

The region has also been subjected to major changes in climatic conditions. During the late Oligocene (*ca*. 25 Mya) to middle Miocene (*ca*. 15 Mya) southern African is thought to have been characterised by wetter and more mesic climatic conditions, and the landscape was probably covered by extensive wetlands and widespread subtropical forests (Zachos *et al*., 2001; Cowling *et al*., 2009). It seems reasonable to expect that many river systems would have been periodically connected during this time. Deterioration and gradual transition of the CFR climate towards steeper moisture and thermal gradients and loss of connectivity among river systems began in the middle Miocene (Zachos *et al*., 2001). Deacon *et al*. (1992) and Tyson & Partridge (2000) suggest that contemporary drier conditions of the CFR were established by the end of the Pliocene (*ca*. 2.6 Ma). In contrast, Partridge *et al*. (1999) inferred wetter climatic conditions for coastal areas of the southern Cape as recently as the Holocene Altithermal (*ca*. 8 000 - 6 000 years ago). Recent river confluences during the Last Glacial Maximum (LGM) about 18 000 years ago are also notable and seem to have had a major influence on the distribution of *Pseudobarbus* genetic lineages in the CFR (Swartz *et al*., 2007, 2009).

The complex geological and climatic history of the CFR has undoubtedly affected the evolution and distribution patterns of the region's endemic biota. Obligate freshwater taxa are likely to be strongly affected, because their dispersal depends on direct connections of freshwater environments, with seawater providing a physiological barrier at each river terminus. Recent molecular studies have revealed both highly restricted and widely distributed genetic lineages of freshwater-dependent fish species in the CFR (Roos, 2004; Van Niekerk, 2004; Swartz *et al.*, 2009; see also Chapter 5). This suggests that some taxa have been isolated for long periods of time, while others have been able to maintain relatively recent gene flow across the CFR despite the apparent isolation of contemporary river systems by marine and terrestrial barriers. Very few studies have rigorously examined various hypotheses explaining the wide distribution of freshwater dependent fishes in the CFR. By far the most comprehensive of these studies were done by Swartz *et al*. (2007, 2009). They examined the influence of sea-level change and river capture events on the distribution of *Pseudobarbus* genetic lineages across the CFR. The majority of previous genetic studies of the CFR's freshwater taxa focused on small geographical scales (e.g. Wishart *et al*., 2006), while others had small sample sizes and large geographical sampling gaps (e.g. Waters & Cambray, 1997; Roos, 2004; Van Niekerk, 2004).

The present study employed a predictive hypothesis-testing approach to assess the extent to which four alternative (but non-exclusive) biogeographic hypotheses would explain genetic variation in a widespread obligate freshwater fish, *Galaxias* 'nebula'. *Galaxias* 'nebula' is one of the recently discovered genetic lineages of the Cape galaxias species complex (currently all classified as *Galaxias zebratus*). The complex consists of several closely related freshwater fishes endemic to the CFR (Swartz in preparation; see also Chapter 5). Although many of the lineages have highly restricted geographic ranges, *Galaxias* 'nebula' is currently understood to be the most widespread obligate freshwater fish in the CFR, occurring across ten proposed palaeoriver systems (Fig 1.4) and 16 currently isolated river systems from the Olifants (eastern tributaries) and Berg River systems on the west coast, across the Breede, Gouritz and smaller coastal systems on the south coast, to the Bitou River system in the eastern parts of the CFR (Fig. 4.1). It is a small (<70 mm) cryptic species which is capable of surviving out of water for a prolonged period of time (Chapter 3 published as Chakona *et al*., 2011) and usually occurs in large numbers in a wide range of habitats from low gradient coastal streams with fine substrates and high water conductivity (range 600 - 4000 $\mu S.cm^{-1}$) to small high elevation fast flowing mountain streams with rocky substratum and low water conductivity (range 10 - 200 $\mu S.cm^{-1}$) (Chapter 2). *Galaxias* 'nebula' also recovers rapidly following disturbances such as bulldozing of the river channel (Swartz and Magellan, in preparation). This species is not utilised and is not used as bait by anglers, probably due to its cryptic nature and small size. It is therefore expected that the present distribution of this species would reflect its natural population history as it is unlikely to have been impacted by human-mediated translocation.

The present study examined the phylogeographic patterns and extent of genetic divergence among *Galaxias* 'nebula' populations using mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) gene sequences to evaluate the relative roles of four biogeographical hypotheses in shaping the evolutionary history of this species. First, if the uplift of the Cape Fold Mountains (Fig. 4.2A) sundered *Galaxias* 'nebula' populations into westward draining systems discharging into the Atlantic Ocean and southward draining systems discharging into the Indian Ocean, then the species would be expected to exhibit deep divergences and phylogeographic structuring between western and southern populations (Fig. 4.2A). Coalescence and divergence time is also expected to correspond with formation of the Cape Fold Mountains. A recent comprehensive study of a widely distributed freshwater crab (*Potamonautes perlatus*) from the CFR revealed a complete split between westward and southward draining river systems separated by the Cape Fold Mountains (CFM), and close relationships within systems (Daniels *et al*., 2006).

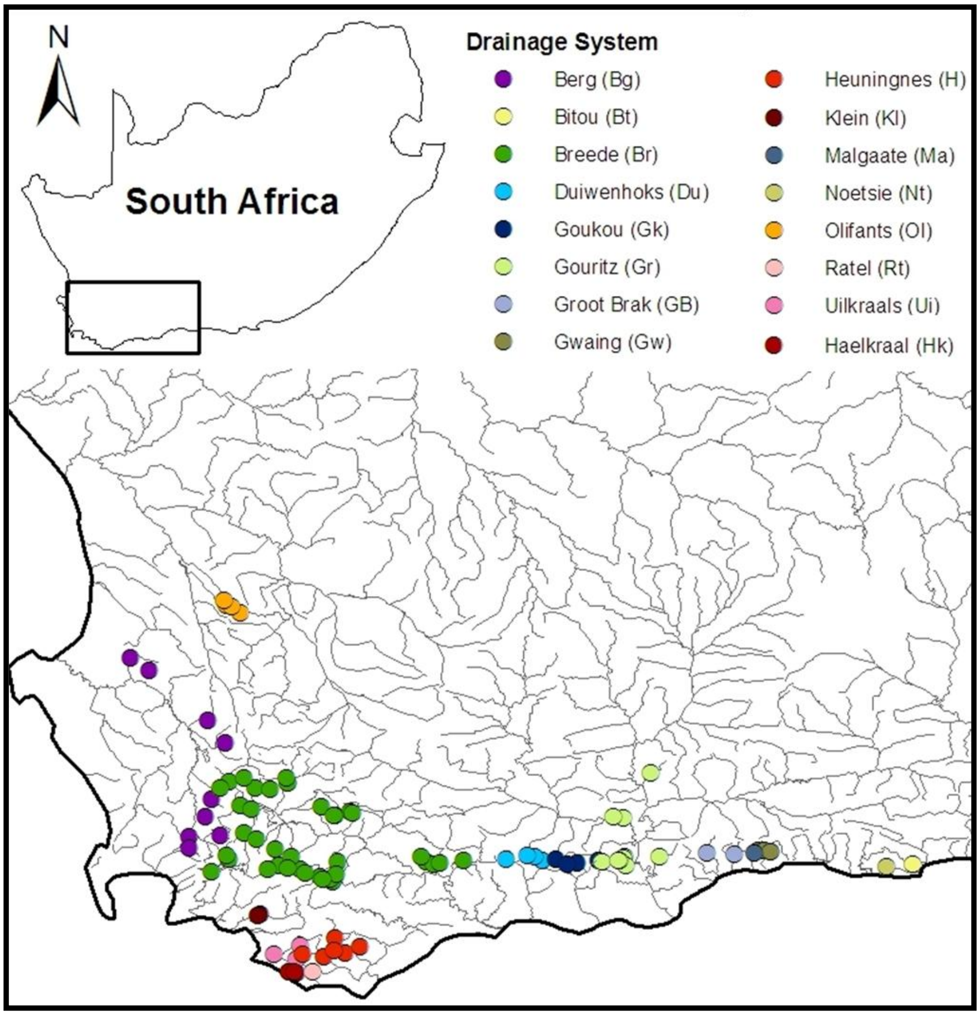


Fig. 4.1: Sampling localities for *Galaxias* ‘nebula’ used for the present study. See Table 4.1 for locality descriptions. The colour codes represent the different river systems.

Second, Swartz *et al*. (2007, 2009) recently provided (using bathymetry and available geological information) empirical evidence for the role of palaeorivers (Fig. 4.2B) in influencing the distribution of redfin (cyprinid minnows of the genus *Pseudobarbus*) genetic lineages in the CFR. The sea-level regression (or palaeorivers) hypothesis would be supported by finding shallow divergences within hypothetical palaeoriver systems and marked differentiation among them (Fig., 4.2B). Thirdly, if present day drainage systems have been unaltered since the Pliocene (*ca*. 5.3 - 2.6 Ma) (reviewed by Cowling *et al*., 2009), *Galaxias* 'nebula' populations can be expected to exhibit shallower divergences within current river systems and deep divergences and structuring among them (Fig. 4.2C) (see Meffe & Vrijenhoek, 1988). Lastly, recent range expansion of *Galaxias* 'nebula' (possibly facilitated by alternative dispersal mechanisms such as 'intermittent' freshwater connections) would be supported by finding low genetic divergence among populations and absence of geographic structuring of alleles among populations across the species' distribution range (Fig. 4.2D).

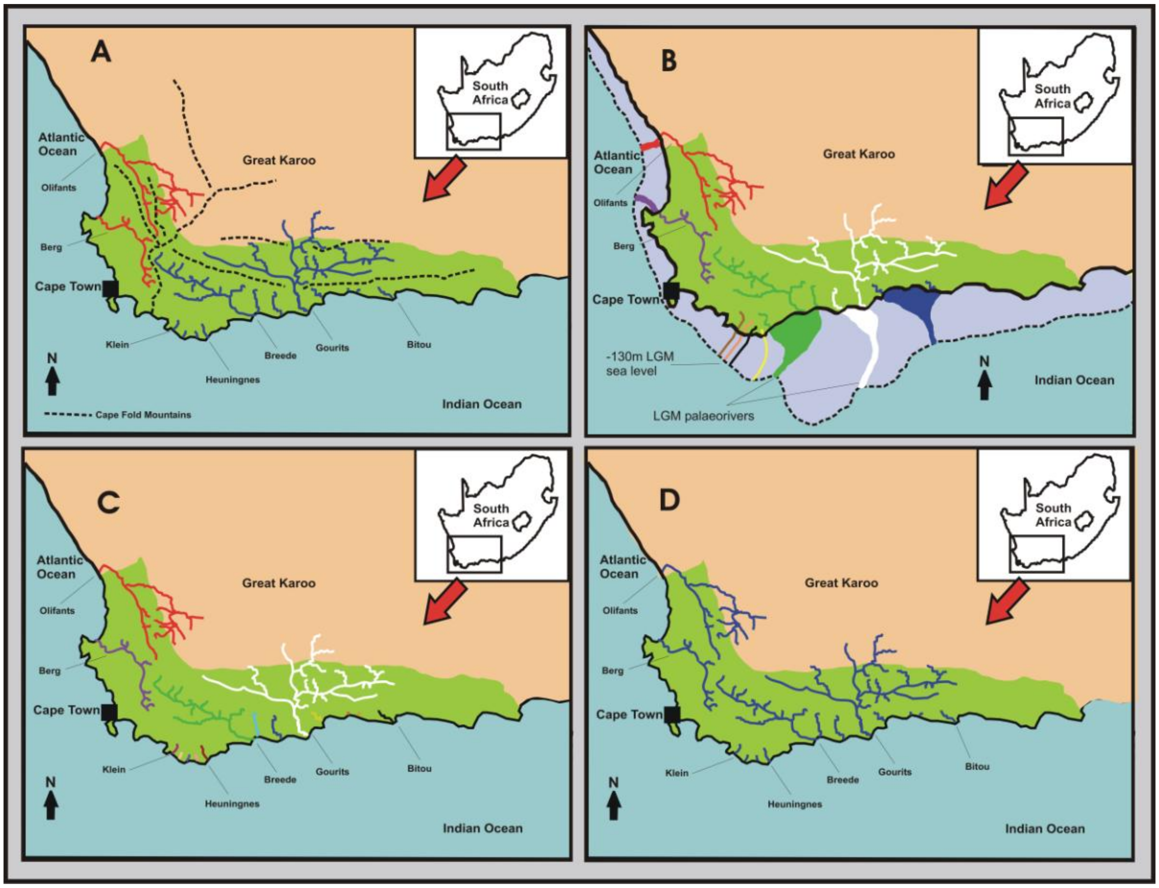


Fig. 4.2: Hypotheses of factors that could have influenced genetic diversity and structure in *Galaxias* ‘nebula’. Panel A illustrates the hypothesis that the uplift of the Cape Fold Mountains (CFMs) caused divergence between populations from western and southern flowing rivers. The location and orientation of the CFMs is shown. Panel B shows the river systems that would have coalesced forming the palaeoriver systems of the Last Glacial Maximum (LGM). The colours indicate the predicted partitions of genetic variation. Panel C describes the hypothesis that genetic variation has been strongly influenced by contemporary river systems (or the stream hierarchy model). This model predicts significant partitioning of genetic variation among river systems. Panel D illustrates the hypothesis that more recent connections have occurred among populations, resulting in low genetic differentiation and no structuring across the species’ distribution range.

4.2. Materials and Methods

Sample collection

Comprehensive sampling of *Galaxias* ‘nebula’ across its entire distribution range in the Cape Floristic Region (Fig 4.1) was done from 2008 to 2010. Additional samples collected during previous field surveys (2004 and 2007) were also used for the present study. Samples were collected using electrofishing, seine netting or snorkelling with a handnet. Fish were anaesthetized using clove oil (0.2 ‰). Small pieces of muscle tissue or whole fish samples were placed in 95% ethanol and stored at ambient temperature in the field, and then at -20 °C in the laboratory. Vouchers of the source specimens have been deposited in the South African National Fish Collection (South African Institute for Aquatic Biodiversity, Grahamstown) for future reference.

DNA extraction, amplification and sequencing

Total genomic DNA was isolated from preserved tissue using the Wizard® Genomic DNA purification kit (Promega, USA) and eluted in a buffer solution. A partial fragment of the mitochondrial COI gene was amplified using primers Vf2-T1 (5' –TGT AAA ACG ACG GCC AGT CAA CCA ACC ACA AAG ACA TTG GCA C- 3') and Vr1-T1 (5' – CAG GAA ACA GCT ATG ACT AGA CTT CTG GGT GGC CAA AGA ATC A- 3') (Ivanova *et al*. 2007) and sequenced using the primer M13F-pUC (5' – GTT TTC CCA GTC ACG AC- 3'). The primers cytb-Glu (5' – GAA AAA CCA CCG TTG TTA TTC A

- 3') and cytb-Thr (5' – CGA CTT CCG GAT TAC AAG ACC - 3') (Waters & Wallis, 2001) were used to amplify the cyt *b* gene which was then sequenced using the cytb-Glu primer. Polymerase chain reactions (PCR) were performed with an Eppendorf Mastercycler (Eppendorf, Germany) and each reaction mixture (50 µl in total) contained 1× reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (ABGene, USA), 20 pmol of each primer, 1 U Super-Therm Taq Polymerase (ABGene, USA) and 100-200 ng of template DNA. The profile for amplification of the COI gene was 94 °C for 3 min followed by 38 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 50 sec, and then final extension at 72 °C for 7 min. The profile for amplification of the cyt *b* gene included an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 30 seconds, 51 °C for 45 seconds and 72 °C for 50 seconds and final extension at 72 °C for 7 minutes. PCR products were sent to Macrogen Inc. (Seoul, Korea) where purification was done using Montáge PCR filter units (Millipore). Some samples were also purified at the South African Institute for Aquatic Biodiversity using a QIAquick® PCR purification kit (QIAGEN) and cycle sequenced using Big Dye® Cycle Sequencing Kit (Applied Biosystems, USA). Automated sequencing was done at Macrogen Inc. (Seoul, Korea) using an Applied Biosystems 3730xl DNA Analyser (Applied Biosystems, Foster City, CA, USA) and the sequencing facility at Rhodes University using an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Sequences were assembled, cleaned and trimmed to equal lengths, using the program SEQMAN ver 7.2.1 (DNASTAR, Lasergene, Seqman Pro).

Sequence diversity analysis

Analyses were conducted separately for the COI and cyt *b* sequences and then for the combined sequences of the two genes. Sequences were aligned using CLUSTALX 2 (Larkin *et al*., 2007). The program DNASP version 5.10 (Librado & Rozas, 2009) was used to translate nucleotide sequences to amino acid sequences. No stop codons or gaps were detected. The same program was used to identify shared and unique alleles, to determine the number of polymorphic sites and the number of haplotypes (N_H). Prior to analysis, a model of sequence evolution that best fitted the data was estimated in MODELTEST version 3.7 (Posada & Crandall, 1998) using Akaike's Information Criterion (AIC) (Burnham & Anderson, 2002). ARLEQUIN (Excoffier *et al*., 2005) was used to calculate haplotype (H_D) and nucleotide (π) diversity and Tajima's (1989) D and Fu's (1997) tests of neutrality. The significance of the demographic parameters were assessed using 10 000 simulations. Significant negative values for D and Fs indicate recent demographic expansion (Ramos-Onsins & Rozas, 2002). Model-corrected distances between unique alleles were calculated using PAUP* (Swofford, 2003). These values were then used to calculate sequence divergences between populations (treating distances between shared alleles as zero).

Population structure and phylogeographic history analyses

Analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.0 (Excoffier *et al*., 2005) was used to explore how genetic variation is partitioned across the geographic landscape. The best models of sequence evolution selected using MODELTEST were: the HKY+I (Hasegawa *et al*., 1985) for the COI gene (molecular evolution parameter values: ti/tv ratio = 16.5538; A = 0.2489; C = 0.2363; G = 0.1724; T = 0.3423; α = 0.0145; I = 0.8064); the GTR (Lanave *et al*., 1984) plus G for the cyt *b* gene (molecular evolution parameter values: A = 0.2420; C = 0.2231; G = 0.1671; T = 0.3678; α = 0.2127) and the TrN (Tamura & Nei, 1993) plus I for the combined sequence data set (molecular evolution parameter values: A = 0.2460; C = 0.2291; G = 0.1683; T = 0.3566; α = 0.0063). The Tamura and Nei (1993) model was the most similar model available in ARLEQUIN to those chosen with MODELTEST. The AMOVA was done with 20 000 permutations to assess the significance of differences between estimated values and those expected when alleles are randomly assigned to populations. To evaluate a possible split between western and southern flowing river systems, sampling sites were divided into 2 groups: the first comprising of all populations from rivers draining into the Atlantic Ocean (Olifants and Berg River systems) and the other comprising of all populations from rivers draining into the Indian Ocean. Secondly, to evaluate the influence of sea-level regression on *G*. 'nebula' genetic variation, the populations were divided into ten groups based on the proposed palaeoriver systems (Swartz *et al*., 2009; Figs. 1.4 & 4.2B): Olifants, Berg, Klein, Uilkraals, Haelkraal, Ratel, Breede-Heuningnes-Duiwenhoks, Gouritz-Goukou, and Wilderness Lakes Region (Klein Brak, Groot Brak, Maalgate, Gwaing to Noetsie) and Keurbooms (Bitou) (Fig. 4.2B). Finally, to evaluate the role of current river hierarchies, sampling sites were divided into 16 groups corresponding to currently isolated drainage systems where the species occur. AMOVA partitions observed variation among samples into within-population (Φ_{ST}), within-group (Φ_{SC}) and among-group (Φ_{CT}) components. Pairwise analyses of Φ_{ST} were also performed in ARLEQUIN to estimate levels of genetic differentiation between river systems.

Nested Clade Phylogeographic Analysis (NCPA) (Templeton *et al*., 1995) was used to make inferences about the population history of *Galaxias* ‘nebula’. This method is most suited for differentiating between historical and contemporary processes (e.g. range expansion, restricted gene flow, past fragmentation) that are responsible for shaping the observed distribution of haplotypes (Templeton, 1998). Although NCPA has been criticised in recent years (e.g. Panchal & Beaumont, 2007; Petit, 2008), Templeton (2008) clarified the misunderstandings raised by these authors and argued that NCPA is still a useful method for making strong phylogeographic inferences, especially when used in conjunction with other methods (Templeton, 2009). A 95% minimum-spanning network was constructed using the statistical parsimony method implemented in TCS 1.21 (Clement *et al*., 2000). Haplotypes in the network were manually converted into a hierarchical nested clade design following the rules of Templeton *et al*. (1987, 1995) and Templeton (1998). The nesting procedure started from the tips to the interior, treating hypothetical intermediate haplotypes the same as extant haplotypes (Cunningham, 2002).

Ambiguities (represented by loops) in the haplotype-network were resolved using predictions based on coalescent theory (Posada & Crandall, 2001a). The decisions for choosing among alternative solutions of the loops followed Pfenninger and Posada’s (2002) criteria: (1) Frequency criterion: alleles are more likely to be connected to older alleles (those with higher frequency in the population) than to younger alleles (or singletons); (2) Topological criterion: singletons are likely to be connected to non-singletons (or interior alleles) than to other singletons (or alleles on the tips). Alleles are also more likely to be connected to extant than to hypothetical alleles; (3) Geographical criterion: alleles are more likely to be connected to alleles from the same geographic region or population than to geographically distant populations.

There were no differences in phylogeographic inferences when either the current coastline or the -200 m coastline of the LGM was used (Swartz, 2005). Therefore, for NCPA analyses, riverine distances between sampling localities were measured along the river courses and current coastline using GIS. User defined riverine distances were incorporated in GEODIS ver. 2.4 (Posada *et al*., 2000) which was used to calculate the different NCPA distances and to test whether the null hypothesis of no association between alleles or clades and their geographical distribution could be rejected. Observed χ^2 values from permutation contingency tests for each level of nesting hierarchy (nested clade) were compared with χ^2 values generated from 10 000 random permutations of the original data (Posada *et al*., 2000). The most recent GEODIS inference key (http://darwin.uvigo.es/download/geodisKey_April2009.pdf, verified October, 2010) was used to infer the processes responsible for the observed distributions of haplotypes for each clade where significant geographical association was found (Posada & Templeton, 2005).

To interpret the observed patterns in relation to historical geological and palaeoclimatic events, divergence times between populations were estimated using two different approaches. The first approach, MDIV (Nielsen & Wakeley, 2001), uses Bayesian inference to estimate the time to the most recent common ancestor (TMRCA), posterior distributions of scaled migration rates ($M = 2N_e m$), time since divergence ($T = T_{\mathrm{div}}/2N_e$) and a measure of genetic diversity ($\theta = 2N_e\mu$) for two populations specified *a priori*. The parameter estimates are the modes of the respective posterior distributions. The underlying assumption of MDIV is that a previously connected population is sundered into two populations which may then either continue to experience gene flow for some time or become completely isolated. Divergence times were estimated between western draining *vs* southern draining systems, among palaeoriver systems and among contemporary river systems. Analyses were run under the finite (HKY) model of sequence evolution using the CBSU Web Computing Resources (http://ser-loop.tc.cornell.edu/cbsu/mdiv.htm). For every sequence set, default priors ($M = 10$ and $T = 5$) were first used with 2 000 000 Markov Chain generations with 500 000 generations as burn-in to identify appropriate parameter values for further analyses (Nielsen & Wakeley, 2001). The program was then re-run for 2 000 000 generations (with the first 500 000 generations discarded as burn-in) across 5 replicate searches. The values from the 5 runs were then averaged to calculate mean values for T and θ. These estimates of T and θ were then used to calculate divergence time as:

$$T_{\mathrm{pop}} = \left[\frac{T\theta}{2L} \times \frac{1}{\mu g}\right]$$

where T and θ represent the mode of the posterior distribution, L is the length of the sequence used, μ is the mutation rate or substitution rate and g is the generation time (see Brito, 2005; Brown *et al*., 2007).

Burridge *et al*. (2008b) recently quantified substitution rates for galaxiids based on known geological isolation histories. For the purpose of the present study, these values were considered to be the most credible and plausible estimates currently available for galaxiid mtDNA. The study used five alternative substitution rates (0.011, 0.01876, 0.026, 0.031 and 0.125 changes/site) to incorporate the time dependent rate of molecular evolution observed in galaxiid fishes (Burridge *et al*., 2008b). Since the generation time for African galaxiids is currently unknown, a generation time of one year was used based on studies of New Zealand galaxiids following Burridge *et al*. (2008b).

A second approach to estimate divergence times between populations used a variable molecular clock recently calibrated using sequence divergences from galaxias populations with known geological isolation histories in New Zealand (Craw *et al*., 2008a). The authors proposed that this could be a potential dating tool for evolutionary processes in the late Quaternary in the Southern Hemisphere (Craw *et al*., 2008a). Divergence times (T_{div}) were computed using the following equation:

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

4.3. Results

Sequence variation

A total of 167 individuals from 91 localities (Table 4.1) were sequenced and yielded 600 base pairs of the COI gene. Seventy-three of these sites were polymorphic. The polymorphic sites resulted in 48 unique alleles (Table 4.2). Six of these alleles were shared between currently isolated river systems: allele 4 occurred in the Uilkraals, Haelkraal, Ratel and Heuningnes, allele 6 was shared between the Haelkraal and the Gouritz, allele 9 was shared between the Duiwenhoks and the Maalgate, allele 10 occurred in the Uilkraals and the Haelkraal, allele 19 was shared between the Breede and the Groot Brak and allele 35 occurred in the Noetsie and the Bitou (Table 4.2).

For cyt *b*, a total of 148 individuals from 84 localities (Table 4.1) were sequenced and yielded 627 base pairs with 95 polymorphic sites which resulted in 63 unique alleles (Table 4.3). Only 3 of these alleles occurred in more than one river system: allele 3 occurred in the Breede and the Groot Brak, allele 38 occurred in the Uilkraals and the Heuningnes, while the Ratel and the Heuningnes shared allele 41 (Table 4.3).

Table 4.1: Sample codes, locality description, drainage system, geographical position and sample sizes for individuals of *Galaxias* ‘nebula’ analysed for mitochondrial cytochrome *c* oxidase subunit I (COI), cytochrome *b* (cyt *b*) and combined cytochrome *c* oxidase subunit I and cytochrome *b* sequences.

Sample code	River	Drainage system	Latitude	Longitude	COI	Cyt b	COI + cyt b
GZ14	Driehoeks (Dwars)	Olifants (Ol)	-32.50127778	19.25258333	1	1	1
GZ10	Driehoeks (Eikeboom)	Olifants (Ol)	-32.45530556	19.17011111	1	1	1
GZ15	Driehoeks (Perdekloof)	Olifants (Ol)	-32.46361111	19.19611111	1		
GZ16	Driehoeks southern	Olifants (Ol)	-32.43111111	19.15000000	1	1	1
AC10A07	Boesmans	Berg (Bg)	-32.77438889	18.58213889	1	1	1
AC09B57	Dwars	Berg (Bg)	-33.85552778	18.94111111	1	2	1
GZ23	Dwars	Berg (Bg)	-33.92252778	18.94141667	1	1	1
AC10A03	Hugos	Berg (Bg)	-33.73488889	19.03833333	1	2	1
AC10A08	Kleinberg	Berg (Bg)	-33.29108333	19.15575000	4	4	4
AC10A02	Krom	Berg (Bg)	-33.63033333	19.07994444	3	2	2
AC10A04	Leeuw	Berg (Bg)	-33.15591667	19.05058333	4	3	3
ES10A01	Olifants	Berg (Bg)	-33.84597222	19.12941667		2	
AC10A06	Platkloof	Berg (Bg)	-32.85302778	18.70141667	2	1	1
AC09C05	Hartbees Tributary	Klein (Kl)	-34.32511111	19.37447222	2	2	1
AC09C07	Hartbees	Klein (Kl)	-34.33419444	19.35850000		2	
AC09A34	Boesmans	Uilkraals (Ui)	-34.59836111	19.59222222	2	2	2
GZ42	Slang	Uilkraals (Ui)	-34.51338889	19.61216667	1	1	
AC09A32	Uilkraals flower valley	Uilkraals (Ui)	-34.56222222	19.45513889	2	2	2
AC09A30	Haelkraal	Haelkraal (Hk)	-34.67683333	19.58697222	1	2	1
ES07A38	Haelkraal	Haelkraal (Hk)	-34.67083333	19.54300000	4	2	2
AC09A28	Haelkraal tributary	Haelkraal (Hk)	-34.66763889	19.57527778	3	3	3
AC09C19	Ratel	Ratel (Rt)	-34.67011111	19.69138889	2	3	2
AC09C15	Kars Tributary	Heuningnes (Hn)	-34.46783333	19.82311111	1		
AC09C12	Koue	Heuningnes (Hn)	-34.56136111	19.63094444	2	2	2
AC09A27	Nuwejaars	Heuningnes (Hn)	-34.57836111	19.75758333	1		
AC09A25	Nuwejaars tributary	Heuningnes (Hn)	-34.55497222	19.88583333	1	1	1
AC09A26	Nuwejaars tributary	Heuningnes (Hn)	-34.54230556	19.81916667	1		
AC09B51	Van Zyl Farm tributary	Heuningnes (Hn)	-34.51836111	19.97591667	1	1	1
AC09B35	Agter-Vink	Breede (Br)	-33.67155556	19.74255556	1	1	1
AC08A32	Amandel	Breede (Br)	-33.98900000	19.18402778	2		
ES07A21	Baviaans	Breede (Br)	-34.02705556	19.55588889		2	
AC09B41	Bereaville	Breede (Br)	-34.02705556	19.48236111	2	2	1
ES07	Boskloof	Breede (Br)	-33.52894444	19.54008333	1		
AC09C81	Bothaspruit	Breede (Br)	-33.56247222	19.34075000	1	1	1
AC09D09	Breede	Breede (Br)	-33.52002778	19.18552778	2	2	2
AC09D41	Buffeljags	Breede (Br)	-33.99916667	20.60455556	1	2	1
AC09B25	Doring	Breede (Br)	-33.93294444	19.46161111	1	1	1

Table 4.1: continued…

Sample code	River	Drainage system	Latitude	Longitude	COI	Cyt b	COI + cyt b
AC09C82	Du Toit	Breede (Br)	-33.96702778	19.16566667	1	1	1
gz33	Elandskloof	Breede (Br)	-34.05202778	19.41627778	1	1	
AC09A74	Ganskraal	Breede (Br)	-34.11183333	19.81530556	2	1	1
AC09A76	Ganskraal	Breede (Br)	-34.12547222	19.81147222	2	2	2
AC09C59	Gobos	Breede (Br)	-34.05344444	19.61366667	2	2	2
gz32	Happy Valley	Breede (Br)	-34.09650000	19.73605556	1	1	
AC09C79	Hartbees	Breede (Br)	-33.56483333	19.43175000	3	3	2
AC08A19	Hex	Breede (Br)	-33.52894444	19.54008333	3	1	1
AC08A18	Hex trib	Breede (Br)	-33.49877778	19.53061111	1	2	1
AC08A14	Hoeks	Breede (Br)	-34.00963889	19.83836111	1	1	
AC09B33	Holsloot tributary	Breede (Br)	-33.82883333	19.27161111	2	2	2
AC09A51	Keissies	Breede (Br)	-33.70352778	19.91086111	4	3	2
AC09A50	Keissies	Breede (Br)	-33.71050000	19.93105556	1	1	1
AC09C63	Keurbooms	Breede (Br)	-34.00441667	20.37713889	2	2	2
AC09C69	Klippe	Breede (Br)	-34.02463889	20.41805556	1	2	1
AC09C77	Koornings	Breede (Br)	-34.01230556	20.45458333	2	1	1
AC09C67	Krom Riviers	Breede (Br)	-34.11166667	19.79566667	1	1	1
AC09C71	Leeu Breede	Breede (Br)	-33.97855556	20.34202778	1	1	1
AC09A78	Oubos	Breede (Br)	-34.07963889	19.82952778	4	4	4
AC09B20	Poesjenels	Breede (Br)	-33.97916667	19.55761111	2	1	1
AC09B44	Riviersonderend	Breede (Br)	-34.06741667	19.07566667	2	2	2
AC09C57		Breede (Br)	-34.07666667	19.64325000	2	2	2
AC09C56		Breede (Br)	-34.04236111	19.53716667	2	2	2
AC09D33		Breede (Br)	-33.66619444	19.24638889	1	2	1
AC09D32	Smallblaar	Breede (Br)	-33.69050000	19.31622222	2	2	2
AC09C65	Soetmelks	Breede (Br)	-34.11238889	19.74552778	2	1	1
AC09B27	Stettyn	Breede (Br)	-33.86977778	19.34750000	2	1	1
AC08A10	Vink	Breede (Br)	-33.73022222	19.82355556	1	2	1
AC08A11	Vink tributary	Breede (Br)	-33.72494444	19.81547222	2	1	1
AC09D20	Wabooms	Breede (Br)	-33.50252778	19.27063889	1	2	1
AC09A42	Wolwekloof	Breede (Br)	-33.56413889	19.12933333	3	3	3
AC09C32	Duiwenhoks	Duiwenhoks (Du)	-33.99480556	21.07519444	2	2	2
AC09C33	Duiwenhoks	Duiwenhoks (Du)	-33.97819444	21.03244444	2	1	1
AC09C35	Duiwenhoks	Duiwenhoks (Du)	-33.97041667	20.98783333	2	2	2
AC09C36	Duiwenhoks	Duiwenhoks (Du)	-33.99211111	20.85430556	2	1	1
AC09B01	Goukou	Goukou (Gk)	-34.00036111	21.41800000	2	2	2
AC09B05	Kleinkruis	Goukou (Gk)	-34.01719444	21.29058333	4	2	2
AC09C31	Korinte	Goukou (Gk)	-33.98883333	21.15794444	2	2	1
AC08A58	Vette	Goukou (Gk)	-34.02125000	21.22500000	4	4	4
ASG(?)	Assegaibos	Gouritz (Gr)	-33.73861111	21.56472222	2		
BRGZ2	Bos	Gouritz (Gr)	-33.73055556	21.50611111	2		
AC09A02		Gouritz (Gr)	-33.97313889	21.57227778	2	2	1
AC09A03		Gouritz (Gr)	-33.98652778	21.56911111	2		

Table 4.1: continued…

Sample code	River	Drainage system	Latitude	Longitude	COI	Cyt b	COI + cyt b
Es07	Gouritz	Gouritz (Gr)	-33.99675000	21.54602778	1		
ES07	Gouritz	Gouritz (Gr)	-33.98652778	21.56911111	1	1	
AC09A01	Langtou	Gouritz (Gr)	-33.97786111	21.78283333	2	2	2
AC08A01	Nels	Gouritz (Gr)	-33.46805556	21.73566667	1	2	1
AC09B03	Vals	Gouritz (Gr)	-34.00700000	21.43575000	2	2	2
AC09A05		Gouritz (Gr)	-34.02500000	21.58394444	2	2	2
AC09A06		Gouritz (Gr)	-33.99672222	21.54611111	2	2	2
AC09A07		Gouritz (Gr)	-33.99883333	21.53497222		1	
ES07A11	Groot Brak	Groot Brak (Gb)	-33.95986111	22.24133333	3	1	1
ES07A15	Klein Brak	Groot Brak (Gb)	-33.95202778	22.07358333	3	2	2
ES07A09	Gwaing	Gwaing (Gw)	-33.93902778	22.38516667	3	1	1
ES07	Gwaing	Gwaing (Gw)	-33.94730556	22.45866667	1		
gb01	Malgas	Gwaing (Gw)	-33.93722222	22.42194444		1	
ES07	Rooi	Gwaing (Gw)	-33.94730556	22.45866667		2	
ES07A10	Maalgate	Maalgate (Ma)	-33.95669444	22.35666667	3		
GZ45	Noetsie	Noetsie (Nt)	-34.03866667	23.15936111	1	1	1
GZ46	Bitou Tributary	Bitou (Bt)	-34.01977778	23.31894444	1	2	

A total of 114 individuals (1227 base pairs) from 73 localities (Table 4.1) were used for the combined analyses, which yielded 132 polymorphic sites and 63 unique alleles (Table 4.4). None of these alleles were geographically widespread. Only 2 haplotypes were shared between river systems (one between the Breede and Groot Brak and another between the Ratel and the Heuningnes) (Table 4.4).

Table 4.2: Distribution of mitochondrial cytochrome *c* oxidase subunit I (COI) haplotypes across river systems for *Galaxias* ‘nebula’. Abbreviations for drainage systems are given in Table 4.1.

	Ol	Bg	Kl	Ui	Hk	Rt	Hn	Br	Du	Gk	Gr	Gb	Ma	Gw	Nt	Bt
n	(4)	(17)	(2)	(5)	(8)	(2)	(7)	(68)	(8)	(12)	(19)	(6)	(3)	(4)	(1)	(1)
Allele																
1	2															
2	1															
3	1															
4				2	2	2	6									
5							1									
6					1						1					
7				1												
8														1		
9									5				1			
10				1	4											
11					1											
12				1												
13									2							
14			1													
15			1													
16													1			
17													1			
18								1								
19								51				3				
20								1								
21												1				
22								2								
23								3								
24								2								
25								1								
26												1				
27												1				
28								2								
29								1								
30								1								
31								2								
32								1								
33											6					
34											3					
35															1	1
36											2					
37											4					
38											2					
39														3		
40											1					

Table 4.2: continued…

	Ol	Bg	Kl	Ui	Hk	Rt	Hn	Br	Du	Gk	Gr	Gb	Ma	Gw	Nt	Bt
n	(4)	(17)	(2)	(5)	(8)	(2)	(7)	(68)	(8)	(12)	(19)	(6)	(3)	(4)	(1)	(1)
Allele																
41										1						
42										8						
43										1						
44										2						
45									1							
46		14														
47		2														
48		1														
Total	**4**	**17**	**2**	**5**	**8**	**2**	**7**	**68**	**8**	**12**	**19**	**6**	**3**	**4**	**1**	**1**

Table 4.3: Distribution of mitochondrial cytochrome *b* (cyt *b*) haplotypes across river systems for *Galaxias* ‘nebula’. Abbreviations for drainage systems are given in Table 4.1.

	Ol	Bg	Kl	Ui	Hk	Rt	Hn	Br	Du	Gk	Gr	Gb	Gw	Nt	Bt
n	(3)	(18)	(4)	(5)	(7)	(3)	(4)	(65)	(7)	(10)	(13)	(3)	(4)	(1)	(1)
Allele															
1										7					
2										1					
3								11				2			
4								2							
5								1							
6								1							
7												1			
8								7							
9								1							
10								1							
11								1							
12								1							
13								2							
14								6							
15								1							
16								3							
17								4							
18								5							
19								3							
20								10							
21								1							
22								1							
23								1							
24								1							
25								1							
26											1				
27											1				
28											2				
29															1
30														1	
31											1				
32											6				
33										2					
34									5						
35									1						
36									1						
37				2											
38				1			1								
39							1								
40							1								

Table 4.3: continued...

	Ol	Bg	Kl	Ui	Hk	Rt	Hn	Br	Du	Gk	Gr	Gb	Gw	Nt	Bt
n	(3)	(18)	(4)	(5)	(7)	(3)	(4)	(65)	(7)	(10)	(13)	(3)	(4)	(1)	(1)
Allele															
41						3	1								
42					2										
43					1										
44					2										
45					1										
46					1										
47				2											
48			1												
49			1												
50			2												
51													4		
52											2				
53		8													
54		1													
55		3													
56		1													
57		2													
58		1													
59		1													
60		1													
61	1														
62	1														
63	1														
Total	**3**	**18**	**4**	**5**	**7**	**3**	**4**	**65**	**7**	**10**	**13**	**3**	**4**	**1**	**1**

Table 4.4: Distribution of haplotypes based on combined mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) sequences of *Galaxias* ‘nebula’ across river systems. Abbreviations for drainage systems are given in Table 4.1.

	Ol	Bg	Kl	Ui	Hk	Rt	Hn	Br	Du	Gk	Gr	Gb	Gw	Nt
n	(3)	(13)	(1)	(4)	(6)	(2)	(4)	(52)	(6)	(9)	(9)	(3)	(1)	(1)
Allele														
1										6				
2										1				
3										1				
4		3												
5		1												
6		3												
7		1												
8		2												
9		1												
10		1												
11		1												
12							1							
13							1							
14							1							
15				1										
16				1										
17					2									
18						2	1							
19				1										
20				1										
21					2									
22					1									
23					1									
24			1											
25								7				1		
26												1		
27								1						
28								1						
29								1						
30								1						
31								3						
32								1						
33								7						
34								2						
35								1						
36												1		
37								1						
38								2						
39								1						
40								8						

Table 4.4: continued…

River *n*	Ol (3)	Bg (13)	Kl (1)	Ui (4)	Hk (4)	Rt (2)	Hn (4)	Br (52)	Du (6)	Gk (9)	Gr (9)	Gb (3)	Gw (1)	Nt (1)
Allele														
41								1						
42								3						
43								3						
44								1						
45								3						
46								2						
47								1						
48								1						
49											1			
50											1			
51											2			
52														1
53											2			
54											2			
55									4					
56										1				
57									1					
58									1					
59													1	
60											1			
61	1													
62	1													
63	1													
Total	**3**	**13**	**1**	**4**	**6**	**2**	**4**	**52**	**6**	**9**	**9**	**3**	**1**	**1**

Diversity and demographic parameter estimates for the COI, cyt *b* and combined data sets are presented in Tables 4.5, 4.6 and 4.7, respectively, for the various hierarchical arrangements of populations. Estimates of *F*s and *D* for populations in the Breede River system, the Breede-Heuningnes-Duiwenhoks palaeoriver system and the combined southern draining river systems differed from those expected under demographic stability (Tables 4.5 - 4.7).

Table 4.5: Genetic diversity measures and demographic estimates based on the mitochondrial cytochrome c oxidase subunit I (COI) gene for *Galaxias* 'nebula' for the current river systems, palaeorivers and regions. * Indicates statistically significant values.

Hierarch level	*n*	Polymorphic sites	N_H	H_D	π	Fu's *F*	Tajima's *D*
Current river systems							
Olifants	4	3	3	0.833 ± 0.222	0.00278 ± 0.0024	-0.133 (0.240)	0.168 (0.732)
Berg	17	2	3	0.324 ± 0.136	0.00056 ± 0.0007	-1.038 (0.052)	-1.069 (0.205)
Klein	2	1	2	1.000 ± 0.500	0.00167 ± 0.0024		
Haelkraal	8	6	4	0.750 ± 0.139	0.00393 ± 0.0027	0.491 (0.565)	0.087 (0.582)
Uilkraals	5	5	4	0.900 ± 0.161	0.00433 ± 0.0034	-0.567 (0.230)	0.562 (0.709)
Ratel	2	0	1				
Heuningnes	8	1	2	0.250 ± 0.180	0.00042 ± 0.0006	-0.182 (0.200)	-1.055 (0.202)
Breede	69	17	13	0.454 ± 0.076	0.00161 ± 0.0012	-9.150 (0.001)*	-2.163 (0.001)*
Duiwenhoks	8	4	3	0.607 ± 0.164	0.00196 ± 0.0016	0.506 (0.522)	-1.030 (0.213)
Goukou	12	6	4	0.561 ± 0.154	0.00298 ± 0.0021	0.659 (0.649)	-0.382 (0.367)
Gouritz	18	13	6	0.830 ± 0.054	0.00495 ± 0.0030	0.643 (0.626)	-0.802 (0.239)
Maalgate	3	5	3	1.000 ± 0.272	0.00556 ± 0.0048	-0.077 (0.225)	
Gwaing	4	2	2	0.500 ± 0.265	0.00167 ± 0.0017	1.099 (0.619)	-0.710 (0.295)
Groot Brak	6	4	4	0.800 ± 0.172	0.00289 ± 0.0022	-0.341 (0.184)	-0.057 (0.469)
Bitou	2	0	1				
Palaeorivers							
Olifants	4	3	3	0.833 ± 0.222	0.00278 ± 0.0024	-0.133 (0.240)	0.168 (0.732)
Berg	17	2	3	0.324 ± 0.136	0.00056 ± 0.0007	-1.038 (0.052)	-1.069 (0.205)
Klein	2	1	2	1.000 ± 0.500	0.00167 ± 0.0024		
Uilkraals	5	5	4	0.900 ± 0.161	0.00433 ± 0.0034	-0.567 (0.230)	0.562 (0.709)
Haelkraal	8	6	4	0.750 ± 0.139	0.00393 ± 0.0027	0.491 (0.565)	0.087 (0.582)
Ratel	2	0	1				
Breede-Dui-Heu	87	21	18	0.635 ± 0.059	0.00306 ± 0.0020	-9.313 (0.001)*	-1.661 (0.022)*
Gouritz-Goukou	31	21	11	0.882 ± 0.034	0.00774 ± 0.0043	-0.445 (0.473)	-0.408 (0.374)
Wilderness LR	14	16	10	0.934 ± 0.051	0.00661 ± 0.0040	-3.389 (0.032)*	-0.868 (0.190)
Bitou (Keurbooms)	1	0	1				
Regions							
West Coast	21	16	6	0.557 ± 0.124	0.00776 ± 0.0044	2.563 (0.869)	0.173 (0.628)
South Coast	146	53	42	0.850 ± 0.027	0.00572 ± 0.0033	-26.042 (0.001)*	-1.959 (0.01)*

Table 4.6: Genetic diversity measures and demographic estimates based on mitochondrial cytochrome *b* (cyt *b*) gene for *Galaxias* ‘nebula’ for the current river systems, palaeorivers and regions. * Indicates statistically significant values.

Hierarch level	*n*	Polymorphic sites	N_H	H_D	π	Fu's *F*	Tajima’s *D*
Current river systems							
Olifants	3	9	3	1.000 ± 0.272	0.00957 ± 0.0078	0.588 (0.429)	
Berg	18	15	8	0.791 ± 0.087	0.00367 ± 0.0024	-2.006 (0.108)	-1.792 (0.024)*
Klein	4	2	3	0.833 ± 0.222	0.00186 ± 0.0018	-0.658 (0.173)	0.592 (0.840)
Uilkraals	5	9	3	0.800 ± 0.164	0.00829 ± 0.0056	2.385 (0.866)	1.448 (0.934)
Haelkraal	7	10	5	0.905 ± 0.103	0.00790 ± 0.0050	0.276 (0.482)	1.145 (0.900)
Ratel	3	0	1				
Heuningnes	4	5	4	1.000 ± 0.177	0.00425 ± 0.0034	-1.414 (0.057)	-0.212 (0.562)
Breede	65	20	22	0.923 ± 0.015	0.00359 ± 0.0022	-14.525 (0.000)*	-1.428 (0.059)
Duiwenhoks	7	4	3	0.524 ± 0.209	0.00182 ± 0.0015	0.263 (0.506)	-1.434 (0.054)
Goukou	10	8	3	0.511 ± 0.164	0.00429 ± 0.0028	2.776 (0.903)	-0.212 (0.469)
Gouritz	13	20	6	0.782 ± 0.105	0.00935 ± 0.0054	1.757 (0.808)	-0.380 (0.390)
Groot Brak	3	1	2	0.667 ± 0.314	0.00106 ± 0.0013	0.201 (0.370)	
Gwaing	4	0	1				
Noetsie	1						
Bitou	1						
Palaeorivers							
Olifants	3	9	3	1.000 ± 0.272	0.00957 ± 0.0078	0.588 (0.429)	
Berg	18	15	8	0.791 ± 0.087	0.00367 ± 0.0024	-2.006 (0.108)	-1.792 (0.024)*
Klein	4	2	3	0.833 ± 0.222	0.00186 ± 0.0018	-0.658 (0.173)	0.592 (0.840)
Uilkraals	5	9	3	0.800 ± 0.164	0.00829 ± 0.0056	2.385 (0.866)	1.448 (0.934)
Haelkraal	7	10	5	0.905 ± 0.103	0.00790 ± 0.0050	0.276 (0.482)	1.145 (0.900)
Ratel	3	0	1				
Breede-Dui-Heu	76	35	29	0.944 ± 0.011	0.00747 ± 0.0041	-13.312 (0.000)*	-1.077 (0.147)
Gouritz-Goukou	23	27	9	0.846 ± 0.051	0.01181 ± 0.0064	1.819 (0.782)	0.047 (0.560)
Wilderness LR	8	16	4	0.750 ± 0.139	0.11221 ± 0.0067	3.123 (0.919)	0.720 (0.803)
Bitou (Keurbooms)	1						
Regions							
West Coast	21	42	11	0.848 ± 0.067	0.01365 ± 0.0073	0.353 (0.585)	-1.055 (0.124)
South Coast	127	66	52	0.972 ± 0.005	0.12105 ± 0.0063	-24.728 (0.000)*	-1.190 (0.107)

Table 4.7: Genetic diversity measures and demographic estimates based on the combined mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) genes for *Galaxias* 'nebula' for the current river hierarchies, palaeorivers and regions. * Indicates statistically significant values.

Hierarch level	*n*	Polymorphic sites	N_H	H_D	π	Fu's *F*	Tajima's *D*
Current river systems							
Olifants	3	12	3	1.000 ± 0.272	0.00652 ± 0.0052	0.901 (0.440)	
Berg	13	14	8	0.910 ± 0.056	0.00232 ± 0.0015	-2.392 (0.060)	-1.532 (0.070)
Klein	1						
Uilkraals	4	14	4	1.000 ± 0.177	0.00679 ± 0.0048	0.118 (0.280)	0.926 (0.800)
Haelkraal	6	13	3	0.867 ± 0.129	0.00538 ± 0.0034	1.719 (0.77)	0.974 (0.830)
Ratel	2	0	1				
Heuningnes	4	6	4	1.000 ± 0.177	0.00258 ± 0.0020	-1.157 (0.090)	-0.315 (0.550)
Breede	52	28	22	0.936 ± 0.016	0.00212 ± 0.0013	-14.542 (0.000)*	-1.910 (0.010)*
Duiwenhoks	6	8	3	0.600 ± 0.215	0.00217 ± 0.0016	1.574 (0.770)	-1.408 (0.040)*
Goukou	9	14	4	0.583 ± 0.183	0.00254 ± 0.0016	1.380 (0.800)	-1.901 (0.010)*
Gouritz	9	29	6	0.917 ± 0.073	0.00729 ± 0.0042	1.304 (0.720)	-0.810 (0.220)
Groot Brak	3	3	3	1.000 ± 0.272	0.00163 ± 0.0015	-0.693 (0.120)	0.000 (0.890)
Gwaing	1						
Noetsie	1						
Palaeorivers							
Olifants	3	12	3	1.0000±0.2722	0.00652 ± 0.0052	0.901 (0.430)	
Berg	13	14	8	0.9103±0.0559	0.00232 ± 0.0015	-2.392 (0.080)	-1.532 (0.060)
Klein	1						
Uilkraals	4	14	4	1.0000±0.1768	0.00679 ± 0.0048	0.118 (0.280)	0.926 (0.800)
Haelkraal	6	13	3	0.8667±0.1291	0.00538 ± 0.0034	1.719 (0.770)	0.974 (0.830)
Ratel	2	0	1				
Breede-Dui-Heu	62	50	29	0.9534±0.0115	0.00509 ± 0.0027	-10.099 (0.010)*	-1.370 (0.060)
Gouritz-Goukou	18	14	10	0.8824±0.0626	0.00954 ± 0.0051	1.481 (0.780)	-0.073 (0.530)
Wilderness LR	5	24	5	1.0000±0.1265	0.00897 ± 0.0058	-0.213 (0.280)	-0.336 (0.460)
Regions							
West Coast	16	56	11	0.9417±0.0406	0.0123 ± 0.0065	0.647 (0.620)	-0.462 (0.390)
South Coast	98	97	52	0.9745±0.0061	0.00849 ± 0.0043	-22.734 (0.000)*	-1.471 (0.040)*

Mean sequence divergences between current river systems ranged from 0 to 2.53 % for COI (Table 4.8), 0 to 5.60 % for cyt *b* (Table 4.9) and 0 to 3.89 % for the combined cyt *b* and COI data set (Table 4.10). These values were consistently higher for comparisons between western draining river systems (Olifants and Berg) and the rest, compared to between southern draining systems (Tables 4.8 – 4.10).

Table 4.8: Mitochondrial cytochrome *c* oxidase subunit I (COI) sequence divergence values for *Galaxias* 'nebula' populations across river systems of the Cape Floristic Region. The values represent means and their ranges are given in parentheses.

	River system	1	2	3	4	5	6	7	8
1	Olifants								
2	Berg	2.53 (2.24-2.89)							
3	Klein	1.97 (1.66-2.28)	2.14 (1.84-2.45)						
4	Uilkraals	1.65 (1.25-2.05)	1.72 (1.24-2.21)	0.79 (0.52-1.06)					
5	Haelkraal	1.70 (1.25-2.05)	1.86 (1.42-2.22)	0.83 (0.52-1.25)	0.69 (0.34-1.05)				
6	Ratel	1.45 (1.25-1.64)	1.61 (1.42-1.81)	0.79 (0.70-0.88)	0.00				
7	Heuningnes	1.45 (1.25-1.64)	1.52 (1.23-1.81)	0.88 (0.70-1.06)	0.63 (0.34-0.87)	0.69 (0.52-0.87)			
8	Breede	2.05 (1.45-2.47)	2.22 (1.62-2.63)	1.00 (0.70-1.25)	0.90 (0.51-1.24)	0.91(0.51- 1.43)	0.90 (0.69-1.05)	0.99 (0.69-1.24)	
9	Duiwenhoks	1.58 (1.25-1.86)	1.75 (1.43-2.03)	0.6 (0.34-1.06)	0.50 (0.17-0.87)	0.58 (0.17-0.88)	0.46 (0.34-0.52)	0.54 (0.34-0.70)	0.78 (0.34-1.24)
10	Goukou	1.60 (1.26-2.04)	2.07 (1.43-2.63)	0.92 (0.69-1.25)	0.80 (0.51-1.24)	0.76 (0.51-1.05)	0.82 (0.69-1.05)	0.92 (0.69-1.24)	1.04 (0.69-1.43)
11	Gouritz	2.15 (1.45-2.90)	2.26 (1.81-2.66)	1.16 (0.70-1.63)	1.03 (0.51-1.44)	1.01 (0.51-1.43)	1.06 (0.69-1.43)	1.11 (0.52-1.62)	0.93 (0.17-1.43)
12	Groot Brak	2.09 (1.64-2.47)	2.12 (1.82-2.45)	1.07 (0.70-1.44)	0.96 (0.51-1.43)	1.01 (0.51-1.63)	0.96 (0.69-1.24)	1.06 (0.69-1.43)	0.63 (0.34-1.23)
13	Maalgate	1.99 (1.25-2.47)	2.16 (1.43-2.64)	0.91 (0.34-1.25)	0.81 (0.17-1.24)	0.80 (0.17-1.05)	0.81 (0.34-1.05)	0.91 (0.34-1.24)	1.05 (0.34-1.43)
14	Gwaing	1.65 (1.45-1.85)	1.75 (1.62-2.01)	0.61 (0.52-0.70)	0.54 (0.34-0.69)	0.59 (0.34-0.87)	0.51 (0.51-0.51)	0.60 (0.51-0.69)	0.72 (0.51-0.87)
15	Noetsie	2.47 (2.25-2.68)	2.49 (2.42-2.63)	1.34 (1.25-1.44)	1.17 (1.05-1.24)	1.17 (1.05-1.24)	1.24 (1.24-1.24)	1.33 (1.24-1.43)	1.05 (0.69-1.24)
16	Bitou	2.47 (2.25-2.68)	2.49 (2.42-2.63)	1.34 (1.25-1.44)	1.17 (1.05-1.24)	1.17 (1.05-1.24)	1.24 (1.24-1.24)	1.33 (1.24-1.43)	1.05 (0.69-1.24)

Table 4.8: continued…

	River system	9	10	11	12	13	14	15
10	Goukou	0.71 (0.34-1.24)						
11	Gouritz	0.94 (0.34-1.62)	1.18 (0.69-1.81)					
12	Groot Brak	0.85 (0.34-1.43)	1.10 (0.69-1.62)	1.06 (0.34-1.63)				
13	Maalgate	1.00 (0.88-1.24)	0.95 (0.34-1.43)	1.16 (0.34-1.81)	1.09 (0.34-1.62)			
14	Gwaing	0.45 (0.17-0.69)	0.64 (0.51-0.87)	0.78 (0.51-1.24)	0.73 (0.51-1.05)	0.73 (0.17-0.87)		
15	Noetsie	1.12 (0.87-1.42)	1.38 (1.23-1.62)	0.00	1.15 (0.87-1.43)	1.37 (0.87-1.62)	0.99 (0.87-1.05)	
16	Bitou	1.12 (0.87-1.42)	1.38 (1.23-1.62)	0.00	1.15 (0.87-1.43)	1.37 (0.87-1.62)	0.99 (0.87-1.05)	0.00

Table 4.9: Mitochondrial cytochrome *b* (cyt *b*) sequence divergence values for *Galaxias* ‘nebula’ populations across river systems of the Cape Floristic Region. The values represent means and their ranges are given in parentheses.

	River system	1	2	3	4	5	6	7	8
1	Olifants								
2	Berg	5.60 (4.80-6.55)							
3	Klein	3.72 (3.20-4.35)	2.67 (2.15-3.23)						
4	Uilkraals	4.17 (3.41-5.06)	3.09 (2.35-3.89)	0.91 (0.16-1.57)					
5	Haelkraal	4.03 (3.18-4.82)	3.034 (2.55-3.67)	0.77 (0.33-1.20)	1.02 (0.16-1.95)				
6	Ratel	3.94 (3.64-4.34)	2.82 (2.55-3.22)	0.84 (0.67-1.02)	0.84 (0.84-0.84)	0.82 (0.16-1.38)			
7	Heuningnes	4.34 (3.64-5.08)	3.22 (2.55-3.91)	1.14 (0.67-1.57)	1.02 (0.84-1.38)	1.16 (0.16-1.95)	0.00		
8	Breede	4.45 (3.65-5.34)	3.80 (2.97-4.61)	1.63 (1.02-2.15)	1.87 (1.20-2.34)	1.96 (1.38-2.55)	1.81 (1.38-2.15)	2.11 (1.38-2.76)	
9	Duiwenhoks	4.08 (3.46-4.90)	2.85 (2.38-3.17)	0.91 (0.50-1.40)	1.19 (0.67-1.98)	1.24 (0.85-1.78)	1.09 (0.85-1.40)	1.37 (0.85-1.98)	1.78 (1.21-2.59)
10	Goukou	3.30 (2.56-4.40)	2.80 (2.34-3.27)	1.02 (0.68-1.38)	1.26 (0.85-1.59)	1.35 (1.03-1.75)	1.20 (1.10-1.38)	1.48 (1.03-1.95)	1.85 (1.38-2.34)
11	Gouritz	3.67 (2.97-5.07)	3.23 (2.54-3.90)	1.56 (1.01-1.95)	1.88 (0.84-2.53)	1.83 (1.01-2.35)	1.71 (1.37-1.94)	2.04 (1.37-2.55)	2.25 (1.37-3.18)
12	Groot Brak	4.30 (3.87-4.83)	3.64 (3.18-4.13)	1.48 (1.20-1.76)	1.72 (1.38-1.95)	1.81 (1.56-2.14)	1.66 (1.56-1.75)	1.95 (1.56-2.34)	0.49 (0.33-0.83)
13	Gwaing	3.26 (2.97-3.63)	2.66 (2.34-3.00)	0.93 (0.84-1.02)	1.38 (1.02-1.75)	1.27 (0.84-1.57)	1.20	1.51 (1.20-1.76)	2.01 (1.57-2.34)
14	Noetsie	3.57 (3.19-4.10)	3.08 (2.75-3.43)	1.37 (1.19-1.56)	1.75 (1.37-2.13)	1.71 (1.56-1.94)	1.56	1.85 (1.56-2.14)	1.98 (1.56-2.34)
15	Bitou	3.35 (2.98-3.88)	3.30 (2.96-3.66)	1.56 (1.37-1.75)	1.94 (1.56-2.33)	1.90 (1.74-2.13)	1.74	2.04 (1.74-2.33)	2.18 (1.75-2.54)

Table 4.9: continued…

	River system	9	10	11	12	13	14
10	Goukou	1.15 (0.33-0.1.78)					
11	Gouritz	1.84 (1.2-2.38)	1.90 (1.39-2.33)				
12	Groot Brak	1.63 (1.21-2.18)	1.73 (1.56-1.94)	2.11 (1.56-2.75)			
13	Gwaing	1.28 (1.03-1.59)	1.14 (1.01-1.21)	1.87 (1.55-2.13)	1.85 (1.75-1.95)		
14	Noetsie	1.65 (1.39-1.97)	1.75 (1.58-1.94)	1.03 (0.49-2.54)	1.85 (1.75-1.94)	1.75	
15	Bitou	1.84 (1.58-2.17)	1.94 (1.77-2.13)	0.93 (0.33-2.74)	2.04 (1.94-2.13)	1.94	0.49

Table 4.10: Combined mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) sequence divergence values for *Galaxias* ‘nebula’ populations from river systems across the Cape Floristic Region. The values represent means and their ranges are given in parentheses.

	River system	1	2	3	4	5	6	7	8
1	Olifants								
2	Berg	3.89 (3.42 - 4.46)							
3	Klein	2.61 (2.47 - 2.79)	2.18 (1.97 - 2.36)						
4	Uilkraals	2.75 (2.46 - 3.09)	2.27 (1.96 - 2.66)	0.63 (0.33 - 0.94)					
5	Haelkraal	2.81 (2.56 - 3.20)	2.36 (2.05 - 2.76)	0.59 (0.42 - 0.76)	0.66 (0.08 - 1.29)				
6	Ratel	2.60 (2.46 - 2.78)	2.15 (1.96 - 2.35)	0.68	0.54 (0.33 - 0.76)	0.68 (0.08 - 1.02)			
7	Heuningnes	2.81 (2.56 - 3.10)	2.34 (2.06 - 2.56)	0.88 (0.76 - 1.03)	0.62 (0.08 - 1.11)	0.92 (0.33 - 1.39)	0.36 (0.25 - 0.50)		
8	Breede	3.09 (2.77 - 3.42)	2.83 (2.35 - 3.19)	1.10 (0.94 - 1.21)	1.27 (0.94 - 1.58)	1.32 (1.02 - 1.57)	1.27 (1.11 - 1.39)	1.49 (1.21 - 1.76)	
9	Duiwenhoks	2.72 (2.37 - 3.12)	2.22 (1.87 - 2.48)	0.62 (0.42 - 0.94)	0.84 (0.42 - 1.21)	0.85 (0.50 - 1.30)	0.77 (0.59 - 0.94)	0.97 (0.68 - 1.31)	1.19 (0.85 - 1.68)
10	Goukou	2.35 (1.97 - 3.01)	2.37 (1.97 - 2.65)	0.85 (0.68 - 0.94)	1.02 (0.68 - 1.30)	1.07 (0.76 - 1.30)	1.03 (0.85 - 1.11)	1.18 (0.85 - 1.48)	1.40 (1.11- 1.67)
11	Gouritz	2.78 (2.26 - 3.21)	2.63 (2.15 - 2.98)	1.15 (0.85 - 1.39)	1.33 (0.67 - 1.86)	1.33 (0.76 - 1.76)	1.33 (1.02 - 1.58)	1.55 (1.11 - 1.96)	1.45 (0.93 - 1.96)
12	Groot Brak	3.00 (2.77 - 3.31)	2.71 (2.45 - 2.97)	1.03 (0.94 - 1.12)	1.20 (0.93 - 1.48)	1.22 (1.02 - 1.39)	1.20 (1.11 - 1.30)	1.42 (1.20 - 1.67)	0.30 (0.16 - 0.67)
13	Gwaing	2.39 (2.26 - 2.56)	2.14 (1.96 - 2.26)	0.68	0.89 (0.67 - 1.11)	0.89 (0.76 - 1.02)	0.85	1.06 (0.94 - 1.21)	1.27 (1.12 - 1.39)
14	Noetsie	2.96 (2.68 - 3.21)	2.76 (2.56 - 2.89)	1.21	1.44 (1.20 - 1.67)	1.43 (1.30 - 1.57)	1.39	1.61 (1.48 - 1.77)	1.44 (1.30 - 1.58)

Table 4.10: continued…

	River system	9	10	11	12	13
10	Goukou	0.92 (0.25 - 1.40)				
11	Gouritz	1.31 (0.76 - 1.87)	1.49 (1.03 - 1.86)			
12	Groot Brak	1.12 (0.85 - 1.58)	1.34 (1.12 - 1.57)	1.39 (0.93 - 1.87)		
13	Gwaing	0.83 (0.59 - 1.12)	0.89 (0.85 - 0.94)	1.21 (1.02 - 1.39)	1.20 (1.11 - 1.30)	
14	Noetsie	1.37 (1.12 - 1.68)	1.58 (1.40 - 1.67)	0.86 (0.50 - 1.96)	1.39 (1.30 - 1.48)	1.21

Pairwise Φ_{ST} values revealed significant genetic heterogeneity among rivers (Tables 4.11 – 4.13), indicating that there is no contemporary gene flow across the landscape. The coastal river Uilkraals, Haelkraal, Ratel and Heuningnes River systems located in the low lying Agulhas Plain consistently recorded the lowest pairwise Φ_{ST} values, with negative values for COI (Tables 4.11 – 4.13). The Breede *vs* Groot Brak, Bitou *vs* Noetsie and the Gouritz *vs* Bitou also had very low Φ_{ST} values (Tables 4.11 – 4.13), but these could have been affected by the small sample sizes for the Groot Brak, Bitou and Noetsie (see Table 4.1).

Table 4.11: Pairwise Φ_{ST} values for *Galaxias* ‘nebula’ populations based on mitochondrial cytochrome *c* oxidase subunit I (COI) sequences.

	River system	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Olifants															
2	Berg	0.993														
3	Klein	0.973	0.993													
4	Uilkraals	0.918	0.975	0.552												
5	Haelkraal	0.919	0.961	0.471	-0.141											
6	Ratel	0.926	0.992	0.922	-0.120	-0.029										
7	Heuningnes	0.961	0.992	0.947	0.289	0.310	-0.318									
8	Breede	0.982	0.985	0.860	0.803	0.768	0.852	0.869								
9	Duiwenhoks	0.951	0.982	0.630	0.233	0.042	0.609	0.733	0.721							
10	Goukou	0.931	0.977	0.647	0.568	0.506	0.643	0.751	0.843	0.499						
11	Gouritz	0.912	0.952	0.501	0.446	0.442	0.451	0.587	0.617	0.339	0.639					
12	Groot Brak	0.970	0.986	0.800	0.685	0.611	0.805	0.886	0.263	0.690	0.759	0.445				
13	Maalgate	0.962	0.988	0.648	0.541	0.409	0.648	0.853	0.869	0.548	0.678	0.567	0.765			
14	Gwaing	0.960	0.989	0.761	0.374	0.195	0.796	0.872	0.776	0.356	0.551	0.217	0.733	0.568		
15	Noetsie	0.982	0.997	0.938	0.771	0.712	1.000	0.983	0.899	0.870	0.842	0.132	0.839	0.783	0.853	
16	Bitou	0.982	0.997	0.938	0.771	0.712	1.000	0.983	0.899	0.870	0.842	0.132	0.839	0.783	0.853	0.000

Table 4.12: Pairwise Φ_{ST} values for *Galaxias* ‘nebula’ populations based on mitochondrial cytochrome *b* (cyt *b*) sequences.

	River system	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Olifants														
2	Berg	0.918													
3	Klein	0.858	0.861												
4	Uilkraals	0.784	0.833	0.326											
5	Haelkraal	0.787	0.822	0.236	0.148										
6	Ratel	0.874	0.878	0.859	0.302	0.291									
7	Heuningnes	0.844	0.872	0.704	0.173	0.389	0.495								
8	Breede	0.909	0.898	0.775	0.758	0.770	0.811	0.818							
9	Duiwenhoks	0.895	0.873	0.738	0.561	0.523	0.852	0.767	0.791						
10	Goukou	0.827	0.848	0.635	0.525	0.556	0.726	0.708	0.802	0.667					
11	Gouritz	0.713	0.775	0.446	0.437	0.433	0.522	0.546	0.747	0.542	0.592				
12	Groot Brak	0.870	0.901	0.887	0.642	0.653	0.967	0.839	-0.002	0.887	0.803	0.578			
13	Gwaing	0.879	0.877	0.903	0.644	0.595	1.000	0.849	0.832	0.889	0.721	0.577	0.976		
14	Noetsie	0.720	0.871	0.860	0.514	0.525	1.000	0.760	0.812	0.874	0.774	0.000	0.942	1.000	
15	Bitou	0.698	0.881	0.879	0.568	0.579	1.000	0.785	0.831	0.890	0.800	-0.127	0.948	1.000	1.000

Table 4.13: Pairwise Φ_{ST} values for *Galaxias* ‘nebula’ populations for combined mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) sequences.

	River system	1	2	3	4	5	6	7	8	9	10	11	12	13
1	Olifants													
2	Berg	0.908												
3	Klein	0.718	0.882											
4	Uilkraals	0.725	0.835	-0.111										
5	Haelkraal	0.767	0.843	0.079	0.037									
6	Ratel	0.815	0.892	1.000	0.142	0.320								
7	Heuningnes	0.828	0.884	0.675	0.193	0.451	0.330							
8	Breede	0.910	0.913	0.791	0.767	0.782	0.830	0.835						
9	Duiwenhoks	0.849	0.882	0.568	0.417	0.469	0.735	0.706	0.790					
10	Goukou	0.828	0.887	0.689	0.593	0.635	0.779	0.773	0.832	0.704				
11	Gouritz	0.707	0.810	0.312	0.414	0.468	0.519	0.582	0.748	0.529	0.640			
12	Groot Brak	0.844	0.908	0.833	0.595	0.649	0.906	0.829	0.060	0.795	0.819	0.547		
13	Gwaing	0.696	0.880	1.000	0.206	0.371	1.000	0.731	0.820	0.680	0.696	0.356	0.857	
14	Noetsie	0.747	0.904	1.000	0.495	0.600	1.000	0.822	0.841	0.814	0.827	0.053	0.875	1.000

From the AMOVA it was clear that *Galaxias* 'nebula' populations are highly structured. The analyses revealed significant genetic variation among contemporary drainage basins: COI (Φ_{CT} = 0.89, $P < 0.001$; Table 4.14), cyt *b* (Φ_{CT} = 0.78, $P < 0.001$, Table 4.15) and combined COI and cyt *b* (Φ_{CT} = 0.77, $P < 0.001$, Table 4.16). The COI results also revealed significant structuring among groups under the palaeoriver (Φ_{CT} = 0.80, $P < 0.001$) and the western *vs* southern draining rivers (Φ_{CT} = 0.81, $P < 0.01$) arrangements (Table 4.14), although corresponding values were much lower for both cyt *b* (Table 4.15) and the combined COI and cyt *b* analyses (Table 4.16).

Table 4.14: Results of the AMOVA tests evaluating three competing hypotheses to explain the partitioning of genetic variation in *Galaxias* 'nebula' based on mitochondrial cytochrome *c* oxidase subunit I sequences.

Level of partitioning (or hypothesis)	% Variance	Phi-value	*P*-value
Region (southern draining *vs* western draining rivers)			
Between regions	82.23	$\Phi_{CT} = 0.82$	< 0.001
Among populations within regions	14.51	$\Phi_{SC} = 0.97$	< 0.001
Within populations	3.26	$\Phi_{ST} = 0.82$	< 0.001
Contemporary river systems (Olifants, Berg, Klein, Uilkraals, Haelkraal, Ratel, Heuningnes, Breede, Duiwenhoks, Goukou, Gouritz, Klein Brak, Groot Brak, Maalgate, Gwaing, Noetsie, Bitou)			
Among river systems	89.73	$\Phi_{CT} = 0.89$	< 0.001
Among populations within river systems	2.76	$\Phi_{SC} = 0.27$	< 0.001
Within all populations	7.51	$\Phi_{ST} = 0.92$	< 0.001
Ten palaeoriver systems (Olifants, Berg, Klein, Uilkraals, Haelkraal, Ratel, Breede-Heuningnes-Duiwenhoks, Gouritz-Goukou, Wilderness Lakes Region, Bitou (Keurbooms))			
Among palaeorivers	81.14	$\Phi_{CT} = 0.81$	< 0.001
Among populations within palaeorivers	12.10	$\Phi_{SC} = 0.64$	< 0.001
Within populations	6.77	$\Phi_{ST} = 0.93$	< 0.001

Table 4.15: Results of the AMOVA tests evaluating three competing hypotheses to explain the partitioning of genetic variation in *Galaxias* ‘nebula’ based on mitochondrial cytochrome *b* sequences.

Level of partitioning (or hypothesis)	% Variance	Phi-value	*P*-value
Region (southward draining *vs* westward draining rivers)			
Between regions	37.45	$\Phi_{CT} = 0.37$	< 0.05
Among populations within regions	47.38	$\Phi_{SC} = 0.76$	< 0.001
Within populations	15.18	$\Phi_{ST} = 0.85$	< 0.001
Contemporary river systems (Olifants, Berg, Klein, Uilkraals, Haelkraal, Ratel, Heuningnes, Breede, Duiwenhoks, Goukou, Gouritz, Groot Brak, Gwaing, Bitou, Noetsie)			
Among river systems	78.41	$\Phi_{CT} = 0.78$	< 0.001
Among populations within river systems	9.97	$\Phi_{SC} = 0.46$	< 0.001
Within all populations	11.62	$\Phi_{ST} = 0.88$	< 0.001
Ten palaeoriver systems (Olifants, Berg, Klein, Uilkraals, Haelkraal, Ratel, Breede-Duiwenhoks-Heuningnes, Gouritz-Goukou, Wilderness Lakes Region, Bitou (Keurbooms))			
Among palaeorivers	32.28	$\Phi_{CT} = 0.32$	< 0.05
Among populations within palaeorivers	48.25	$\Phi_{SC} = 0.71$	< 0.001
Within populations	19.47	$\Phi_{ST} = 0.81$	< 0.001

Table 4.16: Results of the analysis of molecular variance tests evaluating three competing hypotheses to explain the partitioning of genetic variation in *Galaxias* ‘nebula’ based on combined mitochondrial cytochrome *c* oxidase subunit I and cytochrome *b* sequences.

Level of partitioning (or hypothesis)	% Variance	Phi-value	*P*-value
Ocean region (West coast *vs* South coast)			
Between regions	56.04	$\Phi_{CT} = 0.56$	< 0.001
Among populations within regions	37.46	$\Phi_{SC} = 0.85$	< 0.001
Within populations	6.50	$\Phi_{ST} = 0.93$	< 0.001
Contemporary river systems (Olifants, Berg, Klein, Uilkraals, Haelkraal, Ratel, Heuningnes, Breede, Duiwenhoks, Goukou, Gouritz, Groot Brak, Gwaing, Noetsie)			
Among river systems	77.29	$\Phi_{CT} = 0.77$	< 0.001
Among populations within river systems	13.20	$\Phi_{SC} = 0.58$	< 0.001
Within all populations	9.51	$\Phi_{ST} = 0.90$	< 0.001
Ten palaeoriver systems (Olifants, Berg, Klein, Uilkraals, Haelkraal, Ratel, Breede-Duiwenhoks-Heuningnes, Gouritz-Goukou, Wilderness Lakes Region, Bitou (Keurbooms))			
Among palaeorivers	59.16	$\Phi_{CT} =0.59$	< 0.001
Among populations within palaeorivers	31.81	$\Phi_{SC} = 0.78$	< 0.001
Within populations	9.03	$\Phi_{ST} = 0.91$	< 0.001

Evolutionary history

The haplotype networks for COI (Fig. 4.3 & 4.4), cyt *b* (Fig. 4.5 & 4.6) and combined COI and cyt *b* (Fig. 4.7 & 4.8) analyses consisted of three disjointed clades. The first clade comprised of all the Olifants River system samples, the second clade contained all the Berg River system samples and a third clade comprised of all the samples from the southern draining river systems. The star-like radiation pattern of unique alleles that are closely related to high-frequency alleles (only for the Breede River system) and the sharing of alleles between currently isolated and geographically distant river systems suggest recent contact between populations in the southern draining river systems (Figs. 4.3, 4.5 and 4.7).

Haplotype networks for the three data sets were not fully resolved. The COI network had two loops (Fig. 4.3), while the cyt *b* and the combined COI and cyt *b* networks had three loops each (Figs 4.5 and 4.7). For each of the three networks, alternative branches that could be broken to resolve the loops are shown with a numbered solid arrow. For COI loop 1 (Fig. 4.3), there are two alternative places to break the loop (1A and 1B), although the resulting cladogram does not lead to different nesting designs. The presented network (Fig. 4.4) corresponds to removal of connection 1A based on the topology criterion. Allele 34 is more likely to be connected to the extant allele 30 than to the hypothetical allele (connection 1A). In loop 2, the inferred haplotype is more likely to be connected to the more frequent allele 9 according to the frequency criterion. Allele 9 co-occurs with allele 13 in the Duiwenhoks River system.

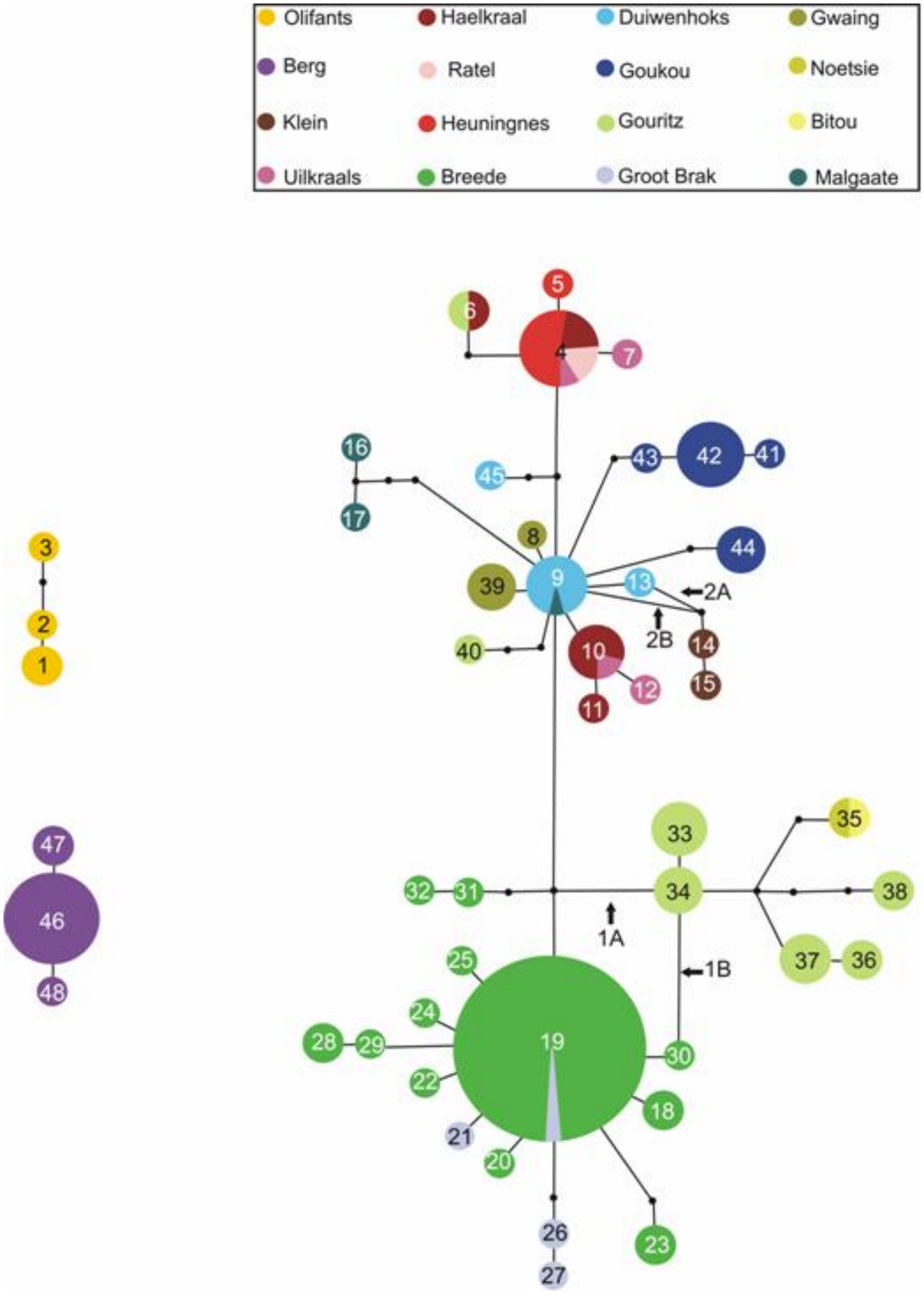


Fig. 4.3: Mitochondrial cytochrome *c* oxidase subunit I (COI) TCS haplotype network for *Galaxias* 'nebula' showing potential connections to resolve loops (arrows). The numbers indicate haplotypes described in Table 4.2. Solid black circles represent hypothetical haplotypes that were not sampled or that are extinct. Colour codes correspond to the 16 river systems where the species occurs.

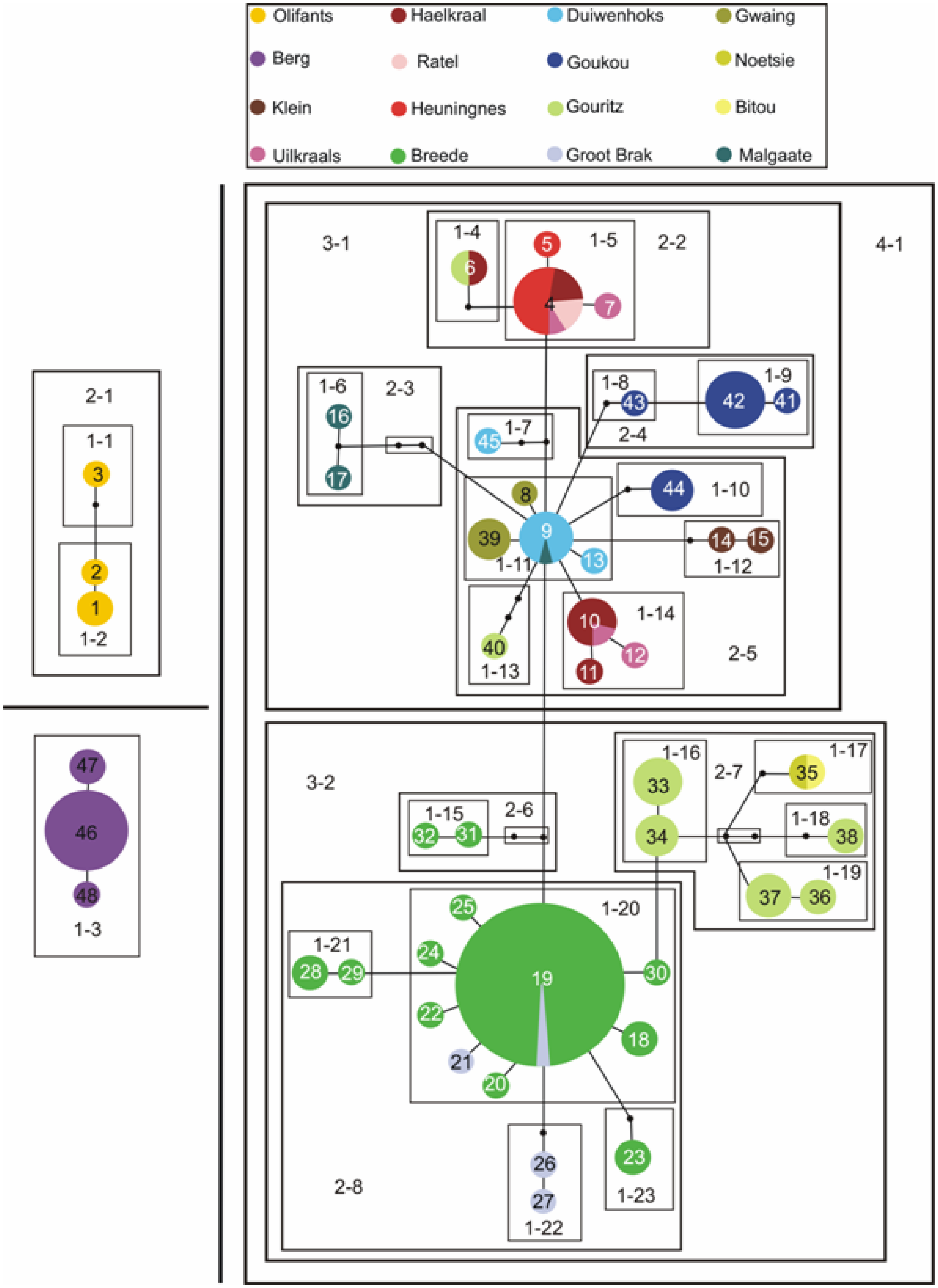


Fig.4.4: Mitochondrial cytochrome *c* oxidase subunit I (COI) TCS haplotype network for *Galaxias* ‘nebula’ showing nesting levels used in Nested Clade Phylogeographic Analysis (NCPA).

For cyt *b* (Fig. 4.5) the connection between allele 5 and allele 6 seems least likely based on the frequency and topology criteria. Allele 6 is more likely to be connected to the more frequent and interior allele 3. Loops 2 and 3 were solved using the frequency criterion, where haplotypes 22 and 23 are more likely to be connected to the more frequent and extant alleles 20 and 14 respectively. Thus, loop 2 was resolved by breaking connection 2C and loop 3 was resolved by breaking connection 3D (Fig. 4.6).

Loop 1 in the combined COI and cyt *b* network (Fig. 4.7) was resolved by breaking connection 1 as allele 30 is more likely to be connected to the extant allele 29 than to the hypothetical allele. Loop 2 was solved by breaking connection 2A based on the frequency criterion as allele 47 is more likely to be connected to the more frequent allele 40. Loop 3 could be broken at 2 alternative places (3A and 3B in Fig. 4.7) which result in almost identical nesting designs. The presented network (Fig. 4.8) corresponds to breaking of connection 3B.

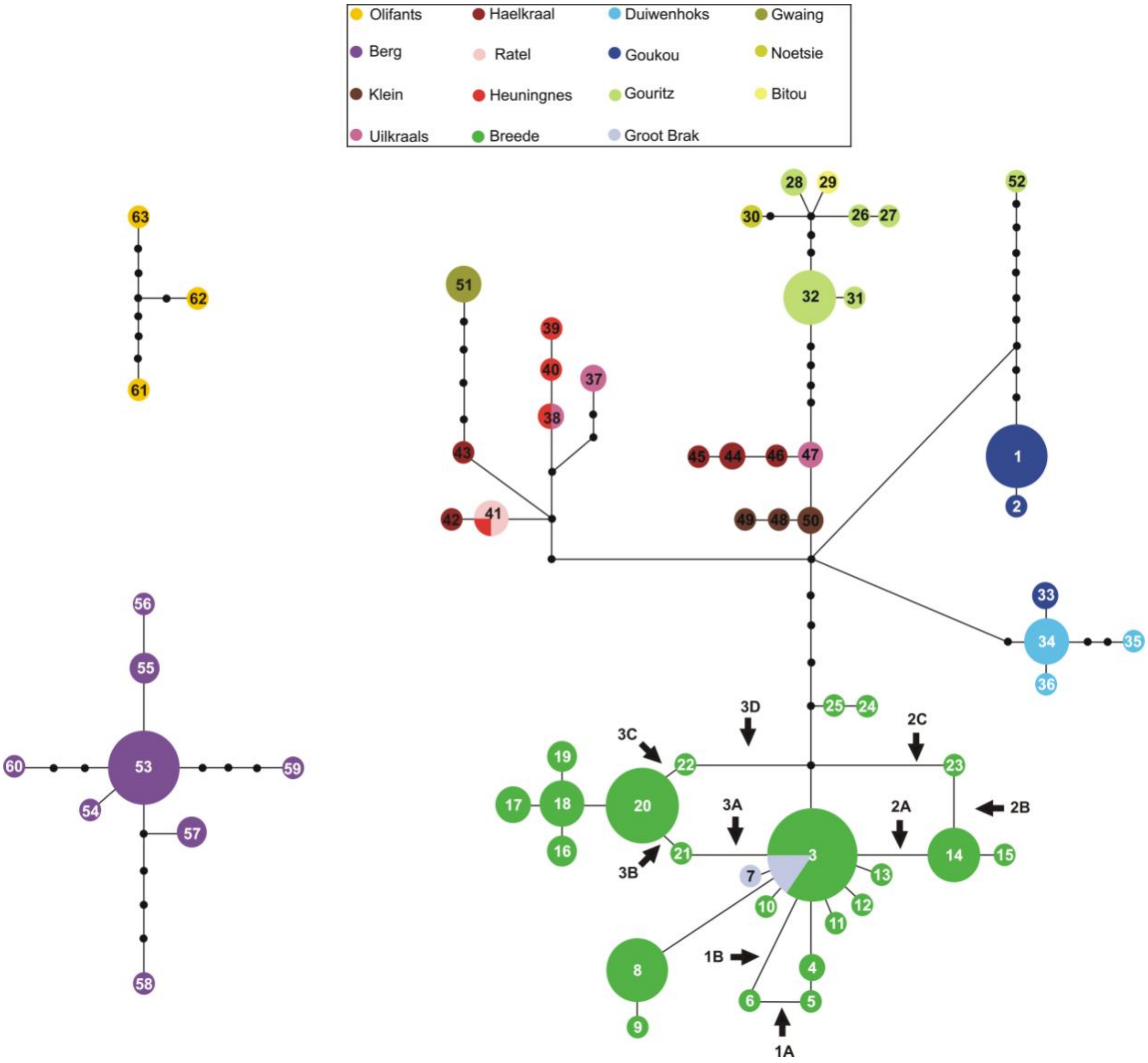


Fig. 4.5: Mitochondrial cytochrome *b* (cyt *b*) TCS haplotype network for *Galaxias* ‘nebula’ showing potential connections to resolve loops (arrows). The numbers indicate haplotypes described in Table 4.3. Solid black circles represent hypothetical haplotypes that were not sampled or that are extinct. Colour codes correspond to the river systems where the species occurs.

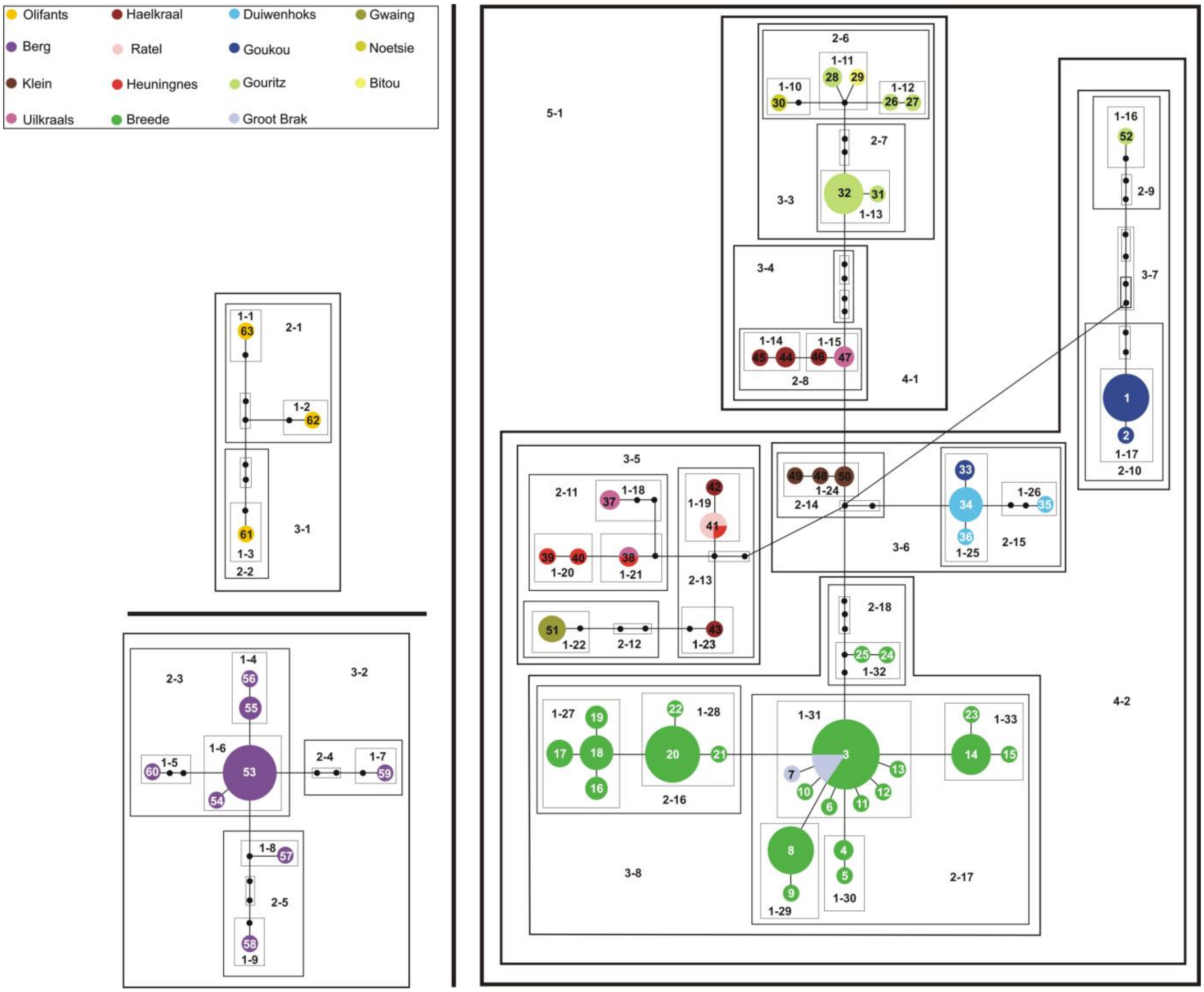


Fig. 4.6: Mitochondrial cytochrome *b* (cyt *b*) TCS haplotype network for *Galaxias* 'nebula' showing nesting levels used in Nested Clade Phylogeographic Analysis (NCPA).

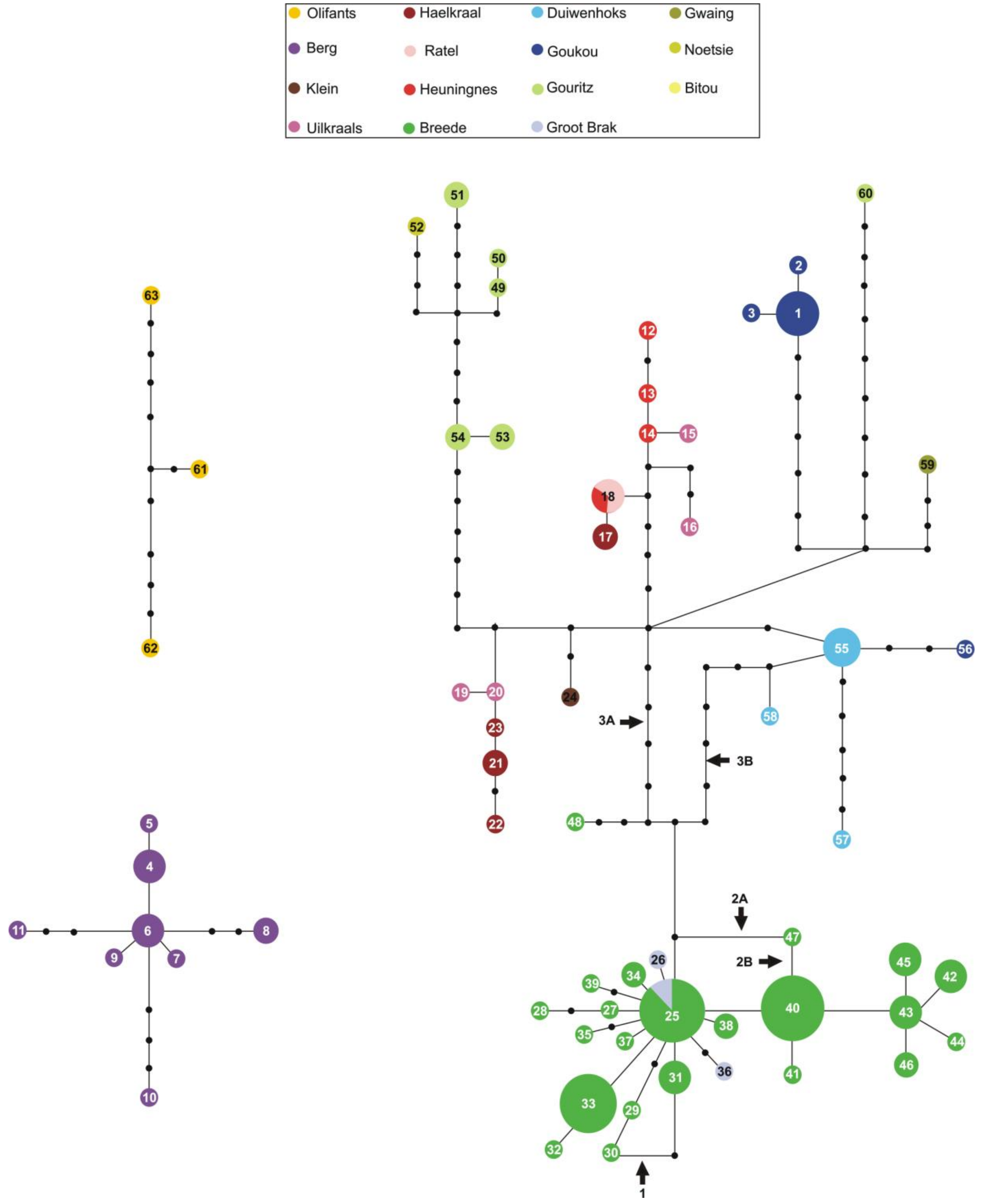


Fig. 4.7: Combined mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) TCS haplotype network for *Galaxias* 'nebula' showing potential connections to resolve loops (arrows). The numbers indicate haplotypes described in Table 4.4. Solid black circles represent hypothetical haplotypes that were not sampled or extinct. Colour codes correspond to the river systems where the species occurs.

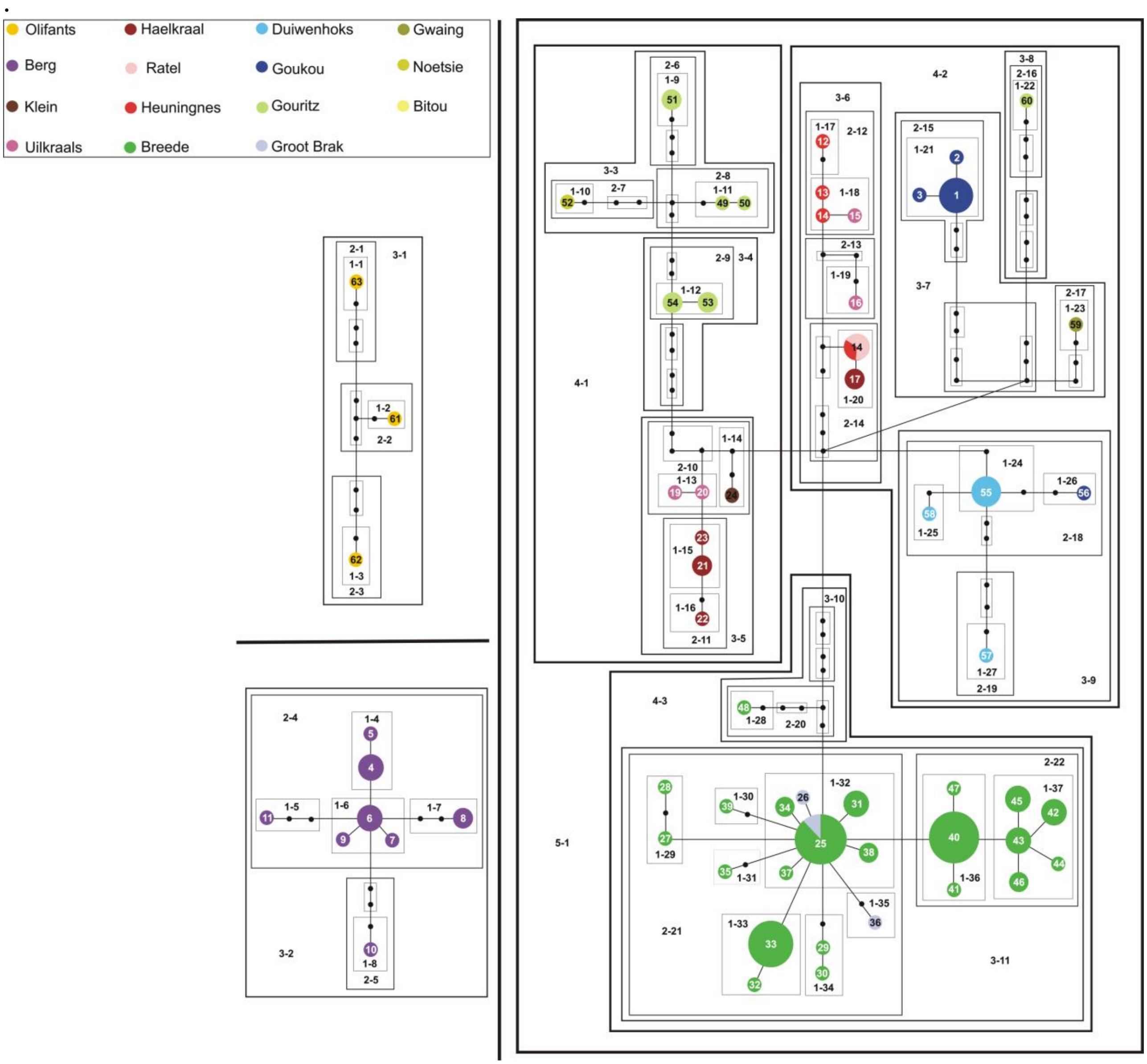


Fig. 4.8: Combined mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt *b*) TCS haplotype network for *Galaxias* 'nebula' showing nesting levels used in Nested Clade Phylogeographic Analysis (NCPA).

NCPA inferences of contemporary and historical processes that may have influenced the phylogeography of *Galaxias* 'nebula' are given in Tables 4.17 - 4.19. NCPA inferences based on the three data sets (COI, cyt *b* and combined COI-cyt *b* sequences) revealed the role of migration, historical fragmentation and isolation by distance events over various geographic scales (Tables 4.17 - 4.19). There was concordance among the three data sets that the geographic association of lineages across the species' range could be due to past fragmentation or long distance colonization (Tables 4.17 – 4.19). Within the southern lineage (clade 4-1 in Fig. 4.4), (clade 5-1 in Fig. 4.6) and (clade 5-1 in Fig. 4.8), contiguous range expansion across the southern draining systems was inferred (Tables 4.17 – 4.19).

For COI, the inferred processes for clade 3-2 (which comprised of haplotypes from the Breede, Gouritz, Groot Brak, Bitou and Noetsie; Fig. 4.4) was either historical fragmentation or long distance colonization or a combination of these processes (Table 4.17). The process inferred for clade 3-1, which was comprised of haplotypes from all the coastal rivers of the Agulhas Plain, as well as samples from the Duiwenhoks, Goukou, Gouritz and Wilderness Lakes Region, was restricted dispersal with isolation by distance. This process was also inferred for the lower level clade 2-5. The outcome for clades 1-20 (Breede and Groot Brak haplotypes) and 1-11 (Gwaing, Maalgate and Duiwenhoks haplotypes) was inconclusive.

For cyt *b*, historical isolation and long distance migration was inferred for clade 4-2 (which comprised of haplotypes from the Klein, all the Agulhas plain rivers, Breede, Duiwenhoks, Goukou, Gouritz, Groot Brak and Gwaing) (Table 4.18; Fig. 4.6). Migration type processes coupled with isolation by distance were also inferred for clades 2-16 to 3-6 and 3-8 to 4-1

(Table 4.18). For clades 1-25 and 3-7 (Fig. 4.6), no distinction between historical and contemporary processes was possible (Table 4.18).

The inferred processes based on the combined data set were restricted gene flow with isolation by distance for clade 3-11 (Breede and Groot Brak), contiguous range expansion in the southern drainages and past fragmentation for the total cladogram (Table 4.19).

Table 4.17: Biological processes influencing the geographic distribution of genetic variation in *Galaxias* 'nebula' based on mitochondrial cytochrome *c* oxidase subunit I inferred using Nested Clade Phylogeographic Analysis.

Clade level	*P*-value	Inference chain	Outcome
1-11	< 0.01	1-2-11-17	Inconclusive outcome
1-20	< 0.01	1-2-11-17	Inconclusive outcome
2-5	< 0.01	1-2-3-4	Restricted gene flow with isolation by distance
2-7	< 0.01	1-2-11-12	Contiguous Range Expansion
2-8	< 0.01	1-2-11-17	Inconclusive Outcome
3-1	< 0.01	1-2-3-4	Restricted gene flow with isolation by distance
3-2	<0.05	1-2-3-5-15	Past fragmentation and or long distance colonisation
4-1	<0.05	1-2-11-12	Contiguous range expansion.
Total cladogram	<0.05	1-2-3-5-15	Past fragmentation/Long distance colonisation

Table 4.18: Biological processes influencing the geographic distribution of genetic variation in *Galaxias* 'nebula' based on mitochondrial cytochrome *b* sequences inferred using Nested Clade Phylogeographic Analysis.

Clade level	*P*-value	Inference chain	Outcome
1-25	< 0.01	1-2-11-17	Inconclusive outcome
2-16	< 0.05	1-2-3-4	Restricted gene flow with isolation by distance
2-17	< 0.01	1-2-3-4	Restricted gene flow with isolation by distance
3-3	< 0.01	1-2-11-12	Contiguous Range Expansion
3-5	< 0.01	1-2-11-12	Contiguous Range Expansion
3-6	<0.05	1-2-11-12	Contiguous Range Expansion
3-7	< 0.05	1-19-20-2	Inconclusive outcome
3-8	< 0.01	1-2-3-4	Restricted gene flow with isolation by distance
4-1	< 0.01	1-2-11-12	Contiguous range expansion
4-2	< 0.001	1-2-3-5-15	Past fragmentation/Long distance colonisation
5-1	< 0.001	1-2-11-12	Contiguous range expansion
Total cladogram	< 0.001	1-2-3-5-15	Past fragmentation/Long distance colonisation

Table 4.19: Biological inferences of processes influencing the geographic distribution of genetic variation in *Galaxias* ‘nebula’ based on combined mitochondrial cytochrome *c* oxidase subunit I and cytochrome *b* sequences inferred using Nested Clade Phylogeographic Analysis (NCPA).

Clade level	*P*-value	Inference chain	Outcome
1-37	< 0.05	1-2-3-4	Restricted gene flow with isolation by distance
2-4	< 0.05	1-2-11-17	Inconclusive outcome
2-21	< 0.05	1-2-11-17	Inconclusive outcome
3-11	< 0.001	1-2-3-4	Restricted gene flow with isolation by distance
4-1	< 0.001	1-2-11-12	Contiguous Range Expansion
4-2	< 0.001	1-2-11-12	Contiguous Range Expansion
5-1	< 0.001	1-2-11-12	Contiguous range expansion
Total clade	< 0.001	1-2-3-5-15	Past fragmentation and/or Long distance colonisation

Estimates of the TMRCA and population divergence times

Estimates of the TMRCA and divergence times based on COI, cyt *b* and combined COI and cyt *b* sequences are presented in Tables 4.20 - 4.22. Assuming that the TMRCAs of populations are a reasonable estimate of their age, MDIV estimates placed the ages of the majority of *Galaxias* 'nebula' populations between the Pliocene and early Quaternary, with only a few of the populations dating to the late Miocene.

Estimates of θ and T were used to determine when populations began to diverge from each other using a wide range of plausible substitution rates (0.011 to 0.125) (Tables 4.20 – 4.22). The MDIV estimates (T_{div}) suggested that divergences between *Galaxias* 'nebula' populations occurred during the Pleistocene. Populations in the Olifants and Berg Rivers seem to have been the most isolated as they had the highest estimates of divergence times when compared with all the other populations. The oldest split in *Galaxias* 'nebula' was found between the Olifants and the Berg and was estimated to have occurred between 64 000 years ago (assuming the fastest rate of evolution of 0.125/My) and 730 000 years ago (assuming the slowest rate of evolution of 0.011/My) based on COI (Table 4.20). For cyt *b* the same split was dated to have occurred between 120 000 and 1 400 000 years ago (Table 4.21). Estimates from the combined COI and cyt *b* data placed the same split between 104 000 and 1 200 000 years ago (Table 4.22). The southern populations on the other hand had more recent divergence times, with the majority of the populations having diverged from one another between 4 300 years ago (assuming the fastest rate of evolution of 0.125/My) and 500 000 years ago (assuming the slowest rate of evolution, 0.011/My) (Tables 4.20 – 4.22).

However, for some of the systems, small sample sizes, particularly those with less than 5 samples, could have affected reliable estimation of divergence times using MDIV.

Results of the variable molecular clock confirmed that the divergence of *Galaxias* ‘nebula’ populations occurred during the Pleistocene. The three data sets (COI, cyt *b* and the combined COI and cyt *b* analyses) also indicated that all the southern populations have more recent divergence times than those from the western draining rivers (Tables 4.20 – 4.22). Interestingly, the minimum divergence times for some populations, particularly those from the low-lying Agulhas Plain (Uilkraals, Haelkraal, Ratel and Heuningnes) coincided with the Holocene (Tables 4.20 and 4.21), with some (i.e. Uilkraals *vs* Heuningnes, Uilkraals *vs* Haelkraal, Haelkraal *vs* Ratel) that have minimum divergence times as recent as 4 000 years ago (Table 4.22).

Table 4.20: Estimates of divergence times (years) between *Galaxias* ‘nebula’ populations mitochondrial cytochrome *c* oxidase subunit I (COI) sequences. The columns represent the population pairs that are being compared, TMRCA, *T*, θ, MDIV estimates of divergence time (T_{pop}) for different substitution rates (μ), mean % sequence divergences (% Div) and estimates of divergence time (T_{div} (with range in brackets)) based on Craw *et al.*’s (2008a) variable molecular clock. Samples from the Wilderness Lakes Region (Groot Brak, Maalgate, Gwaing and Noetsie) were pooled for MDIV calculations.

		MDIV								Craw *et al*. 2008a	
					μ						
		TMRCA	θ	T	0.0110	0.0188	0.0260	0.0310	0.1250	% Div	*T*div (range)
Olifants	Berg	9.82	1.1340	8.4800	728538	427181	308228	258513	64111	2.53	236700 (184000 - 324000)
	Klein									1.97	145600 (110300 - 190500)
	Uilkraals	3.32	2.3312	2.7800	490975	287885	207720	174217	43206	1.65	109300 (73700 - 156100)
	Haelkraal	3.75	2.2729	2.4600	423593	248376	179213	150307	37276	1.70	114400 (73700 - 156100)
	Ratel									1.45	90400 (73700 - 108300)
	Heuningnes	5.32	1.3274	4.6800	470634	275958	199115	166999	41416	1.45	90400 (73700 - 108300)
	Breede	3.74	3.2681	2.5600	633804	371634	268148	224898	55775	2.05	156100 (90400 - 224600)
	Duiwenhoks	4.43	1.6391	3.5600	442071	259210	187030	156864	38902	1.58	102400 (73700 - 132100)
	Goukou	4.53	1.9095	3.3400	483149	283296	204409	171440	42517	1.60	104300 (74500 - 154800)
	Gouritz	2.78	3.7894	1.9200	551187	323191	233194	195582	48504	2.15	170300 (90400 - 326600)
	Groot Brak									2.09	161600 (108300 - 224600)
	Maalgate									1.99	148100 (73700 - 224600)
	Gwaing									1.65	109300 (90400 - 131000)
	Noetsie									2.47	224600 (185600 - 269700)
	Bitou									2.47	224600 (185600 - 269700)
	Wilderness	2.00	5.9345	1.2600	566474	332154	239662	201007	49850		
Berg	Klein									2.14	168800 (129800 - 220900)
	Uilkraals	6.29	1.3497	4.3800	447842	262594	189472	158912	39410	1.72	116500 (72900 - 179300)
	Haelkraal	6.35	1.4460	5.1400	563060	330153	238218	199796	49549	1.86	132130 (87700 - 182000)
	Ratel									1.61	105300 (87700 - 126400)
	Heuningnes	10.09	0.6748	11.2000	572580	335735	242246	203174	50387	1.52	96700 (72060 - 126400)
	Breede	5.38	2.4340	3.6600	674881	395719	285527	239474	59390	2.22	182000 (106300 - 259000)
	Duiwenhoks	7.12	1.1282	6.5400	558967	327752	236486	198343	49189	1.75	119700 (88600 - 153400)
	Goukou	7.15	1.3200	6.0000	600015	351821	253853	212909	52801	2.07	158500 (88600 - 259000)

Table 4.20: continued...

		MDIV								Craw *et al*. 2008a	
					μ						
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Berg	Gouritz	4.51	2.7216	3.1400	647418	379616	273908	229729	56973	2.26	187300 (126400 - 265200)
	Groot Brak									2.12	165900 (127500 - 220900)
	Maalgate									2.16	171700 (88600 - 260500)
	Gwaing									1.75	119700 (106300 - 150800)
	Noetsie									2.49	228700 (215100 - 259000)
	Bitou									2.49	228700 (215100 - 259000)
	Wilderness	3.53	3.6946	2.4600	688545	403731	291307	244322	60592		
Klein	Uilkraals									0.79	41590 (25800 - 59500)
	Haelkraal									0.83	44040 (25800 - 73700)
	Ratel									0.79	41600 (36100 - 47300)
	Heuningnes									0.88	47300 (36100 - 59500)
	Breede									1.00	55300 (36100 - 73700)
	Duiwenhoks									0.60	30300 (16300 - 59500)
	Goukou									0.92	49900 (35500 - 73700)
	Gouritz									1.16	66800 (36100 - 107300)
	Groot Brak									1.07	60200 (36100 - 89500)
	Maalgate									0.91	49200 (16300 - 73700)
	Gwaing									0.61	30840 (25800 - 36100)
	Noetsie									1.34	80900 (73700 - 89500)
	Bitou									1.34	80900 (73700 - 89500)
	Wilderness										
Uilkraals	Haelkraal									0.69	35500 (16300 - 58800)
	Ratel									0.00	
	Heuningnes									0.63	32000 (16300 - 46600)
	Breede	2.13	3.0489	1.2600	291029	170646	123128	103268	25611	0.90	48600 (25250 - 72900)
	Duiwenhoks	2.39	1.5468	0.8200	96088	56342	40653	34096	8456	0.50	24700 (7900 - 46600)
	Goukou	2.77	1.8813	1.2600	179574	105294	75974	63720	15803	0.80	42200 (25250 - 72900)
	Gouritz	2.07	3.4448	1.2000	313166	183626	132493	111124	27559	1.03	57400 (25250 - 89500)
	Groot Brak									0.96	52600 (25250 - 88600)

Table 4.20: continued...

		MDIV								Craw *et al*. 2008a	
					μ						
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Uilkraals	Maalgate									0.81	42780 (7900 - 72900)
	Gwaing									0.54	26890 (16300 - 35500)
	Noetsie									1.17	67500 (58800 - 72900)
	Bitou									1.17	67500 (58800 - 72900)
	Wilderness										
Haelkraal	Ratel									0.00	
	Heuningnes									0.69	35500 (25800 - 46600)
	Breede	2.22	3.0934	1.3800	323404	189629	136825	114756	28460	0.91	49200 (25250 - 88600)
	Duiwenhoks	2.60	1.5333	0.7800	90607	53128	38334	32151	7973	0.58	29150 (7900 - 47260)
	Goukou	2.90	1.8045	1.4200	194124	113825	82129	68883	17083	0.76	39700 (25250 - 58800)
	Gouritz	2.39	2.7646	1.1600	242951	142455	102787	86209	21380	1.01	55300 (25250 - 88600)
	Groot Brak									1.01	56000 (25250 - 107300)
	Maalgate									0.80	42200 (7900 - 58800)
	Gwaing									0.59	29700 (16300 - 46600)
	Noetsie									1.17	67500 (58800 - 72900)
	Bitou									1.17	67500 (58800 - 72900)
	Wilderness	1.50	4.6744	0.7000	247887	145349	104875	87960	21814		
Ratel	Heuningnes										
	Breede									0.90	48600 (35500 - 58800)
	Duiwenhoks									0.46	22530 (16300 - 25800)
	Goukou									0.82	43410 (35500 - 58800)
	Gouritz									1.06	59500 (35500 - 88600)
	Groot Brak									0.96	52600 (35500 - 72900)
	Maalgate									0.81	42780 (16300 - 58800)
	Gwaing									0.51	25250
	Noetsie									1.24	72900
	Bitou									1.24	72900
	Wilderness										

Table 4.20: continued…

		MDIV									
					μ					Craw *et al*. 2008a	
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Heuningnes	Breede	2.66	2.4353	1.7000	313642	183905	132695	111292	27601	0.99	54600 (35500 - 72900)
	Duiwenhoks	3.53	0.8414	1.6200	103268	60552	43690	36643	9088	0.54	26900 (16300 - 36100)
	Goukou	4.08	1.1742	2.5800	229505	134571	97098	81437	20196	0.92	49900 (35500 - 72900)
	Gouritz	2.72	2.6180	1.4600	289561	169786	122507	102748	25481	1.11	63100 (25800 - 106300)
	Groot Brak									1.06	59500 (35500 - 88600)
	Maalgate									0.91	49200 (16300 - 72900)
	Gwaing									0.60	30300 (25250 - 35500)
	Noetsie									1.33	80070 (72900 - 88600)
	Bitou									1.33	80070 (72900 - 88600)
	Wilderness	1.70	4.0225	0.6600	201126	117931	85092	71367	17699		
Breede	Duiwenhoks	2.12	2.9306	1.3400	297502	174442	125866	105565	26180	0.78	41000 (16300 - 72900)
	Goukou	2.03	3.8413	1.0000	291007	170633	123118	103261	25609	1.04	58200 (35500 - 88600)
	Gouritz	2.04	3.8115	1.1400	329176	193014	139267	116805	28968	0.93	50600 (7900 - 88600)
	Groot Brak									0.63	32000 (16300 - 72100)
	Maalgate									1.05	58800 (16300 - 88600)
	Gwaing									0.72	37300 (25250 - 46600)
	Noetsie									1.05	58800 (35500 - 72900)
	Bitou									1.05	58800 (35500 - 72900)
	Wilderness	1.73	4.3935	0.5200	173077	101484	73225	61414	15231		
Duiwenhoks	Goukou	3.07	1.5052	1.4600	166482	97618	70435	59074	14650	0.71	36700 (16300 - 72900)
	Gouritz	2.13	3.0697	0.8400	195346	114542	82646	69316	17190	0.94	51200 (16300 - 106300)
	Groot Brak									0.85	45360 (16300 - 88600)
	Maalgate									1.00	55300 (47300 - 72900)
	Gwaing									0.45	22000 (7900 - 35500)
	Noetsie									1.12	63800 (46600 - 87700)
	Bitou									1.12	63800 (46600 - 87700)
	Wilderness	1.66	4.1280	0.4200	131345	77015	55569	46606	11558		

Table 4.20: continued...

		MDIV									
					μ					Craw *et al*. 2008a	
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Goukou	Gouritz	2.35	3.1900	1.1400	275500	161540	116558	97758	24244	1.18	68300 (35500 - 126400)
	Groot Brak									1.10	62300 (35500 - 106300)
	Maalgate									0.95	51900 (16300 - 88600)
	Gwaing									0.64	32600 (25250 - 46600)
	Noetsie									1.38	84300 (72060 - 106300)
	Bitou									1.38	84300 (72060 - 106300)
	Wilderness	1.73	4.1934	0.7400	235087	137844	99460	83418	20688		
Gouritz	Groot Brak									1.06	59500 (16300 - 107300)
	Maalgate									1.16	66800 (16300 - 126400)
	Gwaing									0.78	41000 (25250 - 72900)
	Noetsie									0.00	
	Bitou									0.00	
	Wilderness	1.50	5.3813	0.3400	138609	81274	58642	49184	12198		
Groot Brak	Maalgate									1.09	61600 (16300 - 106300)
	Gwaing									0.73	37900 (25250 - 58800)
	Noetsie									1.15	66000 (46600 - 88600)
	Bitou									1.15	66000 (46600 - 88600)
Maalgate	Gwaing									0.73	37900 (7900 - 46600)
	Noetsie									1.37	83500 (46600 - 106300)
	Bitou									1.37	83500 (46600 - 106300)
Gwaing	Noetsie									0.99	54600 (46600 - 58800)
	Bitou									0.99	54600 (46600 - 58800)
Noetsie	Bitou									0.00	

Table 4.21: Estimates of divergence times (years) between *Galaxias* ‘nebula’ populations based on mitochondrial cytochrome *b* (cyt *b*) sequences. The columns represent the population pairs that are being compared, TMRCA, *T*, θ, MDIV estimates of divergence time (T_{pop}) for different substitution rates (μ), mean % sequence divergences (% Div) and estimates of divergence time (T_{div} (with range in brackets)) based on Craw *et al.*’s (2008a) variable molecular clock. Samples from the Wilderness Lakes Region (Groot Brak, Maalgate, Gwaing and Noetsie) were pooled for MDIV calculations.

		MDIV									
					μ					Craw *et al*. 2008a	
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Olifants	Berg	4.30	6.5214	2.9600	1399407	820548	592057	496564	123148	5.60	1370000 (1040000 - 1740000)
	Klein	3.42	4.8137	2.2200	774717	454258	327765	274900	68175	3.72	610000 (420000 - 860000)
	Uilkraals	3.05	6.1554	2.1280	949589	556795	401749	336951	83564	4.17	790000 (495000 - 1145000)
	Haelkraal	3.39	5.7545	2.3160	966169	566517	408764	342834	85023	4.03	735000 (414000 - 1050000)
	Ratel									3.94	699000 (582000 - 855000)
	Heuningnes	3.20	6.0578	2.2320	980209	574749	414704	347816	86258	4.34	855000 (582000 - 1154000)
	Breede	3.38	6.9361	2.2960	1154514	676954	488448	409666	101597	4.45	902000 (585000 - 1256000)
	Duiwenhoks	4.30	4.1943	3.4320	1043563	611897	441507	370297	91834	4.08	754000 (514000 - 1082000)
	Goukou	4.25	3.8573	3.0760	860160	504358	363914	305218	75694	3.30	465000 (243000 - 881000)
	Gouritz	3.29	6.1515	2.4600	1097044	643256	464134	389274	96540	3.67	592500 (347000 - 1148000)
	Groot Brak									4.30	842000 (670000 - 1052000)
	Gwaing									3.26	441000 (347000 - 577000)
	Noetsie									3.57	554000 (417000 - 761000)
	Bitou									3.35	473000 (350000 - 674000)
	Wilderness	2.79	6.7532	1.8930	926933	543511	392164	328912	81570		
Berg	Klein	3.27	4.6524	2.2560	760894	446153	321917	269995	66959	2.67	268000 (170200 - 431000)
	Uilkraals	3.20	5.2671	2.0640	788122	462119	333436	279656	69355	3.09	384000 (202500 - 678000)
	Haelkraal	3.31	5.2313	2.3920	907147	531909	383793	321891	79829	3.03	366200 (241000 - 593000)
	Ratel	3.52	4.3411	2.3440	737672	432537	312092	261754	64915	2.82	304700 (241000 - 427000)
	Heuningnes	3.37	5.1419	2.2440	836478	490472	353895	296815	73610	3.22	427000 (241000 - 678300)
	Breede	3.35	6.4242	2.3040	1073026	629173	453972	380751	94426	3.80	643000 (347000 - 964500)
	Duiwenhoks	3.75	4.2614	2.5080	774797	454305	327799	274928	68182	2.85	312700 (207800 - 410000)
	Goukou	3.82	4.0705	2.5320	747165	438103	316108	265123	65751	2.80	299000 (201000 - 444500)

Table 4.21: continued...

		MDIV								Craw *et al.* 2008a	
					μ						
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Berg	Gouritz	3.32	5.5194	2.4730	989659	580291	418702	351169	87090	3.23	430500 (239000 - 682000)
	Groot Brak									3.64	581000 (413000 - 774000)
	Gwaing									2.66	265000 (201000 - 356000)
	Noetsie									3.08	381000 (287000 - 502000)
	Bitou									3.30	455000 (344000 - 588500)
	Wilderness	3.06	5.7906	2.3680	994071	582877	420568	352735	87478		
Klein	Uilkraals									0.91	49200 (7390 - 101400)
	Haelkraal	2.35	2.3763	0.9080	156419	91717	66177	55504	13765	0.77	403000 (15750 - 69800)
	Ratel									0.84	44700 (34300 - 56670)
	Heuningnes	2.50	2.0549	1.5840	235967	138360	99832	83730	20765	1.14	65300 (34300 - 101400)
	Breede	1.83	5.6577	1.1080	454455	266471	192269	161258	39992	1.63	107300 (56670 - 170200)
	Duiwenhoks	3.05	1.5458	1.8520	207541	121693	87806	73644	18264	0.91	49200 (24700 - 86000)
	Goukou	3.76	1.5509	1.6520	185737	108907	78581	65907	16345	1.02	56670 (349000 - 84300)
	Gouritz	3.20	3.5119	1.5080	383932	225120	162433	136234	33786	1.56	100400 (56000 - 143000)
	Groot Brak									1.48	93010 (69800 - 120800)
	Gwaing									0.93	50600 (44700 - 56670)
	Noetsie									1.37	83400 (69000 - 100400)
	Bitou									1.56	100400 (83400 - 119800)
	Wilderness	2.70	3.3268	0.9640	232492	136323	98362	82497	20459		
Uilkraals	Haelkraal	2.32	2.9548	0.5880	125956	73855	53289	44694	11084	1.02	56670 (7390 – 143000)
	Ratel									0.84	447000
	Heuningnes									1.02	56670 (44700 - 84300)
	Breede	1.97	5.9727	1.3960	604454	354424	255731	214484	53192	1.87	133300 (69800 - 201000)
	Duiwenhoks	2.64	2.7172	1.5520	305719	179260	129343	108481	26903	1.19	69000 (34300 - 146800)
	Goukou	2.89	2.6040	1.5680	296008	173566	125234	105035	26049	1.26	74500 (45300 - 103300)
	Gouritz	2.70	4.5425	1.3760	453125	265692	191707	160786	39875	1.88	134500 (44700 - 236700)
	Groot Brak									1.72	116500 (84300 - 143000)

Table 4.21: continued...

		MDIV									
					μ					Craw *et al*. 2008a	
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Uilkraals	Gwaing									1.38	84300 (56670 - 119800)
	Noetsie									1.75	119800 (83400 - 167400)
	Bitou									1.94	141800 (100400 - 199000)
	Wilderness	2.12	4.8537	0.9800	344832	202193	145890	122360	30345		
Haelkraal	Ratel									0.82	43450 (7390 - 84300)
	Heuningnes									1.16	66800 (7390 - 143000)
	Breede	2.03	5.9481	1.3280	572646	335773	242273	203197	50393	1.96	144300 (84300 - 241000)
	Duiwenhoks	2.59	2.7625	1.5240	305210	178961	129127	108300	26858	1.24	72900 (45310 - 123000)
	Goukou	2.88	2.7204	1.4960	295031	172993	124821	104688	25963	1.35	81730 (57330 - 119800)
	Gouritz	2.75	4.4574	1.5000	484708	284210	205069	171993	42654	1.83	128600 (56000 - 202400)
	Groot Brak									1.81	126400 (100400 - 168800)
	Gwaing									1.27	75220 (44700 - 101400)
	Noetsie									1.71	115450 (100400 - 141800)
	Bitou									1.90	136860 (118690 - 167400)
	Wilderness	2.25	4.5788	1.1360	377089	221108	159538	133806	33184		
Ratel	Heuningnes									0.00	0
	Breede	2.02	5.6475	1.2720	520781	305362	220330	184793	45829	1.81	126400 (84300 - 170300)
	Duiwenhoks	3.86	1.3659	2.7530	272632	159859	115345	96741	23992	1.09	61600 (45310 - 86100)
	Goukou	4.07	1.5313	2.3400	259771	152318	109903	92177	22860	1.20	69800 (62330 - 84300)
	Gouritz	3.36	3.3690	1.6640	406413	238302	171944	144211	35764	1.71	115450 (83400 - 141800)
	Groot Brak									1.66	110300 (100400 - 119800)
	Gwaing									1.20	69800
	Noetsie									1.56	100400
	Bitou									1.74	118690
	Wilderness	2.72	3.3466	0.9400	228054	133720	96484	80922	20069		

Table 4.21: continued...

		MDIV									
					μ					Craw *et al*. 2008a	
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Heuningnes	Breede	2.08	6.0564	1.3440	590095	346005	249656	209389	51928	2.11	164500 (84300 - 289400)
	Duiwenhoks	3.00	2.2458	2.0270	329968	193478	139602	117085	29037	1.37	83400 (45310 - 146800)
	Goukou	3.32	2.2810	2.1560	356526	209050	150838	126509	31374	1.48	93010 (57330 - 143000)
	Gouritz	2.93	4.4085	1.6640	531809	311828	224996	188706	46799	2.04	154800 (83400 - 241000)
	Groot Brak									1.95	143000 (100400 - 201000)
	Gwaing									1.51	95820 (69800 - 120800)
	Noetsie									1.85	131000 (100400 - 168800)
	Bitou									2.04	154800 (118690 - 199000)
	Wilderness	2.33	4.4187	1.3130	420709	246684	177992	149284	37022		
Breede	Duiwenhoks	2.17	5.5396	1.4200	570265	334377	241266	202352	50183	1.78	123000 (70520 - 249400)
	Goukou	2.34	5.2036	1.6080	606596	355680	256637	215244	53380	1.85	131000 (84300 - 201000)
	Gouritz	2.54	6.1029	1.4160	626482	367340	265050	222300	55130	2.25	185700 (83400 - 413000)
	Groot Brak									0.49	24160 (15750 - 44100)
	Gwaing									2.01	151000 (101400 - 201000)
	Noetsie									1.98	146800 (100400 - 201000)
	Bitou									2.18	174800 (119800 - 239000)
	Wilderness	2.14	5.6213	1.3800	562373	329750	237927	199552	49489		
Duiwenhoks	Goukou									1.15	66000 (15750 - 123000)
	Gouritz	3.41	3.5218	1.7720	452415	265275	191406	160534	39813	1.84	129800 (69800 - 207800)
	Groot Brak									1.63	107300 (70550 - 174800)
	Gwaing									1.28	76050 (57330 - 103300)
	Noetsie									1.65	109300 (85150 - 145600)
	Bitou									1.84	129800 (102400 - 173300)
	Wilderness	2.78	3.5115	1.2280	312607	183298	132257	110925	27509		

Table 4.21: continued...

		MDIV									
					μ					Craw *et al*. 2008a	
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Goukou	Gouritz	3.32	3.5504	1.5280	393292	230608	166393	139555	34610	1.90	137000 (85150 - 199000)
	Groot Brak									1.73	117600 (100400 - 141800)
	Gwaing									1.14	65300 (56000 - 70520)
	Noetsie									1.75	119800 (102400 - 141800)
	Bitou									1.94	141800 (121900 - 167400)
	Wilderness	3.01	3.3549	1.2560	305473	179115	129239	108394	26882		
Gouritz	Groot Brak									2.11	164500 (100400 - 287000)
	Gwaing									1.87	133300 (99500 - 167400)
	Noetsie									1.03	57330 (24160 - 239000)
	Bitou									0.93	50600 (15750 - 284400)
	Wilderness	2.70	4.6429	1.4280	480650	281831	203352	170553	42297		
Groot Brak	Gwaing									1.85	131000 (119800 - 143000)
	Noetsie									1.85	131000 (119800 - 141800)
	Bitou									2.04	154800 (141800 - 167400)
Gwaing	Noetsie									1.75	119800
	Bitou									1.94	141800
Noetsie	Bitou									0.49	24160

Table 4.22: Estimates of divergence times (years) between *Galaxias* 'nebula' populations based on combined mitochondrial cytochrome *c* oxides subunit I (COI) and cytochrome *b* (cyt *b*) sequences. The columns represent the population pairs that are being compared, TMRCA, *T*, θ, MDIV estimates of divergence time (T_{pop}) for different substitution rates (μ), mean % sequence divergences (% Div) and estimates of divergence time (T_{div} (with range in brackets)) based on Craw *et al.*'s (2008a) variable molecular clock. Samples from the Wilderness Lakes Region (Groot Brak, Maalgate, Gwaing and Noetsie) were pooled for MDIV calculations.

		MDIV								Craw *et al.* 2008a	
					μ						
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Olifants	Berg	5.13	7.7463	4.1400	1188030	696606	502628	421559	104547	3.89	677000 (498000 – 905000)
	Klein									2.61	254000 (225000 – 296900)
	Uilkraals	2.53	10.7118	1.5200	603171	353672	255188	214028	53079	2.75	287000 (223000 – 386000)
	Haelkraal	3.57	7.7355	2.6600	762260	446954	322495	270479	67079	2.81	303000 (243000 – 420000)
	Ratel	3.02	8.2944	3.1200	958677	562124	405594	340176	84364	2.60	252000 (223000 – 295000)
	Heuningnes	3.32	8.4245	2.1000	655384	384287	277278	232556	57674	2.81	303000 (243000 – 386000)
	Breede	3.52	9.5296	2.4800	875506	513356	370406	310663	77044	3.09	384000 (292000 – 499000)
	Duiwenhoks	3.90	6.7995	2.9200	735514	431272	311179	260989	64725	2.72	279200 (206000 – 393600)
	Goukou									2.35	203000 (145000 – 356000)
	Gouritz	2.87	10.2004	2.1800	823772	483022	348519	292306	72492	2.78	295000 (187200 – 424000)
	Groot Brak									3.00	356000 (292000 – 459000)
	Gwaing									2.39	209600 (187200 – 243000)
	Noetsie									2.96	343800 (270000 – 424000)
	Wilderness	2.13	12.6972	1.3000	611482	358545	258704	216978	53810		
Berg	Klein									2.18	175000 (145000 – 204000)
	Uilkraals	3.76	6.6589	2.4400	601903	352928	254651	213578	52967	2.27	189000 (144000 – 265000)
	Haelkraal	4.49	5.7938	3.5000	751217	440479	317822	266561	66107	2.36	204000 (156000 – 290000)
	Ratel	4.35	5.6779	3.4600	727772	426732	307904	258242	64044	2.15	170000 (144000 – 203000)
	Heuningnes	4.40	5.9632	3.3600	742253	435223	314030	263380	65318	2.34	201000 (158000 – 243000)
	Breede	3.95	8.2122	2.9200	888337	520880	375835	315216	78174	2.83	308000 (203000 – 415000)
	Duiwenhoks	4.74	5.1501	3.4887	665586	390269	281594	236176	58572	2.22	181000 (133000 – 226000)
	Goukou	5.02	5.2209	4.0400	781373	458161	330581	277261	68761	2.37	205000 (145000 – 263000)

Table 4.22: continued...

		MDIV									
					μ					Craw *et al*. 2008a	
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Berg	Gouritz	3.94	7.6634	3.1867	904677	530461	382748	321014	79612	2.63	259000 (170000 - 350000)
	Groot Brak									2.71	277000 (221000 – 346800)
	Gwaing									2.14	169000 (144000 – 188000)
	Noetsie									2.76	290000 (264000 – 325000)
	Wilderness	3.34	9.2489	2.2733	778906	456714	329537	276386	68544		
Klein	Uilkraals									0.63	32000 (15800 – 51300)
	Haelkraal									0.59	29800 (20500 – 39800)
	Ratel									0.68	35000
	Heuningnes									0.88	47500 (37000 – 57000)
	Breede									1.10	62000 (51200 – 70500)
	Duiwenhoks									0.62	31500 (20400 – 51300)
	Goukou									0.85	45500 (35000 – 51000)
	Gouritz									1.15	66000 (45500 – 85200)
	Groot Brak									1.03	57300 (51200 – 63800)
	Gwaing									0.68	35000
	Noetsie									1.21	70500
	Wilderness										
Uilkraals	Haelkraal	2.54	4.3267	0.5400	86553	50750	36618	30712	7617	0.66	33600 (3750 – 77000)
	Ratel	2.25	3.8470	0.3400	48455	28412	20500	17194	4264	0.54	27000 (15800 – 39800)
	Heuningnes	2.13	4.2347	0.3400	53338	31275	22566	18926	4694	0.62	31360 (3750 – 63000)
	Breede	1.81	8.7252	1.5000	484839	284287	205124	172040	42666	1.27	75300 (51300 – 102500)
	Duiwenhoks	2.01	5.3839	1.1200	223381	130980	94507	79264	19658	0.84	44800 (20500 – 70500)
	Goukou	2.43	5.1044	1.2000	226912	133051	96001	80517	19968	1.02	56500 (35000 – 77000)
	Gouritz	2.00	7.9466	1.3600	400363	234754	169384	142064	35232	1.33	80200 (34300 – 132000)
	Groot Brak									1.20	70000 (50600 – 93000)

Table 4.22: continued...

		MDIV									
					μ					Craw *et al*. 2008a	
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Uilkraals	Gwaing									0.89	48000 (34300 – 63000)
	Noetsie									1.44	89000 (70000 – 34300)
	Wilderness	1.48	10.9057	0.5200	210081	123182	88881	74545	18487		
Haelkraal	Ratel									0.68	34900 (3750 – 56800)
	Heuningnes									0.92	50000 (1500 – 85000)
	Breede	1.93	7.9792	1.5800	467037	273849	197593	165723	41099	1.32	79300 (56800 – 101400)
	Duiwenhoks	2.50	4.2022	1.7400	270867	158824	114598	96114	23836	0.85	45600 (24700 – 77700)
	Goukou	2.98	4.2847	1.5800	250791	147052	106104	88990	22070	1.07	60000 (40000 – 77200)
	Gouritz	2.44	7.4918	1.3000	360796	211554	152644	128024	31750	1.33	80100 (39800 – 121000)
	Groot Brak									1.22	71600 (56600 – 85100)
	Gwaing									0.89	48000 (39800 – 56800)
	Noetsie									1.43	88900 (78000 – 101400)
	Wilderness	1.82	8.8037	0.8000	260908	152984	110384	92580	22960		
Ratel	Heuningnes									0.36	17300 (11500 – 24800)
	Breede									1.27	75300 (63000 – 85200)
	Duiwenhoks									0.77	40300 (29700 – 51300)
	Goukou									1.03	57000 (45500 – 63000)
	Gouritz									1.33	80100 (56800 – 102500)
	Groot Brak									1.20	70000 (63000 – 77700)
	Gwaing									0.85	45500
	Noetsie									1.39	85200
	Wilderness										

Table 4.22: continued…

		MDIV									
					μ					Craw *et al*. 2008a	
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div (range)
Heuningnes	Breede	2.04	8.2758	1.4000	429213	251671	181590	152301	37771	1.49	94000 (70500 – 121000)
	Duiwenhoks	2.66	3.7116	1.8800	258495	151569	109363	91724	22748	0.97	53000 (35000 – 78200)
	Goukou	3.28	3.5499	2.1800	286683	168098	121289	101726	25228	1.18	68000 (45500 – 93000)
	Gouritz	2.55	6.9837	1.6200	419117	245751	177319	148719	36882	1.55	99000 (63000 – 144000)
	Groot Brak									1.42	87700 (70000 – 111000)
	Gwaing									1.06	59500 (51000 – 70500)
	Noetsie									1.61	105000 (93000 – 122000)
	Wilderness	1.79	8.2124	0.9200	279890	164115	118415	99316	24630		
Breede	Duiwenhoks	1.93	7.9542	1.3267	390922	229219	165390	138714	34401	1.19	69000 (45400 – 112300)
	Goukou	2.29	7.4318	1.6267	447844	262595	189473	158913	39410	1.40	86000 (63000 – 111000)
	Gouritz	2.37	8.9600	1.3400	444780	260799	188176	157825	39141	1.45	90300 (50600 – 144400)
	Groot Brak									0.30	14400 (7500 – 34400)
	Gwaing									1.27	75300 (63800 – 85200)
	Noetsie									1.44	89800 (77700 – 102300)
	Wilderness	1.80	8.6523	1.1600	371813	218014	157305	131934	32720		
Duiwenhoks	Goukou	3.64	3.2551	1.2600	151936	89088	64281	53913	13370	0.92	50000 (11700 – 86000)
	Gouritz	2.57	6.6688	1.4400	355748	208594	150509	126233	31306	1.31	78400 (39700 – 133300)
	Groot Brak									1.12	63800 (45300 – 102300)
	Gwaing									0.83	44100 (29700 – 63800)
	Noetsie									1.37	83400 (63800 – 112300)
	Wilderness	1.97	7.6143	0.8600	242583	142239	102631	86078	21347		

Table 4.22: continued…

		MDIV									
					μ					Craw *et al*. 2008a	
		TMRCA	θ	T	0.011	0.01876	0.026	0.031	0.125	% Div	*T*div
Goukou	Gouritz	2.70	6.1054	1.5400	348314	204235	147364	123595	30652	1.49	94000 (57000 – 132000)
	Groot Brak									1.34	81000 (63800 – 101400)
	Gwaing									0.89	48000 (45100 – 51000)
	Noetsie									1.58	102000 (86000 – 111000)
	Wilderness	2.29	6.8563	0.8600	218435	128080	92415	77509	19222		
Gouritz	Groot Brak									1.39	85100 (50500 – 133300)
	Gwaing									1.21	70500 (56800 – 85100)
	Noetsie									0.86	46000 (24700 – 144400)
	Wilderness	1.84	9.7807	0.5200	188411	110476	79712	66856	16580		
Groot Brak	Gwaing									1.20	70000 (63000 – 77700)
	Noetsie									1.39	85100 (77700 – 93000)
Gwaing	Noetsie									1.21	70500

4.4. Discussion

Phylogeographic patterns

Analyses of mitochondrial COI, cyt *b* and combined COI and cyt *b* sequences revealed both deep and shallow phylogeographic patterns for *Galaxias* 'nebula'. The deep phylogeographic pattern of this taxon is supported by the occurrence of three distinct and geographically exclusive lineages corresponding to the Olifants, Berg and all the southern draining river systems. Estimates of the TMRCA for *Galaxias* 'nebula' also indicate that the majority of populations inhabiting present day river systems evolved during the Pliocene, a period when contemporary drainage basins were being established, with a few of the populations founded during the late Miocene. These results suggest that this species has been an important component of the CFR freshwater ichthyofauna for a considerable period of time.

Results of the present study also indicate that there has been more recent dispersal between river systems across the CFR. This is evident from low inter-basin divergences, suggesting that populations of this species diverged from one another during the mid- to late Pleistocene, well after the establishment of contemporary drainage systems and landscape features. The sharing of some haplotypes between geographically distant river systems on the south coast provides further evidence for recent dispersal.

Phylogeographic patterns of *Galaxias* 'nebula' were generally similar to those reported by Daniels *et al*. (2006) for *Potamonautes perlatus* (a freshwater crab species). They used the

16S rRNA mtDNA gene region to analyse samples collected across a comparable geographic range to the present study. They found close relationships between populations of *P. perlatus* from the southern draining rivers and a few haplotypes were shared among currently isolated river systems on the south coast. However, whilst Daniels *et al*. (2006) found close phylogenetic relationships between individuals from the Olifants and Berg River systems, the present study indicated that *Galaxias* ‘nebula’ populations in these river systems are genetically distinct. Because the present study and Daniels *et al*. (2006) used different mtDNA segments, it is difficult to compare levels of divergence within *Galaxias* ‘nebula’ to those within *P. perlatus* across the two species’ common distribution range. Crabs are assumed to be capable of terrestrial dispersal. Despite this, populations of *P. perlatus* from the western draining river systems and those from the southern draining river systems appear to have been isolated since the Pliocene (Daniels, 2003) compared to *Galaxias* ‘nebula’ whose populations are inferred to have diverged as recent as the Pleistocene.

Uplift of the Cape Fold Mountains

Previous researchers have inferred the role of the Cape Fold Mountains in explaining the biogeographical patterns and evolutionary diversification of freshwater-dependent taxa in the Cape Floristic Region (Barnard, 1943; Skelton, 1986; Wishart & Hughes, 2001; Daniels, 2003; Roos, 2004; Daniels *et al.*, 2006; Gouws *et al.*, 2010). There is clear evidence from two independent approaches that divergence among *Galaxias* ‘nebula’ populations postdates the formation of the Cape Fold Mountains (CFMs) about 65 million years ago. This data may either imply that (i) *Galaxias* ‘nebula’ were able to maintain gene flow across the CFMs until more recent times, or (ii) individuals of a historically isolated population dispersed across the CFR relatively recently followed by recent fragmentation. If tectonic activities influenced the evolutionary history of *Galaxias* 'nebula', then the events of the Post-African II period are likely to have had a greater impact than the earlier Cenozoic and the Post-African I events (Hendey, 1983; Partridge & Maud, 1987; 2000).

Given the historic cycles of extreme arid and wet conditions in the CFR (Marlow *et al*., 2000; Zachos *et al*., 2001), maintenance of gene flow across the CFMs over a prolonged period of time is difficult to envisage. Chance movements of individuals from a historically isolated population possibly facilitated by intermittent connections of adjacent basins during wetter and humid periods, therefore appears to be a more plausible explanation than panmixia. Indeed, NCPA inferences indicated that both historical isolation and long distance colonization appear to be the dominant factors that could have influenced the evolutionary history of *Galaxias* 'nebula'. It is clear that the uplift of the CFMs did not form an effective barrier to *Galaxias* 'nebula' populations inhabiting western draining and southern draining river systems as more recent dispersal of this species has occurred across these prominent mountain ranges.

These results are rather unusual for a freshwater-restricted fish, especially given that genetic diversity in other freshwater taxa in the CFR including *Sandelia capensis* (Roos, 2004), *Pseudobarbus* species (Swartz, 2005; Swartz *et al*., 2007, 2009), *Potamonautes perlatus* (Daniels *et al*., 2006) and *Alporia barnardi* (Wishart & Hughes, 2001) seem to have been strongly influenced by the CFMs. Also, the high number of endemic freshwater fishes restricted to the Olifants system on the west coast suggests that this region has been isolated from the rest of the CFR for a significant period of time. The CFMs have even been found to be a formidable barrier to terrestrial insects (Price *et al*., 2007, 2010) that could otherwise be thought to have high dispersal potential. Findings of the present study are, however, not unique to this region as recent molecular studies of other freshwater-restricted fishes occurring on opposite sides of some of the world's major collisional mountain ranges have also reported similar results. For instance, Šlechtová *et al*. (2004) found close relationships between representatives of *Cottus ferrugeneus* across the European Alps, suggesting active transfer of haplotypes across this prominent mountain range from the glacial cycles to more recent times. There is also evidence of closer genetic relationships between a number of species pairs of freshwater taxa inhabiting both sides of the Great Dividing Range in Australia (McGlashan & Hughes, 2001; Murphy & Austin, 2004; Thacker *et al*., 2008). Burridge *et al*. (2008a) presented strong genetic evidence showing that *Gobiomorphus breviceps* recently dispersed across the Southern Alps via intermittent wet connections. These findings challenge the traditional emphasis of the role of vicariant orogenic processes in explaining occurrence of related taxa on opposite sides of mountain ranges (reviewed by Craw *et al*., 2008b). These authors and the present study highlight the important role that low-lying mountain passes played in facilitating movement of taxa across prominent mountain ranges.

Certainly, portions of the Cape Fold Mountains do form impassable barriers to dispersal, but corridors that traverse these mountains do exist. The most obvious of these is the low lying area between the Little Berg and Michell's Pass (300 m above sea-level) which represents a potential dispersal corridor between the Berg and Breede River systems. Similarly, the valley between the upper Olifants and Koekedou River (884 m above sea-level) represents a potential dispersal corridor between the Olifants and Breede River systems. In addition, there are numerous low passes that were left at the level of captured river beds across the CFR (see Skelton, 1980a). It is therefore likely that recent dispersal of *Galaxias* 'nebula' across the Cape Fold Mountains could have been facilitated by episodic inundation of these low-lying passes, possibly during Pleistocene pluvial/interglacial periods.

Sea-level regression and palaeoriver systems

Swartz (2005) and Swartz *et al*. (2007, 2009) recently provided empirical evidence indicating that coalescence of rivers during low sea-levels of the most recent glacial maximum about 18 000 years ago played an important role in facilitating dispersal of species of the genus *Pseudobarbus* (redfin minnows) across currently isolated drainage systems in the CFR. However, results of the present study revealed that *Galaxias* 'nebula' has clearly not been contained by the proposed palaeoriver systems. NCPA results suggest that contiguous range expansion occurred across river systems that did not coalesce during the LGM. In addition, recent divergence times were found between rivers that belonged to different palaeoriver systems, which can only be explained by alternative and relatively recent dispersal mechanisms. While these results certainly do not exclude the role of sea-level regression in aiding migration between currently isolated river systems, they provide evidence that this mechanism alone is not sufficient to explain the observed genetic patterns in *Galaxias* 'nebula'.

Isolation in contemporary river systems

It was predicted that if *Galaxias* 'nebula' has been confined to currently isolated river systems since the establishment of the CFR drainage systems during the Pliocene, then genetic subdivision should conform to the River Hierarchy Model (RHM) (Meffe & Vrijnhoek, 1988) where populations within the same drainage system would be expected to be more similar to each other than to those in different drainage basins. AMOVA results showed that genetic variation in *Galaxias* 'nebula' has been largely structured by contemporary river systems. These results were supported by the significantly large Φ_{ST} values and very low number of geographically widespread haplotypes, which is consistent with confinement of this species to distinct river systems isolated by terrestrial and marine barriers. However, the overall phylogeographic pattern of this species, particularly the sharing of some haplotypes between some southern draining river systems separated by large geographic distances and estimates of inter-basinal divergence times suggest recent connectivity of populations inhabiting currently isolated river systems. Daniels *et al*., (2006) also found some alleles that were shared among river crab populations from currently isolated river systems on the south coast of the CFR.

The occurrence of closely related freshwater fishes in currently isolated river systems across the CFR has been traditionally attributed to drainage rearrangements or river capture events (Barnard, 1943; Skelton, 1980a, 1986). More recently, Swartz *et al*. (2007) considered river capture to be the most plausible explanation for the close relationships between *Pseudobarbus phlegethon* restricted to the Olifants River system on the west coast and the

Forest lineage of *P. afer* on the south coast. However, river capture alone is unlikely to adequately explain the recent divergence between populations of *Galaxias* ‘nebula’ across the CFR. River captures would have had to occur relatively recently, across a wide area and at a high frequency. This is unlikely since drainage rearrangements are rare in geologically stable landscapes (Bishop, 1995). The CFR landscape has been stable for a long period of time, with the last major tectonic activities occuring during the Pliocene (Partridge & Maud, 1987, 2000; Cowling *et al*., 2009), thereby making frequent and recent river captures unlikely.

Alternative dispersal mechanisms

From the above discussion, it is clear that *Galaxias* ‘nebula’ experienced more recent dispersal across the CFR, but current biogeographic hypotheses are not adequate to explain the phylogeographic patterns of this species. This suggests that alternative dispersal mechanisms could have played a role. Since *Galaxias* ‘nebula’ is confined to freshwater, the close relationship of its populations across the entire distribution range is likely to be a result of interdrainage dispersal following episodic drainage connections, possibly during Pleistocene and Holocene pluvial periods. Several headwater tributaries in the CFR arise in close proximity to each other and are likely to discharge to adjacent systems during periods of intense rainfall. There are also several low-lying mountain passes that could act as dispersal corridors when they become inundated (drainage over-topping) following heavy flooding events. *Galaxias* ‘nebula’ penetrates to higher altitudes in mountain tributaries of the CFR compared to other native fishes (see Chapter 3). Opportunities for temporary interbasin connections are more likely in these areas compared to lower parts of the basin. Experimental evidence shows that this species can tolerate aerial exposure for prolonged periods of time (Chakona *et al.*, 2011) and it can maintain viable populations even in very shallow habitats (personal observations). Such intrinsic ecological attributes can facilitate movement across drainage divides during wet and humid conditions.

The results of the present study showed that the youngest split in *Galaxias* 'nebula' is between populations inhabiting coastal rivers of the low lying Agulhas plain, and revealed that many of these populations were connected as recent as the Holocene Altithermal. NCPA inferred contiguous range expansion across the southern draining systems, which was also supported by results of Fu's *F* and Tajima's *D* tests. These results are consistent with Partridge *et al.*, (1999) inferences of wetter climatic conditions for the coastal areas of the southern CFR during the Holocene Altithermal. The close relationship between southern populations of *Galaxias* 'nebula' is not surprising as the south coast of the CFR experienced relatively recent wetter conditions while the western parts have been drier for a much longer period (Partridge *et al.*, 1999). The inferred fragmentation of *Galaxias* 'nebula' between the Olifants, Berg and southern flowing river systems can therefore be attributed to loss of connectivity among river systems due to higher aridity on the west coast compared to the south coast.

The occurrence of shared alleles between geographically distant river systems is not readily explainable. Recent exchange during low sea-levels is an unlikely explanation because the Breede and Groot Brak, for example, belonged to different palaeoriver systems. Similarly, the Gouritz and the Haelkraal did not form part of the same palaeoriver system. A possible explanation could be incomplete lineage sorting (or retention of ancestral polymorphisms), where long distance colonization could have occurred and then the populations were subsequently fragmented due to more recent intensification of dry climatic conditions. However, if this was the case, this scenario would have been expected to involve a few more river systems, although smaller sample sizes could have concealed the presence of shared haplotypes. Further studies using faster evolving markers such as microsatellites are needed to provide a better understanding of the evolutionary history of *Galaxias* 'nebula'.

Galaxias 'nebula' shows more recent and extensive dispersal events than any other freshwater species in the CFR. The scenarios of mountain uplift, river capture events, palaeorivers and contemporary river systems must have had an influence, but they do not adequately explain the phylogeographic patterns of *Galaxias* 'nebula'. Inter-basin dispersal via intermittent freshwater connections (through either overtopping of low drainage divides or low-lying mountain passes and headwater connections) during Pleistocene and Holocene pluvial periods, possibly augmented by other opportunities such as drainage rearrangements and sea-level regression, seems to be the most credible explanation for the extensive distribution and the patterns of genetic variation in *Galaxias* 'nebula'. These results indicate that the processes that shaped the phylogeography and biogeography of this species are complex and that for a complete picture to be obtained phylogeographic studies should integrate both geological and climatic history, as well as understanding the ecological attributes of the study species. Further studies of *Galaxias* 'nebula' employing faster-evolving genetic markers such as microsatellites and further ecological studies will help shed more light on whether *Galaxias* ‘nebula’ recently expanded its range across the CFR or maintained a wide distribution throughout most of its current range prior to recent fragmentation.

Chapter 5

Evolutionary drivers of lineage diversification and distribution of sympatric freshwater fishes from the south-western Cape Floristic Region of South Africa

Abstract

This study used phylogenetic analyses of mitochondrial cytochrome *b* sequences to investigate genetic diversity within three broadly co-distributed freshwater fish genera (*Galaxias*, *Pseudobarbus* and *Sandelia*) from the south-western CFR to shed some light on the processes that promoted lineage diversification and shaped geographic distribution patterns. A total of 205 sequences of *Galaxias*, 177 sequences of *Pseudobarbus* and 98 sequences of *Sandelia* from 146 localities across the Breede, Duiwenhoks, Goukou, Heuningnes, Ratel, Haelkraal, Uilkraals, Klein and Palmiet river systems in the south-western CFR were used. The data were analysed using phylogenetic and allele network methods and divergence times for the clades retrieved were estimated using three independent approaches. Nine extremely divergent (3.5 – 25.3%) lineages were found within *Galaxias*. Similarly, deep phylogeographic divergence was evident within *Pseudobarbus*, with four markedly distinct (3.8 – 10.0%) phylogroups identified. *Sandelia* has two deeply divergent (5.5 – 5.9%) lineages, but seven minor lineages with strong geographical congruence were also identified. The Miocene-Pliocene major sea-level transgression and the resultant isolation of populations in upland refugia appear to have driven widespread allopatric divergence within the three genera. Subsequent coalescence of rivers during the Pleistocene major sea-level regression as well as intermittent drainage connections during wet periods is proposed to have facilitated postspeciation dispersal of lineages that currently occur across isolated river systems. The high degree of genetic differentiation recovered from the present and previous studies suggest that freshwater fish diversity within the south-western CFR may be vastly underestimated, and taxonomic revisions are required.

5.1. Introduction

The central goal of phylogeographic studies is to determine the evolutionary processes underlying patterns of biotic diversification and the geographic distribution of biodiversity (Avise, 2000). Traditionally, such inferences were based on single-species studies (e.g. Near *et al*., 2001; Pfenninger & Posada, 2002; Murphy & Austin; 2004; Daniels *et al*., 2006). However, given the broad-scale variation in species-specific characteristics such as habitat preferences (see Chapter 3), ecological constraints and potential mechanisms of dispersal (see Chapter 4), it remains difficult to determine the extent to which patterns from a single phylogeographical example can be projected to other biotic components within regions or ecosystems. Comparative phylogeography, the integration of phylogeographic information from multiple co-distributed taxa, provides a more rigorous approach for investigating the historical events that shaped regional species diversity and distribution patterns (e.g. Bermingham & Martin, 1998; Bermingham & Moritz, 1998; Avise, 2000).

The comparison of phylogeographic patterns across many disparate species may help to reveal similar (e.g. Castoe *et al*., 2009; McCulloch *et al*., 2010) or independent responses of species to evolutionary forces (e.g. Hodges *et al*., 2007; Hare *et al*., 2008; Polihronakis & Casterino, 2010). Identification of spatially and temporally congruent patterns of genetic structure across taxonomically and ecologically diverse species allows for more powerful inferences of the major events that have influenced the evolutionary history of regional biota (e.g. Bermingham & Martin, 1998; Castoe *et al*., 2009; McCulloch *et al*., 2010). Conversely, dissimilar patterns of genetic structure in co-distributed species may suggest species-specific responses to shared history due to differences in ecology, life history or dispersal capabilities (e.g. Bermingham & Martin, 1998; Lourie *et al*., 2005; Hare *et al*., 2008; Burridge *et al*., 2008a). Such comparative phylogeographic studies are especially enlightening for regions where geological, tectonic and climatic information are poorly documented (e.g. Arbogast & Kenagy, 2001).

River systems of the Cape Floristic Region (CFR) have relatively low fish diversity, but high levels of endemicity south of the Zambezi River (Thieme *et al*., 2005). Several studies have used molecular phylogenetic approaches to explore the genetic diversity of freshwater fishes of this region (Waters & Cambray, 1997; Bloomer & Impson, 2000; Roos, 2004; van Niekerk, 2004; Swartz, 2005; Wishart *et al*., 2006; Swartz *et al*., 2004, 2007, 2009; present study). These studies have revealed considerable levels of population structuring in almost all the species from the CFR investigated thus far, indicating that taxonomic diversity and endemism may be vastly underestimated. The south-western CFR, in particular, has several unique genetic lineages in the genera *Galaxias*, *Pseudobarbus* and *Sandelia* (Roos, 2004; van Niekerk, 2004; Swartz, 2005; present study). Differentiation among the three *P. burchelli* lineages was suggested to be the result of ecological differences between river systems and existence of in-stream physical barriers (Swartz, 2005).

Accumulating evidence shows that repeated sea-level fluctuations have played an important role in driving speciation and shaping the distribution patterns of several extant freshwater taxa in other regions of the world, including the Neotropics (e.g. Montoya-Burgos, 2003), North America (e.g. Near *et al*., 2003; Near & Bernard, 2004) and the Indo-Australian

Archipelago (e.g. De Bruyn & Mather, 2007). Similar fluctuations have also been experienced in southern Africa (Siesser & Dingle, 1981). Major sea-level changes have occurred along the south coast of South Africa since the Oligocene through the Miocene and Pliocene, to the last glacial maximum (LGM) about 18 000 years before present (Siesser & Dingle, 1981). The last major sea-level transgression in southern Africa occurred during the middle Miocene (*ca*. 15 Mya) to the early Pliocene (*ca*. 5 Mya) (Siesser & Dingle, 1981). This transgression reached between +200 and +300 m above present-day sea-levels (Siesser & Dingle, 1981), and several low altitude river systems on the south coast of South Africa would have been submerged during this period. Sea-levels began to recede again in the late Pliocene (*ca*. 2.6 Ma), and the last major regression which occurred during the LGM reached between -100 and -200 m below current levels (Siesser & Dingle, 1981; Hendey, 1983). The southern African region also experienced complex climatic conditions marked by extremes of wet and dry periods (Partridge *et al*., 1999; Partridge & Maud, 2000; Marlow *et al*., 2000; Zachos *et al*., 2001).

A unique feature of the south-western CFR is the Agulhas Bank, a shallow continental shelf that would have been periodically exposed and submerged due to repeated fluctuations in sea-levels (Siesser & Dingle, 1981; Compton, 2011). This could have resulted in the recurrent connection and isolation of adjacent river systems (Swartz, 2005; Swartz *et al*., 2009). Repeated contraction and expansion of freshwater habitats could have had a significant impact on the diversification and distribution patterns of freshwater taxa in the CFR. Marine incursions during periods of high sea-levels have been identified as major vicariant events that led to allopatric divergence and speciation of Central and North

American freshwater fishes (e.g. Montoya-Burgos, 2003; Near *et al*., 2003; Near & Bernard, 2004). Coalescence of rivers during periods of low sea-level has been linked to range expansion (dispersal) of freshwater restricted taxa and the colonisation of currently isolated river systems (e.g. Montoya-Burgos, 2003; Near *et al*., 2003; Near & Bernard, 2004; Swartz, 2005; Swartz *et al*., 2007, 2009).

Swartz (2005) and Swartz *et al*. (2007, 2009) proposed that the most recent sea-level regression during the LGM was the major evolutionary mechanism underlying the widespread distribution of some extant *Pseudobarbus* lineages in the CFR. Swartz (2005) discovered three historically isolated lineages of *Pseudobarbus burchelli* from the Breede and associated river systems. Two of the lineages, *Pseudobarbus* 'Tradou' and *Pseudobarbus* 'Heuningnes' have restricted geographic ranges, while *Pseudobarbus* 'Breede' occurs in the currently isolated Breede, Duiwenhoks and Goukou River systems (Swartz, 2005). The occurrence of *Pseudobarbus* 'Breede' in the Breede and Duiwenhoks River systems was suggested to be the result of the connection of these rivers during the LGM (Swartz, 2005; Fig. 1.4). However, the occurrence of this lineage in the Goukou River system is not consistent with the sea-level regression hypothesis, since the Goukou formed part of a different palaeoriver system (Swartz, 2005; Figure 1.4). Further, the relationship between *Pseudobarbus* 'Breede' and *Pseudobarbus* 'Heuningnes' is not consistent with the confluence of the Breede and Heuningnes river systems during the most recent major sea-level regression because isolation since the LGM may not be sufficient to produce the level of divergence observed between these two lineages (2.1 – 3.3% for control region and 2.2 – 3.6% for cytochrome *b*) (Swartz, 2005). It is also surprising that *Pseudobarbus* 'Breede' does not occur in the Heuningnes River system and *Pseudobarbus* ‘Heuningnes’ does not occur in the Breede River system despite these rivers having shared a common confluence during the LGM. Swartz (2005) proposed river capture events, ecological differentiation and in-stream physical barriers as possible explanations for these unexpected patterns.

The present study extends previous research by combining environmental variables, mitochondrial DNA analysis and lineage divergence time estimates to identify the underlying drivers of diversification in three co-distributed primary freshwater fish genera (*Galaxias*, *Pseudobarbus* and *Sandelia*) from the south-western CFR (Fig. 1.3). In this study area, *Galaxias* and *Sandelia* are broadly co-distributed from the Goukou, through the Duiwenhoks, Breede, Heuningnes, Haelkraal, Uilkraals and Klein River systems to the Palmiet River system. *Pseudobarbus* is restricted to the Goukou, Duiwenhoks, Breede and Heuningnes River systems and has never been recorded from the smaller coastal river systems (Ratel to the Palmiet river systems) (Fig. 5.6). The current taxonomy of *Galaxias*, *Pseudobarbus* and *Sandelia* recognizes only one species for each of these genera (currently *G. zebratus, P. burchelli* and *S. capensis*) occurring across these isolated river systems in the south-western CFR (Skelton, 2001).

The present study aims (i) to identify unique lineages and map their distribution, and (ii) to determine the factors that drove lineage diversification and contemporary distribution of these taxa. It was hypothesised that marine incursions during periods of elevated sea-levels (Fig. 1.4) could have isolated populations in upland refugia and thereby driving allopatric divergence. Subsequent coalescence of adjacent rivers during periods of lower sea-levels

(Fig. 1.4) could have facilitated post-speciation range expansion. If alternative mechanisms (such as overland dispersal during wetter climatic periods and river capture events) also facilitated post-speciation dispersal of lineages, then unique lineages would be expected to have distribution ranges that span different palaeoriver systems.

According to Birmingham & Martin (1998), evidence of shared history across multiple co-distributed taxa would probably reflect the role of extrinsic climatic or landscape history in shaping contemporary biogeographic patterns, while different patterns would probably reflect the influence of intrinsic biological or ecological differences. The goal of the present study was therefore to determine if patterns observed in the three species can be used to formulate an explicit hypothesis of lineage diversification and distribution of freshwater fishes in the south-western CFR.

5.2. Materials and methods

Sampling

Specimens of the three species were collected from 146 localities across the south-western CFR (Table 5.1) between November 2008 and December 2009. The Breede, Duiwenhoks and Goukou River systems were sampled using a combination of electric fishing, seine netting, snorkelling with a handnet and fyke nets. Samples from the peat-stained or high-conductivity coastal drainages were collected using a 3 m seine net and fyke nets. The fish were anaesthetized with clove oil (0.2 ‰). A small piece of muscle tissue or whole specimen was preserved in 95% ethanol. Genetic samples were kept at ambient temperature in the field and were transferred to -20 °C upon returning to the laboratory. Source specimens (as well as additional vouchers) were fixed in 10% formalin in the field and were transferred to 70% ethanol upon returning to the laboratory. All samples collected for the present study have been deposited at the South African Institute for Aquatic Biodiversity.

DNA extraction, amplification and sequencing

DNA was extracted from preserved tissue using the Wizard® Genomic DNA purification kit (Promega, USA) following the manufacturer's protocol. A partial fragment of the mitochondrial cytochrome *b* gene was amplified. For *Galaxias*, the protocol outlined in Chapter 4 was followed. The primers GluF (5'- AAC CAC CGT TGT ATT CAA CTA CAA - 3') and ThrR (5' – ACC TCC GAT CTT CGG ATT ACA AGA CCG - 3') (Machordom & Doadrio, 2001) were used for *Pseudobarbus* and the PCR protocol was: 94 °C for 2 minutes, 35 cycles of 94 °C for 30 seconds, 54 °C for 30 seconds and 72 °C for 45 seconds, followed by 72 °C for 5 minutes and final storage at 4 °C. The primers used for *Sandelia* were L14841 (5' – CCA TCC AAC ATC TCA GCA TGA TGA AA - 3') (Kocher *et al*., 1989) and H16091 (5' – GTA TCA TTC TGG TTT GAT GTG - 3') (Roos, 2004) and the PCR protocol was similar to that of *Pseudobarbus* except that the denaturing and annealing temperatures were 93 °C and 55 °C, respectively. Sequencing, alignment and editing of sequences were done as outlined in Chapter 4.

Table 5.1: Tributaries sampled for *Galaxias zebratus* (*Gal*), *Pseudobarbus burchelli* (*Pse*) and *Sandelia capensis* (*San*) from the south-western CFR. Locality codes and geographic coordinates are given. The number of individuals that were sequenced per locality is indicated. Blank space indicates that the species was not collected at that locality.

	Sample Code	River	System	Latitude	Longitude	*Gal*	*Pse*	*San*
1	AC08A07	Vink	Breede	-33.73422222	19.82030556		2	
2	AC08A10	Vink	Breede	-33.73022222	19.82355556	2		
3	AC08A11	Noree	Breede	-33.72494444	19.81547222	1		
4	AC08A12	Hoeks	Breede	-34.02316667	19.83852778			1
5	AC08A13	Hoeks	Breede	-34.01091667	19.83922222	1		1
6	AC08A14	Hoeks	Breede	-34.00963889	19.83836111	1	2	
7	AC08A17	Noree	Breede	-33.73277778	19.81361111		2	
8	AC08A18	Hex	Breede	-33.49877778	19.53061111	2		
9	AC08A19	Hex	Breede	-33.52894444	19.54008333	3	2	2
10	AC08A20	Amandel	Breede	-33.52402778	19.54266667	2	2	
11	AC08A21	Amandel	Breede	-33.50661111	19.49497222			1
12	AC08A31	Amandel	Breede	-33.99380556	19.18263889		2	
13	AC08A32	Amandel	Breede	-33.98900000	19.18402778	2		2
14	AC08A38	Amandel	Breede	-33.97447222	19.19175000	3	2	
15	AC08A42	Amandel	Breede	-33.98297222	19.18963889	2		
16	AC08A52	Wit	Breede	-33.57322222	19.13797222		2	2
17	AC09A08	Tradou	Breede	-33.95677778	20.70788889		1	
18	AC09A09	Tradou	Breede	-33.94155556	20.70733333		2	
19	AC09A42	Wolwekloof	Breede	-33.56413889	19.12933333	4		
20	AC09A43	Wolwekloof	Breede	-33.56505556	19.13144444		2	2
21	AC09A46	Titus	Breede	-33.40533333	19.41805556		2	2
22	AC09A50	Keissies	Breede	-33.71050000	19.93105556	2		
23	AC09A51	Keissies	Breede	-33.70352778	19.91086111	3		
24	AC09A54	Keissies	Breede	-33.71050000	19.93105556	1		
25	AC09A58	Koo	Breede	-33.59450000	19.75811111		2	2
26	AC09A61	Die Brak	Breede	-33.57644444	19.75163889		2	
27	AC09A63	Elandskloof	Breede	-34.03055556	19.39000000		2	
28	AC09A64	Elandskloof	Breede	-34.03461111	19.39327778		2	
29	AC09A65	Baviaans	Breede	-34.02705556	19.55588889		2	2
30	AC09A70	Gobos	Breede	-34.03661111	19.63613889	2	2	2
31	AC09A71	Gobos	Breede	-34.03833333	19.62844444	1		
32	AC09A72	Gobos	Breede	-34.03638889	19.62575000	1	2	
33	AC09A73	Ganskraal	Breede	-34.11577778	19.81394444		2	1
34	AC09A74	Happy Valley	Breede	-34.11183333	19.81530556	2	2	
35	AC09A75	Happy Valley	Breede	-34.11222222	19.69797222	2		1
36	AC09A76	Ganskraal	Breede	-34.12547222	19.81147222	2		
37	AC09A78	Boks	Breede	-34.07963889	19.82952778	4		
38	AC09A79	Boks	Breede	-34.09602778	19.82841667		2	
39	AC09B14	Boesmans	Breede	-34.01113889	20.00722222		1	
40	AC09B15	Boesmans	Breede	-34.05100000	19.95300000			2

Table 5.1: continued...

	Sample Code	River	System	Latitude	Longitude	*Gal*	*Pse*	*San*
41	AC09B17	Boesmans	Breede	-34.04158333	19.96205556		1	
42	AC09B18	Poesjenels	Breede	-33.96547222	19.58336111			2
43	AC09B20	Poesjenels	Breede	-33.97916667	19.55761111	1		
44	AC09B22	Dorn	Breede	-33.85333333	19.47580556			1
45	AC09B23	Riesvlei	Breede	-33.89316667	19.44344444			1
46	AC09B25	Dorn	Breede	-33.93294444	19.46161111	1		
47	AC09B27	Stettyn	Breede	-33.86977778	19.34750000	1		
48	AC09B30	Stettyn	Breede	-33.86847222	19.34725000		2	
49	AC09B33	Holsloot	Breede	-33.82883333	19.27161111	2		2
50	AC09B35	Agter-Vink	Breede	-33.67155556	19.74255556	2		
51	AC09B38	Spreeudrifspruit	Breede	-33.98316667	19.32925000		2	2
52	AC09B39	Bereaville	Breede	-34.05930556	19.47736111			2
53	AC09B41	Bereaville	Breede	-34.02705556	19.48236111	5		
54	AC09B42	Bereaville	Breede	-34.03036111	19.48377778		2	
55	AC09B43	Riviersonderend	Breede	-34.06227778	19.07013889		2	
56	AC09B44	Riviersonderend	Breede	-34.06741667	19.07566667	4		
57	AC09B46		Breede	-34.05486111	19.09744444	2		
58	AC09C25		Breede	-34.37052778	20.64566667		4	1
59	AC09C26		Breede	-34.38255556	20.67505556			1
60	AC09C27		Breede	-34.38694444	20.68622222			1
61	AC09C56		Breede	-34.04236111	19.53716667	2		
62	AC09C57		Breede	-34.07666667	19.64325000	2		
63	AC09C59	Gobos	Breede	-34.05344444	19.61366667	2		
64	AC09C61		Breede	-34.06538889	19.65883333	2		2
65	AC09C63	Keurbooms	Breede	-34.00441667	20.37713889	4		
66	AC09C65	Soetmelks	Breede	-34.11238889	19.74552778	2		
67	AC09C66	Soetmelksvlei	Breede	-34.10330556	19.74122222		2	
68	AC09C67	Krom	Breede	-34.11166667	19.79566667	1	2	
69	AC09C69	Klip	Breede	-34.02463889	20.41805556	2	2	2
70	AC09C71	Leeu	Breede	-33.97855556	20.34202778	1		
71	AC09C72	Leeu	Breede	-33.99508333	20.33719444		2	
72	AC09C74	Keurbooms	Breede	-33.98561111	20.37300000		3	
73	AC09C75	Keurbooms	Breede	-33.99644444	20.37375000	2		
74	AC09C77	Koornings	Breede	-34.01230556	20.45458333	2	1	2
75	AC09C79	Hartbees	Breede	-34.01230556	20.45458333	3	1	
76	AC09C81	Bothaspruit	Breede	-33.56247222	19.34075000	2	5	1
77	AC09C82	Du Toit	Breede	-33.96702778	19.16566667	4		2
78	AC09C84	Slanghoek	Breede	-33.61155556	19.22402778	2		
79	AC09C85	Slanghoek	Breede	-33.65522222	19.21797222			1
80	AC09D08	Breede	Breede	-33.68705556	19.42683333	4		

Table 5.1: continued…

	Sample Code	River	System	Latitude	Longitude	*Gal*	*Pse*	*San*
81	AC09D09	Breede	Breede	-33.52002778	19.18552778	2		
82	AC09D16	Du Toit	Breede	-33.93722222	19.16800000		4	
83	AC09D18	Bothaspruit	Breede	-33.59952778	19.32330556		1	
84	AC09D20	Wabooms	Breede	-33.50252778	19.27063889	2		
85	AC09D23	Koekedou	Breede	-33.35911111	19.29547222			2
86	AC09D25	Wit tributary	Breede	-33.53744444	19.14302778		2	
87	AC09D28	Krom	Breede	-33.72277778	19.11294444		6	
88	AC09D32	Smallblaar	Breede	-33.69050000	19.31622222	2		
89	AC09D33	Slanghoek	Breede	-33.66619444	19.24638889	2		
90	AC09D34	Riviersonderend	Breede	-34.06230556	19.07050000		8	
91	AC09D39	Sandrifs Dam	Breede	-33.43613889	19.57088889			2
92	AC09D41	Buffeljags	Breede	-33.99916667	20.60455556	2		
93	ES07A21	Baviaans	Breede	-34.02705556	19.55588889	2		
94	gz32	Happy Valley	Breede	-34.09650000	19.73605556	1		
95	gz33	Elandskloof	Breede	-34.05202778	19.41627778	1		
96	AC09B13	Duiwenhoks	Duiwenhoks	-33.99766667	21.10583333			2
97	AC09C32	Duiwenhoks	Duiwenhoks	-33.99480556	21.07519444	2		
98	AC09C33	Duiwenhoks	Duiwenhoks	-33.97819444	21.03244444	2		2
99	AC09C35	Duiwenhoks	Duiwenhoks	-33.97041667	20.98783333	2		2
100	AC09C36	Duiwenhoks	Duiwenhoks	-33.99211111	20.85430556	1		2
101	AC09C38	Duiwenhoks	Duiwenhoks	-34.00705556	20.88383333		8	
102	AC09C39	Duiwenhoks	Duiwenhoks	-34.01963889	20.93455556		4	2
103	AC08A58	Vette	Goukou	-34.02125000	21.22500000	4	2	2
104	AC09B01	Goukou	Goukou	-34.00036111	21.41800000	7		2
105	AC09B04	Kleinkruis	Goukou	-34.01719444	21.29058333	2	2	
106	AC09B06	Kleinkruis	Goukou	-33.99900000	21.29138889			2
107	AC09B07	Kleinkruis	Goukou	-34.00272222	21.29222222		4	
108	AC09B10	Kleinkruis	Goukou	-33.96108333	21.30677778			2
109	AC09B11	Vette	Goukou	-33.94430556	21.25419444			2
110	AC09B12	Vette	Goukou	-33.98491667	21.20716667		8	
111	AC09C31	Korinte	Goukou	-33.98883333	21.15794444	2		2
112	AC09A28	Haelkraal	Haelkraal	-34.66763889	19.57527778	3		
113	AC09A29	Haelkraal	Haelkraal	-34.66775000	19.57791667			2
114	AC09A30	Haelkraal	Haelkraal	-34.67683333	19.58697222	6		
115	ES07A38	Haelkraal	Haelkraal	-34.67083333	19.54300000	2		
116	AC08A22	Kars	Heuningnes	-34.41316667	19.82041667	2	2	
117	AC08A23	Kars	Heuningnes	-34.41330556	19.82080556			4
118	AC08A24	Kars	Heuningnes	-34.36372222	19.78255556		2	1
119	AC08A25	Kars	Heuningnes	-34.36855556	19.78308333			1
120	AC09A23	Grashoek	Heuningnes	-34.58238889	19.96841667			2

Table 5.1: continued…

	Sample Code	River	System	Latitude	Longitude	*Gal*	*Pse*	*San*
121	AC09A24	Grashoek	Heuningnes	-34.57400000	19.94888889	1	3	
122	AC09A25	Nuwejaars	Heuningnes	-34.55497222	19.88583333	1		
123	AC09A26	Nuwejaars	Heuningnes	-34.54230556	19.81916667	2		2
124	AC09A27	Nuwejaars	Heuningnes	-34.57836111	19.75758333	3	4	2
125	AC09B51	Van Zyl farm tributary	Heuningnes	-34.51836111	19.97591667	1		
126	AC09C12	Koue	Heuningnes	-34.56136111	19.63094444	2		
127	AC09C15	Kars	Heuningnes	-34.46783333	19.82311111	2		1
128	AC09C16	Kars	Heuningnes	-34.45166667	19.90116667	3		2
129	AC09C17	Kars	Heuningnes	-34.41677778	19.80119444		1	
130	AC09B50	Klein	Klein	-34.27619444	19.52513889			2
131	AC09C05	Hartbees	Klein	-34.32511111	19.37447222	4		
132	AC09C07	Hartbees	Klein	-34.33419444	19.35850000	4		1
133	ES07A42	Onrus	Onrus	-34.37825000	19.24000000	4		
134	AC09B49	Palmiet	Palmiet	-34.06919444	19.05072222			2
135	AC09B55	Palmiet	Palmiet	-34.09308333	19.05136111	2		
136	AC09C18	Ratel	Ratel	-34.70725000	19.69733333	2		
137	AC09C19	Ratel	Ratel	-34.67011111	19.69138889	4		
138	ES07A36	Ratel	Ratel	-34.75330556	19.73047222	4		
139	AC09A32		Uilkraals	-34.56222222	19.45513889	2		
140	AC09A33	Uilkraals	Uilkraals	-34.57366667	19.47944444	4		1
141	AC09A34	Boesmans	Uilkraals	-34.59836111	19.59222222	4		
142	AC09A36	Uilkraals	Uilkraals	-34.54366667	19.51519444			1
143	AC09A37	Uilkraals	Uilkraals	-34.52697222	19.53927778	6		
144	AC09A38	Uilkraals	Uilkraals	-34.46708333	19.59711111			1
145	AC09C11	Uilkraals	Uilkraals	-34.51363889	19.61202778	2		
146	GZ42	Slang	Uilkraals	-34.51338889	19.61216667	1		
Outgroups	*G*. ‘mollis’	Leeu	Berg	-33.15591667	19.05058333			
	S. ‘berg’	Leeu	Berg	-33.15591667	19.05058333			
	P. asper	Olifants	Gouritz	-33.62150000	21.94819444			
	P. tenuis	Olifants	Gouritz	-33.62150000	21.94819444			

Genetic analyses

A total of 439 sequences (*Pseudobarbus burchelli*: *n* = 136; *Galaxias zebratus*: *n* = 205 and *Sandelia capensis*: *n* = 98) from 146 localities were generated for the present study (Table 5.1). Forty-one additional sequences of *Pseudobarbus burchelli* generated in a previous study (Swartz, 2005) were also included in the present study. No stop codons were observed when examining amino acid translations, indicating that all sequences obtained were consistent with being mitochondrial copies. Shared and unique alleles for each genus were identified using the program DNASP ver 5.10 (Librado & Rozas, 2009). The most appropriate model of sequence evolution for each data set was selected using MODELTEST 3.7 (Posada & Crandall, 1998) under the AIC criterion (Burnham & Anderson, 2002). The model of sequence evolution selected for *Galaxias* was the general time-reversible model (GTR) (Lanave *et al*., 1984) with a specified proportion of invariable sites (I = 0.468) and gamma shape parameter (GTR + I + Γ). For *Pseudobarbus*, the GTR with a specified proportion of invariable sites (GTR + I) was selected. For *Sandelia*, the transitional model (TIM) (Posada & Crandall, 2001b) with a specified proportion of invariable sites and gamma shape parameter (TIM + I + Γ) was the best model selected. These models were used to calculate pairwise sequence distances for each taxon in PAUP4.0b10 (Swofford, 2003).

Evolutionary relationships among alleles within each genus were inferred using Maximum Likelihood (ML) and Bayesian Inference (BI) methods based on the selected model. *Pseudobarbus tenuis* and *P. asper* were used as outgroups for the *Pseudobarbus* phylogeny. Sequences of *P. burgi* were also included to test the relationships among the *Pseudobarbus* species in the study area. *Galaxias* ‘mollis’ (Swartz, unpublished) and *Sandelia* ‘Berg’ (Roos, 2004) both from the Leeu River (Berg River system) were used to root the *Galaxias* and *Sandelia* phylogenies, respectively. The ML analyses were done in PAUP4.0b10, using heuristic tree searches and applying the tree-bisection-reconnection (TBR) branch-swapping algorithm with 10 random addition replicates.

Bayesian analyses were performed using the Bayesian Markov Chain Monte Carlo (BMCMC) algorithm implemented in MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). As per the defaults, two parallel BMCMC analyses and four simultaneous Markov chains (three heated, one cold) per run were used. Each analysis was run for 5 million generations and sampled every 100th generation to obtain 50 000 sampled trees. The burn-in value was determined by plotting the average standard deviation of split frequencies, tree length and log-likelihood scores against generation time using the program TRACER v. 1.4 (Rambaut and Drummond, 2007). These plots showed that stationarity was reached within the first 10% of the trees obtained. Hence, the first 5000 trees were discarded as burn-in. The remaining 45 000 sampled trees were used to estimate a 50% majority rule consensus tree and Bayesian posterior probabilities. Posterior probability values greater than or equal to 95% were considered to represent significant support for the relationships reflected in the trees (Huelsenbeck & Ronquist, 2001; Wilcox *et al*., 2002). For each data set, four separate BI runs were done, and the topology and nodal support of the resulting trees were compared. This was done to assess whether the chains converged to the same point. In all cases, the four independent runs gave identical topologies among historically isolated lineages and only slight differences in branch support between runs, indicating that the chain lengths were sufficient.

In addition, the program TCS version 1.21 (Clement *et al*., 2000) was used to accommodate non-bifurcating relationships among the lineages within each genus. This allows for alternative connections or loops that are usually collapsed into unresolved polytomies (Posada & Crandall, 2001a) to be visualised.

Divergence time estimation

Estimates of divergence time between lineages can shed light on whether cladogenesis in the different groups arose contemporaneously or over different time scales, and thus help to link observed patterns of divergence with known historical climatic or geological events. Three independent methods were used to estimate divergence time among lineages within *P. burchelli*, *G. zebratus* and *S. capensis*.

First, the Bayesian Markov chain Monte Carlo (BMCMC) algorithm implemented in the program BEAST v. 1.5.4 (Drummond & Rambaut, 2007) was used to estimate the time to the most recent common ancestor (TMRCA) for the different lineages of each genus using the best-fit model estimated using MODELTEST 3.7 (Posada & Crandall, 1998). Because fossil evidence for southern African freshwater fishes is lacking (reviewed by Stewart, 2001), ‘internal’ rate calibrations could not be made. Therefore, the present study used ‘external’ rates for calibration. This was achieved by setting the boundaries of divergence times (using the ucld.mean prior) based on the lowest and highest molecular evolution rates reported in the literature. The rates used in the current study were 0.011 changes/site/million years and 0.031 changes/site/million years reported by Burridge *et al*. (2008b) for New Zealand galaxiids. These were considered to be the best available estimates of mitochondrial DNA evolutionary rates for galaxiid fishes. The use of these rates for *Pseudobarbus* and *Sandelia* was justified by the fact that the range of these rates (0.011 – 0.031) encompasses the 0.02 mean rate for the vertebrate mtDNA cyt *b* gene (Brown *et al*., 1979) that has been used by a number of researchers for fishes whose substitution rates are currently unknown (e.g. Aboim *et al*., 2005). Analyses were run for 40 million generations, with samples logged every 1000 steps. Four independent sets of analyses using a relaxed molecular clock were carried out for each data set.

The BEAST results were visualised using the program Tracer v. 1.4 (Rambaut & Drummond, 2007), specifically to assess the frequency plots for acceptable mixing and stationarity of the MCMC chain, to determine appropriate burn-in and to assess the adequacy of the effective sample sizes (ESS) for each estimated parameter. The first 4000 trees (10 %) were discarded as burn-in because the plots showed that stationarity was reached well within the first four million generations.

Secondly, Craw *et al*.'s (2008a) variable molecular clock was used to estimate divergence times between lineages within each genus. Divergence times (T_{div}) were computed using the following equation:

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

Thirdly, the net divergence estimate was used. This was calculated using the formula:

$$\text{Net divergence}\ (N_{\mathrm{D}}) = \frac{\%\ \text{divergence}}{\mu}$$

Where μ = mutation rate per million years (MY). The two rates (0.011 and 0.031) from Burridge *et al*. (2008b) were used for this analysis.

Environmental data

For each locality sampled, a range of environmental variables were measured or visually assessed. Topographical data (elevation and gradient) were calculated using GIS spatial analyst in ArcView based on the GPS coordinates that were recorded at each site in the field. Water temperature and conductivity were measured *in situ* using an HI 98312 portable meter and pH was measured *in situ* using an HI 98128 portable meter (Hanna Instruments®, USA). The dominant substratum at each locality was visually assessed and scored as: silt and mud (0), sand (1), cobbles (2), boulders and bedrock (3). Aquatic vegetation was also visually assessed and scored as: absent (0), slight (1), moderate (2), abundant (3). Water flow velocity was characterised as static (0), slow (1), moderate (2) and fast (3).

The environmental data from these eight variables were subjected to principal components analysis (PCA) using SPSS v. 16.0 (SPSS Inc.). The PCA scatter plot revealed two distinct groups, the first comprising of sites in the Heuningnes, Ratel, Haelkraal and Uilkraals river systems (referred to as coastal sites) and the second comprising localities in mountain tributaries of the Breede, Duiwenhoks and Goukou river systems (referred to as inland sites).

Multivariate analysis of variance (MANOVA) was performed to examine the significance of differences in environmental variables between coastal and inland river systems using SPSS v. 16.0 (SPSS Inc.), with PCA axis scores as dependent variables and ‘region’ (i.e. coastal or inland) as the fixed factor.

5.3. Results

Galaxias zebratus *lineage diversity and distribution*

The edited alignment of mtDNA cyt *b* sequences comprised of 648 base pairs of which 183 were variable. These variable sites resulted in 74 unique alleles (Fig. 5.1 and 5.2). Both overall haplotype (H_D) and nucleotide (π) diversity were high (H_D = 0.977; π = 0.05326). The relationships among historically isolated lineages of the cytochrome *b* gene trees from BI and ML methods were identical. The ML tree is presented with posterior probability support values from the BI analysis (Fig. 5.1). The analyses revealed deep divergences among the ingroup taxa, and eight historical lineages were identified, excluding the outgroup *Galaxias* ‘mollis’ (Fig. 5.1). Individuals from the Onrus River system were closely related to the outgroup and were therefore referred to as *Galaxias* ‘mollis’. The other lineages were called *Galaxias* ‘nebula’, *G.* ‘rectognathus’, *G.* ‘Heuningnes’, *G.* ‘Klein’, *G.* ‘slender’, *G.* ‘Riviersonderend’, *G.* ‘Breede’ and *G.* ‘Goukou’. Alleles grouped within these lineages with high Bayesian posterior probability values (0.99 - 1.00) for those lineages that had multiple alleles. Relationships among these lineages were not well resolved, but *G.* ‘rectognathus’ was resolved as sister to *G.* ‘nebula’ (Bayesian probability = 1.00) and *G.* ‘Heuningnes’ was resolved as sister to *G*. ‘klein’ (Bayesian probability = 1.00). The clade containing *G*. ‘Heuningnes’ and *G*. ‘Klein’ was resolved as sister to the clade containing *G*. ‘rectognathus’ and *G*. ‘nebula’ (Bayesian probability = 0.98). It is, however, uncertain how *G*. ‘slender’, *G*. ‘Riviersonderend’, *G*. ‘Breede’ and *G*. ‘Goukou’) are related to each other and to the clade containing *G*. ‘nebula’, *G*. ‘rectognathus’, *G*. ‘Heuningnes’ and *G*. ‘Klein’ (Fig. 5.1).

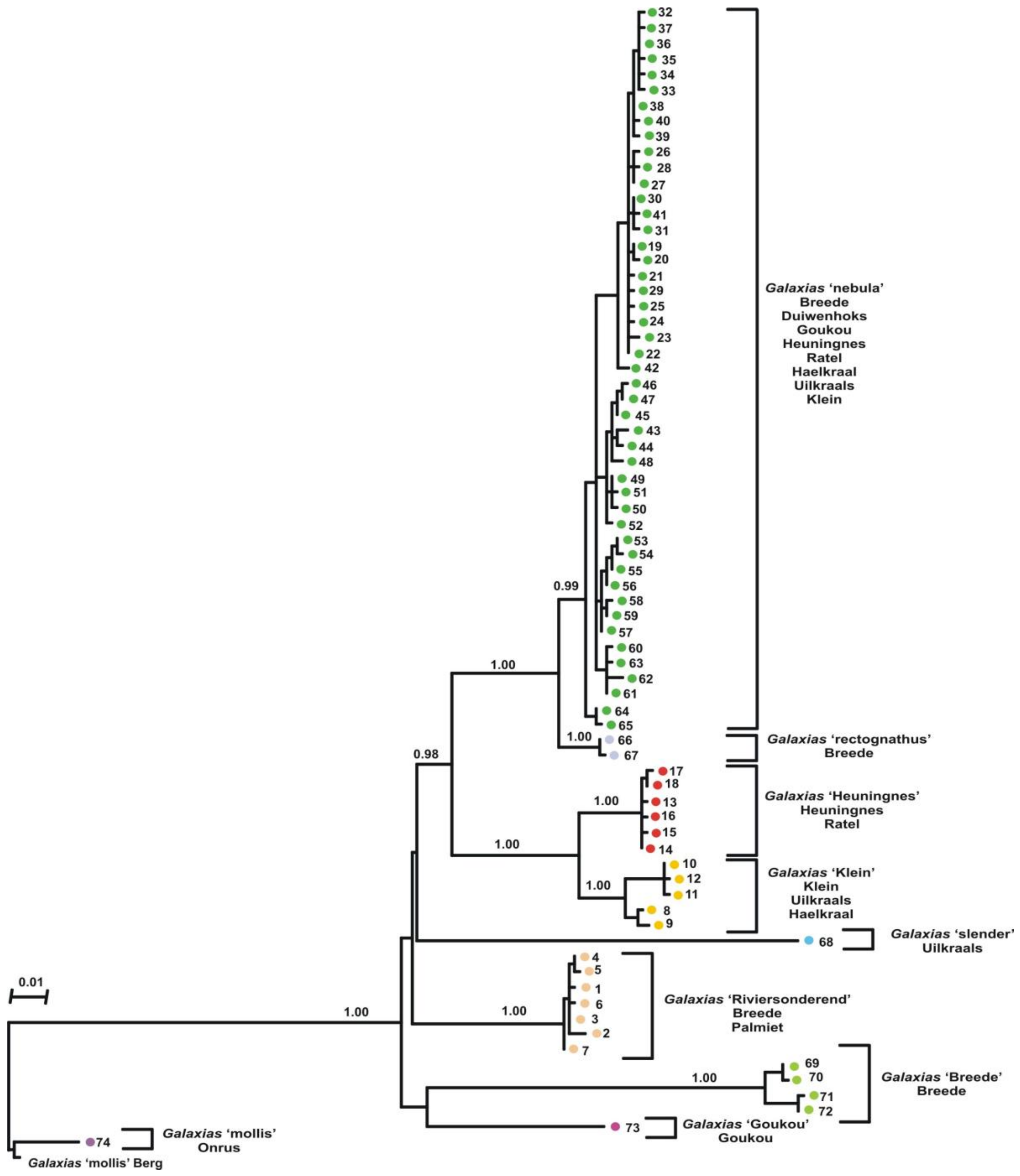


Fig. 5.1: Maximum Likelihood phylogenetic estimate of relationships among mitochondrial cytochrome *b* alleles of *Galaxias zebratus* from the south-western CFR based on the general time-reversible (GTR) model with a specified proportion of invariable sites (I) and gamma shape parameter (Γ) (GTR + I + Γ). Bayesian posterior probabilities are given on the branches. The numbers (1-74) represent unique alleles and the colours indicate lineages. Their distribution ranges are presented in Fig. 5.3. River systems in which the lineages occur are listed below the lineage names.

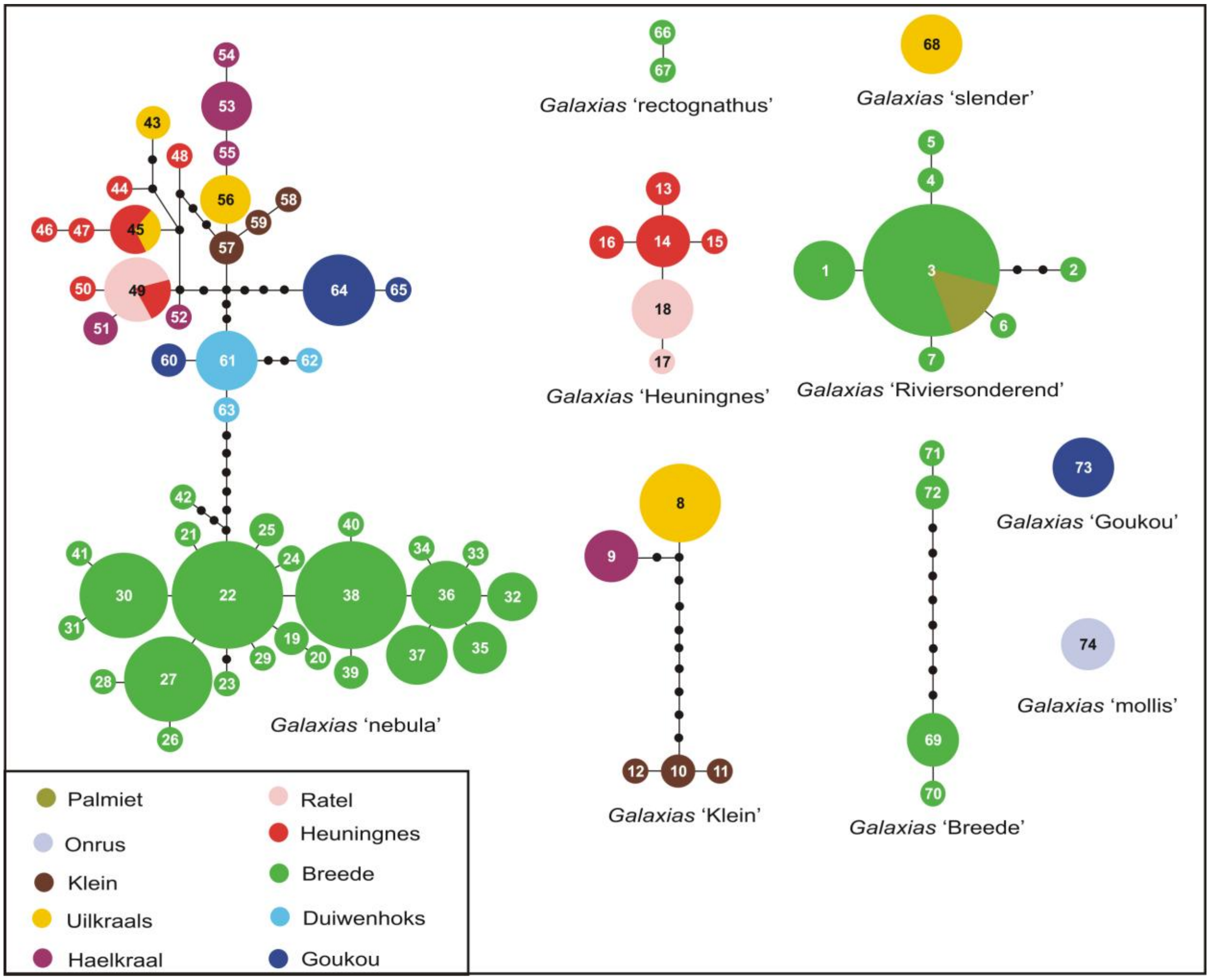


Fig. 5.2: TCS network of cyt *b* alleles (1-74) from individuals of *Galaxias zebratus* from the south-western CFR. Alleles are colour coded by river system. The sizes of circles are proportional to allele frequency. Black dots represent missing alleles in the network. Each branch represents one mutation step.

The TCS allele network (Fig. 5.2) consisted of six distinct clades and three disconnected alleles, reflecting the same lineages as the BI and ML phylogenies. Deep phylogenetic divergences were found among lineages (3.49 – 25.27 %) while values within lineages were much lower (0.00 – 1.37 %) (Table 5.2).

Table 5.2: Mean and range of model-corrected genetic distances between *Galaxias zebratus* lineages from the south-western CFR. The ranges of the values are given in brackets. Within lineage divergences are shown in bold.

	Lineage	1	2	3	4	5	6	7	8	9
1	‘nebula’	**1.29 (0-2.56)**								
2	‘rectognathus’	3.49 (2.38-4.25)	**0.16**							
3	‘Heuningnes’	11.16(10.04-12.47)	11.04 (10.62-11.41)	**0.30 (0-0.31)**						
4	‘Klein’	11.46 (9.43-12.93)	10.67 (10.29-11.07)	4.40 (3.84-4.84)	**1.35 (0-2.06)**					
5	‘slender'	17.71 (16.41-18.88)	17.15 (16.81-17.50)	18.23 (17.80-18.56)	17.07 (16.51-17.39)	**0.00**				
6	‘Riviersonderend’	12.12 (10.91-13.90)	9.59 (9.03-10.32)	12.62 (11.41-14.11)	11.91 (10.50-13.36)	16.91 (16.49-17.96)	**0.38 (0-0.79)**			
7	‘Breede’	18.24 (15.42-21.39)	18.76 (17.97-19.87)	18.56 (17.63-19.76)	17.79 (16.58-19.93)	24.86 (24.26-25.82)	18.02 (17.10-19.45)	**1.37 (0-1.86)**		
8	‘Goukou'	13.72 (12.26-14.76)	13.89 (13.60-14.18)	12.45 (12.15-12.89)	13.22 (12.83-13.59)	17.61	11.37 (10.72-12.24)	16.65 (16.18-17.25)	**0.00**	
9	‘mollis’	20.50 (18.85-22.51)	19.63 (19.23-20.03)	20.10 (19.83-20.48)	19.37 (18.51-19.92)	24.51	20.14 (19.56-21.19)	25.27 (24.19-26.81)	17.25	**0.00**

Galaxias ‘nebula’ is the most widespread of the nine lineages, occurring in all river systems of the south-western CFR, except the Onrus and Palmiet (Fig. 5.3). It occurs in sympatry with six of the *Galaxias* lineages that have highly restricted geographic ranges. *Galaxias* ‘rectognathus’ (Fig. 5.1 and 5.2) is restricted to two tributaries of the Riviersonderend, namely the Du Toit and the Amandel Rivers (Fig. 5.3). *Galaxias* ‘Heuningnes’ (Fig. 5.1 and 5.2) was collected from seven localities in the Heuningnes River system and from two localities in the Ratel River system (Fig. 5.3.). *Galaxias* ‘Klein’ (Fig. 5.1 and 5.2) occurs in the Klein, Uilkraals and Haelkraal (Fig. 5.3). *Galaxias* ‘slender’ (Fig. 5.1 and 5.2) is restricted to the Uilkraals River system (Fig. 5.3). *Galaxias* ‘Riviersonderend’ (Fig. 5.1 and 5.2) occurs in the upper Riviersonderend and six of its tributaries, namely the Du Toit, Amandel, Gobos, three unnamed tributaries between Greyton and Riviersonderend, and the Keurbooms River just below the confluence of the Breede and Riviersonderend (Fig. 5.3). This lineage also occurs in the Palmiet River system despite this system being separated from the Riviersonderend catchment by the Hottentots Holland Mountain Range (Fig. 5.3).

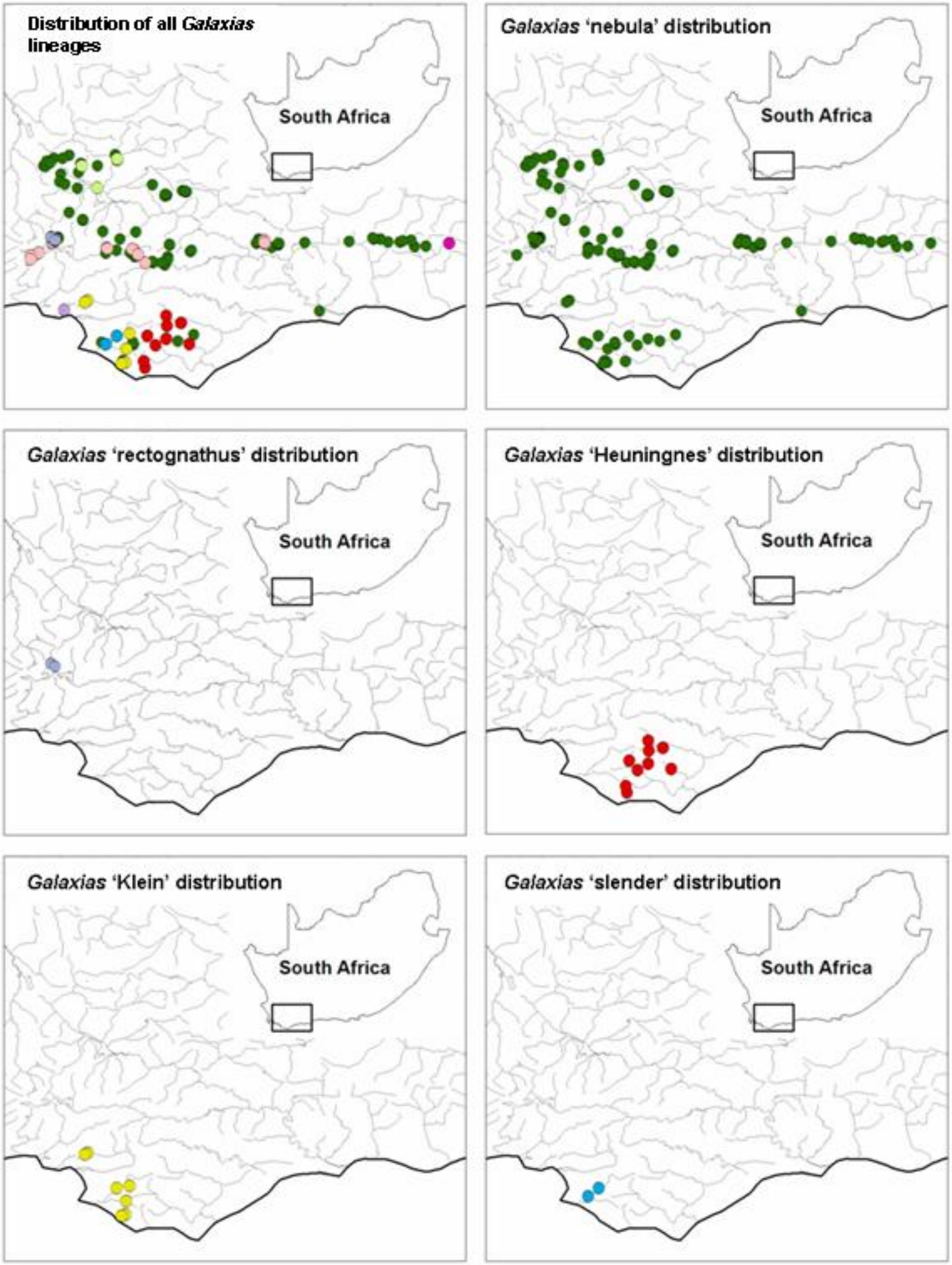
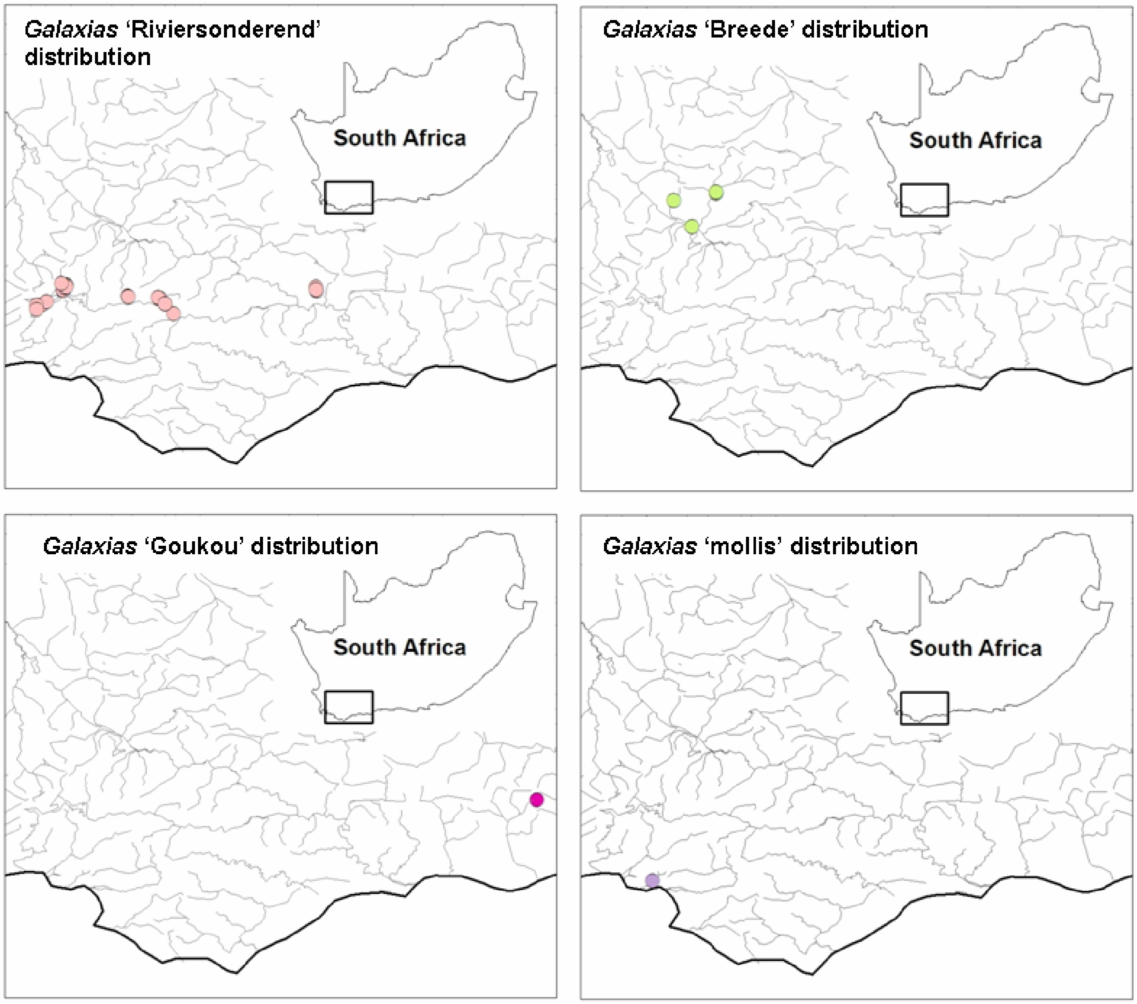


Fig.5.3: Distribution of *Galaxias zebratus* lineages in the south-western CFR. The colour codes are as denoted in Fig. 5.1.


Galaxias 'Riviersonderend' distribution

Galaxias 'Breede' distribution

Galaxias 'Goukou' distribution

Galaxias 'mollis' distribution

Fig. 5.3: continued…

Galaxias 'Breede' (Fig. 5.1 and 5.2) was collected from a single locality in the main-stem Breede River and two of its tributaries, the Hex and Bothaspruit (Fig. 5.3). *Galaxias* 'Goukou' (Fig. 5.1 and 5.2) is restricted to the Goukou River system where it was collected from a single locality (Fig. 5.3). *Galaxias* 'mollis' occurs in the Onrus River system in the south-western CFR (Fig. 5.3) as well as in the Leeu River, a tributary of the Berg River system on the west coast.

Pseudobarbus burchelli *lineage diversity and distribution*

The edited alignment of mtDNA cyt *b* sequences from 177 individuals was 676 base-pairs long and 95 of these characters were variable. This resulted in 47 unique alleles (Fig. 5.4 and 5.5). Haplotype (H_D = 0.885) and nucleotide (π = 0.021) diversity were high. BI and ML analyses resulted in identical relationships among historically isolated lineages. The ML tree is presented with Bayesian posterior probabilities indicating branch support (Fig. 5.4). The monophyly of the double-barbed redfins (*P. burchelli* and *P. burgi* species groups) is strongly supported (Bayesian posterior probability = 1.00). The analyses revealed substantial genetic structuring within *P. burchelli*. Four distinct lineages: *Pseudobarbus* 'Breede', *Pseudobarbus* 'Heuningnes', *Pseudobarbus* 'Tradou' and *Pseudobarbus* 'giant' were identified.

TCS analysis produced four distinct clades (Fig. 5.5) reflecting the same lineages as the BI and ML phylogenies (Fig. 5.4). Deep phylogenetic divergences were found among lineages (2.58 – 9.99%), while minor genetic divergences were found within lineages (0.30 – 0.71%) (Table 5.3).

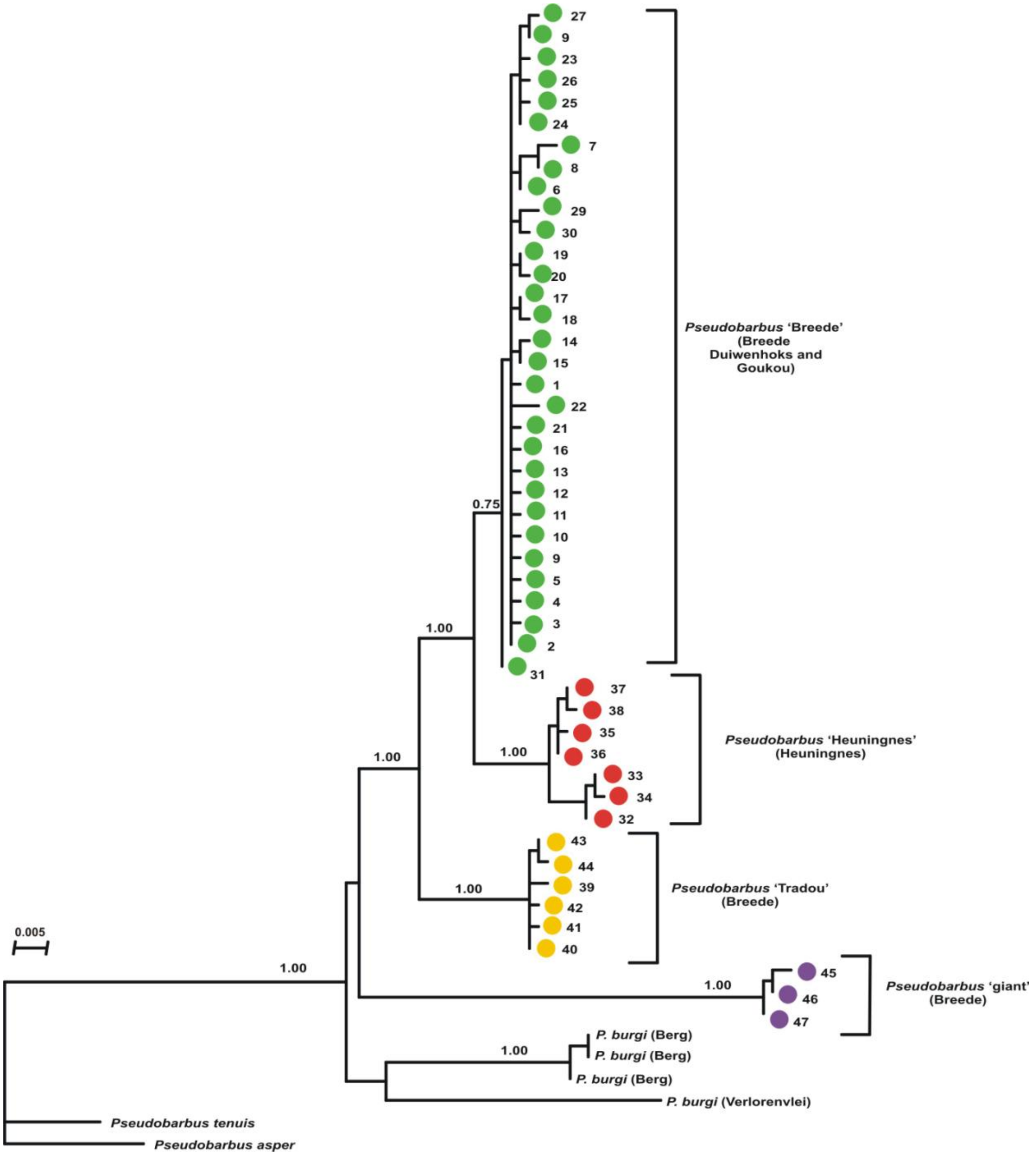


Fig. 5.4: Maximum Likelihood analysis of phylogenetic relationships among mitochondrial cyt *b* alleles identified in *Pseudobarbus burchelli* from the south-western CFR based on the general time-reversible (GTR) model with a specified proportion of invariable sites (I) (GTR+ I). Bayesian posterior probabilities are given on the branches. The numbers (1-47) represent unique alleles and the colours represent unique lineages. Distribution ranges of these lineages are presented in Fig. 5.6. River systems in which the lineages occur are given in parentheses.

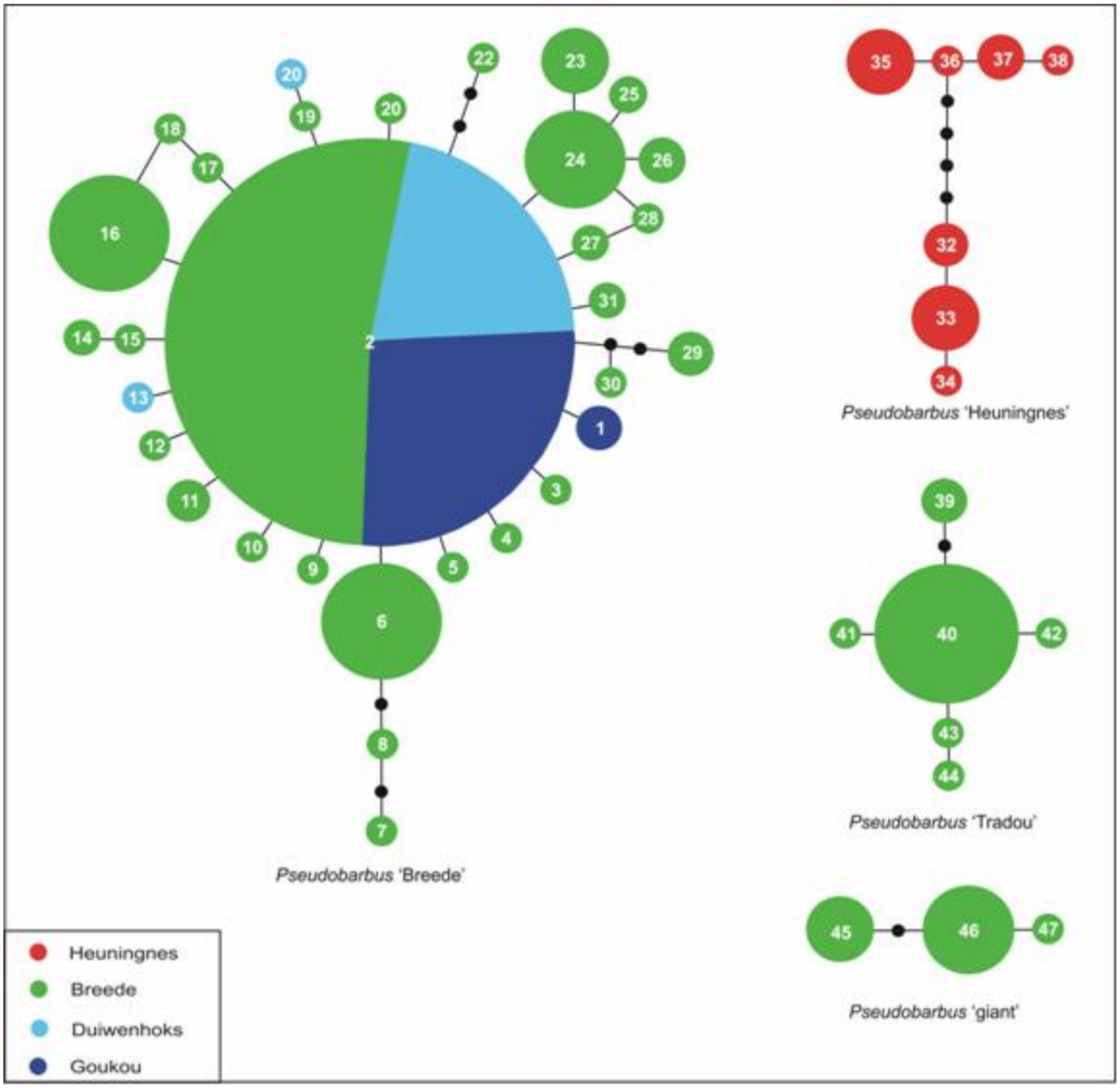
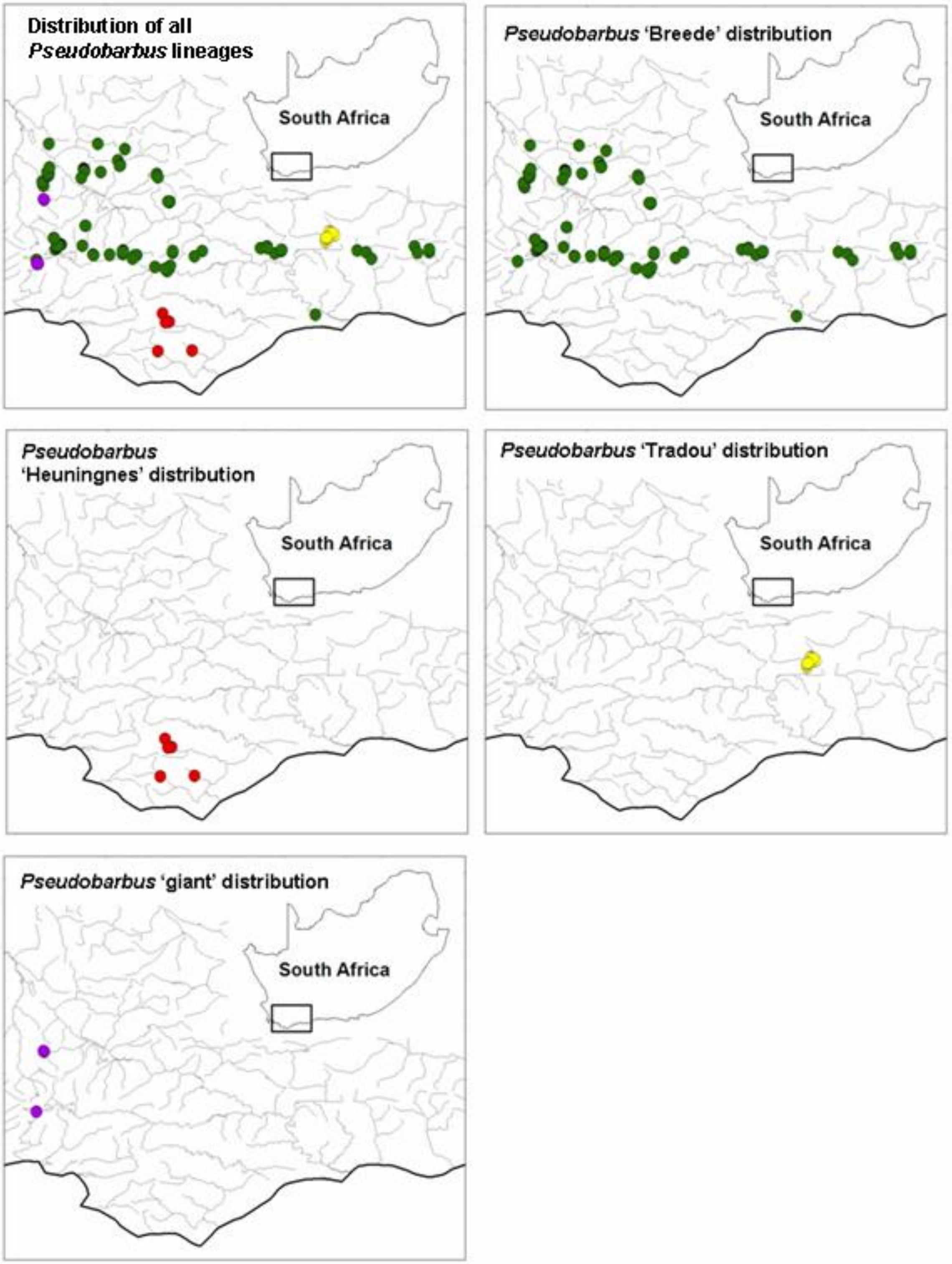


Fig. 5.5: TCS network of cyt *b* alleles (1-47) from individuals of *Pseudobarbus burchelli* from the south-western CFR. Alleles are colour coded by river system. The sizes of circles are proportional to allele frequency. Black dots represent missing alleles in the network. Each branch represents one mutation step.

Table 5.3: Mean and range of model-corrected genetic distances between *Pseudobarbus burchelli* lineages from the south-western CFR. The ranges of the values are given in brackets. Within lineage divergences are given in bold.

	Lineage	1	2	3	4
1	'Breede'	**0.43 (0-0.91)**			
2	'giant'	9.99 (9.25-10.97)	**0.30 (0-0.45)**		
3	'Tradou'	3.77 (3.20-4.27)	9.29 (8.79-9.98)	**0.33 (0-0.60)**	
4	'Heuningnes'	2.58 (1.86-3.20)	9.92 (9.25-10.45)	4.75 (4.09-5.40)	**0.71 (0-1.38)**

Pseudobarbus 'Breede' is widely distributed across the currently isolated Breede, Duiwenhoks and Goukou River systems (Fig. 5.6). *Pseudobarbus* 'Heuningnes' was collected from three tributaries of the Heuningnes River system, namely the Nuwejaars, Grashoek and Kars (Fig. 5.6). *Pseudobarbus* 'Tradou' is restricted to the Tradou catchment, a tributary of the Breede River system (Fig. 5.6). *Pseudobarbus* 'giant' was collected from only two localities, the upper Riviersonderend and the Krom River (tributary of the Molenaars) in the upper Breede (Fig. 5.6).



Distribution of all *Pseudobarbus* lineages

Pseudobarbus 'Breede' distribution

Pseudobarbus 'Heuningnes' distribution

Pseudobarbus 'Tradou' distribution

Pseudobarbus 'giant' distribution

Fig. 5.6: Distribution of *Pseudobarbus burchelli* lineages in the south-western CFR. The colour codes are as denoted in Fig. 5.4.

Sandelia capensis *lineage diversity and distribution*

DNA sequencing resulted in 98 sequences of the mtDNA cyt *b* gene and the edited alignment was 621 bp long. The sequences contained 66 variable sites which resulted in 30 alleles (Fig. 5.7 and 5.8) with H_D = 0.948 and π = 0.01228. BI and ML phylogenetic reconstructions recovered four major lineages. *Sandelia* 'eastern' had marginal support (Bayesian probability = 0.95), *Sandelia* 'Agulhas' was poorly supported while *Sandelia* 'DG' had good support (Bayesian probability = 1.00) (Fig. 5.7). Groups of alleles (denoted i – vii) within the four major groups had strong geographical affinities (described below).

The TCS output consisted of one large network and a disconnected singleton allele from the Klein River system (allele 30) (Fig. 5.8). Within the large allele network, seven lineages (i – vii) with strong geographical congruence can be identified. The first lineage, *Sandelia* 'Duiwenhoks' (i), (well-supported in the phylogenetic tree) comprised of alleles that are restricted to the Duiwenhoks River system, and the second lineage, *Sandelia* 'Goukou' (ii), is restricted to the Goukou River system (Fig. 5.9). The third lineage, *Sandelia* 'Breede' (iii), comprised of alleles collected from multiple sites in the Breede, Duiwenhoks and Goukou River systems (Fig. 5.9). The alleles which comprised the phylogenetically well-supported *Sandelia* 'Riviersonderend' (lineage iv) were restricted to the Riviersonderend and Palmiet Rivers (Fig. 5.9). *Sandelia* 'Koekedou' (lineage v), also well-supported in the tree, was restricted to localities in the Koekedou and Titus Rivers, both tributaries of the upper Breede (Fig. 5.9). *Sandelia* 'Agulhas' (lineage vi) (supported with a Bayesian posterior probability of 1.00) comprised of alleles from the Heuningnes, Haelkraal and Klein River systems, while *Sandelia* 'Uilkraals' (lineage vii) is restricted to the Uilkraals River system.

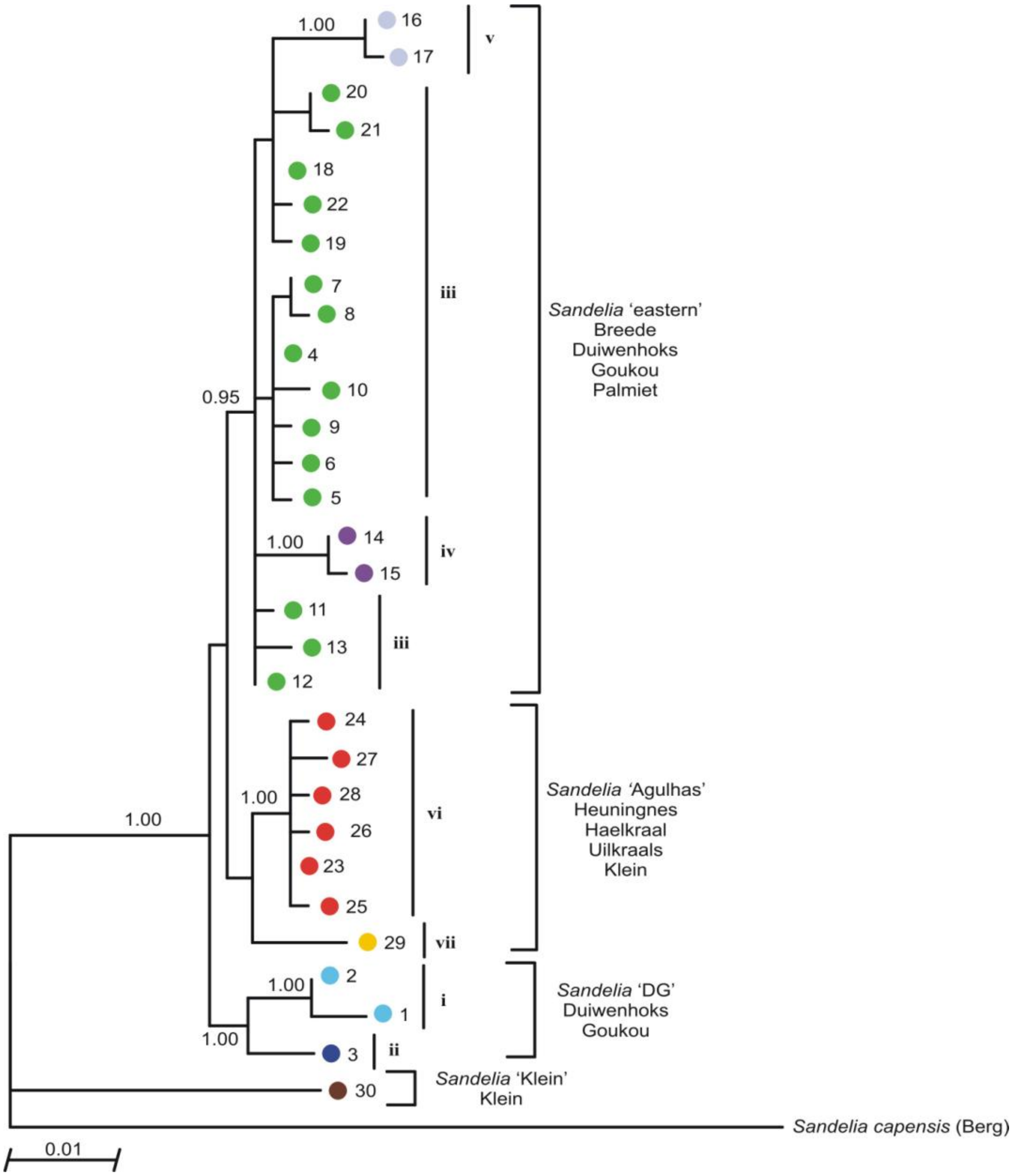


Fig. 5.7: Maximum Likelihood phylogenetic analysis of relationships among mitochondrial cyt *b* alleles (1-30) identified in *Sandelia capensis* from the south-western CFR based on the transitional (TIM) model with a specified proportion of invariable sites (I) and gamma shape parameter (Γ) (TIM + I + Γ). Bayesian posterior probabilities are given on the branches. The colours denote lineages (i – vii) that had strong geographic affinities and their distribution ranges are presented in Fig. 5.9. River systems in which the lineages occur are listed below the lineage names.

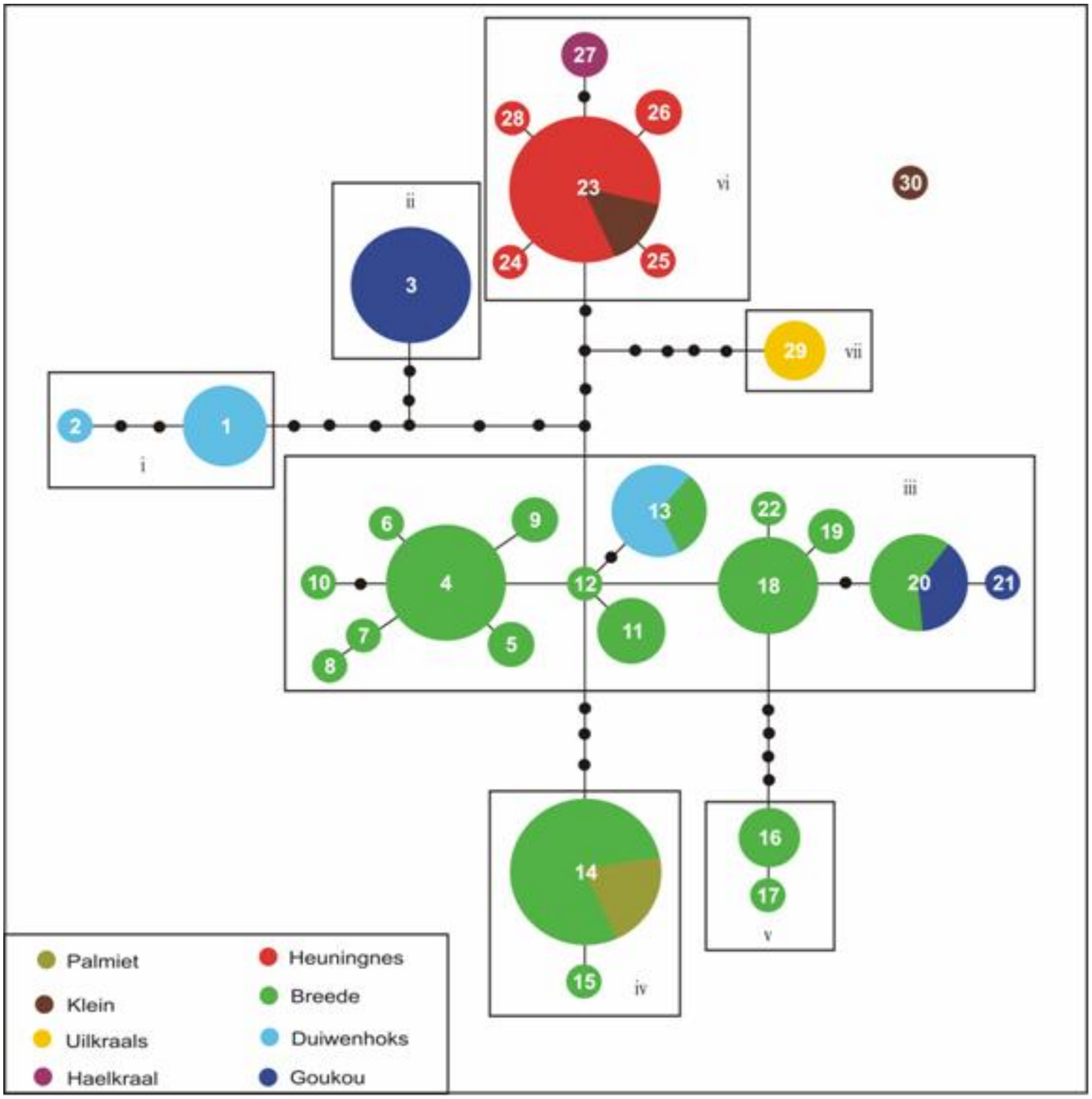


Fig. 5.8: TCS network of cyt *b* alleles (1-30) of *Sandelia capensis* from the south-western CFR. Alleles are colour coded by river system. The sizes of circles are proportional to allele frequency. Black dots represent missing alleles in the network. Each branch represents one mutation step. The roman numerals (i – vii) correspond to the lineages as denoted in Fig. 5.7.

Three of the four major lineages had shallow divergences among them (1.58 – 1.94%), with allele 30 from the Klein River system being the only deeply divergent lineage within *Sandelia capensis* (5.53 – 5.92%) compared to all the other lineages (Table 5.4).

Table 5.4: Mean and range of model-corrected genetic distances between four major lineages identified within *Sandelia capensis* from the south-western CFR. Within lineage divergences are given in bold.

		1	2	3	4
1	‘eastern’	**0.83 (0-2.10)**			
2	‘Agulhas’	1.58 (0.99-2.66)	**0.62 (0-1.53)**		
3	‘DG’	1.88 (1.18-3.27)	1.94 (1.52-2.31)	**1.12 (0-1.70)**	
4	‘Klein’	5.53 (4.90-6.47)	5.72 (5.53-5.98)	5.92 (5.57-6.47)	**0.00**

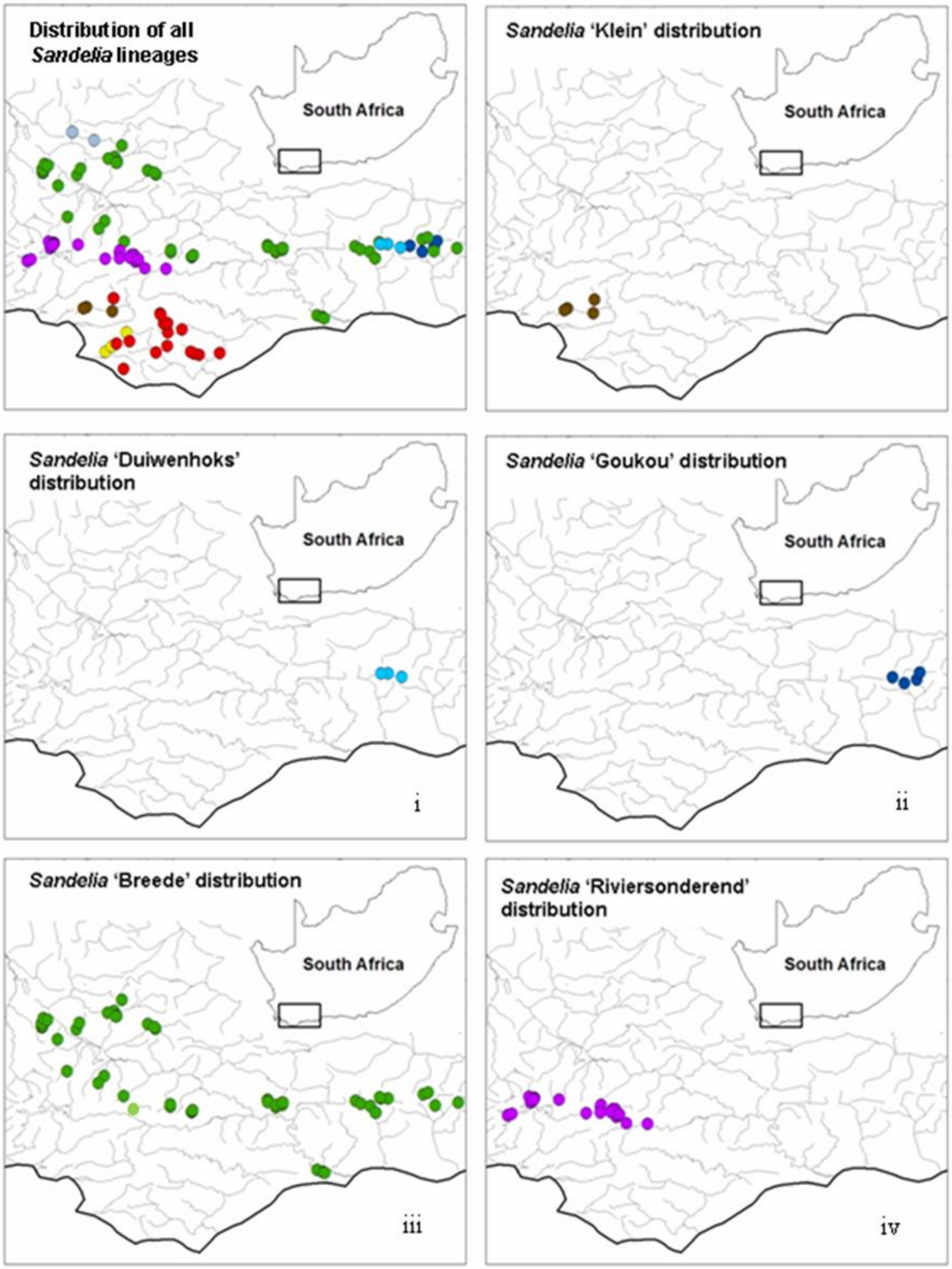


Fig. 5.9: Distribution of *Sandelia capensis* lineages in the south-western CFR. The colour codes and the roman numerals (i – vii) correspond to the lineages as denoted in Figs. 5.7 and 5.8.

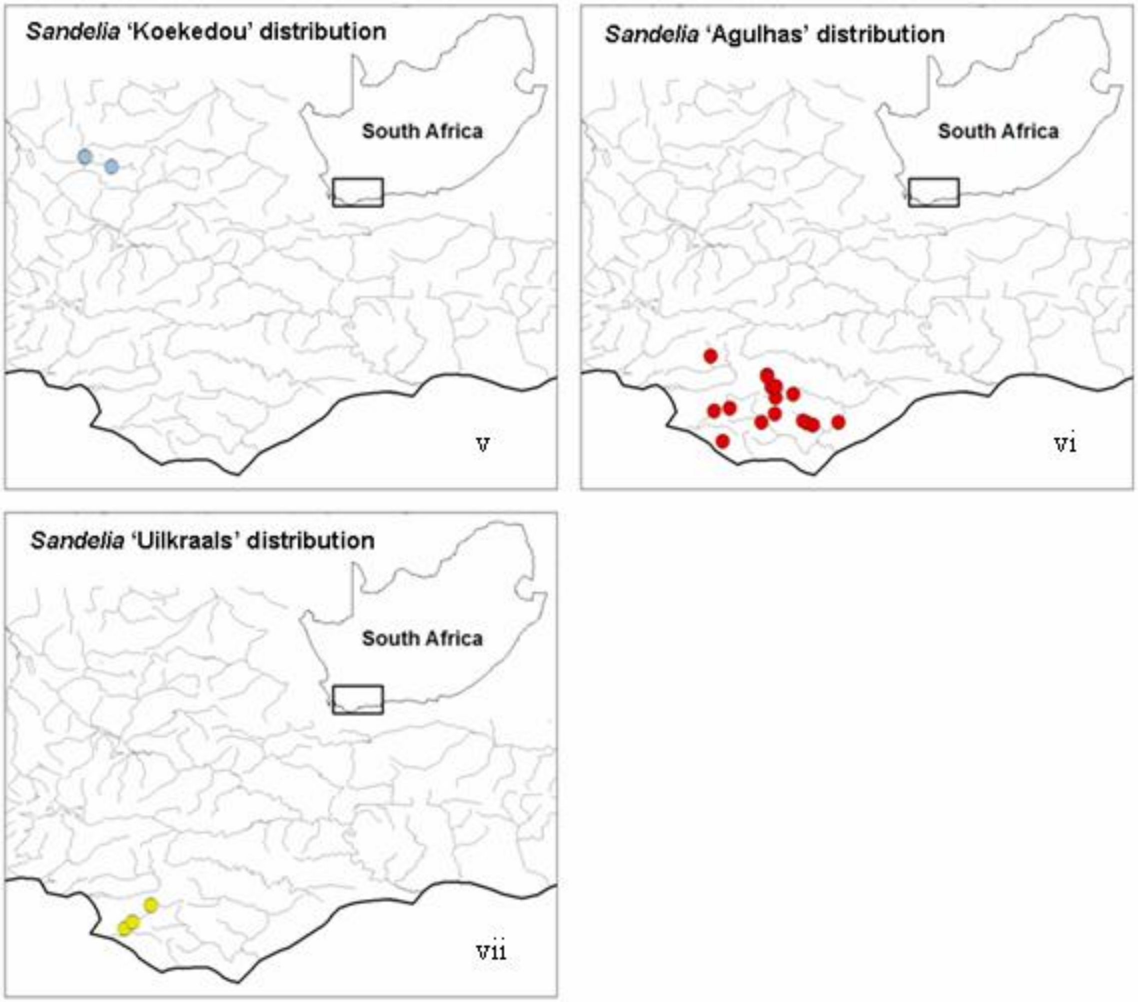


Fig. 5.9: continued…

Estimates of divergence times

Dating estimates from three independent methods (BEAST, Craw *et al*.’s (2008a) variable molecular clock and net divergence) are given in Figs 5.10A – 5.10C and Table 5.5. Net divergence estimates (N_D) based on the slower rate of evolution (1.1%) were about two to three times higher than the other two estimates. Values obtained from net divergence using the higher rate of evolution and those from the other approaches were generally comparable. The ranges of divergence estimates of *Galaxias* and *Pseudobarbus* lineages fell within a period bounded by the late Miocene or early Pliocene and the Pliocene-Pleistocene boundary (Table 5.5; Fig. 5.10 A and B). Dating suggested that the deepest split within *Sandelia* occurred at the end of the Pliocene (Table 5.5; Fig. 5.10 C), but much of the phylogeographic structuring within this genus occurred during the Pleistocene (Table 5.5; Fig. 5.10 C).

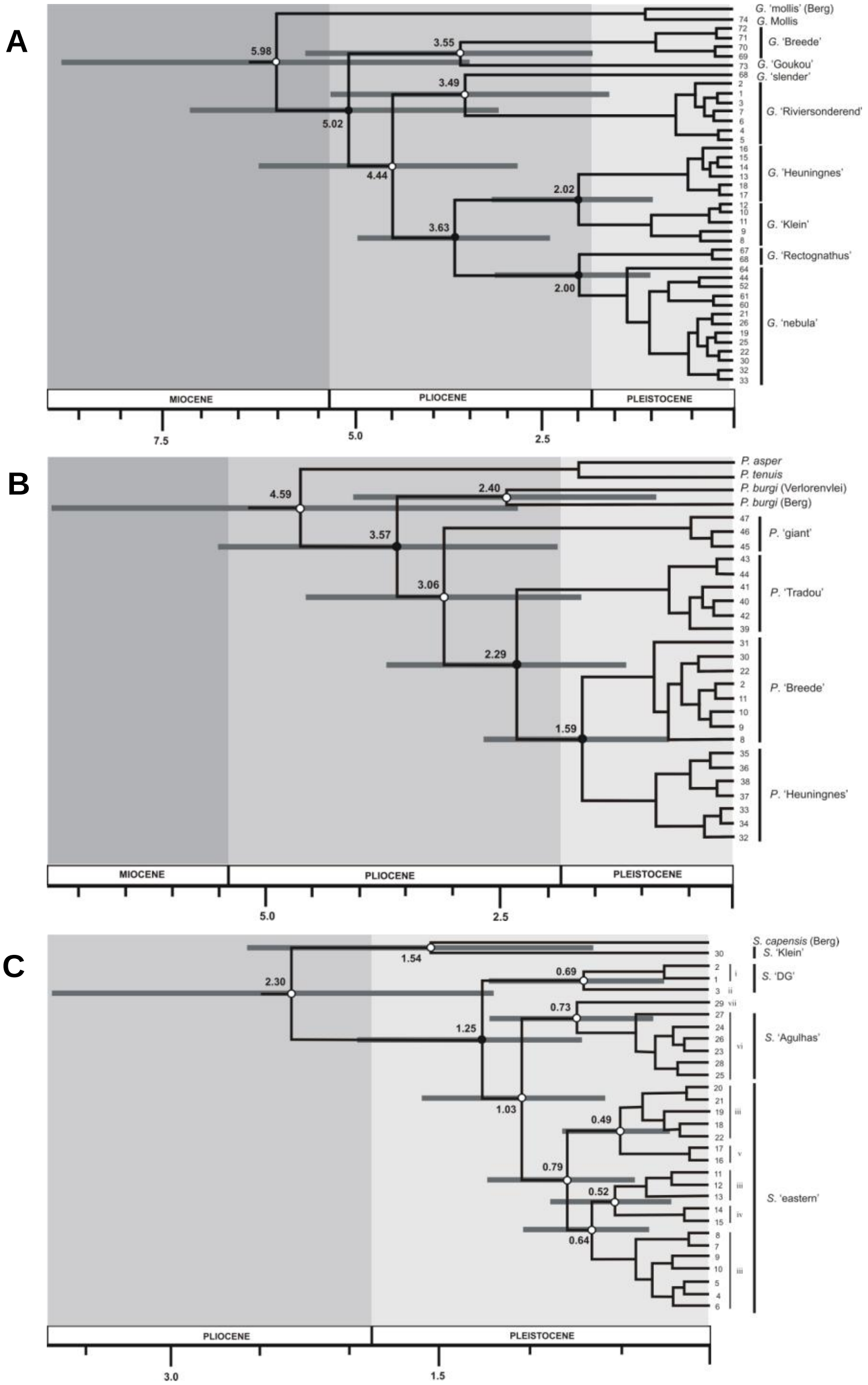


Fig. 5.10: Chronogram with estimates of divergence times (Million years ago) for major lineages of *Galaxias zebratus* (A), *Pseudobarbus burchelli* (B) and *Sandelia capensis* (C) inferred using Bayesian coalescent analyses implemented in BEAST. Values on the nodes represent the estimated mean divergence dates for the lineages, and bars represent 95% highest posterior densities for divergence estimates. Solid circles represent posterior probability values greater or equal to 0.95 and open circles represent values less than 0.95.

Table 5.5: Time of divergence between *Galaxias*, *Pseudobarbus* and *Sandelia* lineages based on Craw *et al*.'s (2008a) variable molecular clock (T_{div}) and net divergence (N_{div}). Comparisons were made hierarchically in cases where the sister group relationships among the lineages were supported, but all comparisons were considered for the respective lineages whose relationships were not supported. The values were calculated using the mean and range of the divergence between the clades or lineages.

	Craw *et al*. (2008a)	Net divergence	
	MYA (range)	MYA (range) at 1.1%	MYA (range) at 3.1%
Galaxias			
[neb] [rec]	0.53 (0.21-0.82)	3.18 (2.17-3.86)	1.13 (0.77-1.37)
[kle] [heu]	0.88 (0.66-1.06)	4.00 (3.49-4.40)	1.42 (1.24-1.56)
[neb+rec] [kle+heu]	3.63 (2.89-4.28)	10.26 (8.57-11.76)	3.64 (3.04-4.17)
[sle] [neb+rec+kle+heu]	6.20 (5.68-6.68)	16.08 (14.92-17.17)	5.71 (5.29-6.09)
[riv] [neb+rec+kle+heu]	3.96 (2.73-4.77)	10.98 (8.21-12.83)	3.89 (2.91-4.55)
[bre] [neb+rec+kle+heu]	6.42 (5.29-7.67)	16.59 (14.02-19.44)	5.89 (4.98-6.90)
[gou] [neb+rec+kle+heu]	4.54 (3.98-5.03)	12.33 (11.04-13.42)	4.37 (3.92-4.76)
[mol] [neb+rec+kle+heu+sle+riv+bre+gou]	7.25 (6.52-8.13)	18.48 (16.82-20.47)	6.56 (5.97-7.26)
[sle] [riv]	3.88 (3.32-4.47)	10.83 (9.55-12.15)	3.84 (3.39-4.31)
[sle] [bre]	6.23 (5.75-7.09)	16.17 (15.07-18.12)	5.74 (5.35-6.43)
[sle] [gou]	4.40 (4.25-4.56)	12.02 (11.67-12.35)	4.26 (4.14-4.38)
[riv] [bre]	6.33 (5.96-6.90)	16.39 (15.55-17.69)	5.81 (5.52-6.28)
[riv] [gou]	3.67 (3.40-4.01)	10.33 (9.75-11.13)	3.67 (3.46-3.95)
[bre] [gou]	5.78 (5.59-6.02)	15.13 (14.71-15.68)	5.37 (5.22-5.56)
Pseudobarbus			
[bre] [heu]	0.25 (0.13-0.42)	2.35 (1.69-2.91)	0.83 (0.60-1.03)
[tra] [heu+bre]	0.70 (0.42-1.28)	3.59 (2.91-4.91)	1.27 (1.03-1.74)
[gia] [tra+heu+bre]	3.08 (2.64-3.51)	8.99 (7.99-9.97)	3.19 (2.84-3.54)
[bre] [tra]	0.63 (0.42-0.83)	3.43 (2.91-3.88)	1.22 (1.03-1.38)
[bre] [gia]	3.12 (2.82-3.51)	9.08 (8.41-9.97)	3.22 (2.98-3.54)
[heu] [tra]	1.02 (0.76-1.28)	4.32 (3.72-4.91)	1.53 (1.32-1.74)
[heu] [gia]	3.09 (2.82-3.30)	9.02 (8.41-9.50)	3.20 (2.98-3.37)
[tra] [gia]	2.84 (2.64-3.11)	8.45 (7.99-9.07)	3.00 (2.84-3.22)
Sandelia			
[eas] [agu]	0.10 (0.05-0.27)	1.41 (0.90-2.42)	0.50 (0.32-0.86)
[eas] [dg]	0.14 (0.07-0.45)	1.77 (1.07-2.97)	0.63 (0.38-1.06)
[dg] [agu]	0.14 (0.10-0.20)	1.75 (1.38-2.10)	0.62 (0.49-0.74)
[kle] [eas+agu+dg]	1.33 (1.08-1.71)	5.03 (4.45-5.88)	1.78 (1.58-2.09)

Environmental data

The first principal component (PC1) explained 41.29% of the variance in the environmental parameters (Table 5.6). The major contributing variables to this component were dominant substratum, abundance of aquatic vegetation, elevation, conductivity and water velocity (Table 5.6). PC2, which explained 19.45 % of the variance, was dominated by water temperature and pH (Table 5.6).

MANOVA revealed significant separation between coastal and inland sites based on PC1 scores ($F_{1, 134} = 207.99$; $P < 0.001$), indicating differences in dominant substratum, presence of aquatic vegetation, elevation, conductivity, velocity and gradient between the two regions. No significant differences were found between inland and coastal sites based on PC2 scores ($F_{1, 134} = 0.24$; $P > 0.05$). The PCA scatter plot showed clear separation of coastal sites from inland sites, but there was considerable overlap of sites within regions (Fig. 5.11). Both MANOVA and PCA scatter plot results showed that river systems that originate within or beyond the coastal mountain ranges and those that originate within the Agulhas Plain belong to distinct ecological regions. These systems have been called inland (drain fold mountains and beyond) and coastal (originate within the coastal Agulhas Plain) river systems for convenience.

Table 5.6: PC1 and PC2 factor loadings for environmental variables measured in the present study.

	PC Component	
Variable	1	2
pH	-0.317	0.749
Conductivity	-0.723	0.275
Temperature	-0.032	0.775
Gradient	0.575	0.416
Elevation	0.745	0.257
Dominant substratum	0.821	0.172
Aquatic vegetation	-0.772	-0.055
Water velocity	0.723	-0.213
% variance explained	**41.29**	**19.45**

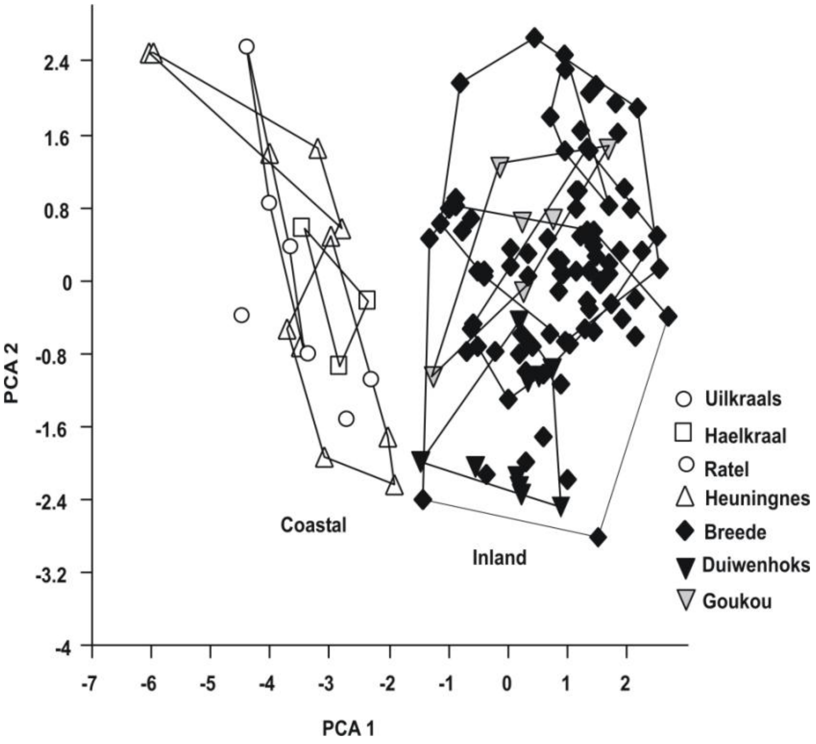


Fig. 5.11: PCA scatter plot of environmental variables from coastal and inland sites in the south-western CFR.

5.4. Discussion

Lineage diversity and distribution

Substantial mitochondrial differentiation is evident in the study area for *Galaxias zebratus* and *Pseudobarbus burchelli*, and at least two deeply divergent lineages were found for *Sandelia capensis*. These results are consistent with data based on the mitochondrial cytochrome *c* oxidase subunit I for *Galaxias zebratus* (Swartz, unpublished) and cytochrome *b* and control region for *Pseudobarbus burchelli* (Swartz, 2005). While detailed morphological studies and genetic analysis of nuclear genes are needed to substantiate the taxonomic status of the newly discovered lineages, results of the present study indicate that the south-western CFR represents a previously unrecognized centre of freshwater fish diversity and microendemism. Descriptions of the newly discovered lineages of *Galaxias*, *Pseudobarbus* and *Sandelia* have been initiated with various collaborators.

Galaxias zebratus, *Pseudobarbus burchelli* and *Sandelia capensis* show broad similarities in the distribution of lineages across the south-western CFR because they all have: (1) genetically distinct lineages restricted to the coastal river systems of the Agulhas Plain and inland lineages restricted to the Breede, Duiwenhoks and Goukou River systems, (2) some lineages that show low or no differentiation across currently isolated river systems and (3) deeply divergent lineages that occur in sympatry. *Galaxias zebratus* and *S. capensis*, in particular, have distinct lineages that largely co-occur. For example, they both have distinct lineages that are restricted to the Goukou, Riviersonderend and Palmiet, Uilkraals and Klein. Common phylogeographic patterns in multiple sympatric species across a wide geographic range may suggest similar responses to common regional scale physical processes (Avise, 2000; Bermingham & Martin, 1998). However, for patterns to maximally represent simultaneous responses to evolutionary forces, they must also be temporally concordant (Donoghue & Moore, 2003). Temporal patterns of divergence between *G. zebratus* and *S. capensis* are, however, not consistent with spatial patterns because the species showed substantial differences in the timing of cladogenesis. This may suggest the influence of similar evolutionary forces, such as historical isolation followed by subsequent range expansion, but these events could have affected the two species at different time periods.

Alternatively, the lack of temporal concordance between *S. capensis* compared to *G. zebratus* and *P. burchelli* could be a result of differences in the mutation rate of their mitochondrial DNA. If this is the case, then *S. capensis* has a much slower mutation rate than that reported for other groups of fishes. There is strong evidence for variation in the rates of nucleotide substitution among divergent taxonomic groups due to differences in metabolic rates, generation time and body size (Martin & Palumbi, 1993). Significant differences in the rates of molecular evolution have also been reported among the anabantoid fishes (Rüber *et al*., 2006), which includes the genus *Sandelia*. This presents an interesting case that warrants further investigation.

The occurrence of much higher lineage diversity in *Galaxias* than both *Pseudobarbus* and *Sandelia* is difficult to explain. Predation and competition from introduced alien fishes could have had stronger impacts on the larger species (*Pseudobarbus* and *Sandelia*) while the smaller sized and more cryptic *Galaxias* species could find concealment and evade predation pressure. Therefore, the lower lineage diversity in both *Pseudobarbus* and *Sandelia* could be a result of extinctions of some of the lineages due to alien predation. The most likely explanation is, however, that *Galaxias* have been in the south-western CFR for a much longer period than *Pseudobarbus* and *Sandelia*. This hypothesis is supported by the existence of older lineages in *Galaxias*. It is also noteworthy that relatives of this genus occur in all Gondwanan continents in the Southern Hemisphere (McDowall, 1973), while the closest relatives of the other two genera are in Africa (Swartz, 2005; Ruber *et al*., 2006). This suggests that the African *Galaxias* could have a history that is associated with the breakup of Gondwana and therefore an older history of occurrence in the CFR than *Pseudobarbus* and *Sandelia*. The innate adaptability of lineages, and the ability to disperse and occupy different habitats could also have influenced diversification among the three genera.

Biogeography and lineage diversification: the role of middle Miocene to early Pliocene marine transgressions

Divergence time estimates inferred from three independent approaches showed that diversification within *Galaxias zebratus* and *Pseudobarbus burchelli* occurred in the late Miocene through to the Pliocene-Pleistocene boundary. This coincides with the period of the last major sea-level transgression in southern Africa when sea-levels reached +200 to +300 m above present day levels along the south coast of South Africa (Siesser & Dingle, 1981). This transgression resulted in the drowning of small-low altitude coastal systems, such as the Ratel and Haelkraal, while river systems with headwaters in higher altitude regions, namely the Palmiet, Klein, Uilkraals, Heuningnes, Breede, Duiwenhoks and Goukou were considerably fragmented (Siesser & Dingle, 1981; Hendey, 1983; Fig. 1.4). The high sea-levels persisted until the late Pliocene when sea-levels gradually started to recede (Siesser & Dingle, 1981). Isolation of small populations of freshwater taxa in highland refugia for such a prolonged period of time could explain the diversification of the lineages in *G. zebratus, P. burchelli* and *S. capensis*, especially those that have strong geographic affinities.

Restriction of the deeply divergent lineage, *Sandelia* 'Klein', to the Klein River system is consistent with the expectation that the upper reaches of the Klein provided important refuge to freshwater taxa during the last major sea-level transgression. Similarly, restriction of *Galaxias* 'slender' and *Galaxias* 'Goukou' to the Uilkraals and Goukou River systems, respectively, is evidence that both river systems served as important refugia for freshwater fishes during the Miocene-Pliocene high sea-levels. The distribution limits of *Galaxias* 'Riviersonderend' and *Galaxias* 'Breede' suggest that these lineages could have evolved in allopatry due to vicariance caused by possible isolation of the Breede and Riviersonderend catchments during the Miocene-Pliocene marine incursion (Fig. 1.4). Similarly, *Pseudobarbus* 'Breede', *Pseudobarbus* 'Tradou' and *Pseudobarbus* 'giant' could have diverged through allopatric isolation due to possible vicariance of the Breede, Riviersonderend and Tradou Rivers, followed by post-speciation dispersal for *Pseudobarbus* 'Breede' and *Pseudobarbus* 'giant' to attain their present day distributions. Alternatively, because the Breede is a large river system with diverse habitats, it may also be possible that either *Pseudobarbus* 'giant' or *Pseudobarbus* 'Breede' or both could have evolved in sympatry to exploit empty niches (Kawata, 2002).

Biogeography and lineage diversification: the role of the last glacial maximum (LGM) sea-level regression

The Miocene-Pliocene transgression was followed by a major regression during the last glacial maximum (LGM) (Siesser & Dingle, 1981). Exposure of the Agulhas Bank during this period resulted in the confluence of several adjacent rivers before reaching the sea (Fig. 1.4). The palaeorivers of the LGM have been proposed as the plausible explanation for the common occurrence of *Pseudobarbus* lineages across currently isolated river systems in the CFR (Swartz, 2005; Swartz *et al.* 2007, 2009). The present study also shows some support for the role of palaeoriver systems in facilitating dispersal of freshwater fishes. The confluence of the Breede and Duiwenhoks River systems during the LGM (Swartz, 2005; Fig. 1.4) could have facilitated dispersal of *Pseudobarbus* 'Breede' and *Sandelia* 'Breede' which now occur in both river systems.

The lack of sharing of lineages (except *Galaxias* 'nebula') between the Breede and Heuningnes was surprising, because these river systems shared a common confluence as recently as the LGM (Swartz, 2005). PCA and MANOVA revealed that the Breede and Heuningnes belong to distinct ecological regions. Thus, ecological differences between these systems could have hampered dispersal of Breede lineages to colonise the Heuningnes River system and vice versa. This is assuming, of course, that the current ecological differences were present and maintained when the river systems shared a common confluence.

The role of alternative dispersal mechanisms

Galaxias ‘Heuningnes’ shows no differentiation between the Heuningnes and Ratel River systems despite these systems being currently isolated by both marine and terrestrial barriers. Drowning of the Ratel during the Miocene-Pliocene transgression is likely to have caused extinction of freshwater taxa in this river system. Consequently, populations of freshwater taxa in this system are likely to be recent immigrants from adjacent refugial populations. *Galaxias* ‘Heuningnes’ is likely to have survived and evolved in the Heuningnes River system and then dispersed to the Ratel River system. Since the Ratel and Heuningnes did not share a common confluence during the LGM, dispersal via intermittent freshwater connections is the most plausible explanation for the lack of differentiation between these two river systems. The lack of a discernible drainage divide between the Ratel and a western tributary of the Heuningnes could have allowed movement following episodic connections during periods of heavy flooding.

Similarly, *Galaxias* ‘Klein’ has closely related alleles across the Klein, Uilkraals and Haelkraal, despite the current hydrological isolation of these river systems. These river systems did not coalesce during the LGM (Fig. 1.4). Overland dispersal via intermittent freshwater connections during pluvial periods is therefore the most likely explanation for the current distribution of *Galaxias* ‘Klein’. Since the Haelkraal is likely to have been submerged during the Miocene-Pliocene transgression, *Galaxias* ‘Klein’ is likely to have survived in either the Klein or the Uilkraals. The restriction of the genetically distinct *Galaxias* ‘slender’ to the Uilkraals and the relatively smaller size of this river system suggests that *Galaxias*

‘Klein’ and *Galaxias* ‘slender’ could have evolved in allopatry, and their co-occurrence in the Uilkraals could be a result of secondary contact. It is therefore logical to suggest that *Galaxias* ‘Klein’ could have evolved in the Klein River system due to possible vicariant isolation by the middle Miocene to early Pliocene marine incursions. This would then be consistent with the *Sandelia* ‘Klein’ scenario discussed earlier.

Sandelia ‘Agulhas’ also occurs across several currently isolated river systems that did not coalesce during the LGM regression (Fig. 5.9). Low genetic differentiation between *Sandelia* ‘Agulhas’ across the Heuningnes, Haelkraal, Uilkraals and Klein suggests either recent disruption of gene flow or recent range expansion. Historical panmixia is unlikely, particularly given the historical oscillations between extremes of dry and wet conditions that were experienced in southern Africa (Zachos *et al*., 2001). A more plausible explanation is, therefore, that *Sandelia* ‘Agulhas’ could have evolved in isolation and only recently expanded its range across river systems draining the Agulhas Plain. Based on the existence of distinct lineages of *Sandelia* in the Klein and Uilkraals, and the fact that the Haelkraal was drowned during the Miocene-Pliocene transgression, it is more likely that *Sandelia* ‘Agulhas’ evolved in the Heuningnes River system. The low genetic differentiation in this lineage across these river systems may be evidence for more recent dispersal via low drainage divides during wet climatic periods. This mechanism was used to explain the wide geographic range of *Galaxias* ‘nebula’ across the CFR (see Chapter 4). Thus, the sympatric occurrence of two lineages of *Sandelia capensis* in the Uilkraals and the Klein could be a result of secondary contact due to post-speciation dispersal of *Sandelia* ‘Agulhas’.

The occurrence of *Pseudobarbus* 'Breede' and *Sandelia* 'Breede' in the Goukou River system is not consistent with the sea-level regression hypothesis because the Goukou belonged to a different palaeoriver system (Swartz, 2005; Fig. 1.4). This suggests the role of alternative dispersal mechanisms such as a rare river capture event or episodic connection possibly during inundation of low drainage divides during wetter climatic periods. The upper Duiwenhoks and the Korinte River (a tributary of the Goukou) are separated by a low drainage divide which could have allowed interdrainage movement during pluvial periods. Partridge *et al*., (1999) inferred wetter climatic conditions for coastal areas of the southern Cape as recently as the Holocene Altithermal (*ca*. 8 000 - 6 000 years ago). Connections of low drainage divides during wetter climatic periods have been proposed to have facilitated interdrainage movement of freshwater restricted taxa elsewhere (e.g. Thacker *et al*., 2007; Craw *et al*., 2008b).

Human-mediated translocations could have also influenced the distribution patterns of *Sandelia* 'Breede' and *Pseudobarbus* 'Breede'. A comprehensive study of the habitat associations of *Galaxias* 'nebula', *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' indicated that *Sandelia* 'eastern' is restricted to river reaches below 425 m altitude (see Chapter 3). *Sandelia* 'eastern' is also not capable of scaling major in-stream physical barriers (i.e. waterfalls) while *Pseudobarbus* 'Breede' always occurred at higher elevations than *Sandelia* (see Chapter 3). This pattern is reversed in the Goukou, with *Sandelia* 'Breede' occurring above major waterfalls that are effective barriers for both *Pseudobarbus* 'Breede' and *Galaxias* 'nebula'. This and the fact that individuals of *Sandelia* 'Breede' collected from populations above one of the major waterfalls in the Goukou contained Breede alleles suggests that *Sandelia* ‘Breede’ in the Goukou could have been introduced from the Breede River system. Since *Sandelia* ‘Breede’ and *Pseudobarbus* ‘Breede’ co-occur in large numbers within the Breede, it is possible that they could have both been introduced into the Goukou River system. This is subject for further investigation with additional markers such as microsatellites that will help to discriminate between natural and human aided movements.

The occurrence of *Galaxias* ‘Riviersonderend’ and *Sandelia* ‘Riviersonderend’ in the Palmiet River system was unexpected because the Riviersonderend and Palmiet are separated by a high mountain barrier. This suggests possible human-mediated translocation from the Riviersonderend. If this is the case, it is surprising that *Pseudobarbus* ‘Breede’ was not translocated as well, because individuals of this species attain larger sizes and are more conspicuous (due to the red/orange fins) than *Galaxias* ‘Riviersonderend’. *Pseudobarbus* ‘Breede’ seems to avoid small streams and river reaches that are overgrown with aquatic vegetation (see Chapter 3). Thus, the redfins could have failed to establish in the Palmiet after introduction because it is a much smaller habitat and the river channel is almost entirely covered with *Prionium serratum* vegetation.

Results of the present study are consistent with the palaeogeographic hypothesis invoked to explain phylogeographic patterns from other regions of the world (e.g. Bermingham & Martin, 1998; Near *et al*., 2003; De Bruyn & Mather, 2007). These studies provided evidence that sea-level fluctuations played a major role in driving diversification and influencing biogeographic patterns of extant freshwater taxa in the Neotropics (Bermingham & Martin, 1998), North America (Near *et al*., 2003; Near & Bernard, 2004) and the Indo-Australian

Archipelago (De Bruyn & Mather, 2007). Along similar lines, diversification of freshwater fishes within the south-western CFR appears to have been generated predominantly by passive vicariance of river systems during the Miocene-Pliocene transgression. Coalescence of adjacent rivers due to the subsequent sea-level regression during the LGM facilitated postspeciation range expansion (dispersal) of lineages to colonise currently isolated river systems. Dispersal could have also been facilitated by rare events such as intermittent wet connections during pluvial periods. Thus, the occurrence of deeply divergent lineages of the three genera in sympatry could be a result of secondary contact through dispersal from Miocene-Pliocene refugial populations.

This paleogeographic and dispersal hypothesis for freshwater fishes of the south-western CFR is summarised in Figs 5.12 – 5.14. Further testing (and refining) of this hypothesis with other freshwater restricted taxa within the south-western CFR, for example macroinvertebrate groups with low dispersal abilities, is required. Knowledge of ecological attributes is crucial as it can help to identify disparities in the distribution patterns, and may help to discriminate between patterns arising from natural processes and possible human translocations. Further studies using multiple taxa, ecological niche modeling, assessment of ecological speciation and evolution of functional traits will help to improve our understanding of the relative roles that geography, climate change and ecology played in the evolution, composition and distribution of extant taxa (reviewed by Hickerson *et al*., 2010; Chan *et al*., 2011).

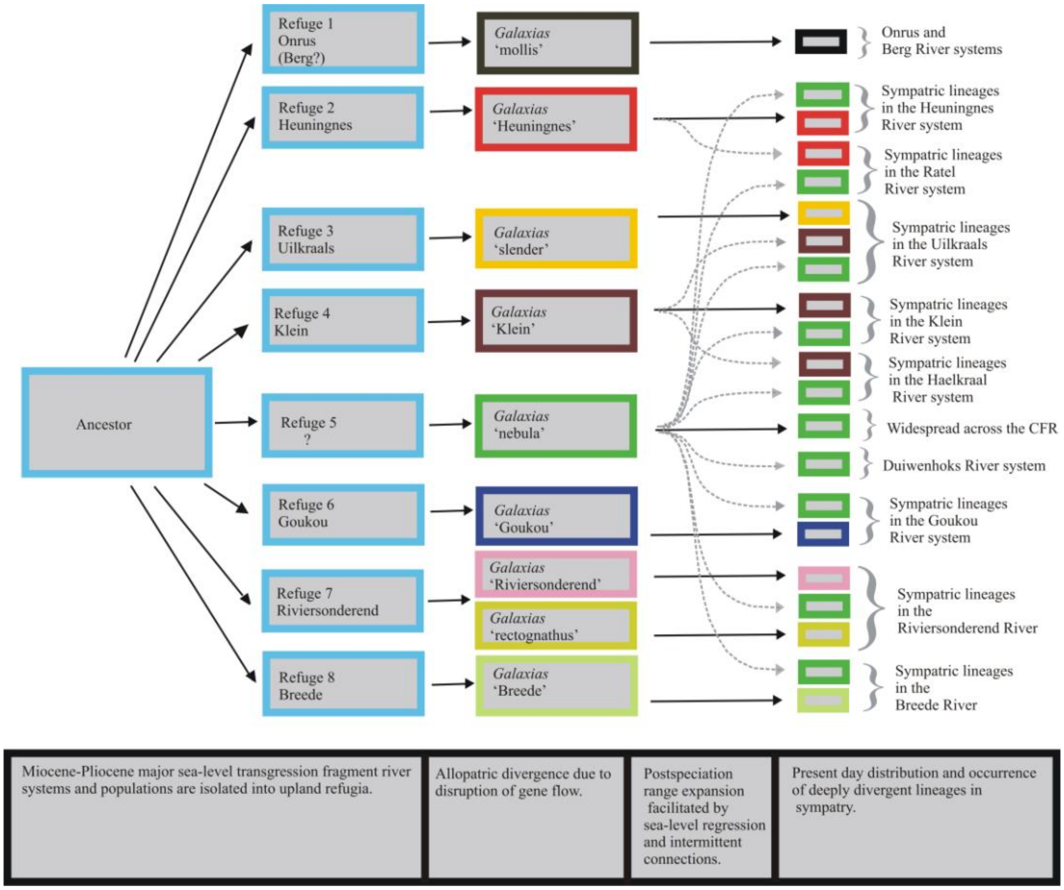


Fig. 5.12: Conceptual speciation and distribution hypothesis for *Galaxias* in the south-western CFR.

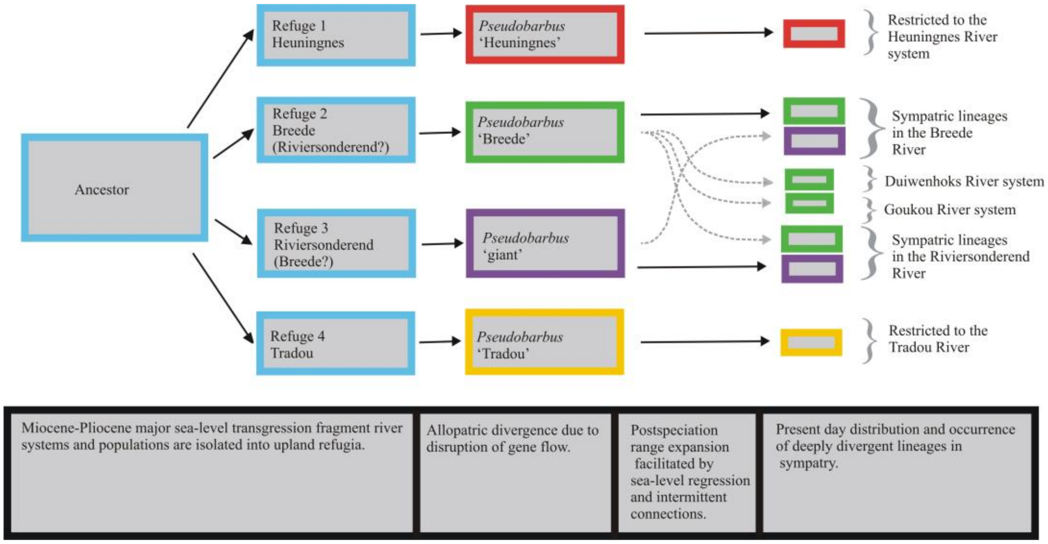


Fig. 5.13: Conceptual speciation and distribution hypothesis for *Pseudobarbus* in the south-western CFR.

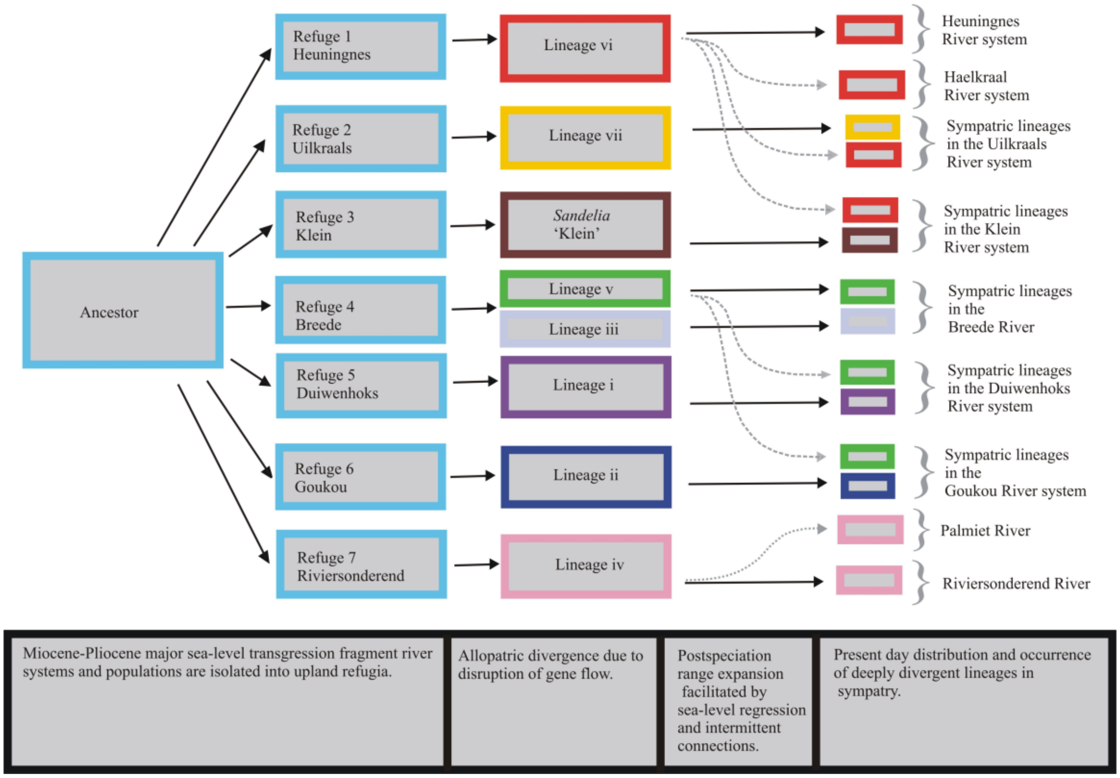


Fig. 5.14: Conceptual speciation and distribution hypothesis for *Sandelia* in the south-western CFR.

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Chapter 6

General conclusion and conservation recommendations

There are clear differences in habitat use among three widely-distributed lineages, *Galaxias* 'nebula', *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' (Chapter 2). *Sandelia* 'eastern' is a lower reach specialist with an upstream boundary at about 425 meters above sea-level, while both *Galaxias* 'nebula' and *Pseudobarbus* 'Breede' penetrate into higher elevations. These differences in habitat use appear to be linked to differences in the lineages' morphological traits that can affect performance of important tasks such as swimming (Videler and Wardle, 1991) and feeding (Nilsson and Broenmark, 2000). Future research should compare swimming performances of *Galaxias* 'nebula', *Pseudobarbus* 'Breede' and *Sandelia* 'eastern' to substantiate these conclusions. Other factors such as competition, predation, temporal variation in habitat use and availability of resources should also be considered in future research to get a better understanding of the factors that influence habitat selection in these fishes. Such factors have been found to influence distribution patterns of stream fishes elsewhere (Angermeier & Karr, 1983; Gasith & Resh, 1999). The present study was therefore exploratory, but nevertheless provides the first quantitative information on habitat preferences of three freshwater fishes in the south-western CFR.

The coexistence of some of the newly discovered lineages of *Galaxias*, *Pseudobarbus* and *Sandelia* (Chapter 5) creates a high potential for competition. It is predicted that competition prevents sympatric species from having completely overlapping niches (Stewart *et al*., 2002).

Resource partitioning is therefore fundamental for the co-existence of species, particularly if they are closely related. Resource partitioning in ecological communities occurs along three niche axes: spatial separation (e.g. use of different habitats), temporal avoidance and dietary differences (e.g. Ben-David *et al*., 1996; Stewart *et al*., 2002). Habitat preferences of most of the newly discovered lineages could not be assessed following the approach used in Chapter 3, because of their limited distribution in undisturbed areas. However, the use of stable isotope analysis (reviewed by Inger & Bearhop, 2008) may provide insights into the mechanisms of resource partitioning between sympatric lineages of *Galaxias*, *Pseudobarbus* and *Sandelia* that were discovered in the present study. The biology (e.g. reproduction and growth) of the newly discovered lineages also needs to be investigated in future research.

The present research documented, for the first time, a case of amphibious capabilities in an African galaxiid, *Galaxias* 'nebula' (Chapter 3). This finding has implications for the interpretation of this lineage's widespread distribution and phylogeographic pattern. Future research should examine the physiology and histology of *Galaxias* 'nebula' (e.g. blood composition, metabolic adjustments, mechanisms of ammonia excretion, structure of the skin and gills) to determine the specific adaptations and modifications that allow this fish to survive out of water for an extended period of time (e.g. Park, 2002; Ip *et al*., 2004; Ong *et al*., 2007; Richards, 2010). This research is also required for the other African galaxias lineages to determine if this is a common phenomenon or not.

There is evidence for the role of tectonics, sea-level fluctuations and intermittent freshwater connections in shaping the evolutionary history of the CFR's most widespread freshwater fish, *Galaxias* ‘nebula’ (Chapter 4). This lineage has intrinsic ecological attributes (emersion tolerance and slender body shape) (Chapters 2 & 3) that could have allowed it to exploit a range of dispersal mechanisms, hence its present-day wide distribution range across the CFR (Chapter 4).

The present study extensively sampled the river systems of the south-western CFR to identify all unique lineages of *Galaxias*, *Pseudobarbus* and *Sandelia* and map their distribution (Chapter 4; Chapters 5). Some of the lineages were already known (Roos, 2004, van Niekerk, 2004; Swartz, 2005), but these have been mapped much more accurately than before. The present study (Chapter 4; Chapter 5) revealed further diversity and confirms the suggestion by Linder *et al*. (2010) that the current taxonomy vastly underestimates the diversity of freshwater fishes of the CFR. The study area, representing only a portion of the wider CFR, has nine genetically distinct lineages of *Galaxias*, four lineages of *Pseudobarbus* and at least two lineages of *Sandelia* (Chapter 5). Most of these historical lineages are likely to be elevated to full species status following taxonomic revisions of these groups. Tests of reproductive isolation can be done for some of the lineages, particularly for those that occur in sympatry.

Although mtDNA is an informative marker that has been widely used for phylogeographic studies, it is constrained by its relatively low mutation rate compared to some nuclear markers (Sunnucks, 2000; Zhang & Hewitt, 2003). This limits its ability to disclose processes on a very recent time scale. Mitochondrial DNA also only reflects maternal gene flow, and strong incongruence between mitochondrial and nuclear gene trees has been reported for several taxa (e.g. Sota & Vogler, 2001; Shaw, 2002; Spinks & Shaffer, 2009). To draw firm phylogeographic conclusions, the use of multiple genes has been emphasised (Chan & Levin, 2005). It is also important that more rapidly evolving markers such as microsatellites be used to study the range-wide gene flow patterns of *Galaxias* 'nebula'. Because of their extremely high levels of polymorphism, microsatellites are commonly used to study genetic differentiation among closely-related populations (Selkoe & Toonen, 2006). *Sandelia capensis* showed unexpected low levels of divergences among populations from distant geographic areas (Chapter 5). The use of microsatellite markers in future research will help to further identify genetic diversity within this species. Possible cases of human-mediated translocations have also been identified (Chapter 5). Future investigations using additional markers will help to discriminate between natural and human aided movements (e.g. Bryja *et al.*, 2010).

Genetic data has revealed that several of the lineages that currently have fragmented distributions (*Galaxias* 'nebula', *Galaxias* 'Breede', *Galaxias* 'Riviersonderend', *Galaxias* 'Heuningnes', *Galaxias* 'Klein', *Pseudobarbus* 'Breede', *Pseudobarbus* 'giant', *Pseudobarbus* 'Heuningnes', *Sandelia* 'eastern', *Sandelia* 'Agulhas' and *Sandelia* 'Klein') (Chapter 5) may have had continuous ranges connected through the main-stem habitats. This is supported by habitat use data for *Galaxias* 'nebula', *Pseudobarbus* 'Breede' and *Sandelia* 'eastern'. The data presented here therefore provide a more robust and objective approach to reconstruct historical distribution ranges of native fishes in the study area prior to invasion of the rivers by alien fishes. The current disjunct distributions of these lineages can be attributed to the impact of invasive alien predators and competitors that now dominate in the main-stems (personal observations). Alien fishes have also caused significant impacts on native fishes in Australia and New Zealand (reviewed by Crowl *et al*., 1992).

Whilst invasion by alien predators and competitors is the most serious threat, habitat degradation through land use changes, the construction of weirs and complete water abstraction in the mountain tributaries are contributing to the decline of indigenous fish populations. The impacts of predation are highest in the downstream reaches that are more susceptible to invasion by aliens from the main-stems. In addition, because low gradient valleys in the middle and downstream reaches are most suitable for the development of intensive farming activities, in-stream habitats in the lower reaches of all streams in the study area have been severely degraded. In most instances, all the water from the tributaries is diverted for irrigation and domestic purposes. Most reaches become dry downstream of water take-off points during the dry season. With the changes in global climatic conditions projected to cause drier conditions in southern Africa (IPCC, 2007), more streams in the CFR are likely to be dammed and weirs may be moved further upstream. This will have severe impacts on the south-western CFR’s fish fauna because many of the lineages are confined to lower tributary reaches. The only exceptions are *Galaxias* ‘nebula’ and *Pseudobarbus* ‘Breede’ that penetrate into higher elevations and steeper gradients. Most of the lineages also have very restricted geographic ranges and probably small population sizes. Many of them are only known from a few localities, while others are restricted to a single locality or tributary (Chapter 5). Given the comprehensive sampling approach in the present study, these probably closely represent their remaining natural ranges.

Small fragmented populations have been shown to have a higher probability of extinction due to inbreeding which can result in reduced reproduction and survival (reviewed by Frankham, 2005). This risk may be highest for *Pseudobarbus* ‘giant’, *Galaxias* ‘Breede’, *Galaxias* ‘Goukou’, *Galaxias* ‘rectognathus’, *Galaxias* ‘slender’, *Galaxias* ‘Klein’ and *Sandelia* ‘Klein’, because they have low population densities. Conservation measures in the CFR should first address more immediate threats that affect the distribution range of these lineages, such as invasion by aliens, habitat degradation and excessive abstraction of water. Once the populations are secured, the risk of inbreeding can be assessed, and critical populations can be enlarged to ensure long-term survival.

Pseudobarbus ‘giant’ is only known from two localities, one in the upper Riviersonderend and one in the Krom River (Chapter 5). The upper Riviersonderend population persists above Theewaterskloof Dam, which has large populations of *Micropterus* spp. and *Clarias gariepinus*. The upper Riviersonderend currently has the highest number of individuals for this lineage. The future survival of this population is, however, uncertain because there is no barrier to prevent upstream migration of aliens. Snorkel counts indicate that the Krom population is represented by less than 120 adult individuals in a stream reach of less than 50 m. Here, the major impact is the rainbow trout that has invaded beyond popular fly-fishing streams lower down in the catchment. Eradication of aliens and building of weirs to prevent re-invasion are the immediate measures required to prevent extinction of *Pseudobarbus* ‘giant’.

Pseudobarbus 'Tradou' only occurs in the Tradou catchment. This lineage is currently listed as Critically Endangered in the International Union for the Conservation of Nature (IUCN) (Tweddle *et al*., 2009) Red Data List. The major threats to this lineage include excessive water abstraction and alien fish invasions (Swartz & Tweddle, 2011). The Municipality of Swellendam has built a canal just above the upper limit of the fish distribution. This canal diverts all the water from the river, and as a result the downstream sections dry out completely during the dry season, apart from occasional return-flow in places. Deep pools in the Tradou gorge probably act as the source from where the fish repopulate upstream during the wet season. Snorkel surveys during the present study indicated that this lineage's range has contracted and shifted (compared to Swartz's 2002 and 2007 surveys) while the ranges of *Micropterus salmoides*, *Tilapia sparrmani* and *Lepomis microchirus* have extended. These aliens probably originate from farm dams in the catchment area. There is also point and diffuse pollution from the town of Barrydale upstream of the Tradou gorge. It was observed during the present study that several *Pseudobarbus* 'Tradou' individuals (mainly juveniles and sub-adults) from localities below the town were heavily parasitised. This could be an indication of poor water quality (Poulin, 1992; Marcogliese, 2005). Heavy parasite prevalence can significantly reduce recruitment due to reduced fitness and increased susceptibility to diseases and predation (Poulin, 1992; Lafferty & Morris, 1996). Urgent conservation of this lineage requires restoration of natural flows as well as physical removal of alien fishes from the river and upper catchment dams. Given the multiple impacts facing the source populations in the Tradou gorge, it is recommended that the upstream section of the river above the town of Barrydale be rehabilitated and used as a sanctuary for this lineage.

Galaxias ‘Riviersonderend’ persists in a few tributaries of the Riviersonderend and one tributary of the lower Breede (Chapter 5). Like *Sandelia* ‘eastern’ this lineage is also restricted to lower reaches below 425 m above sea-level. This lineage can be protected by building barriers that prevent upstream migration of aliens from main-stem areas. The location of such barriers should be carefully chosen to ensure that lower gradient river reaches are included. Since water abstraction is another major threat, there is also need for many water take-off points that are located in the upper river reaches to be moved lower down. Lower reaches of all the tributaries in the study area are heavily degraded by bulldozing and stream bank cultivation. There is a need to restore and secure these habitats to ensure the recovery of lower reach specialists. These measures will also protect *Galaxias* ‘rectognathus’ which co-occurs with both *Galaxias* ‘Riviersonderend’ and *Sandelia* ‘eastern’ in the Du Toit and Amandel rivers (Chapter 5).

Galaxias ‘Breede’ was collected from only three localities in the Hex River, Bothaspruit River and the main-stem Breede River (Chapter 5). The Hex River population seems to be the most viable, because it is the only locality where the lineage was found in large numbers. Only four individuals were caught in the Breede main-stem and one individual was caught in Bothaspruit. The Hex River is, however, heavily bulldozed and has been invaded by both *Oncorhynchus mykiss* and *Clarias gariepinus*. Translocation or captive breeding may have to be considered to prevent extinction of this lineage.

The situation is particularly dire for the coastal river systems across the Agulhas Plain where the alien predators, particularly *Micropterus* spp., have become widespread in the absence of physical in-stream barriers. All the lineages from the Agulhas Plain have highly fragmented distributions (Chapter 5), and in most instances only a few individuals were collected in marginal habitats such as vegetated pockets on stream edges. The most immediate conservation measures required to prevent the extinction of *Galaxias* 'Heuningnes', *Galaxias* 'slender', *Galaxias* 'Klein', *Galaxias* 'mollis', *Pseudobarbus* 'Heuningnes', *Sandelia* 'Agulhas', *Sandelia* 'Klein' and *Sandelia* 'Uilkraals' is the eradication of alien fish predators and the creation of sanctuaries.

Only *Galaxias* 'nebula' and *Pseudobarbus* 'Breede' are not immediately threatened with extinction, because they penetrate streams to higher altitudes where natural barriers often prevent invasion by alien predators. These two lineages have also been less impacted by water abstraction, because most of the weirs are built below their upper limits. In such cases, the weirs can be positive if they secure the habitats of the remaining indigenous fish population from invasion by alien fishes.

The current location of freshwater protected areas in the CFR needs to be reviewed, because distribution ranges of many of the newly discovered and highly threatened lineages fall outside these. A similar approach to the one adopted in the present study needs to be applied to other drainage systems across the CFR to identify the lineages that are most threatened with extinction. It is also clear from the present study that accurate inventory of lineage diversity is a fundamental pre-requisite for ecological and biological studies as well as management and conservation of the CFR’s stream fishes.

The proposed model of the evolution of drainage patterns in the south-western CFR and proposed connections between them (Chapter 5), needs to be tested and refined using other aquatic taxa such as invertebrates with limited mobility. Genetic diversity in other aquatic taxa that have only been diagnosed with phenotypic (morphological) characters needs to be investigated to better understand aquatic biodiversity in the CFR. Future comparative studies with more taxa will provide a more robust assessment of the processes that influenced the evolutionary history of the CFR’s stream biota.

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