EXAMINING CATCHMENTS AS FUNCTIONAL UNITS FOR THE CONSERVATION OF RIVERINE BIOTA AND MAINTENANCE OF BIODIVERSITY

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Water Research Commission

Examining catchments as functional units for the conservation of riverine biota and maintenance of biodiversity

Catchments as the unit for conservation and management, with specific consideration of the implications for the development of Inter-Basin Water Transfers and Water Resources Management

Report prepared for the Water Research Commission

by

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EXECUTIVE SUMMARY

Motivation and Research Objectives

This project has arisen from a number of developments in the conservation and management of water resources. These include the shift toward catchment management philosophies and practices, articulated and reflected in the development of the new South African Water Act of 1998, and the findings and concerns raised by WRC project K665/1/00 reported in Snaddon and Davies (1999). In the first instance, the new Water Act of 1998 gives full powers to the Minister of Water Affairs and Forestry to transfer water from river basins with perceived surpluses to basins and human populations with perceived water deficits. Such transfers can only be achieved by means of a wide variety of inter-basin water transfers (IBTs), a rapidly expanding technology in South Africa. Concerns about the impacts of IBTs on river basin integrity, in terms of faunal and floral biodiversity, were first raised by Petitjean & Davies in 1988. The concerns were subsequently pursued by Davies *et al.* (1992) and finally, through the research of Snaddon & Davies (1998, 1999) and Snaddon *et al.* (1998, 1999), the problems surrounding the genetic integrity of historically isolated river basins and populations and the maintenance of biological processes were first quantified. It was clearly demonstrated that a wide variety of viable organisms could unwittingly be transferred across natural catchment divides via IBTs. In order to address the findings of these researchers and the implications of IBTs for catchment management, and the ultimate conservation of riverine biodiversity, research was initiated to assess the genetic integrity of catchments as management units. The primary object of the research was to quantify the degree of congruence between catchments as management units and natural populations of a variety of riverine organisms.

Following recommendations arising from the first steering committee advising the project reported here, the original aims and objectives were modified in order to broaden the scope of the research and the relevance of the results for management action. The modified objectives of the programme are:

Primary Aims

- To examine the integrity of catchment units within the framework of Evolutionarily Significant Units and to assess the implications of inter-basin water transfers for the conservation of river ecosystem functioning and riverine biodiversity using selected taxa;
- To contribute to the development of a management protocol for use in the assessment of the ecological effects of IBTs on both donor and recipient river basins.

Specific Objectives

- Using various techniques, to assess the levels of genetic differentiation between river basins and to examine the integrity of river basins;
- To identify species through genetic and ecological studies to be used in the development of a management protocol in the assessment of future water transfer projects
- To study the ecology and dispersal capabilities of selected invertebrate taxa within the context of their ability to recover following disturbance or local extinction
- To facilitate technical exchange relating to the ecological functioning of riverine systems between Australia and South Africa. To this end collaboration between the Water Research Commission, the Freshwater Research Unit at the University of Cape Town, the Department of Zoology at the University of Stellenbosch and the Centre for Catchment and In-Stream Research at Griffith University, Queensland, Australia has been fostered.

Achievement of Objectives

The results allowed all of the objectives to be suitably addressed, whilst also providing a large amount of additional information on the distribution of genetic and organismal biodiversity in the aquatic fauna of the south-western Cape region.

Considering the management implications and the development of a protocol for assessing the effects of IBTs (Chapter 8) the results highlight the suitability of two species for use as target species in future assessments of the degree of genetic separation of historically isolated catchments - the net-winged midge, *Elporia barnardi* (Chapter 5) and the Cape galaxiid, *Galaxias zebratus* (Chapter 4).

For some organisms, catchments represent the primary unit within which movement and dispersal is confined (Chapters 4 and 5). For other more mobile organisms, populations effectively cover a wide geographic area that encompasses a number of different catchment units (Chapter 7).

With regard to the last of the specific objectives, collaboration and work was undertaken at each of the institutions. In addition, the project was officially listed as an associated project (B803) within the Restoration Ecology Programme of the Co-operative Research Centre for Freshwater Ecology (CRC FE) in Australia.

Capacity Building and Collaboration

The Water Research Commission has recognised the need to incorporate the development of South Africa's capacity as a part of its research projects. Whilst not part of the initially aims or objectives of this project, we feel that we have undertaken a number of activities that have contributed to the development of personnel and information that will enable South Africa to develop and enable future conservation and management strategies within a sustainable framework. As such we have been involved in:

- teaching at undergraduate level;
- contributed to international and national conferences;
- fostered collaboration, internationally through association with Australia's Cooperative Research Centre for Freshwater Ecology, and locally through a number of institutions;
- published extensively in peer reviewed journals and books, both locally and internationally; and

- involved many students, a number from formally disadvantage backgrounds through the Bishop Desmond Tutu Trust, in the field work associated with this programme.

Results of Genetic Analyses

Our hypothesis was that catchments as biogeographically distinct units would isolate riverine populations, depending upon the dispersal characteristics of each species within a particular community of riverine organisms. Those species with poor powers of dispersal would thus tend to be genetically different from related populations in other catchments, whilst those species with robust dispersal characteristics (strongly flying insects, for instance) would show little genetic differentiation in separate catchments. Quantification of such divergences or similarities would then allow us to establish:

- the identification of organisms suitable as assessment markers for future IBT developments;
- the degree of movement between catchments by species with different dispersal characteristics;
- the degree of genetic mixing which may occur as the result of IBT developments;
- the degree to which water managers and conservationists should be concerned about genetic mixing that may have already occurred in presently operational IBTs; and
- the implications of the development of future IBTs for the conservation of riverine biotas within the context of South Africa's national and international obligations to conserve biological diversity.

The four organisms selected reflected a wide range of dispersal characteristics. They are:

- the Cape galaxiid (*Galaxias zebratus;* Teleostei: Galaxiidae), a small non-migratory fish confined to fresh waters;

- the net-winged midge, *Elporia barnardi* (Diptera: Blephariceridae), an insect whose larvae are strictly confined to mountain cascades and riffles and, hence, have a very limited potential for dispersal;
- the stonefly, *Aphanicerca capensis* (Plecoptera: Notonemouridae), an insect species representing riverine organisms with intermediate dispersal characteristics, whose nymphs are limited to cool mountain streams; and
- the widely distributed dragonfly, *Aeshna subpupillata* (Odonata, Aeshnidae: the South African stream hawker), whose nymphs, and aerial adult phases are robust, with a potential for wide dispersal.

Samples of these organisms were taken from two discontinuous regions - three streams on the Table Mountain massif, and a number of streams in the Jonkershoek catchment of the Hottentots Holland mountain range north of Stellenbosch - based on the assumption that the movement or organisms between catchments will be reflected in the genetic structure of their populations. In order to ascertain degrees of genetic similarity or divergence, some 30 to 50 organisms were genetically analysed using allozyme electrophoresis and direct sequencing of a targeted section of mitochondrial DNA (mtDNA).

The results of the research revealed the following:

- little genetic variability was observed within any of the populations, giving insights into the demographic history of the organisms over geological time as opposed to the more recent geological-speaking "instantaneous" water resources developments;
- movement of individuals as reflected by the degree of genetic population structure mirrored the dispersal characteristics of each species; populations of robust dispersers displayed little genetic differentiation, whilst poor dispersers displayed relatively high degrees of population structure in terms of their genetics;
- the Cape galaxiid and the net-winged midges show no movement of individuals between catchment units, with serious implications for IBTs and their future design, operation and management; and

- the robust disperser, the South African stream hawker, covers a number of catchment units. This also has serious implications for both the use of catchments as management units for the conservation of some riverine organisms, and has serious implications for large-scale, long distance water transfers.

Over and above these findings we discovered a high degree of diversity and genetic divergence within species - the Cape galaxiid, the net-winged midges and the stoneflies - all of which had been thought to be single species. This begs further research and illustrates the fact that the Western Cape is not simply a botanical biodiversity "hotspot", but that in-stream faunas are also rich in endemics. The long period of geological and climatic stability and the isolation of individual catchments has resulted in a unique assemblage of aquatic organisms, many of which remain undescribed and/or undetected. This has obvious implications for management as individual catchments seem to be exhibiting unique signatures in terms of their species complements and genetic structure. In addition:

- five genetically distinct groups were identified in the Cape galaxiid, suggesting there to be a number of as yet unrecognised species;
- the levels of divergence separating these groups are the highest ever recorded for any freshwater fish species;
- similar genetically distinct groups were found in the net-winged midge and the stonefly, alluding to the presence of unique endemic forms associated with the Table Mountain massif and adding to the increasing wealth of knowledge identifying this as one of the world's most important biological regions;
- the synthesis of this and other information is driving a recognition of the Cape fynbos region not only as a region of botanical importance, but as a region with a unique and highly endemic freshwater fauna.

Recommendations for IBT Planning and Management

In light of the results, and South Africa's national and international obligations, the following recommendations are made with reference to the design of a conservation strategy for riverine biodiversity and specific consideration of the implications of

inter-basin water transfers. Further to this, the report serves as a statement of consequence, providing an iteration of the facts and the implications of certain actions, or non-actions, on future conservation strategies. The onus of responsibility for incorporating such considerations into practical management decisions lies with the decision makers and will invariably need to incorporate compromises between conservation considerations and the practical constraints of meeting supply requirements. As such we recommend that:

- all future developments which involve the planning and construction of IBTs should take cognisance of the potential for genetic mixing of riverine biotas in receiving systems;
- the genetic structure of target organisms *such as those identified in this report* be included in all impact assessments associated with IBTs bearing in mind South Africa's commitments to maintain organismal diversity in a sustainable manner as a signatory to the international Convention on Biological Diversity;
- information generated by the research reported here should be incorporated into the design and development of future protected areas for the conservation of riverine organisms as opposed to catchment-based management for water supply; and
- the Water Research Commission should motivate the Department of Environmental Affairs and Tourism to develop, with internationally available funding through organisations such as the Global Environment Fund (GEF), a nationally co-ordinated project to undertake revisions of the country's freshwater biodiversity.

Statement of Consequence

The information contained within this report suggests that the development of water resources could have very serious effects on the future conservation of aquatic biodiversity. Obviously such concerns need to be carefully weighed against national needs for water. The final decisions and onus of responsibility clearly lie with water resources planners and decision makers; specifically the ministers and departments of Water Affairs and Forestry, Environmental Affairs and Tourism and Mines and

Energy Affairs. Therefore, in addition to the specific recommendation, the information contained in this report serves as a statement of consequence.

For some species, the development of IBTs will provide a conduit for the transfer of individuals between geologically separated catchment units and historically isolated populations. The transfer of individuals from such historically isolated populations has the potential to undermine the evolutionary processes important in species formation and thus the generation of biodiversity by providing an avenue for gene flow between genetically discrete populations. As a signatory to a number of international treaties addressing the conservation and management of biodiversity, there is a framework and obligation to take necessary mitigatory measures to ensure the protection of such components of biodiversity for the long-term sustainable utilisation and protection of the countrys' natural heritage.

For other, more mobile species, the distribution of genetic variation and pattern of population sub-division indicates that the effective population covers a wide geographic range. As such IBTs between adjacent catchments would not have an impact on these species. However, it should be noted that there are a number of existing IBTs that transfer water over huge distances from catchments far removed from each other. While beyond the scope of this project, such IBTs may have implications for conservation with the transfer of individuals across different biogeographical zones. Such lack of genetic population structure does have other conservation and management implications. An interbreeding, homogeneous population structure across a wide geographic area would suggest that catchment units may not represent the appropriate scale for conservation of the aquatic fauna.

From the distribution and patterns of genetic variation reflected among these organisms, efforts at conserving the fauna of riverine ecosystems should move beyond individual catchment considerations to incorporate the design of protected areas and management strategies that cover and incorporate a number of adjacent catchments. Such areas or management plans should be replicated and spread through identified biogeographic regions.

The idea of the *Precautionary Principle* is being afforded increasing recognition, having already been incorporated into many international conventions and national legislations. It would therefor seem prudent to incorporate such considerations into the development of conservation strategies. From the distribution and patterns of genetic variation reflected among the taxa examined, efforts at conserving the fauna of riverine ecosystems should move beyond individual catchment considerations to incorporate protected areas and management strategies that cover and incorporate a number of adjacent catchments. Such areas or management plans should be replicated and distributed across identified biogeographic regions. Further consideration should be afforded to the influence of water resources developments on the genetic population structure of aquatic organisms and on the long-term sustainable conservation of aquatic ecosystems and processes therein. As a signatory to a number of international treaties and conventions addressing the conservation and management of biodiversity, South Africa has an obligation to take necessary mitagatory measure to ensure the protection of such components of biodiversity for the long-term sustainable utilisation and protection of the countries natural heritage.

Further Research Needs

Despite careful selection of taxonomically well resolved species, the results of this work have revealed a number of unresolved taxonomic considerations, highlighting the need for more detailed systematic revisions and genetic surveys. Such surveys should be undertaken before further genetic mixing can take place via the transfer of individuals through the new IBTs.

In order to properly assess the implications of future water resources developments such genetic surveys should be extended over a very broad geographic range using carefully selected organisms. Such a wide-scale genetic survey using obligate freshwater species, such as the net-winged midges or freshwater fish, could provide the basis for identifying phylogeographic regions within which to manage future water resource planning. Such information will also allow for taxonomic assessments as well the elucidation and definition of important historical, landscape and evolutionary processes that ultimately underlie the faunal and ecological properties of a system or a bioregion.

Given the climatic characteristics of South Africa, along with the demographic and socio-economic constraints, there is a growing need to provide water. Such provision will continue to involve its redistribution through the development of IBTs. Accordingly, emphasis should be placed on developing techniques and protocols to prevent the transfer of living organisms or their seeds or eggs.

Given the age and antiquity of the south-western Cape region, where the research presented here was undertaken, there is a clear need to expand this type of research to other distinct biogeographical regions. For instance, we regard it as an imperative that similar research be undertaken on Mpumalanga tropical low-veld rivers, high-veld rivers and systems within ranges such as the Drakensberg in KwaZulu/Natal. This in order to establish whether or not there are similar patterns of genetic distinction elsewhere in the country and, hence, the implications for future IBTs in those regions.

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The Steering Committee responsible for this project, consisted of the following persons:

Dr Steve Mitchell	Water Research Commission (Chair)
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Mr DS van der Merwe	Water Research Commission
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Chapter 1

Introduction

General Background

Conservation has progressed from its initial beginnings as a largely emotive issue to a progressive quantitative branch of science that draws on many disciplines. Furthermore, the accumulation and synthesis of knowledge derived from this progression in thought has been formalised into most national legislations. In turn, the over-riding principles enshrined in these national efforts have been governed by the principles outlined and articulated in international agreements such as the *Convention on Biodiversity*. However, there are still many unresolved and pressing issues of fundamental importance within conservation. These are set primarily around the question of what is it that should actually be preserved/conserved or protected and/or managed. Indeed, even beyond the question of what we should be conserving are the questions themselves that remain unresolved; viz - what are our roles and responsibilities in the protection of species, and ecosystems and/or the processes that sustain life?

While habitat destruction is the single largest factor responsible for the erosion of biodiversity, the initial conservation imperative was focused toward the protection of individual species perceived to be under immediate threat. It was soon realised, however, that management schemes directed at individual species are doomed to failure without proper consideration of habitat health (Lovejoy, 1996). Fortunately, conservation has long since moved beyond simple habitat conservation. There has been an increased recognition that the situation is far more complex and requires different approaches leading to ecosystem-based health approaches, while more recently, evolutionary considerations have also emerged. This is reflected in the objectives outlined by the International Union for the Conservation of Nature (IUCN) in its definition of "conservation". The definition includes the following essentials:

· the maintenance of essential ecological processes and planetary life support systems;
· the ensured sustainable utilization of species and ecosystems; and
· the preservation of genetic diversity.

The development of these different schools of thought - from the conservation of individual taxa, through the conservation of ecosystems or of evolutionary potential/variability - reflects the differences that exist between the variety of biological disciples (Bowen, 1999). For example, systematists argue that conservation efforts should be directed toward distinct taxa because these contribute to overall biodiversity on a scale proportional to their morphological or genetic distinctiveness. On the other hand, ecologists maintain that landscapes and ecosystems should be the primary medium for conservation efforts because they provide the essential life-support systems for endangered, as well as for, non-endangered taxa. Recently, however, a relatively new school of thought based on evolutionary theory has argued that conservation efforts should focus on the preservation of the genetic diversity that allows biota to adapt to new conditions (see Frankel,1974; Lande & Shannon, 1996; Lynch, 1996; Crandall *et al*., 2000). This approach can be extended to speciose groups that may be the source of future biodiversity (Erwin, 1991). When viewed in a temporal framework the systematic focus on bioheritage (the past), the ecological focus on ecosystem integrity (the present), and the evolutionary focus on novel adaptations (the future) reveal the temporal nature of conservation biology (Bowen, 1999).

Conservation of Lotic Ecosystems

Lotic ecosystems provide some interesting challenges to conservation planning and protection. The longitudinal and hierarchical nature of the river system, together with the geological structure of the catchments that they drain, creates discrete and functionally independent units that are relatively easily defined. While this overcomes many of the problems associated with ecosystem-based approaches to conservation, it does, however, present a suite of other challenges. For instance, the longitudinal nature of rivers creates a level of connectivity such that disturbances and perturbations are transferred both up and down stream (e.g. Pringle, 1997; Baer & Pringle, 2000). Complete protection and the implementation of a "preservation" philosophy to conservation is also difficult given the inevitable demands of human populations, the size of many catchments, coupled to the intrinsic linkages between rivers and their catchments. For example, while perhaps possible on smaller spatial scales, the preservation approach to the conservation of large international rivers, such as

southern Africa's Zambezi River, would require detailed and coordinated efforts between eight different riparian countries, covering an area of 1,570,000 km². Other constraints to lotic ecosystem conservation include a general lack of taxonomic information coupled with continuing taxonomic confusion surrounding many aquatic taxa. This is confounded by the historical development of conservation efforts which have arisen, not from a recognition of the need to conserve rivers as functioning ecosystems, but more from the perspective of their maintenance as systems for safe, potable water supply, for human consumption, and for transport and other human uses.

Early conservation initiatives directed at lotic systems were driven primarily by the need to provide safe, potable water supplies for both domestic and agricultural purposes, and the efforts of recreational interests. Although there have been some species-specific approaches (river dolphins, beavers, otter), efforts at conserving lotic systems have largely centred on maintaining the physical and structural properties of the system and the processes involved therein (Boon *et al.*, 1992; O'Keeffe & Uys 2000; Clifford, 2001). The implicit underlying assumption has typically been one of surrogacy. Biotic processes and patterns are assumed to be controlled by the physical and structural properties of the stream habitat and will therefore be protected by maintaining the physical and structural attributes that provide the habitat template (*sensu* Southwood 1977) for the biota. These habitats are seen as the result of predictable physical processes, and thus fall between the forces that structure rivers and the biota that inhabit them (Vannote *et al.*, 1980; Frissel *et al.*, 1986; Wevers & Warren, 1986). Habitats also have considerable management value as they reflect activities within the catchment and are easily identifiable, whereas biological processes may not.

There are, however, a number of important considerations required for the maintenance of biological processes and protection of biological diversity that go beyond the physical template. Demographic processes such as dispersal and recolonisation, for example, along with the evolutionary forces of gene flow, genetic drift and natural selection are important not only in structuring and maintaining viable populations but also in the process of species formation. Acknowledging that such processes operate within historical constraints, it is important to consider the spatial

scale at which these processes take place. Such considerations depend on the pool of potential species available for colonisation, along with the life history attributes and population dynamics of individual species. For lotic organisms it is particularly important to consider these with respect to catchment units, which are increasingly being recognised, and defined, as the primary operational unit for the conservation of lotic systems and management of water resources.

It is generally considered that lotic organisms are highly mobile with strong powers of dispersal. This is reflected in the resilience of lotic communities and the ability of stream organisms to recover subsequent to disturbance (Niemi *et al.*, 1990; Wallace, 1990). Further evidence of this potential is cited as the widespread geographic distribution of many species across catchment boundaries. However, the geological structure of the catchment unit and longitudinal and hierarchical nature of the rivers that drain them impose a number of potential barriers to the dispersal of lotic organisms. As such, these isolated units represent islands of habitat for the aquatic fauna within a terrestrial landscape and provide an interesting template for examining the interaction between dispersal and time in population processes and species formation. This has important implications for the development of conservation and management strategies relating to river systems, particularly those relating to the development of inter-basin water transfers and the design of protected areas aimed at preserving populations of lotic organisms.

Genetic Population Structure in Lotic Organisms

While a number of factors can influence the genetic structure of a population it is generally accepted that the magnitude and spatial distribution of genetic differences between populations is, at least in part, a reflection of an organism's dispersal activity through time and space (Slatkin, 1985; Bohonak, 1999). Thus the extent to which catchment units influence the genetic population structure or evolutionary processes will depend upon a number of factors, including the effects of natural selection, genetic drift and mutation along with the movement of individuals among catchment units (i.e. gene flow) (Allendorf & Phelps, 1981). Under the assumption of selective neutrality, examining patterns of genetic differentiation allows inferences to be made as to the extent of gene flow, and thus movement, between geographically disjunct

groups of individuals (Slatkin, 1987). In the absence of any movement, genetic drift and mutation act such that there will be measurable differences in the genetic structure of these units. Alternatively, in the absence of any barriers to movement, the mixing of individuals results in homogeneous genetic structure.

Studies of a number of vertebrate and invertebrate riverine organisms have revealed various levels of genetic differentiation between populations across different scales (Invertebrates: Zera, 1981; McArthur *et al.*, 1992; Robinson *et al.*, 1992; Their, 1994; Hughes *et al.*, 1995; Stewart, 1997; Thomas *et al.*, 1997; Fish: Kraiem, 1993; Machordom & Doadrio, 1993; Berrebi *et al.*, 1995; Waters & Cambray, 1997; Amphibia: Driscoll 1998). The magnitude and the spatial scale at which this differentiation is defined varies, reflecting the palaeo-history of the species and the landscapes within which they are found along with individual species attributes. Genetic structuring among populations of a number of aquatic insects suggests the potential for wide dispersal at both adult and larval stages (diving beetles: Bilton, 1992; waterstriders: Zera, 1981; Preziosi & Fairbairn, 1992; black flies: Snyder & Linton, 1984; mayflies: Sweeney *et al.*, 1986, 1987; Schmidt *et al.*, 1995; caddisflies: Jackson & Resh, 1992; Hughes *et al.*, 1998). For example the caddisfly, *Tasiagma ciliata*, shows low levels of genetic differentiation across large spatial scales, but significant differentiation at smaller spatial scales within reaches in a stream (Hughes *et al.*, 1998). These results suggest that while larval movement is limited within a catchment, adult flight represents an important mechanism for dispersal among catchments. In contrast, the population structure of some other aquatic taxa suggests limited powers of dispersal that are consistent with attributes of those species (amphipods: Thomas *et al.*, 1997; atyid shrimps: Hughes *et al.*, 1996; zooplankton: Boileau, 1991; Boileau & Hebert, 1991; Their, 1994). For example, high levels of differentiation between populations of the atyid shrimps, *Caridina zebra* and *Paratya australiensis*, reflect the fully aquatic habit of these species (Hughes *et al.*, 1995, 1996). Most of these studies have examined patterns of variation in nuclear DNA, using electrophoretic methods, with few studies having looked at patterns of variation in the mitochondrial DNA. Furthermore, none have explicitly examined the effects of catchment units on the genetic structure of aquatic organisms with different dispersal traits.

Models of proposed gene flow began with Wright's (1931) island model, in which there is assumed to be an equal probability of individuals being exchanged between a finite number of sub-populations. However, given the natural spatial and temporal distribution of sub-populations, individuals are typically restricted in their movements between adjacent sub-populations. Recognising this, the stepping-stone model of isolation by distance (Kimura, 1953; Kimura & Weiss, 1964; Slatkin, 1993) attempts to incorporate some of this complexity and proposes that measures of genetic differentiation at neutral loci will increase with geographic distance. Assuming a stepping-stone model, Slatkin (1993) showed that the equilibrium status of populations can be rejected if there is no significant relationship between geographic distance and genetic distance as measure by N_em or M^, which is robust to variable mutation rates among loci.

Just as Frissel *et al.* (1986) proposed a hierarchical framework based on physical characteristics for conceptualising stream classification within the context of the catchment unit, Meffe and Vrijenhoek (1988) proposed the Stream Hierarchy Model (SHM) in an attempt to summarise the genetic relationships among populations and among and within drainages. Based on the assumption that the complex hierarchical branching patterns of riverine systems have the potential to isolate populations between rivers, the SHM proposed that the degree of connectivity is related to the position of a population within a catchment. Populations within sub-catchments or catchments are expected to be genetically more similar than populations in different sub-catchments or catchments, with the greatest degree of differentiation between catchment units. The total genetic variation is considered to be the sum of variation within sites (HS) and the diversity attributed to the different levels of the hierarchy, described by $H_T = H_S + D_{SC} + D_{CD} + D_{DT}$, where:

D_{SC}: diversity of sites within sub-catchments;
D_{CD}: diversity among sub-catchments within catchments;
D_{DT}: diversity among drainages relative to the total.

At equilibrium with gene flow and genetic drift, it is hypothesised that $D_{DT}>D_{CD}>D_{SC}$.

Understanding how an organism's dispersal ability relates to genetic population structure can also provide important insights into the micro-evolutionary processes operating throughout a species history. In the absence of gene flow between con-specific populations across a species range, genetic drift, mutation and natural selection can result in the divergence and differentiation of genetic, morphological and behavioural attributes such that they come to represent distinct and unique assemblages. Given that biological diversity is generated through the differentiation of natural populations and subsequently consolidated through speciation (Tregenza & Bridle 1997), such assemblages are central to the conservation of biodiversity. Indeed, descriptions of components of biodiversity in Annex I of the Convention on Biological Diversity include ecosystems, species and genetic lineages. Irrespective of how such units are defined, or what strategies are subsequently employed to ensure their protection, identification and subsequent protection of these primary units is the ultimate goal of conservation.

Defining Units for Conservation

Central to understanding the concepts behind biodiversity conservation is acknowledgement, acceptance and agreement on the unit upon which the conservation strategy is based. Exactly how to delineate and define such units has been debated for centuries (Darwin, 1859; Mayr, 1957; Avise & Walker, 1999; Hendry *et al.*, 2000). More than 25 different species concepts, each derived from a different set of assumptions about descent with modification, were identified by Mayden (1997). However, concerns have been raised that the delineation of distinct species fails to capture the essence of biological diversity adequately (Hendry *et al.*, 2000). This has led some to assume that species are not real entities but none the less utilise the same techniques and concepts, such as the Phylogenetic Species Concept, to delineate distinct groups that merit conservation (Goldstein *et al.*, 2000; Hendry *et al.*, 2000). Such approaches, however, still do not negate the need to define and delineate exactly what constitutes significant or important differences and thus they suffer from the same problems as conceptual consideration of other taxonomic units.

Similarly, Evolutionarily Significant Units (ESUs) have received a great deal of attention within the conceptual development of identifying units for conservation (Ryder, 1986; Waples, 1991; Moritz, 1994; Pennock & Dimmick, 1997; Paetkau, 1999; Crandall *et al.*, 2000). These were originally defined as population units that represented significant adaptive variation, defined through concordant data derived from a number of different techniques (Ryder, 1986). They were subsequently defined as a population (or group of populations) that is substantially reproductively isolated from other con-specific population units, and represent an important component in the evolutionary legacy of the species (Waples, 1991), introducing reproductive isolation and adaptive potential as definitive criteria. The conceptual development of delineating and defining ESUs has subsequently focussed largely on reproductive isolation, emphasising the evolutionary history of a population. Moritz (1994) argues that ESUs should be reciprocally monophyletic for mitochondrial DNA (mtDNA) alleles and show divergence of allele frequencies at nuclear loci. In contrast, populations connected by low levels of gene flow, such that they exhibit significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles, have been termed management units (MUs). These represent functionally independent populations, considered to be more suitable for short-term management of the more inclusive ESUs, forming a logical unit for population monitoring and demographic studies.

Many of the problems associated with the use of ESUs are derived from the inclusion of adaptive potential into the definition and the problem of trying to determine what will, in future, be of significance in the evolution of new species. While Paetkau (1999) argues that the use of genetic criteria as proposed by Moritz (1994) have been erroneously used to define ESUs, reciprocal monophyly fails to incorporate important information about adaptive potential (see Crandall *et al.*, 2000). Furthermore, the inclusion of monophyly as a criterion in determining ESUs ignores the fact that there are many paraphyletic groups that exhibit local adaptation (Takahata & Slatkin, 1990; Neigel & Avise, 1986; Powell, 1991; Hedin, 1997). There is agreement however, on the need to maintain historical population structure (Moritz, 1994; Crandall *et al.*, 2000) with genetically distinct units having conservation importance. This is especially true if these can be supported by other attributes, be they morphological or associated geological breaks, suggestive of vicariant events. While having separated

populations to an extent where gene flow is limited, examples exist where such separation has not been sufficient for the evolution of attributes warranting specific species status, but where the chance of reconnecting these populations is impossible. Thus it would be prudent to employ a precautionary principle and ensure that the integrity of these units is maintained. Thus, irrespective of definition, ESUs reflect historically isolated populations that can be used to address long term management issues, define conservation priorities and set strategies. Despite debate over the relevance of species units, ESUs, and their definition, primarily focussed around the need to define and quantify adaptive potential, the increasing data available on neutral genetic variation has largely resulted in a reliance upon molecular based criteria for defining primary units for conservation.

Acknowledging the issues surrounding the definition of adaptive potential, many of the problems concerning the use of genetic criteria to define taxonomic units arise from disagreement on the degree of convergence between mtDNA discontinuities and other species attributes, such as morphology and mate recognition systems. For example, Avise and Walker (1999) concluded from their study of mtDNA diversity within 252 taxonomic species of vertebrates that "mtDNA data and traditional taxonomic assignments tend to converge on what therefore may be real biotic units in nature". However, re-analysing their data, Hendry *et al.* (2000) came to the conclusion that mtDNA discontinuities do not match those of recognised taxonomic species. While they advocate abandoning the concept of species in favour of grouping organisms at certain levels by specifying the amount of difference in various traits, this essentially does not negate the problems that exist in determining that which constitutes a significant degree of difference. Both groups acknowledge that there are many problems associated with such comparisons, such as the non-equivalence of taxonomic rank among different types of organisms (Avise & Johns, 1999), the subset of species chosen, their ranges and the subset of samples within these and the use of different genetic markers (Avise & Walker, 1999). As such, they advocate further efforts to extend tests of species realities to larger data sets, other taxa, and to the use of additional analytical techniques.

Integrating Considerations of Genetic Population Structure into Catchment Management Strategies

Given that catchments can influence the genetic structure of natural populations there is a need to examine the relationships between the spatial distribution of genetic variation, species dispersal and time. There is an ever increasing demand being placed upon the earth's water resources to provide water, food and services for growing human populations. Associated with these demands there has been an observed decline in the quality and integrity of river health, along with a rapid decline in and imperilment of the freshwater fauna that inhabit these systems. As a result there is a pressing need to address and to reconcile human needs with the long-term conservation and sustainable use of aquatic ecosystems. This need is being recognised and addressed through various initiatives and developments, such as the development of integrated approaches to catchment management. With the concept of an integrated approach to the management of freshwater resources fundamental to future water resources planning and conservation, river catchments and aquifer units have been identified as the basic management units that should be used in future policy development (see Wishart, 2000). Signed and ratified by 168 countries, the Convention on Biological Diversity's (CBD) Subsidiary Body on Scientific, Technical and Technological Advice (SBSTTA), established Under Article 25, paragraph 1 of the CBD, is charged with providing the Conference of the Parties, the supreme body of the Convention, with advice relating to the implementation of the CBD. Among recommendations made by the Global Biodiversity Forum (GBF) to the SBSTTA was the need to develop mechanisms to allow application of the ecosystem approach to the conservation of inland waters biodiversity within a catchment framework.

Such recommendations are being implemented in national policies and legislation. For example, the South African Water Act (1998) has identified the catchment as the logical unit for effective management. The law calls for the establishment of catchment management agencies who are required to progressively develop catchment management strategies for the water resources within water management areas. The catchment management strategy must set the principles for allocating water to existing and prospective users, taking into account all matters relevant to the protection, use,

development, conservation, management and control of water resources. They must also be in harmony with the national water resources strategy, which amongst other things provides the framework for the conservation and management of water resources for the country as a whole. While similar considerations are recognised in other national legislation, such as Australia, the South African water law has provided a template for the development of ecologically sustainable legislation in other southern African countries. Similar considerations are now being given to the development of, among others, Zimbabwe's proposed new water law.

While ecological principles underlie such legislation, the assumption is that conservation will be facilitated through maintaining the physical and structural templates. While this is increasingly reflected in the development of water resources development strategies and the legal framework within which they are being implemented, the biological relevance of such units remains equivocal. If catchments do impose significant barriers to dispersal, resulting in the formation of distinct units confined within catchment boundaries, the transfer of water between catchments through the development and operation of inter-basin water transfers (IBTs) may pose a threat to the biogeographic integrity of such units (Davies *et al.*, 1992, 2000). IBTs are an accepted set of technologies frequently used to reconcile the problems of water supply and demand. While providing for the transfer of water from one distinct catchment to another, they also provide a conduit for the transfer of organisms between historically isolated catchment units.

Southern Africa is a leader in the development of inter-basin water transfer technologies. Across the region, water is seen as one of the greatest constraints to increased socio-economic development and, as with many dryland (*sensu* Davies *et al.*, 1995) regions across the globe, IBTs are increasingly being used to reconcile and to 'rectify' the problems posed by the uneven distribution and paucity of permanent surface waters. In South Africa, IBTs are stated by the Department of Water Affairs and Forestry to be established sets of technologies that will be used with increasing frequency to redistribute water on a national basis. This increasing use and reliance on IBTs has seen them listed as one of the major threats to the integrity of river and stream ecosystems (e.g. Davies and Petitjean, 1989; Davies *et al.*, 1993, 1995; Snaddon and Davies, 1999a, b; Snaddon *et al.*, 1999; Davies *et al.*, 2000). The

ecological effects, along with some of the social and political implications of IBTs, have been addressed (see Snaddon *et al.*, 1999). Most developments involve a donor and a recipient river in which the effects on the donor are typical of those experienced as a result of in-stream impoundments. While the ecological implications associated with such transfers are many and varied, including, among others, changes in riverine geomorphology, water quality and community structure (O'Keeffe & de Moor, 1988; Snaddon & Davies, 1998; Snaddon *et al.*, 1998), one of the major concerns voiced thus far, but based only on anecdotal evidence (Cambray & Jubb, 1977a, b; Skelton & Merron, 1985), is the transfer of species and/or individuals between river basins. The introduction of species, both indigenous and alien competitors and parasites, into river systems where historically they did not occur has numerous implications, many of which have been previously well documented, such as the effects of trout and carp. More recent concerns however, have focused attention on the transfer of individuals from genetically distinct populations between historically isolated river basins.

Already in South Africa there are a number of extant IBTs. which cross not only adjacent river catchments but which also move water, and potentially individuals and species, across biogeographic boundaries (Davies *et al.*, 2000). With an increasing propensity toward the redistribution of water, and the subsequent crossing of river-basin boundaries and the connection of historically isolated rivers, there is the potential for inadvertently undermining the genetic integrity of our river ecosystems. The extent to which catchment units prevent the exchange of individuals and, therefore, the mixing of individuals, and thus the effect of IBTs on the evolutionary legacy of a species is not really known. Ultimate effects will depend on a number of contemporary and historical factors, including the dispersal traits of individual taxa and the geological history of the system/s in question.

Alternatively, if catchments do not impose significant barriers to dispersal, then the catchment unit may not represent the appropriate scale for developing conservation strategies aimed at protecting biodiversity in lotic ecosystems. For example, the existence of populations in proximate catchments provides a source for recolonisation and a potential buffer against the effects of natural environmental stochasticity. For species that inhabit a shifting mosaic of habitats, such as is found in lotic environments, the existence of several or many populations is considered vital (Picket

& Thompson, 1978). These populations serve as sources for recolonisation and thus act as buffers against natural environmental stochasticity (Weeks *et al.*, 1996). Even rare events of inter-breeding between isolated populations can help in maintaining the overall genetic fitness of species or populations (Gilpin, 1987). So, it may be that the minimum viable population for some species, such as dragonflies, which are found in relatively low densities and have highly developed powers of flight, may operate across spatial scales larger than the catchment unit.

Inferring Dispersal

All such considerations for the conservation and management of lotic ecosystems depend upon the effects of the structural properties of the catchment unit on the dispersal of lotic organisms. The dispersal potential of a species can be variously inferred. Morphological measures, in particular wing structure and length, are important in facilitating and determining the dispersal potential of adult insects (den Boer, 1970; Harrison, 1980; McLachlan, 1985; Malmqvist, 2000). Those species with larger wings typically exhibit greater dispersal and have wider distributions when compared to those species with relatively shorter wings. In comparison, while the aquatic larvae of many insects can readily move, either passively or actively, through drift, crawling and/or swimming, such movements are typically limited to between habitat patches within catchments (Palmer *et al.*, 1996). There have also been various attempts to quantify directly the movement and dispersal in a number of aquatic invertebrates using mark recapture, direct observation, radiotracking and radar (Erman, 1986; Freilich, 1991; Hershey *et al.*, 1993; Nürnberger, 1996; Winterbottom *et al.*, 1997; York *et al.*, 2000). However, such studies are time consuming and are often complicated by the small size and high abundance of lotic invertebrates, along with the physical conditions of the stream environment. Furthermore, rare events that can have significant influence on population processes and structure are easily missed during sampling or observation.

Given the inherent problems associated with inferring or obtaining direct estimates of dispersal for lotic invertebrates, molecular methods provide a valuable indirect method for obtaining a mean estimate of the degree of dispersal among aquatic invertebrate populations over a species history. This is possible because a

population's genetic structure is derived from the combined effects of genetic drift, mutation, natural selection and gene flow, with gene flow resulting from the dispersal of individuals (or gametes) among populations. In the absence of natural selection, which is accepted for many "neutral" molecular markers, organisms with a strong potential for dispersal would be expected to show less genetic population structure than those with a more limited potential for dispersal.

The aim of this project, outlined in more detail at the end of Chapter 2, is therefore, to use molecular techniques to look at dispersal in a number of aquatic organisms and assess the congruence between catchment units and genetic population structure.

Chapter 2

Freshwater Biological Diversity And Conventions Covering Its Protection

What is Biological Diversity?

The term "biological diversity", or biodiversity, is commonly used to describe the variety and variability of species, as well as the ecosystems on the planet. Biological diversity is typically defined at three levels; genetic diversity, species diversity, and ecosystem diversity. *Genetic diversity* refers to the variety of genetic information contained in all of the individual plants, animals and micro-organisms. Such diversity occurs within and between populations of species, as well as between species. *Species diversity* refers to the variety of living species. While *Ecosystem diversity* relates to the tremendous variety of habitats, biotic communities, and ecological processes within ecosystems.

Such diversity is the outcome of over 3,000 million years of evolution. Implicit in the process of evolution is the fact that biological diversity is dynamic. New genetic variation within a species or population, the creation of a new species or formation of a novel ecosystem will cause an increase in biological diversity. Similarly, a decrease in genetic variation, species extinction or the loss of an ecosystem decreases biological diversity. The exact number of the Earth's existing species is still unknown. While an estimated 1.7 million species have been identified, estimates of the number of species vary from anywhere between 5 and 100 million (although a few recent estimates based on marine benthic and pelagic communities push the number to a possible billion species or more). A great many of these species, estimated at between 50 and 90% of this total, inhabit tropical rainforests. About 17 million hectares of tropical forest, an area four times the size of Switzerland, are being cleared annually and it is estimated that at these rates, roughly 5 to 10% of tropical forest species may face extinction within the next 30 years. But such losses of biodiversity are not confined to tropical forests alone. Nearly as much temperate rainforest has been lost and marine and freshwater systems are also facing serious losses and degradation in terms of overall biodiversity. At current rates of extinction, the Earth will have lost about 20% of its presently living species by the year 2020.

The Freshwater Imperative

Despite the fact that inland aquatic ecosystems represent one of the most diverse and as yet largely undescribed group of environments, conservation efforts have historically been directed toward terrestrial, and more recently, marine ecosystems. The neglect of lotic ecosystems ignores the fact that these environments include some of the most threatened ecosystems on earth. For example, it has been estimated that 20% of all freshwater species have become extinct or are in danger (**http://www.watervision.org/**) with the rates of imperilment within major aquatic taxa such as fish, crayfish, and mussels running between three to eight times those for birds and mammals in North America (Masters, 1990; in Angermeier, 1995). This neglect also ignores the fact that while freshwater ecosystems occupy only 0.8% of the Earth's surface (McAllister *et al.*, 1997) they provide habitat for some 44,000 described aquatic species. This represents in the order of 2.4% of all known species (Reaka-Kudla, 1997). Of the animals it has been estimated that 12% of all animal species live in fresh water (Abramovitz, 1996). This hides the fact that there are a great many more species that rely on freshwater ecosystems for survival, humans included. Furthermore, it is estimated that this represents only a small portion of the actual number of freshwater species. In the last 18 years, for example, roughly 309 new freshwater species have been described annually (Nelson, 1976, 1984, 1994 in Revenga *et al.*, 2000) (Table 2.1).

Table 2.1. Distribution of the world's biodiversity by habitat type. Taken from McAllister *et al.* (1997) in Revenga *et al.* (2000).

	Habitat Extent	Percent Known Species*	Relative Sp Richness#
Freshwater	0.8	2.4	3
Terrestrial	28.4	77.5	2.7
Marine	70.8	14.7	0.2

*Sum does not add to 100% because 5.3% of known symbiotic species are excluded.
#Calculated as the ratio between the percent species known and the percent area occupied by the ecosystem.

While data pertaining to freshwater taxa are generally poor, particularly from the world's developing countries (see Wishart *et al.*, 2000) it is generally agreed that the fish fauna are better known. It has been estimated that more than 20% of the world's 10,000 described freshwater fish species have become extinct, threatened, or endangered in recent decades (Moyle and Leidy, 1992). Many more aquatic-dependent species, such as mussels, birds, and plants are highly threatened (Revenga *et al.*, 2000). Given the many as yet undescribed and undiscovered species, along with the revision of previously recognised yet genetically distinct and morphologically conservative cryptic taxa, this is likely to be a considerable underestimate (Bräutigam, 1999). Information regarding the loss of biodiversity in inland waters is hampered by the fact that the taxonomic basis for such assessments is still largely inadequate. For example, a study in the Cross River basin in Cameroon and Nigeria found that fish diversity had been underestimated by 73%, while an impact assessment for a dam project in Laos discovered 60 species new to science. Nearly 100 species of freshwater fish are described each year.

Of the 734 species of fish which are classified as threatened by the IUCN, 84% are freshwater species (IUCN, 1996; McAllister *et al.*, 1997). Some 63% of South Africa's fish fauna are threatened or endangered, while in Europe the figure runs at 42% and in Iran some 22% are threatened or endangered (Moyle and Leidy, 1992). The United States arguably has the best available data. Of the freshwater species there, 37% of fish species, 67% of mussels, 51% of crayfish and 40% of amphibians are threatened or have become extinct (Master *et al.*,1998; see also Karr *et al.*, 2000). The North American freshwater fauna shows that species are being lost at an "ever-accelerating rate" (Moyle and Leidy, 1992). Despite many efforts between 1979 and 1989 no species have been removed from the American Fisheries Society's list of imperilled fish as a result of the implementation of recovery activities. In fact, the rate of fish and mussel endangerment continues to increase (Williams *et al.*, 1989; Allan and Flecker, 1993; Williams *et al.*, 1993; Warren *et al.*, 1994). However, five species have been removed from the list as a result of more detailed information on their distribution, 11 due to taxonomic revisions with a further ten species removed as a result of their extinction. Since the initial assessment, more than three times as many fish taxa (24) have declined compared to those that have improved (seven) in status (Williams *et al.*, 1989).

A recent analysis of the world's ecosystems (Revenga *et al.*, 2000) highlighted the fact that freshwaters are "…in by far the worst condition from the standpoint of their ability to support biological diversity." The ever increasing demand for water and food resources will inevitably continue and the already considerable pressures on freshwaters will place more of the species and ecosystem processes at escalating risk. The available data suggest that future extinction rates will be of the order of five times higher for freshwater animal species than for terrestrial ones (Ricciardi and Rasmussen, 1999). It is estimated that 20% of freshwater fish have recently become extinct, are endangered or vulnerable. Some 30% of America's 979 native freshwater fish species are either extinct or at risk. In Australia, one third of the 193 fish species are considered to be threatened, while in Europe 42% are of concern. In contrast, many attempts at restoration and rehabilitation would appear to ignore the very simple fact that biological stocks are required from which to restore and to rehabilitate natural systems. Rivers by their very nature are dynamic and are resilient, with considerable scope to recover from disturbances, but rehabilitation after major disturbance requires a robust biological source. Within the context of conservation, and considering the various approaches to conservation, it is perhaps interesting to note that only 4% of the threatened and endangered aquatic species in the United States for which there are plans for rehabilitation/preservation, have shown significant recovery (Williams and Neves, 1992: in Angermeier, 1995).

While extinction is a natural process, current losses of biodiversity have both direct and indirect causes most of which have been accelerated through human activities. These include habitat loss and fragmentation, invasion by introduced species, the over-exploitation of resources, pollution, global climate change, and industrial agriculture and agroforestry. While such losses arguably represent an environmental tragedy, they also have profound implications for economic and social development. At least 40% of the world's economy and 80% of the needs of the poor are derived from biological resources. In addition, the richer the diversity of life, the greater the opportunity for medical discoveries, economic development, and adaptive responses to new challenges such as climate change.

The dramatic losses of species and the destruction of ecosystems often obscure the equally large and important threats to genetic diversity within species. Genetic resources have tremendous and growing value as raw material for biotechnology and have always been essential to agriculture. The genetic variation that exists within a species also provides the foundation for the generation of new species and, thus biodiversity, through speciation. Undermining or eroding genetic diversity thus has the potential to impede the creation of new species and the future addition to the overall biodiversity of the planet.

Why Conserve Biological Diversity?

The conservation of biological diversity has ceased to be viewed merely in terms of protecting threatened species or ecosystems. It has emerged as a fundamental part of the movement towards the development of an ecologically sustainable future. Components of biological diversity play an important role in ecosystem functioning and in maintaining important processes, such as water purification in freshwater systems. In inland waters the status of the organisms present can also be used to infer the health of the system. Biodiversity also provides enormous direct and indirect economic benefits. In agriculture, disease- and pest-resistant crop varieties are continually derived from wild relatives of domestic crops. Nearly half the medical prescriptions world wide are based on compounds extracted from wild plants, animals or from micro-organisms. Major resource-based industries, such as fisheries and eco-tourism, also depend heavily on biological diversity.

Inland water ecosystems are increasingly vulnerable because of their inextricable links with, and reliance upon the broader landscape and catchment processes. The importance of protecting and maintaining inland water ecosystems is heightened by virtue of the fact that they provide a critical human resource and are strongly influenced by socio-cultural factors, such as population density and pressure, land tenure, and degree of knowledge, to name a few. They provide potable water supplies, harvestable plants and animals, routes of travel and transport, waste removal and renewable energy. In recognition of the situation in inland waters, the *Global Biodiversity Strategy* has recommended that reforms be made to policies that hasten the loss of biodiversity from freshwater ecosystems. One of the eight priority actions

for the sustainable use of freshwaters outlined in the *Global Biodiversity Strategy* includes the conservation of the diversity of aquatic species and genetic stocks. With much of the world's biodiversity as yet un-documented there is a need to put in place strategies and policies that ensure the protection of natural resources of biodiversity before they are lost. This idea is increasingly being recognised and incorporated into policy through the idea of the Precautionary Principle.

The Precautionary Principle

An increasingly important development in the philosophical consideration of environmental policy and protection is that of the *Precautionary Principle*. In essence, the principle requires action to prevent serious and irreversible damage even before harm can be scientifically demonstrated or economically assessed. Proponents argue that the principle should be applied in situations where both the probability and value of irreversible damage are unknown. The principal was initially formulated and incorporated into legislation to allow regulatory decisions to be made in the face of what are often irreducible uncertainties regarding causal relationships. Its development reflects the need to address the pervasiveness of uncertainty and indeterminacy coupled with the ongoing need to take decisions that facilitate preventative action. In the application of the *Precautionary Principle*, public and private decisions should be governed by careful evaluation, wherever practical, to avoid serious or irreversible damage to the environment and by an assessment of risk-weighted consequences of various options (The Intergovernmental Report on the Environment, May, 1992; cited in Deville and Harding, 1997)

The principle is largely regarded as having emerged from European environmental policies in the late 1970s. The first treaty to reflect the principle was the *Vienna Convention for the Protection of the Ozone Layer*, followed in 1987 by the *Montreal Protocol*. It has subsequently become enshrined in numerous international treaties and declarations, providing the basis for European environmental law through the *Treaty on European Union* (1992) and is increasingly prominent in the development of environmental health policies.

Since its initial development and inception, the *Precautionary Principle* has been integrated into numerous international treaties and legal instruments. Most of these are of global application on environmental matters of broad concern and apply to almost all human activities. The increasing employment of the principle represents a departure from previous philosophical foundations and is an important recognition of the weight and direction of the burden of scientific proof and notions of certainty. Noteworthy examples include, but are not restricted to, the 1995 *Fish Stocks Agreement*, the annex to which includes a specific set of guidelines for the application of "precautionary reference points" in the conservation and management of the stocks concerned. The 1996 *Protocol to the London Dumping Convention* states: "In implementing this protocol, Contracting Parties shall apply a precautionary approach to environmental protection ... when there is reason to believe that wastes or other matter introduced in the marine environment are likely to cause harm even when there is no conclusive evidence to prove a causal relation between inputs and their effects".

The *Precautionary Principle* has also been included in the *World Charter for Nature* (1982), the *Rio Declaration* (1992) and the *International Convention on Biological Diversity*. Others include the *Directive of the Council of the European Communities on Urban Waste Water*, the *Convention for the Protection of the Marine Environment of the North-East Atlantic*, the 1994 *Code of Practice on the Introduction and Transfer of Marine Organisms*, by the International Council for the Exploration of the Seas', *Guidelines for Preventing the Introduction of Unwanted Aquatic Organisms and Pathogens from Ships' Ballast Water and Sediment Discharges*, by the International Monetary Organisation, and the Food and Agricultural Organisation of the United Nations' (FAO/UN) *Guidelines on the Precautionary Approach to Capture Fisheries and Species Introduction*.

Despite its increasing recognition and adoption, the principle suffers from extreme variability in interpretation. For example, one legal analysis identified fourteen different formulations of the principle in treaties and nontreaty declarations. However, it is an important governing principle, shifting the onus of responsibility in such a way as to demand a kind of pro-active, preventative approach. It has also been invoked before the *International Court of Justice*. Judge Weeramantry in his opinion, dissenting from the *Order of the Court of 22 September 1995*, concluded that the

Precautionary Principle was gaining increasing support as part of the international law of the environment. New Zealand has also invoked the principle in support of its application to the *International Court of Justice* to review France's decision to recommence nuclear testing in the South Pacific.

A literal interpretation of the principle could require that all activities and substances which may be harmful to the environment be regulated, and possibly prohibited. This, applies even if no conclusive or overwhelming evidence is available as to the harm or likely harm those activities may cause to the environment. An even more fundamental interpretation would shift the burden of proof in decision-making. This would require a person who wishes to carry out an activity to prove that the activity will not cause harm to the environment.

Relating specifically to the conservation of biodiversity, the *World Charter for Nature* (1982) states that "...where potential adverse effects are not fully understood, the activities should not proceed." The *Rio Declaration* (1992) says that a lack of "...full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation". Furthermore, in its preamble, the *Convention on Biological Diversity* notes "...that where there is a threat of significant reduction or loss of biological diversity, lack of full scientific certainty should not be used as a reason for postponing measures to avoid or minimize such a threat".

In 1997, the fifth session of the *Commission on Sustainable Development* incorporated the *Precautionary Principle* into *Principle 15 of the "Rio Declaration on Environment and Development: Application and Implementation"*. It called on States to apply the principle, according to their capabilities, in order to protect the environment. Lack of full scientific certainty was not to be used as a reason for postponing cost-effective measures to prevent environmental degradation where there are threats of serious or irreversible damage. This obviously raises the question: what constitutes a serious threat, or irreversible damage? But given that most countries, South Africa included, are signatories to the *Rio Convention*, and specifically to the *Convention on Biodiversity*, and have accordingly invested in developing national frameworks, policies and legislation, they have signified their agreement and commitment to the protection of biological diversity.

Central to Principle 15, is the element of anticipation, reflecting a requirement that effective environmental measures need to be based upon actions which take a long-term approach and which might anticipate changes on the basis of scientific knowledge.

The *Precautionary Principle* is now widely accepted as a fundamental concept of many national environmental laws and regulations in order to protect the environment. Developed as a philosophical cornerstone underpinning the theoretical concepts of many international agreements and policies it is now elaborated, for instance, in the *Water Law* and *Planning Law* of Israel, in the *Environmental Protection Act* of the Czech Republic, and is also included in numerous draft environmental laws currently under consideration - for example in the Pakistan draft *Environmental Protection Act* of 1996.

International Conventions and Policies

The conservation of biological diversity and the sustainable use of its components is not a new item on the diplomatic and political agenda. It was highlighted in June 1972 at the *United Nations Conference on the Human Environment*, held in Stockholm. In 1973, the very first session of the *Governing Council* for the new *United Nations Environment Programme* (UNEP) identified the "...conservation of nature, wildlife and genetic resources as a priority area". These ideas were first formalised with the publication of the *World Conservation Strategy* (WCS) bought out by the *International Union for the Conservation of Nature* (IUCN), United Nation Environmental Programme (UNEP) and the *World-wide Fund for Nature* (WWF).

The aim of the WCS was to '*...help advance the achievement of sustainable development through the conservation of living resources*' via three main objectives of living resource conservation:

- the maintenance of essential ecological processes and life-support systems;
- the preservation of genetic diversity; and
- the ensurance of the sustainable utilisation of species and ecosystems.

Since 1980, the WCS has been tested by the preparation of national and sub-national conservation strategies in over 50 countries. In 1987 the *World Commission on Environment and Development* released the publication, *'Our Common Future'*. This publication examined the global interdependence and relationships between economics and the environment. It contributed significantly to the growing recognition of the need for sustainable development and international equity. At the same time, governments also adopted the essentials of the document, *Environmental Perspective to the Year 2000 and Beyond*. This defined a broad framework intended to guide national action and international co-operation for environmentally sound development. Then in June 1992, more than 178 governments meet in Rio de Janeiro at the *United Nations Conference on Environment and Development* (UNCED) to agree on an agenda for the environment and sustainable development into the 21st Century. This became know as *Agenda 21*.

Four agreements emerged from the *Rio Earth Summit* - the *Convention on Biological Diversity*, the *Framework Convention on Climate Change*, the *Convention to Combat Desertification*, and the *Forest Principles*. These call for policies, strategies, and solutions to mitigate the effects of climate change, biodiversity loss, desertification, and forest degradation and conversion, respectively. In particular, each document calls on countries to integrate these four objectives into national and regional development plans, policies, programmes, and strategies. Over 150 countries and the European Union signed the *Global Convention on Biological Diversity* and *Agenda 21* towards sustainability in Rio de Janeiro. They remained open for signature until 4 June 1993, by which time they had received 168 signatures.

Table 2.2. Southern African signatories to the Convention on Biological Diversity.

Country	Signed	Ratified	Country	Signed	Ratified
Angola	1992	1998	Kenya	1992	1994
Botswana	1992	1995	Madagascar	1992	1996
DRC	1992	1994	Malawi	1992	1994
Namibia	1992	1997	Mauritius	1992	1992
Mozambique	1992	1995	Swaziland	1992	1994
South Africa	1993	1995	Tanzania	1992	1996
Zimbabwe	1992	1994	Uganda	1992	1993
Zambia	1992	1993	Lesotho	1992	1995

The Convention was the first global instrument to take a comprehensive approach to the issues of conserving the world's biological diversity and to using its' biological resources in a sustainable way. The objectives of the *Convention on Biological Diversity* (CBD) are:

"*...the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of the benefits arising out of the utilisation of genetic resources.*"

The convention defines *Biological Diversity* as the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species, and of ecosystems. Thus it represents the first comprehensive global agreement to address all aspects of biodiversity, including genetic, species, and ecosystem diversity. Furthermore, it recognises that the conservation of biological diversity is "...a common concern of humankind" and an integral part of the development process, providing a framework for conserving biodiversity.

Most articles of the Convention set out policy guidelines that Parties can follow, rather than establishing precise obligations or setting of targets, with the Conference of the Parties (COP) representing the supreme authority of the Convention. The *Subsidiary Body on Scientific, Technical and Technological Advice* (SBSTTA) was established under Article 25, paragraph 1, of the Convention to provide the COP and, as appropriate, its other subsidiary bodies with advice relating to the implementation of the Convention. SBSTTA is a multidisciplinary body, open to participation by all Parties and comprising government representatives competent in all the relevant fields of expertise. Pursuant to the *modus operandi*, the scientific and technical contribution of non-governmental organisations to the fulfilment of the mandate of SBSTTA is strongly encouraged in accordance with the relevant provisions of the Convention and of the rules of procedure for meetings of the *Conference of the Parties*. The SBSTTA programme of work emanates from Article 25 and the cumulative decisions of each meeting of COP which set out, *inter alia*, the topics on which advice is required for implementing the Convention.

To date, three meetings of SBSTTA have been held and a total of 28 recommendations have been developed and considered by COP. At its third meeting, the SBSTTA produced a document on *Biological Diversity of Inland Waters* (1997). The report was aimed at assisting the SBSTTA in considering the trends and status of biological diversity in inland water ecosystems.

South African Policy Documents on Biodiversity

In recognition of these changes, and like many legal and policy reforms around the world, South Africa has instituted a decentralised programme of water resources management and control, based around the adoption and development of an integrated, watershed or catchment-based approach to water. In it's approach South Africa has been hailed for its long-term vision as well as for the ecological framework within which the new policies and law have been implemented. The new approach is based upon sustainable principles that require maintenance of the natural ecosystem and its functioning (South African Water Act, 1998). An equally great challenge posed by the new water policies is the need for the South African Government to take a multi-disciplinary approach to water management issues. Hydrological and

engineering considerations — for decades, the water department's focus (Department of Water Affairs and Forestry; DWAF) — are now merely pieces of a larger management framework that gives equal consideration to economic, social, and ecosystem issues within the context of the countries legislative, constitutional and international obligations.

South Africa has been identified as one of the world's 17 megadiversity countries that collectively claim more than 60% of the global biodiversity within their borders. South Africa is the only one of these countries without rainforest. There have been a number of different policy documents developed in recognition of this unique national asset subsequent to South Africa's signing (1993) and ratification of the *Convention on Biodiversity* in 1995. These began with the *'Guidelines for the development of a strategy to implement the Convention on Biological Diversity'* that were prepared at a meeting of biologists representing universities, museums, conservation agencies and the *Department of Environmental Affairs and Tourism* (DEAT) held in Pretoria in 1996. This was then followed by the development and publication of a *Green Paper* in October, 1996 which lead to the eventual development and publication of *'The White Paper on the Conservation and Sustainable Use of South Africa's Biological Diversity'* (*Government Gazette*, vol. 385, no. 18163, 28 July 1997. Notice 1095 of 1997). This represents the culmination of all these various documents and the government of South Africa's commitment to conserving biological diversity. In it is outlined a policy for the implementation of the CBD, comprising six goals, namely to:

- conserve the diversity of landscapes, ecosystems, habitats, populations, species and genes;
- use biological resources sustainably and minimise adverse impacts on biological diversity;
- ensure that benefits derived from the use and development of genetic resources serve national interests;
- expand the human capacity to conserve biodiversity, to manage its use, and to address factors threatening it;
- create conditions and incentives that support the conservation and sustainable use of biodiversity; and
- promote the conservation and sustainable use of biodiversity at an international level.

This report attempts to address some of these issues within the context of integrating considerations of biological diversity into catchment management strategies. The conservation of biodiversity has undergone many transitions. Its development has progressed from a relatively emotive issue to a theoretical science that has provided the foundations for the development of international agreements. In turn, these international agreements, underpinned by a shift in the philosophical foundations enshrined within the adoption of the *Precautionary Principle* to conservation, acknowledge the inherent wealth and importance of biodiversity. Often the true worth and importance of this biodiversity is unrealized. Given the crisis facing the biodiversity of freshwater systems, the history of conservation of these systems – focused around the physical and structural template, and the recognition and development of catchment units as the primary functional units - we attempt to determine if the genetic structure of lotic populations is congruent with catchment units. We also examine whether or not they represent an appropriate scale at which to manage water resources, through catchment management policies, for the conservation of lotic biodiversity. Specifically, our aim was to use a comparative phylogeographic approach to examine the genetic population structure of a number of lotic taxa with different dispersal traits across a number of different catchments. It was hypothesized that the genetic population structure would be related to dispersal abilities/traits of individual taxa and that they would show a gradient in the effects of catchment isolation. The aims as outlined in the proposal approved by the Water Research Commission were as follows:

Primary Aims

- To examine the integrity of catchment units within the framework of Evolutionarily Significant Units and to assess the implications of inter-basin water transfers for the conservation of river ecosystem functioning and riverine biodiversity using selected taxa;
- To contribute to the development of a management protocol for use in the assessment of the ecological effects of IBTs on both donor and recipient river basins.

Specific Objectives

- Using various techniques, to assess the levels of genetic differentiation between river basins and to examine the integrity of river basins;
- To identify species through genetic and ecological studies to be used in the development of a management protocol in the assessment of future water transfer projects
- To study the ecology and dispersal capabilities of selected invertebrate taxa within the context of their ability to recover following disturbance or local extinction
- To facilitate technical exchange relating to the ecological functioning of riverine systems between Australia and South Africa.

Chapter 3

General Methods

Study Area: Characteristics of the South-Western Cape, South Africa

The study area is situated in the south-western corner of the African continent, home of the Cape Floral Kingdom (CFK). The most biologically diverse plant Kingdom in the world, the terrestrial flora of this region is both uniquely rich in species (e.g. Bond and Goldblatt, 1984) and exhibits exceptionally high levels of endemism. The smallest of the six floral kingdoms (in terms of land area), the CFK has the highest known levels of local plant endemism outside of tropical forests (e.g. Cowling and Holmes, 1992) and is the only Kingdom to be found within a single country. Comprising roughly 4% of the total land area of southern Africa - 90,000 km^2, the CFK contains around 42% of southern Africa's vascular plants. That about 70% of the estimated 9600 species of plants found in the CFK are endemic to the region (e.g. Goldblatt, 1997) has contributed to South Africa being recognised as one of the world's 17 biological "mega-diversity" countries.

Species richness and endemism are two key attributes of biodiversity that reflect the complexity and uniqueness of natural ecosystems. Many areas, particularly those biodiversity rich areas of Central Africa, South America, and Southeast Asia, exhibit strong similarities in the patterns of endemism and species richness (Revenga *et al.*, 2000). In contrast, analyses from a wide range of phylogenetically independent taxa in South Africa have shown that while faunal species richness tends to be concentrated in the north-east of the country, endemism is highest and concentrated in the south-western Cape region (e.g. anurans - Drinkrow *et al.*, 1995; fish - Skelton *et al.*, 1995; millipedes - Hamer, 1997; tortoises and terrapins - Branch *et al.*, 1995; various vertebrates - Lombard 1995, Mugo *et al.* 1995).

While Pomeroy (1993) states that "endemism at the Cape is high for plants but not for other groups", more recent data suggest that the region supports at least comparable levels of faunal endemism with regard to invertebrates, both terrestrial and aquatic. Thus the CFK is increasingly being recognised as an important global repository of

biodiversity that extends well beyond its floristic component (Picker & Samways, 1996; Van Nieuwenhuizen & Day, 2000). For example, Wishart and Day (2001) conducted a preliminary examination of levels of endemism within aquatic fauna of the Cape Floristic Region. This study shows that well over half (64%) of the freshwater invertebrate species found in the region are found nowhere else and that this group comprises almost a third of South Africa's freshwater invertebrates (Table 3.1). With over 30 times as many species, species richness is much higher in the terrestrial plants. However, given that the total surface area of rivers and wetlands combined is much less than one thousandth that of the total land surface area, the level of endemism, area for area, in freshwater invertebrates must be orders of magnitude greater.

Table 3.1. Numbers of species, and percentages of endemics, in representative freshwater taxa (amphipods, copepods, ostracods, trichopterans, simuliids, ephemeropterans, molluscs, fish and amphibians) in South Africa (SA) as a whole and in the Cape Floristic Region (CFR).

	# in SA	# in CFR	# CFR endemics	% CFR taxa endemic to CFR	% SA taxa endemic to CFR
Animal:					
Families	63	30	6	20	10
Genera	225	103	24	23	11
Species	654	298	190	64	29
Plant Species	~20000	9600	6720	70	33
Riparian Sp.[5]	200	100	15	15	8

Some groups display remarkable levels of endemism and speciation (Table 3.2). For example, all species of amphipod known in the CFK are endemic. Other groups show that this trend is not simply confined to those organisms examined. For instance, of the 27 species of Dytiscidae confined to the Western Cape, 25 species and six of the 12 genera are considered endemic to the region (Omer-Cooper, 1962). Five of these genera are monospecific, whilst the sixth has two species. The dytiscid fauna is dominated by the genus *Canthyporus*. This genus has a very discontinuous distribution with 21 species in the south-western Cape, of which 14 are endemic. In context, there are three species endemic to the Eastern Cape, with single species of

this group found in Namibia, the DRC, Tanzania, Madagascar, Swaziland and Lesotho. Other groups, like the clinging water beetles (Coleoptera: Dryopidae and Elmidae) also display an incredible degree of endemism. *Strina* and *Rapnus* are both endemic genera of the Dryopidae found only in the south-western Cape, including eight of the nine species found in South Africa. While 16 of the 32 elmid species found in South African are endemic to this region.

Table 3.2. Numbers of species and percentages of endemic species for selected aquatic taxa in the Cape Floristic Region (CFR), South Africa (SA).

	# spp in SA	# spp in CFR	# CFR endemics	% CFR spp end. to CFR	% SA spp end. to CFR
Amphipods	43	27	27	100	79
Fish	93	20+	16+	80+	17+
Caddisflies	151	80	61	76	40
Copepods	18	7	5	71	28
Ostracods	156	77	50	65	32
Blackflies	39	22	9	41	23
Mayflies	91	39	15	38	16
Amphibians	109	~45	~25	56	23
Molluscs	72	26	7	27	10
Total	654	298	190	64	29

More recently, genetic diversity and phylogenetic units have also been recognized as fundamental components of biodiversity. These are important not only in defining species boundaries but represent a potentially important component in the evolutionary legacy of a species. Further to this, the use and development of molecular techniques has resulted in the revision of a number of taxa and an increase in the number of recognised endemics and species (Stewart, 1997). Studies on the molecular and morphological systematics of several groups in the south-western Cape region have recently resulted in taxonomic revisions and the identification of a number of new species. Revision of the amphipods, for example, lead to an increase in the number of recognised species from 12 species in one genus to 26 species in three genera (Stewart & Griffiths, in press). Recent revisions of the stonefly genus

Aphanicercella have identified a number of morphologically discrete forms of *A. barnardi* that differ from one another and from other known species of *Aphanicercella* (Stevens & Picker, 1999). These forms display minor yet consistent differences in male and female genitalia, with no evidence of intermediate forms, and have smaller geographical ranges than other more morphologically diverse species. Stevens and Picker (1999) consider these forms to be five valid new species, closely related within the *A. barnardi* species-complex. Clear positive assortative mating within forms, indicative of reproductive isolation, has confirmed the biological species status.

The aquatic fauna of the south-western Cape region was, with ever increasing precaution considered as a southern extension of the Ethiopian faunal Region. However, it is increasingly being recognised that this fauna is descendant from the southern temperate Gondwanaland fauna, and as such is probably the oldest and least disturbed stock of the continent's biome (Endrödy-Younga, 1988). Described as "the relict of the Gondwanaland fauna" (Balinsky, 1962), many of the cold-stenothermal forms appear to belong to a paleao-endemic element constituting the remains of the Mesozoic fauna, with Gondwanaland affinities (Harrison, 1965). With separation of the Gondwanaland continents taking place during the Cretaceous, those common Gondwanaland elements can only be among those groups which had developed by the middle of the Mesozoic. A similar palaeogenic fauna is found in the Lesotho-Drakensberg Highlands to the north-east, the occurrence of which is also considered an original feature of a very old distribution. While the Highlands of this region arose contemporaneously with the Cape Fold system, at the onset of the break up of Gondwanaland, Stuckenberg (1962) expressed the view that the palaeogenic fauna of the Cape should be considered distinct.

Species richness in the terrestrial plants of the CFK is hypothesized by botanists (e.g. Goldblatt, 1997) to have resulted from the presence of a complex mosaic of diverse habitats and steep ecological gradients. These are set against the background of a relatively stable climate and geology after the Mediterranean climate was established following the beginning of the Pliocene. Similarly, the Gondwanan fauna of the Cape, spared the effects of glaciation, has persisted in an ancient landscape with relatively little geological or climatic change in more than 200 million years (e.g. Deacon *et al.*,

1992). The mosaic of sandstone and shale substrata gives rise to a variety of different soil types with local areas of limestone adding to the edaphic diversity. The current climatic conditions are extremely variable across the region, while the mountainous landscape results in steep physical gradients. Combined with the edaphic diversity and the low nutrient levels this creates an unusual patchy mosaic of different local habitats. It was in this mosaic that the highly diverse and endemic flora and fauna of the CFK evolved, and which still seems to represent evolutionary 'hotspots' (*sensu* Myers *et al.* 2000) for many of the region's taxa (e.g. aquatic coleopterans - Omer-Cooper, 1962; amphipods - Stewart & Griffiths, in press; fish - Impson *et al.*, 2000).

The inland waters of the CFK also display a wide range of physical and chemical conditions. Water chemistry is characteristic, the soft oligotrophic waters usually being influenced by secondary compounds leached from the terrestrial vegetation. Nutrient levels range from extremely oligotrophic to mesotrophic (e.g. Britton, 1991), while pH levels range from as low as 4 in highly acid blackwater streams to 7 in clear neutral waters, and wetlands exhibit salinities from practically zero to hypersaline. Furthermore, given the climatic and altitudinal gradients across the region it is not surprising that there is a diversity of hydrological regimes across a range of spatial dimensions. These vary from small temporary pans to large open coastal lakes and estuaries, and from large perennial rivers to small, seasonal or ephemeral first-order streams.

The unusual chemical characteristics, which may have persisted for as long as the flora has been subjected to nutrient and water stress, may have been important in providing strong selective pressures on the genetic composition of the fauna, driving differentiation. As the Karoo sediments eroded to give way to the Table Mountain Sandstones, the different chemical characteristics were reflected in regions various water bodies. With most of the runoff derived mainly from the Table Mountain quartzite, it is thus deficient in calcium and magnesium carbonates, resulting in soft waters low in pH. This would have provided a strong selective pressure upon the local fauna driving the evolution of this now endemic fauna. Similarly, such conditions are bound to have been important in preventing the establishment and spread of other species. Many crustaceans, for instance, are unable to tolerate highly acidic waters,

while the lack of calcium has prevented the establishment of a large or diverse mollusc fauna.

Historical changes in climate, with the expansion and contraction of the tropical zone around the Equator, may also have been a factor in developing and maintaining diversity. Indeed, the Afromontane distribution of species related to those found in the CFK (e.g. Cowling & Holmes 1992) reflects the latitudinal and altitudinal requirements of the terrestrial flora and the aquatic fauna. The division between the Cape Folded Mountains in the west and the east, along with changes in the rainfall patterns, is also reflected in differences in water chemistry. Vicariant events would also appear to have been important in the differentiation and speciation of some groups, as evidenced by the unique and endemic fauna of Table Mountain and the Peninsula Mountain chain.

Post-Palaeozoic (< 248 MYr) erosion, along with geological stability during the Tertiary (~2-65 MY), resulted in erosion of the sandstones between False Bay and Table Bay, creating the Cape Flats and denuding the land bridge that connected the Peninsula range, including Table Mountain, to the closest fold of the Hottentot's Holland (Walker, 1952; Deacon, 1983; Theron *et al.*, 1992; Reid *et al.*, 1998). The Peninsula, including Table Mountain, is an area of about 470km^2 and is recognised as a global hotspot of biodiversity within the CFK for higher plants and invertebrates, with roughly 2285 plant species 90 of which are endemic (Trinder-Smith *et al.*, 1996). Table Mountain, which rises 1,111.4m at Maclears Beacon, usually only has permanent-running water on the Table or in the gorge streams below 300m. Condensation from heavy mountain mists in summer maintains the famous 'Table Cloth' which probably prevents the Table streams and some of the gorge streams from drying up altogether.

Harrison & Barnard (1971) examined the invertebrate fauna of Table Mountain and suggested that the land bridge would have been impassable to some species, such as *Phreatoicus capensis* and the gammarids. Assuming that selective pressures would be similar in both regions, they postulated that there would need to be very little gene flow to prevent speciation of an endemic Table Mountain fauna. The absence of any endemics among the insects in their survey led them to conclude that there must be at

least some active or passive dispersal to and from the mainland. More recent analysis, however, has shown there to be 111 endemic invertebrates to the Peninsula chain, most of which are centred on Table Mountain (Picker & Samways, 1996). Twenty-four of these are insects, of which 12 are aquatic. In all, there are 23 (including the two amphibian species) aquatic species endemic to the Peninsula mountain chain. The majority of these endemics inhabit typical palaeogenic zones: upper-reach forest streams, riverine forests and caves. This endemic fauna most likely represents the richest concentration of endemic invertebrate species for any small area in the world (Picker & Samways, 1996).

Thus it would appear that the aquatic ecosystems of the CFK have been subjected to a long period of stable climatic and geological isolation, both from each other and from those of the interior of the continent. Variability in the physical and chemical characteristics, along with the oligotrophic and often seasonal or ephemeral nature of the aquatic environments, has imposed strong selective pressures on their inhabitants and precluded invasions by exotic fauna. It is these features that provide the backdrop for the remarkable degree of endemism exhibited by the aquatic fauna of the CFK and from which four taxa were selected to examine the effects of catchments on the dispersal and genetic population structure of lotic organisms.

Study Species

Taxa were selected according to their potential for dispersal, which was inferred *a priori* using a number of criteria. It was also decided that those species selected should be well resolved taxonomically and from different Orders, to allow for independent comparisons of evolutionary history. These species also had to be present in the same localities and in sufficient numbers to facilitate rigorous statistical analyses. While a wide range of dispersal profiles exist among lotic organisms, it has proven difficult to try and determine the exact extent of the dispersal (see Palmer *et al.*, 1996). In marine systems, longevity of the larval stage is often equated to dispersal potential, with the end of the larval stage punctuated by settlement. In lotic ecosystems, larval life stages are not so rigid, nor are they punctuated by settlement and such inferences are difficult to draw. In the adult stage, dispersal has been variously inferred from morphological attributes, distribution and life history (den

Boer, 1970; Harrison, 1980; McLachlan, 1985; Malmqvist, 2000). Thus, the dispersal potential of the following taxa was inferred from the geographic range of the species, behaviour, morphology and life history characteristics.

Using these criteria four species were selected exhibiting a wide variety of life history traits, dispersal characteristics and population sizes. These included three winged insects and a fish (Figure 3.1). The dragonfly, *Aeshna subpupillata* (Odonata: Aeshnidae), represents a winged insect capable of wide dispersal. This is true with the larval and adult stages, both of which are robust and large relative to the other two species, being widely spread across large parts of southern Africa. The stonefly, *Aphanicerca capensis* (Plecoptera: Notonemouridae) is intermediate in its size and robustness relative to the other species and in its ability to disperse. Adults of this species are rarely found beyond the high humidity of the well-canopied stream margins while the larvae prefer the fast flowing riffle and run habitats. In contrast, the net-winged midge, *Elporia barnardi* (Diptera: Blephariceridae), is restricted in its distribution to the mountains of the south-western Cape. Representing the third of the insect species it has very limited powers of dispersal. Considered stenobionts, members of this group inhabit only cold, mountain streams where they are restricted to the riffle and cascade habitats at both the larval and adult stages. In addition to these three winged insects, a fourth species, the fish, *Galaxias zebratus* (Teleostei: Galaxiidae) was included. As a non-diadromous fish this species is restricted to movement within catchment units. Further detailed descriptions relating to each of these taxa is given in the introduction to the individual species chapters.

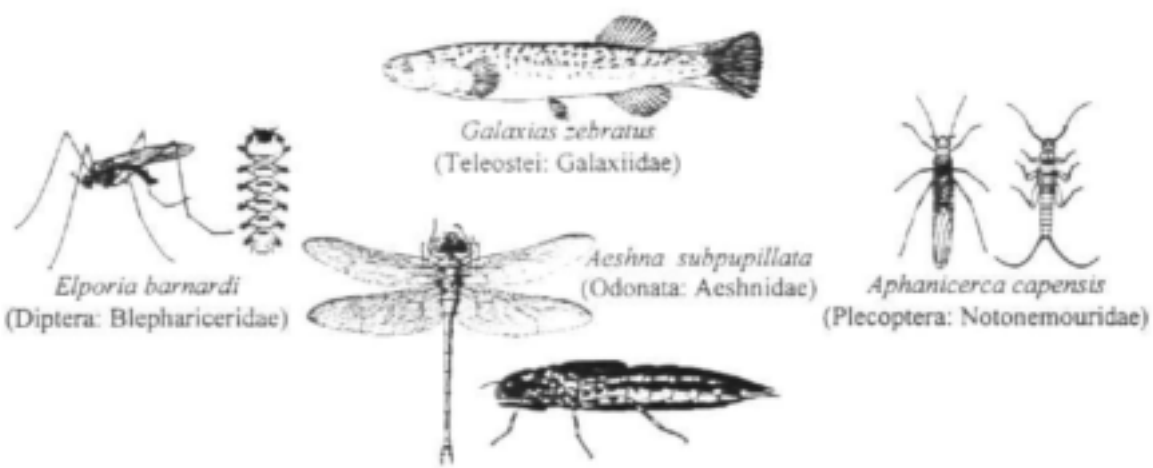

Figure 3.1. Aquatic species selected to reflect the range of dispersal characteristics.

Study Sites

While the sites sampled varied between each of the four taxa, individuals were collected from a minimum number of common sites in order to facilitate phylogenetic comparisons. Details of the exact sampling sites for each species are given in the specific chapters. Streams common to all taxa were located on Table Mountain and in the Jonkershoek catchment of the Hottentot's Holland (Figure 3.2a). Three sites in the Jonkershoek catchment were situated along the Eerste River with samples also collected from within the first order tributaries, Langrivier, and Swartboskloof (Figure 3.2b). Table Mountain is separated from the nearest source of permanent running waters, in the Jonkershoek catchment, by 60km consisting of urban settlement and the Cape Flats. Three streams on Table Mountain common to all taxa were sampled; Skeleton Gorge, Platteklip Stream and Kasteelspoort Stream (Figure 3.2c).

Contemporary climate characteristics of this region are typical of those of a Mediterranean-type climate, with cold, wet winters and warm, dry summers. While this is true, up to 25% of rainfall on the Peninsula can fall in the summer months (October to March). Average rainfall on Table Mountain areas is between 1000-2000 mm yr^{-1}, although rainfall gradients moving away from the mountain are very steep and average only 500-600 mm yr^{-1} on areas of the Cape Flats. Mean annual precipitation in Langrivier averages 2242 mm (Bosch & Hewlett, 1982) and exhibits a strong seasonal signal, with less than 10 % of precipitation falling between the summer months of December and March (van der Zyl, 1971). Temperatures are similarly variable although they do not show strong spatial or temporal variations. This is due largely to the influence of the ocean and the wind regime. Mean average temperatures on the mountain summit are around 16°C and 22°C at sea level. Mean maximum and minimum temperatures vary by about 10°C.

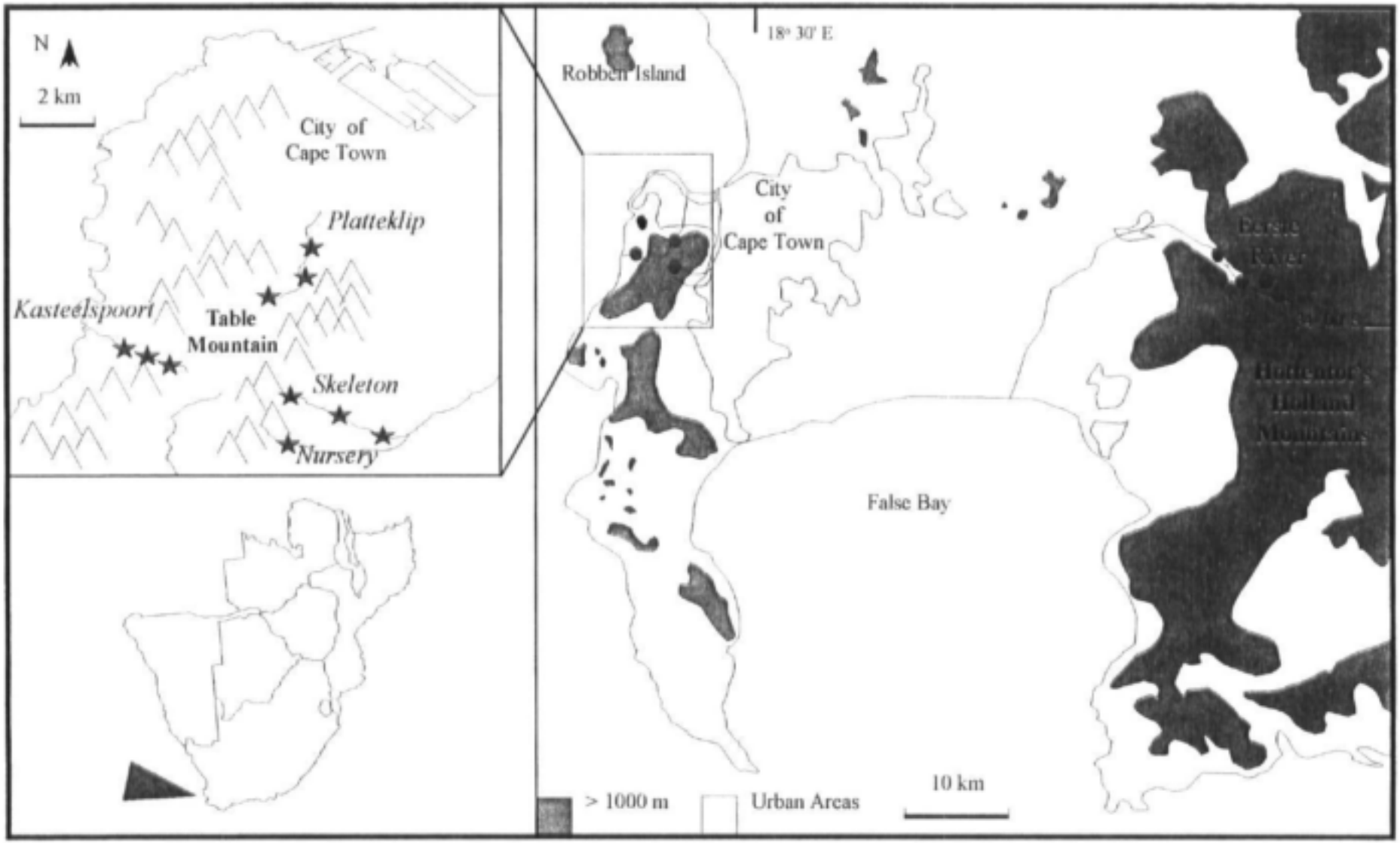

Figure 3.2. Map of the streams in the southwestern Cape, South Africa, from which organisms were collected.

Collection

Due to the density and life history traits of individual species, collections varied slightly. However, in each instance between 30 and 50 individuals of each taxon were collected. Sites included varied slightly depending on the presence or absence of each taxon (see above). Due to the low densities and longer life cycles of the odonate, *Aeshna subpupillata*, and the fish, *Galaxias zebratus*, 30 to 50 individuals were collected from a single site within in the lower foothill zones of each of the streams. However, in order to examine the effects of stream structure, both *E. barnardi* and *A. capensis*, found in greater numbers, were sampled from three sites in each of these streams. The only exception was the first order tributaries in the Jonkershoek catchment from which samples were collected from a single site. These sites were typically no more than 750m stream length apart and represented the upper and lower limits of the species range along with an intermediate site mid-way between these.

Molecular Techniques

While genetic techniques have now long been utilised in the examination of individuals and populations, their extrapolation and employment in the study of freshwater systems has been fairly limited. In particular, research into freshwater insects has been lacking. In an examination of Aquatic Resources CD-Rom based literature database between 1971 and 1989, a total of 1,725 papers were recovered using the search string "freshwater and genetic". Only 85 of these papers were related to invertebrates, with 14 of these having to do with river ecosystems. In comparison, 1,358 records were related to the genetics of freshwater fish. Between 1991 and 1998 there was a marked increase in the number of records (3,053) dealing with the genetics of freshwater ecosystems, although these were still predominately dealing with the genetic structure of fish populations (2,542). Very few records addresses genetic issues relating to freshwater invertebrates (175) and fewer still with freshwater insects (38).

Clearly there has been a bias in the genetic study of freshwater organisms toward fish, with the trend primarily related to commercially important species. This fact is borne out in the high proportion of invertebrate studies that deal with commercially

important species such as crayfish and the like. While the commercial application of these techniques obviously has important management implications relating indirectly to the conservation of species through proper population management and controls, the incorporation of molecular methods in the conservation of freshwater systems has been largely ignored. The incorporation of genetic techniques into conservation has been focussed on large, high profile species and fish stocks that grab the attention of the public at large, with much of the work carried out thus far focussed around the systematics of specific groups.

There are a number of comprehensive texts that explain the techniques used to examine the genetic structure of organisms and their populations and much of the information presented below has been taken from these (i.e. Richardson *et al.*, 1986; Hartl & Clarke, 1989; Speiss, 1989; Hillis *et al.*, 1996; Avise, 2000). The aim is to provide a summary of the information required for an understanding of the techniques used in this project and background information into genetic, or molecular, ecology. Two methods are used in the following report and described here. The first is allozyme electrophoresis, which provides a measure of nuclear DNA diversity, through examination of the mobility of proteins that are used as a reflection of an individuals genetic composition. The second technique within the mitochondrial DNA (mtDNA) examines and compares individual nucleotide sequences.

Deoxyribonucleic Acid (DNA)

Commonly referred to as DNA, deoxyribonucleic acid provides the foundations for the structural organisation of all organisms. While genetic influences over the morphology and inheritance of traits and the influence over variation within natural populations have been of interest since the time of Darwin and Mendel, it was not until the discovery of the structure of DNA by Watson and Crick in 1953 that molecular methods became of importance. It then took more than ten years to develop an understanding of the levels of variation that exist within natural populations. In 1966 two papers (Hubby & Lewontin, 1966; Lewontin & Hubby, 1966) examining the molecular variation in natural populations using protein electrophoresis were published and so the use of molecular methods in population genetics and systematics began.

Four different repeating subunits, or nucleotides code for information which, depending on their arrangement, encode for other biological molecules, such as proteins. Proteins reflect the end point products of a complex series of pathways that begin with the information encoded in deoxyribonucleic acid (DNA), through mRNA to amino acid sequences. The repeating subunits that make up DNA, nucleotides, consist of a deoxyribose molecule, a phosphate and one of four nitrogeneous bases, guanine (G), cytosine (C), adenine (A) or thyamine (T). These bases are always paired in a specific manner, with guanine always paired with cytosine and adenine always paired with thymine. The arrangement of nucleotides along a strand of DNA then codes for amino acids. Nucleotides are arranged such that a group of three nucleotides code for a single amino acid. There are 20 common amino acids in nature and proteins comprise a specific sequence of between 50 and 2,000 amino acids which all combine to create characteristics unique to a particular protein. These triplet units of nucleotides are known as codons and there are 64 possible triplets of these four nucleotides taken three at a time (i.e. CCC, CCA, CAA etc). Since the number of codons exceeds the number of amino acids, more than a single codon may encode the same amino acid. In diploid organisms, each protein is encoded once on each of two homologous chromosomes. Such proteins and other secondary molecules represent the second level of organismal organisation. The developmental and metabolic processes controlled by these secondary molecules then influence the gross morphology of an organism, its behaviour, reproductive biology and habitat requirements etc, representing the third level of organismal organisation.

As the most abundant organic molecule, proteins perform many different functions in the body. Structural proteins, such as collagen and keratin are associated with the skin, connective tissues and hair. Another form of protein function involves the regulation of growth and the transport of elements through body such as oxygen by haemoglobins. The most common form though, and those used for electrophoresis are enzymes. Enzymes are responsible for the catalysis of metabolic reactions such as those involved in the breakdown of starch, or glucose into water and carbon dioxide. Enzymes are highly specific in the reactions they facilitate and the substrata they modify. Enzymes that consist of different primary sequences of amino acids, but which facilitate, or catalyse the same reaction using the same substrate are called *isoenzymes* or *isozymes* (Markert & Moller, 1959). Enzymes encoded by different

alleles of the same locus belong to a class of isozymes called *allozymes*. The information encoded in the DNA for a specific character is loosely referred to as a gene. Variants of this information may exist in a population and these alternate forms are referred to as *alleles*. The position of a gene on a chromosome is called a *locus* (pl. loci). These terms, gene and locus are often used interchangeably.

A given population may include many different forms of a gene at a particular locus, however each individual will only carry two genes, or alleles. If these alleles are the same on the two homologous chromosomes, that individual is said to be *homozygous* for that particular allele. If the alleles are different then the individual is referred to as being *heterozygous*. Thus the frequency and geographic distribution of various alleles allows inferences to be made about a population's genetic structure.

Allozyme Electrophoresis

Allozyme electrophoresis is a technique that examines the migration of proteins (e.g. enzymes) through a gel under the influence of an electric field. The amino acids that make up protein sequences are joined together by covalent peptide bonds to form polypeptides, which are genetically determined. Allozymes are variants of polypeptides representing different allelic alternatives of the same gene locus. Each of the 20 unique amino acids have unique side chains characterised by size, shape and charge. It is these side chains that are responsible for the movement of the proteins through a matrix during electrophoresis.

All electrophoretic techniques involve an electrical power supply, a support matrix (cellulose acetate gel or strips, starch gel etc.), and ionic buffers. The application of an electrical current to opposite ends of the suspension medium via the ionic buffers causes the proteins to move depending upon several factors including size and charge. Those molecules, or proteins, having a net positive charge (cations) migrate to the cathode and negatively charged proteins (anions) migrate to the anode. After application of a current the individual proteins are selectively stained. These histochemical stains provide a specific substrate for the enzyme, or protein, allowing it to catalyse the particular reaction. A dye is then applied that is used to visualise the movement and position of the protein molecule.

Mitochondrial DNA

With the advent of the Polymerase Chain Reaction (Saiki *et al.*, 1985; Mullis *et al.*, 1986) studies of mtDNA variation have become a relatively straight forward and accessible, providing a powerful technique for the examination of genetic structure in natural populations. As a result, there a large number of reviews summarising the major features of mtDNA (Avise, 1986; Avise *et al.*, 1987; Moritz *et al.*, 1987; Harrison, 1989). In short, assuming that mtDNA markers are neutral and that back mutation is negligible, mtDNA provides a sensitive indicator of female mediated gene flow, founder events and other population processes. As a result of these factors mtDNA is expected to show greater differences than nuclear markers between demes, especially where the female individuals are more sedentary than the males and within species diversity is generally expected to be high. Low levels of mtDNA diversity would suggest either frequent dispersal by females or recent bottleneck in the species.

The basis of mtDNA studies is the examination of variation within the individual nucleotide sequence among particular individuals of the same or different taxa. Mutations create variation in the nucleotide sequence of a particular individual. Selection pressure on DNA sequence variation in regions that code for functional proteins or enzymes is higher than on non-coding regions, although selection acts mainly at the amino acid level. Protein sequences are derived from the translation of the nucleotide sequence, with each amino acid in the protein sequence coded by three consecutive nucleotides. However, the genetic code is degenerate which means that the majority of amino acids can be translated from several different combinations, which mostly differ in the third position. The consequence is that at the DNA level, third codon positions often have the least selective constrain and change rather frequently, where as second positions are most conserved.

Within any group of organisms, mutations (permanent changes of the DNA sequence) accumulate over time. As a result, the DNA sequences of two lineages become progressively more different as the time since they shared a common ancestor progresses. The comparison of the nucleotide sequence of the same gene (or DNA region) between species, populations or individuals, therefore allows the reconstruction of their phylogenetic relationships.

The first step is to extract the DNA from either a tissue sample or the whole organism. There a number of different techniques, variously adapted for different taxonomic groups (see Palumbi, 1996 for a review). Samples can be taken from fresh material, dried samples, those fixed in ethanol or in some instances formalin preserved samples. In this process the nucleic acids are separated from the cell proteins, lipids and other organic and inorganic content.

The Polymerase Chain Reaction (PCR) then allows a particular region of the DNA to be amplified several million times. The technique involves three different steps at different temperatures. The first step, denaturation, involves the separation of the double stranded DNA which is follwed by the annealing stage, where a short DNA fragment, the primer, is bound to a specific region of DNA. Then the thermostable *Taq* polymerase enzyme synthesises a complementary strand, starting at the primer. After a single PCR cycle the number of copies of the target region has doubled, after 2 cycles it has increased four times and so on, until after 30 or more cycles the PCR region has been amplified several million times.

The primers are designed specific to particular regions of the DNA. If there is no prior knowledge of the sequence from which specific primers can be designed, 'universal' primers for conserved regions of DNA found across a wide range of taxa can be used to amplify and obtain sequence data from which more specific primers can then be designed. Alternatively there are other techniques available, such as RAPD (Random Amplified Polymorphic DNA), in which short primers bind randomly to several loci in the DNA rather than to a specific site.

The amplified DNA, or PCR product, can then be analysed further using either restriction fragment length polymorphisms (RFLPs) or DNA sequencing. For RFLPs restriction enzymes cut nucleic acids at specific sites, mostly comprising 2 to 4 consecutive nucleotides (e.g. AATT). If a mutation has occurred at a site being specific for this restriction enzyme, the banding pattern of the RFLP changes, and this can be made visible by gel electrophoresis and subsequent staining of the DNA fragments. If the mutation has occurred elsewhere, it will remain undetected. DNA sequencing is nowadays usually done according to the Sanger 'chain termination method' on automated machines. It provides the exact nucleotide sequence of the

entire PCR product by way of specially marked primers or nucleotides and highly sophisticated equipment and software.

Genetic Methods

Allozyme Electrophoresis

Two different methods were used. Cellulose acetate electrophoresis (Titan III plates, Helena Laboratories) was used to examine genetic population structure in *Elporia barnardi*. Individual larvae were homogenised by hand with 20µl of grinding buffer (2.44 g Trizma base, 0.37 g EDTA free acid, 5.36 NH_4Cl, 19.80 g glucose, 20 ml 0.022 M NaN_3 in 1 L H_2O). More detailed descriptions are given in Chapter 5.

Starch gel electrophoresis was used to examine the genetic population structure in *A. capensis*, *A. subpupillata* and *G. zebratus*. Whole individuals or samples of tissue were homogenised in 0.01M Tris buffer, pH 8, using a glass rod attached to a portable, variable speed motor. Samples were centrifuged at 25000 x ***g*** for 5 minutes and then stored in plastic Eppendorf tubes at –80°C until electrophoresis. Filter paper wicks (Whatmans #3) were dipped into the supernatant of the sample and inserted on to a horizontal starch gel (12% hydrolysed potato starch). Gels were run in a refrigerator with a constant temperature of 4°C for between 4 and 5 hours at a current of between 40 and 50mA. Gels were then sliced and the sites of enzymatic activity stained using specific chemical reagents in agar overlays. All stain recipes were taken from Shaw and Prasad (1970). Alleles and loci (when more than one was present) were numbered in order of increasing electrophoretic mobility. Allele designations were confirmed by running presumed homologues side by side on the same gel (i.e., line-up gels, *sensu* Richardson *et al.*, 1986).

MtDNA

Extraction

Total genomic DNA was extracted from randomly selected individuals within each of

the streams. DNA was extracted from small amounts of tissue using either a modified CTAB extraction method (Reineke *et al.*, 1998) or Chelex (Bio-Rad). For Chelex extractions approximately $1mm^2$ of tissue was added to 100µl of Chelex and ground with a sterile pestle. An additional 700µl of 5% Chelex was then added with 10µl 20mg/ml Proteinase K and left overnight at 58°C. Chelex was then boiled at 100°C for 15 minutes and spun for three minutes at 13500rpm.

Polymerase Chain Reaction (PCR) Amplification

The cytochrome oxidase *c* subunit I fragment of the mtDNA was used to infer intra-specific phylogenies in all four taxa. Polymerase chain reaction (PCR) amplification of double-stranded product was carried out in a Perkin Elmer thermocycler using primers designed to amplify a 710-bp fragment of the mitochondrial DNA cytochrome *c* oxidase subunit I (LCO1490, 5′-GGTCAACAAATCATAAAGATATT GG-3′, and HCO2198, 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′: Folmer *et al.*, 1994). In addition, a fragment of the cytochrome *b* region of the mtDNA was examined in *G. zebratus*.

Cycling conditions included an initial denaturing step of 94°C for 5 min, and then 94°C for 30sec, annealing at 50°C for 30 sec, extension at 72°C for 1 min for 35 cycles. A final cycle included a 5-min extension at 72°C. Reactions were carried out in 50 µl reaction volumes containing 5 µl of 10X reaction buffer (100 mm Tris-HCl pH 8.3 and 500 mm KCl) (Biotech International Limited), 1 µl of dNTPs (Biotech International Limited), 2 µl 50mM $MgCl_2$ (manufacturer), 1 unit of *Thermus aquaticus* DNA polymerase (Biotech International Limited), 2 µl of DNA template and 2 µl of each primer (10mM), the balance being made up with double distilled water. Double stranded products were gel purified using Qiagen gel purification columns (Qiagen Pty Ltd, Victoria, Australia) following the manufacturers specifications.

Sequencing

Estimates of the resulting DNA concentrations were made by running purified PCR product on a 1.6% agarose gel (with ethidium bromide) next to a known marker, and

visualised under ultraviolet light. Direct sequencing of purified products was achieved via cycle-sequencing (Perkin Elmer, BigDye terminator, 3.2pmol primer, 5-20ng DNA) and sequenced using an Applied Biosystems 377 automated sequencer. All fragments were sequenced in both directions and manually aligned by eye using BioEdit (Hall, 1999). Useable fragments varied slightly in length between taxa and are listed in the individual chapters.

Statistical Analyses

Allozymes

Data Summary

Allozyme allele frequencies were calculated and from these, average unbiased estimates of genetic identity (*I*) (Nei, 1978) were derived using BIOSYS-2 (Swofford & Selander, 1997). Allele frequencies at each site were represented using pie charts while the unweighted pair group method algorithm (UPGMA) was then used to construct a population tree to represent the relationships between the sites.

Tests for Hardy-Weinberg Equilibrium and Linkage Disequilibrium

Wright's estimate of F_{IS} is the correlation of alleles within individuals within one population and can be used to compare genotypic frequencies with those expected under Hardy-Weinberg equilibrium. Exact P-tests were performed to test for departures from Hardy-Weinberg equilibrium using GENEPOP (Raymond & Rousset, 1995). Genotypic linkage disequilibrium for pairs of loci at each population was also assessed using GENEPOP to test the independence of allozyme loci.

Population Sub-division

The structuring of populations within streams, between streams and between ranges was examined using *F* statistics (Wright, 1965, 1978; Nei, 1977; Weir & Cockerham, 1984). These can be considered a measure of the proportion of the genetic variation that is among populations. Values may vary between zero, indicating no difference in

allele frequencies between populations, and one, reflecting fixed differences between populations. The programme FSTAT (Goudet, 1999) was used to assess the genetic structure of populations using a hierarchical analysis based on the method of Weir and Cockerham (1984). F_{ST} values were calculated for each level of the hierarchy using jack-knifing procedures to obtain standard deviations and determine significance. A permutation procedure in FSTAT assesses significance of F_{ST} comparisons by comparing observed values against a distribution of the null hypothesis (F_{ST} not > 0). Hierarchical *F*-statistics were used to examine levels of differentiation at various levels within and among catchments.

Isolation by Distance

The stepping-stone model of isolation by distance (Kimura, 1953; Kimura & Weiss, 1963; Slatkin, 1993) proposes that measures of genetic differentiation at neutral loci will increase with geographic distance. At equilibrium between gene flow and genetic drift the level of genetic differentiation (measured as $M\hat{}$) should be inversely related to the geographic distance between populations, where $M\hat{}$ is defined by Slatkin (1993) as:

$$M\hat{} = \tfrac{1}{4}(1/F_{ST}-1)$$

Geographic distance was estimated from grid co-ordinates using a global positioning system accurate to within ±100m. Due to the non-independence of pairwise comparisons between geographic distance and $M\hat{}$, significance was determined using Mantel's test (Manly, 1997). Ordinary least-squares regression was used to estimate the slope and intercept of the relationships.

MtDNA

Nucleotide diversity and divergence within and among populations

Sequences were manually aligned using BioEdit (Hall, 1999). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.0 (Kumar *et al.*, 2001). MEGA was used to identify positions of variation among individual

sequences and thus distinguish haplotypes. Preliminary examination of the sequence data revealed intermediate levels of diversity ($0.05<d<0.3$) and transition (TS):transversion (TV) ratios greater than 2. Jukes-Cantor estimates are considered suitable only if there is a low TS:TV ratio. When this ratio is greater than two Kimura's 2-parameter model (1980) is considered more appropriate. As such, net nucleotide divergence between pairs of populations, and nucleotide diversity within each population were calculated using the Kimura 2-parameter model (Kimura, 1980) of substitution and pairwise sequence divergence matrices constructed using MEGA's distance matrix function.

Genetic Distance and Relationships

Mid-point rooted neighbour-joining (NJ) trees (Saitou & Nei 1987) of unique mtDNA haplotypes were constructed in MEGA from the resulting matrix. Bootstrap tests were performed (1000 replicates) to test relative node support for the resulting phylogeny.

Haplotype cladograms were also constructed to provide more detailed examination of the distribution and relationship of haplotypes among the sites. These provide a sensitive means of reflecting the relationships among closely related maternal lineages displaying the number of base pair differences between major haplotypes. These were generated using the TCS computer program (Clement *et al.*, 2000) based on the cladogram estimation algorithm described by Templeton *et al.* (1992). This program collapses sequences into haplotypes and calculates the frequencies of each in the sample. These frequencies are then used to estimate haplotype outgroup probabilities, which subsequently correlate with haplotype age (Donnelly & Tavaré, 1986; Castelloe & Templeton, 1994).

The rate of sequence divergence in the mtDNA has been variously inferred. A survey based on arthropod mtDNA estimated the rate of sequence divergence at 2.3% pairwise divergence per million years (Brower, 1994). However, more recent estimates, derived from *Alpheus* shrimps across the Isthmus of Panama, put the rate of divergence in the COI region at about 1.4% per Myr (Knowlton & Weigt, 1998). The divergence times between lineages were subsequently calculated from uncorrected pairwise values and calibrated using both of these estimates.

Analysis of MOlecular VAriance (AMOVA)

MtDNA sequence data reveals additional information about the evolutionary relationships among haplotypes, as well as their frequency in each population. Using the Arlequin program (version 2.1) (Schneider *et al.*, 1997) mtDNA variation among populations was partitioned using the Analysis of MOlecular VAriance (AMOVA) approach of Excoffier *et al.* (1992). This allows examination of a proposed population structure based upon a hierarchical model of gene flow. Specifically, the proportion of genetic diversity distributed among drainages, among sites within drainages and within sites was determined using mtDNA haplotype frequency data incorporating the Kimura 2-parameter (Kimura, 1980) measure of distance among haplotypes. Such analysis calculates the following Φ statistics;

Φ_{ST} – the proportion of genetic variation between all sampled sites

Φ_{SC} – the proportion of genetic variation between sites within groups

Φ_{CT} – the proportion of variation between groups within the total sample

The significance of the fixation indices was calculated as described in Schneider *et al.* (1997) and references therein.

Chapter 4

The Cape Galaxiid
Galaxias zebratus
(Teleostei: Galaxiidae)

Introduction

The freshwater fish fauna of the south-western Cape is recognised as a centre for a distinct "Cape" component of the African ichthyofauna (Skelton, 1994). While the Cape Floristic Kingdom (CFK) has a relatively low number of species, with only 19 primary freshwater fish species, 16 of these are endemics (84%). In context, only 11% of the South African fish and 5% of the southern African fauna are considered endemic. There are a further 16 species of alien fishes also found in the region, including 12 invasive species. As a results of this and other factors, 11 of the native fish species are critically endangered or endangered, with another three considered vulnerable and one, *Galaxias zebratus* (Figure 4.1), near-threatened.

Figure 4.1. The Cape galaxiid, *Galaxias zebratus* (Teleostei: Galaxiidae)

With about 45 species in 6 genera, the Galaxiidae are most diverse and best represented in Australia and New Zealand. Representatives of the group are also found in South America and in Africa, with a single species, *Galaxias zebratus*, found at the southern tip of Africa. While some galaxiid fishes are freshwater, including the Cape galaxiid, there are some estuarine and diadromous species. Although there have been suggestions that marine dispersal may explain the distribution of the galaxiids (McDowall, 1973), Barnard (1943) and Rosen (1974) have suggested the group had Gondwanan origins. The relict status of this species would indeed appear to be supported by the phylogenetic affinities of *G. zebratus* (Waters unpublished, see Waters & Cambray, 1997).

Restricted to mainly lowland areas, *Galaxias zebratus* is found over roughly 600km, from the Krom River to the Clanwilliam-Oliphants system (Figure 4.2). Occupying a wide range of habitats, from headwater streams to lakes, *G. zebratus* is tolerant of both alkaline and acid conditions. It does, however, favour gentle currents within sheltered banks near the head of pools and, although it is able to penetrate inland and inhabit the foothill and mountain stream zones, is primarily restricted to lowland reaches. This has enabled populations of *G. zebratus* to inhabit the alkaline waters of the Cape Flats, from where the type specimen was described, as well as the acidic mountain streams of the Cape fold mountains.

Much confusion surrounds the taxonomy of *G. zebratus*. Four species were originally described from the south-western Cape region, defined on the basis of morphological differences. These included the *G. zebratus* Castelnau, 1861, *G. punctifer* Castelnau, 1861, *G. capensis* Steindachner, 1894 and *G. dubius* Gilchrist & Thompson 1917. Subsequently, Scott (1936, 1966) recognised two species, while Whitley (1956) noted all four. However, morphological plasticity in galaxiid fishes is common (McDowall & Frankenberg, 1981) and in an extensive revision of the Cape galaxiids Barnard (1943) noted 'extreme difficulty in finding clear-cut and constant characters of specific value'. While Barnard had recognised two forms, *G. punctifer* and *G. zebratus*, he subsequently concluded that the Cape galaxiid comprised a single variable species, *Galaxias zebratus*. This conclusion was subsequently supported through morphological and osteological data (McDowall, 1973). He went on to suggest that the low number of vertebrae (39-41) might justify separate generic ranking. Similarly, the presence of six pelvic fin rays rather than the seven typical of other galaxiids led Scott (1936, 1966) to propose a subgenus. More recently, Waters and Cambray (1997) examined mitochondrial DNA from five populations of the Cape galaxiid across its range. They found some of the highest values of mtDNA sequence divergence recorded for fish, ranging between 5.8 and 13.8%, suggestive of interspecific and intergeneric comparisons.

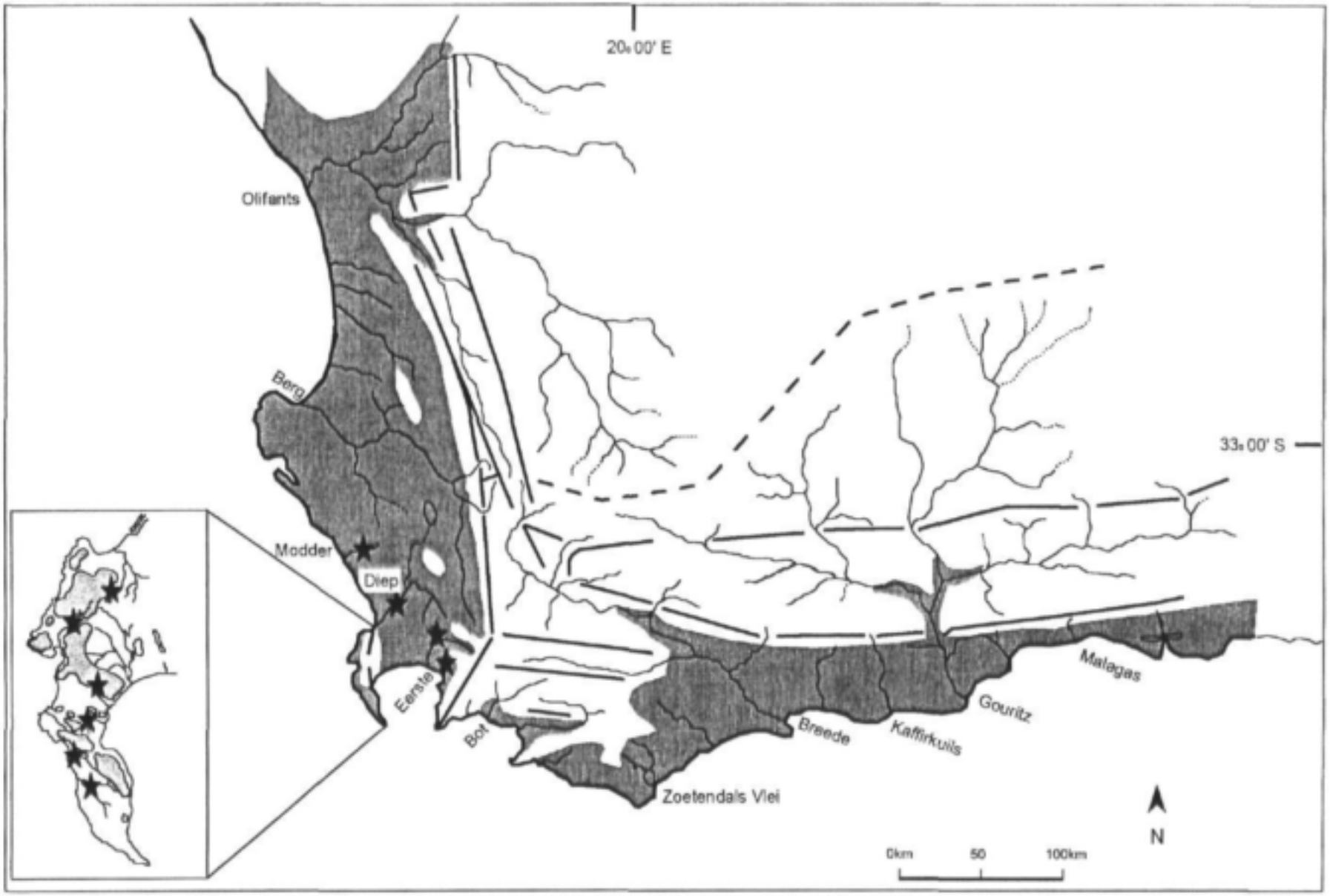

Figure 4.2. Distribution of the Cape galaxiid, *Galaxias zebratus* (Teleostei: Galaxiidae) ★ sampling sites.

The aim of this section was to examine the patterns of genetic variation across small spatial scales among various populations of *G. zebratus*. The use of allozyme electrophoresis and direct nucleotide sequencing of the mtDNA provide independent estimates of the degree of genetic differentiation. Furthermore, the use of estimated rates of divergence in the mtDNA allow inferences to be made as to the possible timing of various events and the processes involved in the formation of these patterns.

Methods

Collection

Between October and November 1999 roughly 30 individuals were collected from six streams on the Cape Peninsula, two streams in the Hottentot's Holland range and another two streams on the west coast (Figure 4.2; Table 4.1). Streams sampled on the Peninsula range included Klaasjagers, Schusters and Glencairn at the southern end of the Peninsula and the Liesbeek, Disa and Silvermine rivers on Table Mountain. In the Hottentot's Holland samples were collected from the Eerste and Lourens rivers. The two streams sampled on the western side of the Cape Flats included the Diep and Silverstroom rivers (Figure 4.2; Table 4.1). Fish were typically collected from along the sheltered and often-vegetated stream margins using a hand-net (950μm-mesh size), returned to the laboratory live and stored at -80°C.

Table 4.1. Descriptive summary of sampling sites for populations of *Galaxias zebratus* (Teleostei: Galaxiidae).

	Longitude	Latitude
Klaasjagers	34°14'	18°24'
Schusters	34°12'	18°24'
Glencairn	34°08'	18°25'
Silvermine	34°06'	18°25'
Disa	34°01'	18°24'
Liesbeek	33°59'	18°25'
Diep	33°47'	18°33'
Silverstroom	33°35'	18°21'
Eerste	33°57'	18°56'
Lourens	34°02'	18°56'

Allozyme electrophoresis

Muscle tissue was removed from the tail, with an excision made from behind the stomach to the base of the caudal fin on one side. Starch gel electrophoresis was used to screen 26 different enzymes. Of these 14 yielded consistently interpretable results giving a total of 18 loci that were retained for further analysis (Table 4.2).

Table 4.2. Enzymes and running conditions used in the allozyme electrophoretic analysis of *Galaxias zebratus* (Teleostei: Galaxiidae). Buffer systems used were: MF: Tris-Borate-EDTA (Markert & Faulhaber, 1965); RW: Lithium Hydroxide-Borate (Ridgway *et al*., 1970); TC: Tris citrate (Whitt, 1970).

Protein	Abbr.	E.C.Number	Run Buffer
Alcohol dehydrogenase	*ADH*	1.1.1.1	TC
Sorbitol dehydrogenase	*SDH*	1.1.1.14	TC
Lactate dehydrogenase	*LDH*	1.1.1.27	RW
Malic enzyme	*ME*	1.1.1.40	MF
Glucose dehydrogenase	*GDH*	1.1.1.47	TC
Glyceraldehyde-3-phosphate dehydrogenase	*GAP/ G3PDH*	1.2.1.12	TC
Aspartate Aminotransferase	*AAT*	2.6.1.1	RW
Esterase	*EST*	3.1.1.1	MF
Peptidase Glycyl-leucine	*PepGL*	3.4.11.-	MF
Peptidase Leucyl-Tyrosine	*PepLT*	3.4.11.-	MF
Aldolase	*ALD*	4.1.2.13	TC
Glucose-6-phosphate isomerase	*GPI*	5.3.1.9	RW
Phosphoglucomutase	*PGM*	5.4.2.2	MF
Leucine amino peptidase	*LAP*		MF

MtDNA

Small tissue samples (~1mm in size) were taken from five individuals from each of the streams. A fragment of the cytochrome *c* oxidase subunit I (COI) and cytochrome *b* regions of the mtDNA was then examined using the protocols outlined in the general methods (Chapter 3). The cytochrome *b* region was examined in order to facilitate comparisons with data provided by Waters and Cambray (1997). They sequenced a single individual from each of five sites across the range of *G. zebratus* (Figure 4.2) and these were obtained from the GenBank Data Library (Accession Nos. U66609-66613).

Results

Allozyme Data

Of the 18 loci retained for further investigation, 12 were considered polymorphic with frequency of the most common allele less than 95%. Eight of these polymorphic loci displayed fixed differences for one of two alleles among sites, while four displayed polymorphism within at least some sites (Appendix 1). No deviations from those frequencies expected under the assumptions of Hardy-Weinberg equilibrium were detected for any of the variable loci in any of the sites (Table 4.3). Similarly, no genotypic disequilibrium was observed.

Table 4.3. F_{IS} values for the four polymorphic loci in populations of *Galaxias zebratus* from streams in the south-western Cape, South Africa.

	PGM	*GPI*	*MDH3*	*ME*
Klaasjagers	-0.01	-0.04	-	-
Schusters	-0.083	-0.18	-	-
Glencairn	-	-	-	-
Silvermine	-	-	-	-
Disa	-0.21	-	0.06	0.04
Liesbeek	-	-	-	-
Diep	-	-0.06	0.15	-
Silverstroom	-	-0.07	-	-
Eerste	-	-	-	-
Lourens	-	-	-	-

Using allele frequency data from the 18 different loci, genetic identity and distance values were calculated (Nei, 1978) and dendrograms constructed. The ten populations separated into two distinct groups (Figure 4.3; Table 4.4), with populations from Klaasjagers, Schusters and Glencairn streams grouping out with those from the Silverstroom and Diep rivers. This group was distinct from those of the second group (Genetic Identity = 0.41) comprising populations from the Table Mountain sites, the Disa, Silvermine and Liesbeek rivers, and the Eerste and Lourens rivers of the

Hottentot's Holland range. The F_{ST} value for among all sites was 0.95 ± 0.02. As a result of the degree of genetic divergence between these two groups it was decided they be treated separately for subsequent analyses. Thus, for each of the two forms F_{ST} values were calculated among streams within a geographic range, this included the Cape Peninsula, Western Cape/Cape Flats, Hottentot's Holland and the Table Mountain groups. F_{ST} values were also calculated among streams within each of these geographic regions.

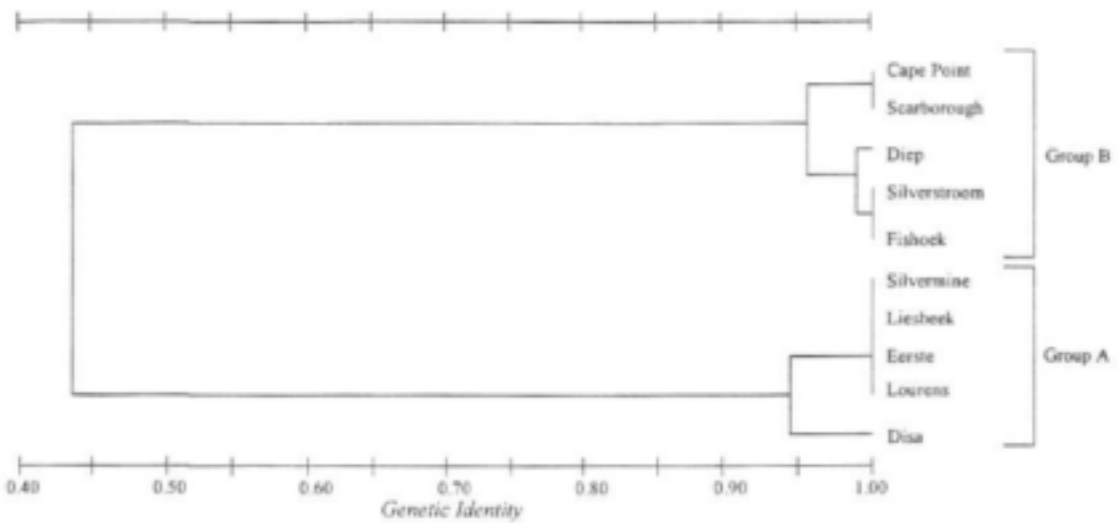

Figure 4.3. Phenogram showing the results of UPGMA analysis based on Nei's genetic identity for *Galaxias zebratus* (Teleostei: Galaxiidae).

As is evident from Table 4.5, significant levels of genetic sub-structuring were observed among streams within each of the two separate forms. The F_{ST} values among streams within each of the both groups were similar. F_{ST} values of 0.68 ± 0.17 and 0.73 ± 0.29, were calculated for Group A and B respectively. Both these groups consist of two geographically disjunct sub-groups (see Figure 4.3). F_{ST} values for among ranges and streams within these sub-groups also show significant levels of population sub-structuring (Table 4.6a and 4.6b). Streams from on Table Mountain and the Hottentot's Holland, comprising Group A, show a relatively lower, yet still significant level of population sub-structuring. This pattern of population sub-structuring in Group A is due to the polymorphic *MDH3* loci in individuals from the Disa River, with individuals from all other streams being monomorphic. The F_{ST} value calculated for between the two geographic sub-regions in Group B, the southern Peninsula and West Coast, display a much higher degree of sub-structuring, due

Table 4.4. Unbiased estimates of Nei's (1978) genetic identity (above diagonal) and genetic distance (below diagonal) calculated between populations of *Galaxias zebratus* (Teleostei: Galaxiidae).

Pop[a]	Klaasjagers	Schusters	Glencairn	Silvermine	Disa	Liesbeek	Diep	Silverstroom	Eerste	Lourens
Klaasjagers	***	0.99	0.95	0.96	0.96	0.44	0.41	0.44	0.44	0.44
Schusters	0.00	***	0.94	0.95	0.95	0.44	0.42	0.44	0.44	0.44
Glencairn	0.06	0.06	***	0.99	0.99	0.37	0.41	0.37	0.37	0.37
Silvermine	0.04	0.05	0.02	***	1.00	0.39	0.40	0.39	0.39	0.39
Disa	0.04	0.05	0.02	0.00	***	0.34	0.40	0.39	0.39	0.39
Liesbeek	0.83	0.81	1.00	0.94	0.94	***	0.94	1.00	1.00	1.00
Diep	0.88	0.88	0.90	0.91	0.93	0.07	***	0.94	0.94	0.94
Silverstroom	0.83	0.81	1.00	0.94	0.94	0.00	0.07	***	1.00	1.00
Eerste	0.83	0.81	1.00	0.94	0.94	0.00	0.07	0.00	***	1.00
Lourens	0.83	0.81	1.00	0.94	0.94	0.00	0.07	0.00	0.00	***

Table 4.5. Overall estimates of F_{ST} values for variation among all streams for each of the two groups recognised in *Galaxias zebratus*. (***NS)

	Group A	**Group B**
PGM	1.00 ± 0.65	0.76 ± 0.10
GPI	-	0.03 ± 0.03***
MDH3	0.93 ± 0.42	0.91 ± 0.40
ME	0.79 ± 0.35	-
Over All Loci	0.68 ± 0.17	0.73 ± 0.29

primarily to a higher degree of polymorphism (Table 4.6b). The higher F_{ST} values for the geographic sub-structuring in Group B is also due to the polymorphic *MDH3* loci which is monomorphic in the Cape Peninsula populations and the lack of polymorphism at the *PGM* loci in the Cape Flats populations.

Table 4.6a. Estimates of F_{ST} values for various geographic comparisons for *Galaxias zebratus* Form A in the south-western Cape, South Africa (# monomorphic comparisons and therefore unable to calculate). * $P < 0.001$

	Between Ranges	Within Table Mountain	Within Hottentot's Holland
PGM	0.23*	1.00 ± 0.54*	#
MDH3	0.12*	0.87 ± 0.36*	#
ME	0.11*	0.74 ± 0.30*	#
Over All Loci	0.19 ± 0.06*	0.68 ± 0.17*	#

Table 4.6b. Estimates of F_{ST} values for various geographic comparisons for *Galaxias zebratus* Form B in the south-western Cape, South Africa (# can not perform jackknifing with only two samples). * $P < 0.001$

	Between Regions	Within Cape Peninsula	Within Cape Flats
PGM	0.74#*	0.40 ± 0.49*	-
MDH3	0.00#	-	0.51#*
GPI	0.26#*	0.10 ± 0.06***	-0.01#
Over All Loci	0.76 ± 0.39*	0.55 ± 0.31*	0.68 ± 0.41*

The Disa River population is again responsible for a high degree of population sub-structuring observed among Table Mountain streams, with an F_{ST} value equivalent to that over all populations in Group A. Both the Lourens and Eerste rivers, comprising the Hottentot's Holland populations are monomorphic across all loci and it is therefor impossible to derive any local estimate of F_{ST}. F_{ST} values among streams within each of the geographic sub-groupings for Group B show higher F_{ST} values than those observed among streams in Group A. This is in part due to higher levels of polymorphism, and the fact that only a single allele was found in Glencairn individuals for both *PGM* and *GPI*, both of which were polymorphic in the Klaasjagers and Schusters populations. West Coast populations show high F_{ST} values indicative of significant population sub-structuring due to polymorphism at the *MDH3* loci in the Diep River population which was fixed for a single allele in the Silverstroom population.

MtDNA

Cytochrome c Oxidase I Sequence Data

Fifty-six 638-bp sequences from the cytochrome oxidase *c* sub unit 1 region COI region of *Galaxias zebratus* representing the ten sampled populations were examined revealing 18 putative haplotypes (Table 4.7). These sequences have been deposited in the GenBank Data Library (Accession Nos. *to be advised*). These were differentiated by a total of 61 variable nucleotide sites, of which 58 were parsimoniously informative with only three were singleton sites. The overall nucleotide composition included a T/C content of 56.3%. Fifty-two of these are 3rd codon position changes, with a single 2nd and eight 1st codon position changes. There was a transition:transversion ratio of 2.3, with 16 transitional and seven transversional pairs. Of these transitional pairs, 13 were in the 3rd position and three at 1st codon positions. All transversions were at 3rd codon positions.

Average distance between haplotypes within groups varied from zero to 0.2%, reflecting the low haplotype diversity, while average net distance between groups ranged from 0.1% to nearly 8% as seen between Klaasjagers and Eerste river populations (Table 4.8). The population tree for *G. zebratus* haplotypes shows two deeply-divided clades, supported by high bootstrap values, with roughly 7% sequence divergence between the two (Figure 4.4). Depending on the estimates of mtDNA divergence used, this corresponds to a period of separation of between 3 and 5 MYrs. The first clade includes populations from Table Mountain and the Hottentot's Holland, along with the Silverstroom population situated on the West Coast. The second clade comprises populations from the southern part of the Cape Peninsula and the Diep River population on the West Coast. This largely supports the pattern observed in the nuclear markers, with the exception of the Silverstroom population from the West Coast. The allozyme data also showed a deep divergence supporting two clades, but based on eight fixed differences, placed the Silverstroom population in the same clade as that including the Diep River population and those from the southern Peninsula.

Table 4.7. Variable nucleotide positions in the 18 *Galaxias zebratus* haplotypes of a 638-bp fragment of the mtDNA cytochrome oxidase *c* subunit I gene identified by DNA sequencing. Dots indicate identity to haplotype G1. Numbers refer to position of base pairs from the start of the fragment. Entire sequences can be retrieved from the GenBank database at accession numbers as indicated in the column to the far right of the table.

	1 1 1 1 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 6 6 6	GenBank
	1 2 3 4 6 7 8 8 5 7 8 9 2 5 6 7 8 8 9 0 0 1 1 2 2 3 3 4 4 6 6 7 8 8 9 9 0 1 1 3 4 5 6 7 8 9 9 0 0 2 2 3 3 3 5 5 7 7 0 1 2	Accession
	5 0 6 3 4 2 1 4 4 7 6 5 5 8 4 9 2 8 7 0 6 2 8 1 7 1 6 2 8 6 9 8 4 5 1 3 8 1 4 8 2 6 5 7 3 2 8 1 7 6 8 1 4 7 5 8 0 9 0 5 7	No[x]
G1	T T A T C A A G A C C G C T T T T A A A G A G A T T A T A A C T G T T A T C T T G T C G G C G T C C C A C C T A A C C A A	
G2	C C . . T	
G3	C	
G4	. . . . A	
G5	. . . . A . . . . G	
G6	. . . . G . . . . A . . . . G .	
G7	. . . . G . . . . A . . . . A . . . . G .	
G8	. . . . G . . . . A . . . . . . A	
G9	. . . . A . . . . . . A	
G10	. . . . C . . . . A . . . . . . A . . . A	
G11	. . . . C . . C . . . . A . . . . . . A . . . A	
G12	. . . . . . G . . . . G . . . . . C . . . . . . A . . . . T . .	
G13	C . T C . G G T . T G . T C C C A G . G A G T T . . C . C . T . A C C T A T C C A C T A T T T G T T A T T T . G G T . . G	
G14	C . T C . . G T . T G . T C C C A G . G A G T T . . C . C . T . A C C T A T C C A C T . T T T G T T A T T T . G G T . . G	
G15	C . T C . . G T . . G . T C C C A G . G . G T T . . C . C . T C A C C T A T C C A C T . T T T G T T A T T T C G G T . . .	
G16	C . T C . . G T . T G . T C C C A G . G . G T T . . C . C T T C A C C T A T C C A C T . T T T A T T A T T T C G G T . . .	
G17	C . T C . . G T . T G . T C C C A G . G . G T T . . C . C . T C A C C T A T C C A C T . T T T G T T A T T T C G G T . . .	
G18	C . T C . . G T . T G A T C C C A G . G . G T T . . C . C . T C A C C T A T C C A C T . T T T G T T A T T T C G G T . . .	

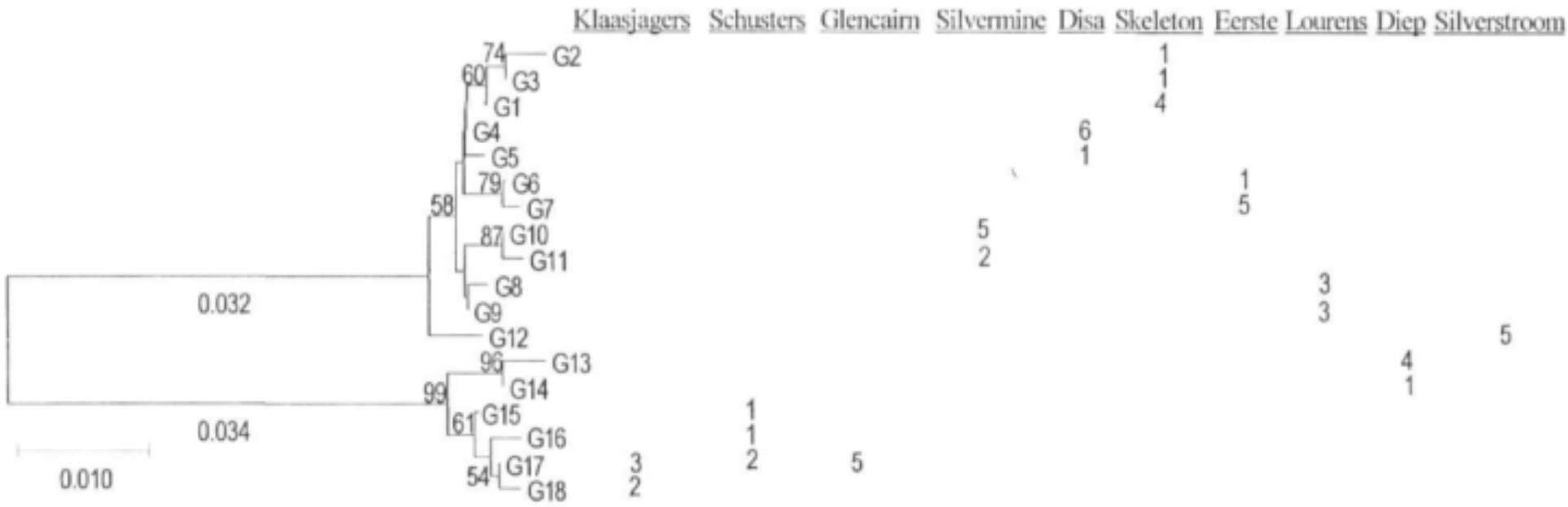

Figure 4.4. A mid-point rooted neighbour-joining tree, constructed using the Kimura-2-parameter (1980) model of substitution, for all COI haplotypes for *Galaxias zebratus* (Teleostei: Galaxiidae).

Table 4.8 Net nucleotide distance between groups (below diagonal) and standard error (above diagonal) and mean nucleotide distance within groups (on the diagonal) for 638-bp fragment of the COI region of the mtDNA. Distance among haplotypes was estimated using Kimura-2-parameter model (1980). See general methods section for more details.

	Klaasjagers	**Schusters**	**Glencairn**	**Silvermine**	**Disa**	**Skeleton**	**Eerste**	**Lourens**	**Diep**	**Silverstroom**
Klaasjagers	**0.000**	0.001	0.001	0.012	0.011	0.012	0.011	0.012	0.003	0.012
Schusters	0.001	**0.002**	0.000	0.011	0.011	0.011	0.011	0.011	0.003	0.011
Glencairn	0.001	0.000	**0.000**	0.012	0.011	0.012	0.011	0.012	0.003	0.012
Silvermine	0.075	0.074	0.075	**0.001**	0.003	0.003	0.003	0.002	0.011	0.004
Disa	0.075	0.073	0.074	0.005	**0.000**	0.002	0.002	0.002	0.011	0.003
Skeleton	0.076	0.074	0.075	0.007	0.002	**0.002**	0.003	0.002	0.011	0.003
Eerste	0.079	0.077	0.078	0.006	0.004	0.006	**0.001**	0.003	0.011	0.004
Lourens	0.074	0.072	0.073	0.004	0.002	0.004	0.006	**0.001**	0.011	0.003
Diep	0.009	0.008	0.008	0.077	0.077	0.078	0.080	0.075	**0.001**	0.011
Silverstroom	0.075	0.073	0.075	0.011	0.006	0.008	0.011	0.008	0.077	**0.001**

Although the support within each of these clades is not as strong, with low bootstrap values, each of the rivers in clade 1 exhibits a unique array of haplotypes, with the exception of a shared haplotype in the Lourens River and Skeleton Gorge populations. In the second of the two clades, the Diep River shows a distinct monophyletic catchment signature with two unique haplotypes, G13 and G14, not observed in any of the other populations. In comparison, the remaining streams that make up this clade share a common haplotype, G17. All three of these streams, Klaasjagers, Schusters and Glencairn, are located on the southern part of the Cape Peninsula.

This pattern is reflected within the haplotype network (Figure 4.5). The number of mutations between the two clades is beyond the resolution of the TCS program. Within the first of the two clades, the haplotype network shows the Disa River population to be central, and ancestral, to the other populations. The Silverstroom population that groups out differently based on the mtDNA analysis is also shown to be derived from the Disa River population. The second clade show haplotype G17 shared and central to all of the southern Peninsula populations with the unique Diep River haplotypes separated by a number of non-sampled haplotypes.

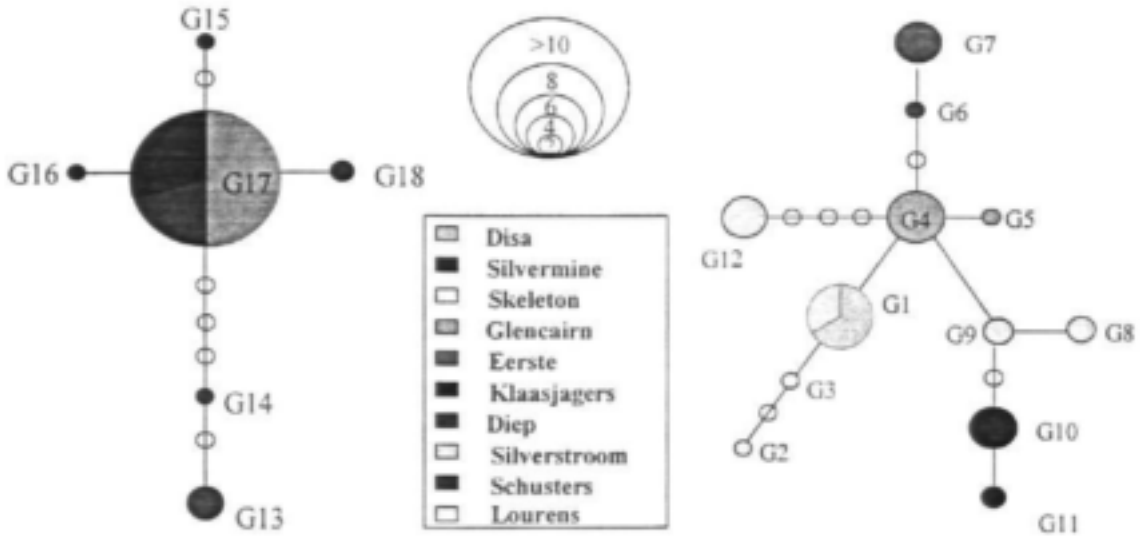

Figure 4.5. A haplotype network showing the relative frequencies and relationships between COI haplotypes for *Galaxias zebratus* (Teleostei: Galaxiidae). The open circles represent missing haplotypes.

Cytochrome b *Sequence Data*

A 416-bp fragment of the cytochrome *b* region of the mtDNA was successfully sequenced from 48 individuals of *G. zebratus* representing the ten sampled populations (Table 4.9). These sequences have been deposited in the GenBank Data Library (Accession Nos. *to be advised*). These were subsequently aligned manually against the five 284-bp sequences obtained for *G. zebratus* from Waters and Cambray (1997). This resulted in 284 comparable base pairs from 52 individuals and revealed 18 putative haplotypes. The overall nucleotide composition included a T/C content of 58%. These haplotypes were differentiated by a total of 67 variable sites. Of these, 59 (88%) were 3rd codon position, seven (10%) were 1st position and one (2%) 2nd position. There was a tranvserion bias of 3.8:1, with 79% of pairwise substitutions transversions and 21% transitional pairs. There was one 1st position transition, no 2nd position and 13 3rd position transitional pairs, with three 3rd position transversions. These changes resulted in seven amino acid changes.

Average pairwise sequence divergence between groups ranged from zero, reflecting the absence of any base pair differences between the Schusters and Glencairn populations, to more than 17% between individuals from the Olifants and Diep river populations (Table 4.10). It is interesting to note that 60 of the 91 pairwise comparisons differed by more than 10%. The mean inter-population divergence is 6·0±0.7%, with a coefficient of differentiation of 97.2±0.8%. These patterns of sequence divergence are reflected in the phylogeny of haplotypes, which clearly shows five well-supported clades (Figure 4.6). Three of these comprise the individual sequences identified by Waters and Cambray (1997), with the Olifants and the Noetsie rivers clearly representing independent lineages. Individuals from the Krom and Kouga rivers comprise the third of these clades, being separated from the Noetsie River by about 7%. While McCune and Lovejoy (1998) estimate cytochrome *b* divergence at about 2.5% per Myr, estimates derived from *Gasterosteus* were 2.8% (Orti *et al.*, 1994). This would suggest that the Krom and Kouga populations have been isolated from the Noetsie for between 2.5 and 2.8 MYrs.

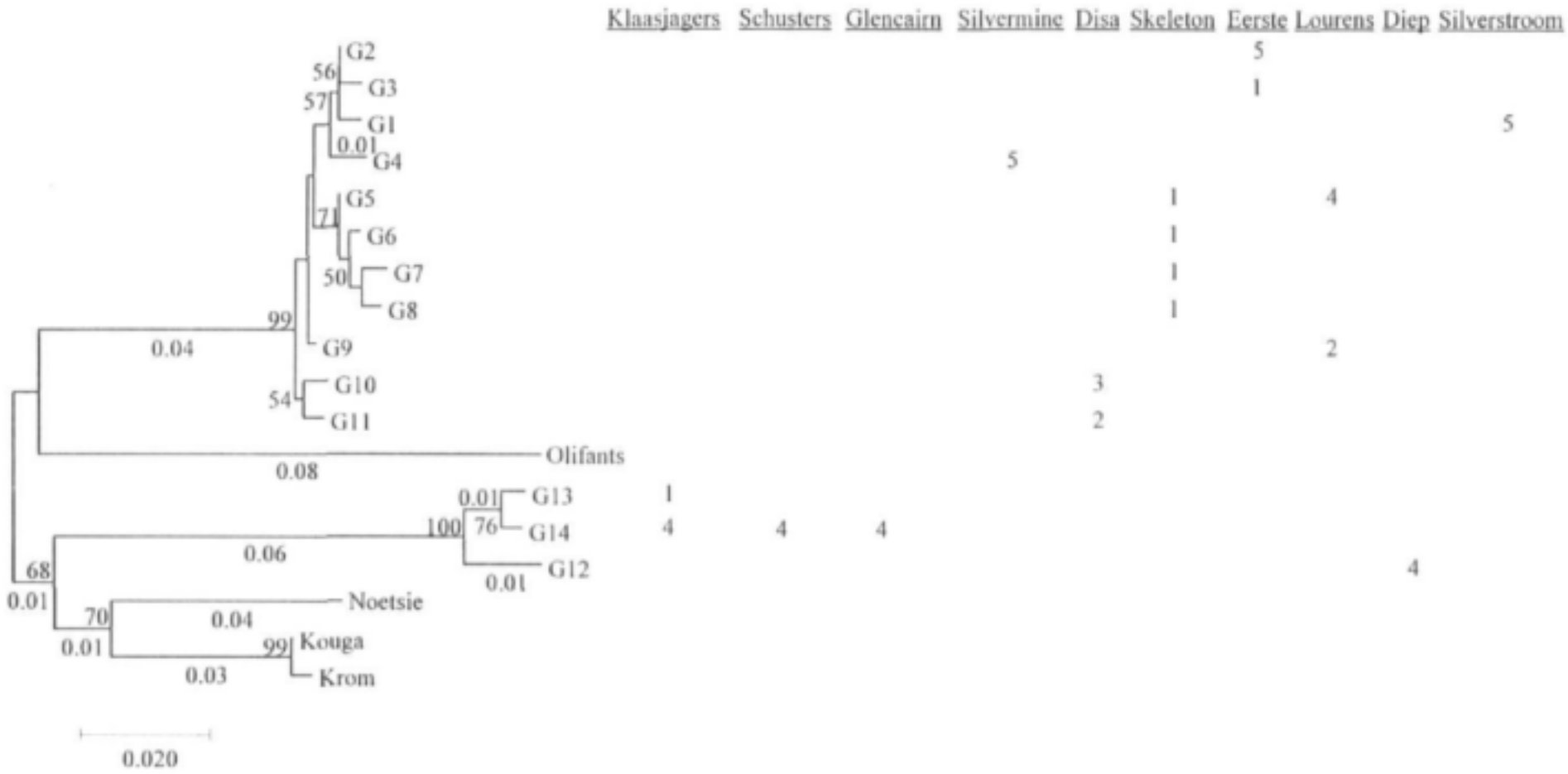

Figure 4.6. A mid-point rooted neighbour-joining tree, constructed using the Kimura-2-parameter (1980) model of substitution, for all cytochrome *b* haplotypes for *Galaxias zebratus* (Teleostei: Galaxiidae).

Table 4.9. Variable nucleotide positions in the 18 *Galaxias zebratus* haplotypes of a 248-bp fragment of the mtDNA cytochrome *b* gene identified by DNA sequencing. Dots indicate identity to haplotype G1. Numbers refer to position of base pairs from the start of the fragment. Entire sequences can be retrieved from the GenBank database at accession numbers as indicated in the column to the far right of the table.

	3	7	9	1 2 5	1 2 7	2 3 6	3 3 9	4 2 2	5 5 5	6 7 0	7 6 2	9 5 0	9 3 6	9 6 9	1 0 5	1 1 8	1 2 1	2 2 4	2 2 0	2 3 2	3 3 3	3 6 5	4 2 3	4 3 5	4 5 1	5 4 4	5 5 7	6 7 0	7 8 3	8 8 6	8 9 8	9 0 1	1 1 7	1 2 9	2 2 2	2 3 5	3 4 8	4 7 2	3 3
G1	T	C	T	T	A	T	A	A	T	A	A	T	G	T	A	C	A	C	T	A	C	T	C	T	C	A	G	A	C	T	A	A	C	A	T	T	G	T	T	C	T	C	T	T	T	A	T	T	T	C	A	C	T	T	G	A	T
G2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
G3	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
G4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
G5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
G6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
G7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
G8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
G9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
G10	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
G11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
G12	C	.	.	C	G	.	G	G	.	G	.	C	.	.	G	.	.	T	C	C	T	C	.	.	.	T	.	G	T	C	G	.	T	.	.	.	A	C	.	.	C	T	C	.	A	G	.	.	.	A	T	.	A	.	.	.	.
G13	C	.	.	C	G	.	G	G	.	G	.	C	.	.	.	.	.	T	C	C	T	.	.	.	.	T	A	G	T	C	G	.	T	.	.	.	A	C	.	.	C	T	C	C	A	G	.	.	.	G	T	.	G	.	.	.	.
G14	C	.	.	C	G	.	G	G	.	G	.	C	.	.	G	.	.	T	C	C	T	.	.	.	.	T	A	G	T	C	G	.	T	.	.	.	A	C	.	.	C	T	C	.	A	G	.	.	.	G	T	.	G	.	.	.	.
G15	C	.	.	.	.	.	.	.	.	.	.	C	.	.	G	T	.	T	T	.	.	C	.	T	.	G	T	C	G	.	.	C	.	.	C	C	.	.	.	.	.	C	G	.	.	C	A	T	T	A	.	A	.	.	.	.	.
G16	C	.	.	.	.	.	.	.	.	.	.	C	.	T	G	T	.	T	T	.	.	C	.	T	.	G	T	C	G	.	.	C	.	.	C	C	.	.	.	.	.	C	G	.	.	C	A	T	T	A	.	A	.	.	.	.	.
G17	C	.	.	.	.	.	.	.	.	G	.	.	C	.	T	.	T	.	T	T	.	.	?	.	T	A	G	T	C	G	.	.	T	.	C	.	C	.	T	.	.	C	.	.	.	.	.	C	A	C	.	.	C	A	.	.
G18	G	T	A	.	.	.	.	C	.	.	.	A	.	T	.	.	.	T	T	.	T	.	T	T	A	G	.	C	G	G	.	.	A	.	.	.	.	C	T	.	.	A	.	C	A	C	G	T	T	.	.	.	C	C

Table 4.9. continued....

	2 4 9	2 5 2	2 5 5	2 5 8	2 6 1	2 6 7	2 7 0	2 7 3	2 7 4	2 7 9	Gene Bank Accession Numbers
G1	A	T	T	G	A	T	T	A	A	A	
G2	.	A	.	.	.	.	.	.	.	.	
G3	.	A	.	.	.	.	.	.	.	.	
G4	.	A	.	.	.	.	.	.	.	.	
G5	.	A	.	.	.	.	.	.	.	.	
G6	.	A	.	.	.	.	.	.	.	.	
G7	.	A	.	.	.	.	.	.	.	.	
G8	.	A	.	.	.	.	.	.	.	.	
G9	.	A	.	.	.	.	.	.	.	.	
G10	.	A	.	.	.	.	.	.	.	.	
G11	.	A	.	.	.	.	.	.	.	.	
G12	G	A	C	T	G	C	.	.	.	.	
G13	G	A	.	T	G	C	.	.	.	.	
G14	G	A	.	T	G	C	.	.	.	.	
G15	.	A	C	T	G	.	.	G	.	.	
G16	.	A	C	T	G	.	.	G	.	.	
G17	.	A	C	T	G	.	.	.	G	.	
G18	.	G	C	T	.	.	A	.	.	T	

Table 4.10. Net nucleotide distance between groups (below diagonal) and standard error (above diagonal) and mean nucleotide distance within groups (on the diagonal) for a 248-bp fragment of the cytochrome *b* region of the mtDNA. Distance among haplotypes was estimated using Kimura-2-parameter model (1980). See general methods section for more details.

	Klaasjagers	Schusters	Glencairn	Silvermine	Disa	Skeleton	Eerste	Lourens	Diep	Silverstroom	Kouga	Noetsie	Krom	Olifants
Klaasjagers	**0.000**	0.001	0.001	0.023	0.022	0.023	0.023	0.022	0.008	0.024	0.022	0.023	0.022	0.026
Schusters	0.001	**0.000**	0.000	0.023	0.022	0.023	0.023	0.022	0.008	0.024	0.022	0.023	0.023	0.026
Glencairn	0.001	0.000	**0.000**	0.023	0.022	0.023	0.023	0.022	0.008	0.024	0.022	0.023	0.023	0.026
Silvermine	0.130	0.130	0.130	**0.000**	0.007	0.007	0.005	0.005	0.024	0.006	0.020	0.021	0.021	0.023
Disa	0.124	0.124	0.124	0.016	**0.004**	0.005	0.005	0.004	0.023	0.006	0.020	0.021	0.020	0.022
Skeleton	0.130	0.130	0.130	0.016	0.010	**0.007**	0.005	0.001	0.023	0.006	0.018	0.020	0.019	0.023
Eerste	0.130	0.130	0.130	0.007	0.009	0.008	**0.001**	0.004	0.024	0.003	0.020	0.021	0.021	0.023
Lourens	0.125	0.125	0.125	0.010	0.007	0.002	0.005	**0.004**	0.023	0.005	0.019	0.019	0.019	0.022
Diep	0.020	0.018	0.018	0.135	0.133	0.135	0.135	0.130	**0.000**	0.024	0.020	0.023	0.021	0.027
Silverstroom	0.134	0.134	0.134	0.011	0.012	0.012	0.004	0.009	0.139	**0.000**	0.021	0.022	0.021	0.023
Kouga	0.115	0.115	0.115	0.100	0.098	0.085	0.100	0.090	0.102	0.104	**n/c**	0.017	0.003	0.022
Noetsie	0.123	0.123	0.123	0.104	0.102	0.098	0.104	0.094	0.123	0.108	0.068	**n/c**	0.016	0.024
Krom	0.119	0.119	0.119	0.104	0.102	0.089	0.104	0.094	0.106	0.108	0.004	0.064	**n/c**	0.022
Olifants	0.162	0.162	0.162	0.124	0.118	0.128	0.128	0.123	0.171	0.128	0.125	0.134	0.120	**n/c**

n/c – not calculable as there is only one individual

The remaining two clades, both well supported by high bootstrap values, are comprised of sequences derived from the ten sampled populations and reflect the same phylogenetic pattern observed for the COI fragment. The first clade is comprised of individuals from Table Mountain and Hottentot's Holland streams, with the inclusion of individuals from Silverstroom. With the exception of a shared haplotype between the Skeleton Gorge and Lourens rivers, G5, each river is monophyletic and represented by a unique array of haplotypes. This group is separated from the Olifants river by around 12% sequence divergence, which again depending on estimates of the rate of divergence that is used, corresponds to about 4.3 to 4.8 MYrs. It is separated from the Noetsie, Kouga, Krom and the second clade by roughly 10% in each instance, corresponding to between 3.4 and 4 MYrs of isolation.

The second clade suggests that the West Coast population from the Diep River is ancestral to populations of the southern Peninsula. These populations from the southern Peninsula all share a common haplotype, G14. These two internal clades are separated by about 2%, indicating a period of separation of roughly 700 to 800 ky. The structure of the haplotype network suggests that we have either not sampled the ancestral haplotype or that it has subsequently been lost, with the TCS programme unable to resolve some of the relationships between the various haplotypes (Figure 4.7). When examined in relation to the haplotype network for the COI fragment some of these relationships can be resolved. For example, we can then hypothesis that the Silvermine haplotype is derived from the Lourens River, which shares ancestral haplotypes with the Skeleton River. All of which could have been derived from an ancestral haplotype that has subsequently been lost from the Disa River. If examined in relation to the haplotype network for the COI fragment, it becomes obvious that the absence of an ancestral cytochrome *b* haplotype from the Disa River is responsible for the poor resolution of relationship among the network.

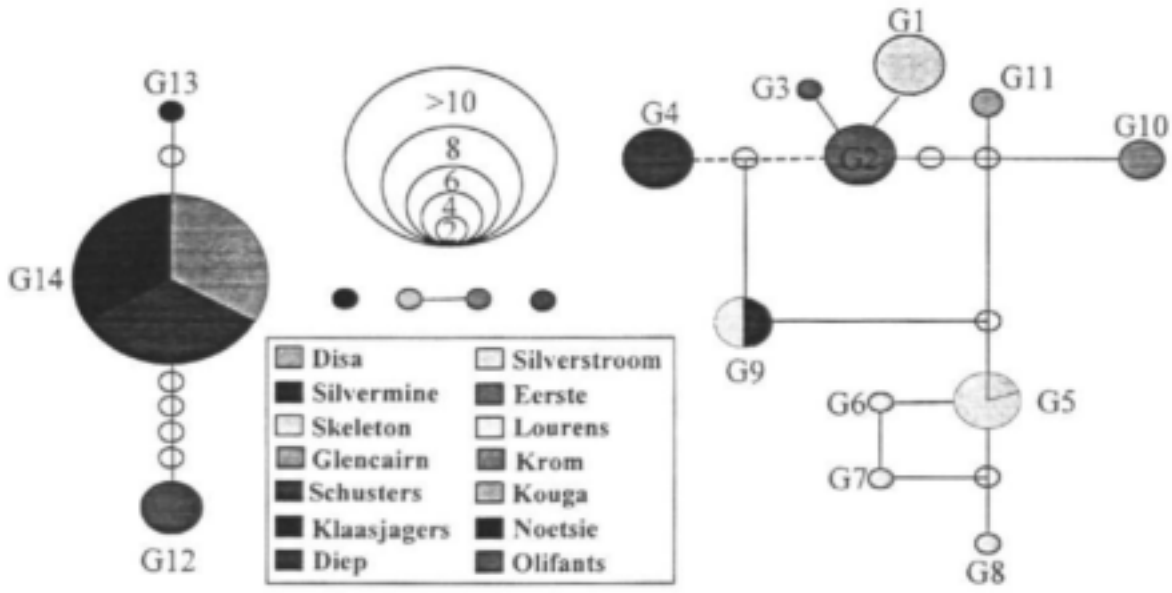

Figure 4.7. A haplotype network showing the relative frequencies and relationships between cytochrome *b* haplotypes for *Galaxias zebratus* (Teleostei: Galaxiidae). The open circles represent missing haplotypes.

Discussion

While there are reportedly about 45 species of galaxiid fishes distributed among six genera world wide, *G. zebratus* is the only species found on the African continent. This pattern of species distribution presumably corresponds to the assumed break-up of Gondwana, where Africa was the first to break out of the super-continent, with Australasia, Antarctica and South America remaining in contact for a much longer period (De Wit *et al.*, 1988). Very little is known about the biology and ecology of *G. zebratus*, however, these results would tend to suggest that galaxiid diversity on the continent has previously been under estimated. Morphological variability within this fairly conservative group would appear to mask the fact that the long period of isolation of the African continent has enabled the differentiation of a number of distinct genetic groups, each with a long, isolated and individual history. Unfortunately it is impossible to ascertain if these genetic forms correspond to those species original described as type localities are now largely degraded and all attempts to collect specimens were unsuccessful.

Variability

As is to be expected for an obligate freshwater species, the distribution of genetic variation exhibits a high degree of structure associated with catchment units. However this distribution does not reflect a simple model of isolation by distance, but a more complex interaction between contemporary processes overlaying the cumulative history of the species and the region. The low levels of polymorphism in any of the loci examined makes it difficult to make any inferences about population level processes. Low levels of variability in nuclear loci seems to be common among lotic organisms of the south-western Cape Region, having been observed among a number of taxa (although see Stewart, 1997; Daniels *et al.*, 2001). Similarly low levels of variation have been observed in the net-winged midge, *Elporia barnardi*, and the dragonfly, *Aeshna subpupillata*, which had only six and four polymorphic loci respectively, while no variable loci were detected among populations of the common Cape stonefly, *Aphanicerca capensis*. Similar low levels of allozymic variation have been observed in other non-diaromous, landlocked species of *Galaxias* (Ovenden *et al.*, 1993). Similarly, Waters and Cambray (1997) postulated that similarities between the Krom and Noetsie river populations suggest that intrapopulational variation may be low. The low haplotype diversity, with most catchments typically represented by a single haplotype, would appear to support this idea.

Given the long, stable geologic history of the region and climatic oscillations that have taken place, the stream fauna is likely to have suffered severe fluctuations in population size, possibly eroding the levels of variability through successive bottlenecks. Over the past two million years – the Pleistocene – there has been a rhythmic growth and retreat of the high latitude ice sheets, with a one hundred thousand year periodicity as a result of orbital forcing (Hays *et al.*, 1976; Tyson *et al.*, 2001). Under the coldest conditions the Cape mountains lay beneath the permanent snowline and therefor did not carry glaciers. The moderate height of the mountains and the reliability of winter rainfall systems have also been important in offsetting the effects of the arid periods (Deacon *et al.*, 1992). For obligate freshwater species confined within catchment units, such climatic oscillations may have reduced the amount of stream length available, forcing upstream retreats and reducing population

sizes. This may have resulted in bottlenecks in the population, successively eroding genetic variability, resulting in the low levels of observed variability.

Catchment Structure

Catchment units have been shown to influence the genetic structure of populations in a number of lotic fish species (Ward *et al.*, 1994). The relatively higher F_{ST} values observed in freshwater lotic species have been variously attributed to both the effects of catchment isolation and the smaller effective population sizes. Comparison of F_{ST} and genetic distance data for a number of diadromous and non-diadromous galaxiid species in New Zealand highlights the effects of catchments as isolating mechanisms (Allibone & Wallis, 1993). Such isolation, leading to higher F_{ST} and genetic distance values among conspecific populations of non-diadromous species, plays a potentially important role in speciation, as well as extinction. Despite the low number of polymorphic loci, the F_{ST} values for both groups are very high reflecting a high degree of structure.

Values of F_{ST} observed within both groups are greater than those observed across the entire range of *G. maculatus*. One of the most widespread freshwater species, *G.maculatus*, is found in Australia, New Zealand, South America along with the Lord Howe, Chatham and Falkland islands. Small scale investigations of populations in the Bay of Plenty, New Zealand, showed very low levels of differentiation (F_{ST} = 0.055) reflecting the diadromous nature of this species (Barker & Lambert, 1988). Populations from across the species range show only moderate levels of differentiation (F_{ST} = 0.14), consistent with a set of historically connected interbreeding populations (Berra *et al.*, 1996). Differentiation in Group A is derived entirely from the Disa River population. All of the 18 loci examined in these populations were fixed for a single allele, with the exception of the Disa River population. Three loci, *MDH3*, *ME* and *PGM* exhibited variation. The Disa River presents an interesting comparison. Other species included in this study were either absent (*Elporia*), found in very low numbers (*Aphanicerca*) or displayed similar differences in genetic structure when compared to other Table Mountain populations (*Aeshna*). Populations in Group B exhibit a greater degree of allozyme variability While the Glencairn population did not exhibit any polymorphic loci, all other

populations displayed variability at the *GPI* loci, Klaasjagers and Schusters at the *PGM* loci and the Diep River at the *MDH3* loci. Negates any comparisons between regions really.

With the exception of the Silverstroom population, the pattern of genetic variation observed in both the cytochrome *b* and COI regions of the mtDNA is congruent with that of the population tree derived from the allozyme data. That the Silverstroom population groups out in a different clade based on mtDNA raises some interesting considerations. This could be due to introgression between ancestral forms and more recently introduced individuals. This could result in the observed anomaly where ancestral patterns of genetic variation are detected using allozyme electrophoresis with incorporation of mtDNA lineages from recently introduced individuals.

In terms of structure, mtDNA provides a more sensitive marker with greater resolution. The neighbour-joining population tree for shows a number of monophyletic clades, each corresponding to individual catchment units. That is, each catchment contains its own unique array of haplotypes suggesting an absence of gene flow and a period of independent isolated evolution. There are two exceptions, the southern Peninsula populations share a common COI haplotype, while the Lourens River and Skeleton Gorge populations share a common cytochrome *b* haplotype. Accepting that an ancestral cytochrome *b* haplotype may have been lost, the pattern of variation in both the population tree and haplotype networks would suggest the Disa River haplotype ancestral to the rest.

Taxonomic Issues

The genetic distance between the two groups suggests the presence of two different taxa. It has been suggested that genetic information can be used to resolve the species status of a population if other criteria are unsuccessful (Thorpe, 1982). Given the morphological variation and historical taxonomic uncertainties relating to *G. zebratus*, genetic techniques could provide an important distinguishing tool for the systematics of the group. Thorpe (1982) suggested allopatric populations with a genetic distance above 0.16 might reflect different species. Avise and Aquadro (1982) examined inter-generic genetic distances and found distances between species of about 0.36, while

Hänfling and Brandl (1998) propose populations of the bullhead, *Cottus gobio*, separated by a distance of 0.36 represent two taxa. Watts *et al.* (1995) found genetic distances between populations of the Western Minnow, *G. occidentalis*, in Australian of 0.40 suggesting two different species. The number of fixed differences between populations has also been used to infer species status, with species having about 50 % of loci fixed differences (Avise, 1994). Populations of *G. zebratus* were separated by a genetic distance of more than 0.85 with two thirds of loci exhibiting fixed differences.

Intraspecific cytochrome b sequence divergences are typically around 1% in fishes (Meyer *et al.*, 1990; McVeigh *et al.*, 1991; Finnerty & Block, 1992; Patarnello *et al.*, 1994; Taylor & Dodson, 1994). In a review of the levels of sequence divergence among sister taxa of various fish species, McCune and Lovejoy (1998) report a maximum intraspecific divergence in the cytochrome b region of 5.7% observed in *Mallotus villosus*. The maximum interspecific divergence observed between sister taxa is reportedly 7.2%, between *Fundulus heteroclitus* and *F. grandis*. Levels of cytochrome *b* sequence divergence among South American species of the genus *Cynolebias* (Cyprinodontiformes, Rivulidae) ranged from 4.5% to 25.9% (Garcia *et al.*, 2000). While others have reported "exceptionally high" values of cytochrome *b* sequence divergence that "equal or surpass those reported between many fish species and even some genera" in the Leatherside which displayed two evolutionarily distinct clades with sequence divergence of approximately 8% (Kocher & Stepien 1997).

While the Galaxiids typically exhibit higher degree of sequence divergence than that observed in other fishes, the values observed *G. zebratus* are greater than any of those previously reported. The levels of sequence divergence are comparable to other interspecific and some intergeneric comparisons within the Galaxiidae (see Waters & Cambray, 1997) and within the osmeroids, thought to be closely related to the galaxiids (Begle, 1991; Patterson & Johnson, 1995). For example, genetic divergence among the Olifants River individual and populations from the southern Cape Peninsula (~16%) surpasses the 13% sequence divergence between the Olifants and Noetsie rivers reported by Waters and Cambray (1997) as the largest of any fish species. McDowall (1973) found differences in the vertebral counts were associated or corresponded to geographic location and although the initial pattern of genetic

variation in *G. zebratus* displayed a pattern of isolation by distance (Waters & Cambray, 1997), the small-scale distribution of genetic variation observed among streams observed here shows no geographic concordance. The results reveal five highly divergent and distinct genetic forms of *G. zebratus*. In the absence of any information on reproductive behaviour or success and given the inherent morphological variation, it may be prudent to afford specific recognition of these discrete forms.

Why so ?

The Cape galaxias can be considered part of the ancient Gondwanan palaeoendemic fauna characteristic of much of the Cape Floristic Region. While its current distribution reflects geological processes and history, more contemporary factors such as marine dispersal, human translocation, river capture and sea level fluctuations may also prove important in explaining some of the patterns of genetic variation. There is no evidence for diadromy in *G. zebratus*, which would tend to be supported by the high levels of divergence and lack of concordance with a model of isolation by distance.

Drainage evolution is thought to have played a major role in the distribution of fishes within the Cape Fold mountains (Barnard, 1936, 1943), with eustatic sea level changes considered to have been important in aiding the dispersion of *G. zebratus* (Skeleton, 1980, 1994). While sea levels did not rise more than a few meters above present levels during the Pleistocene (Van Andel, 1989), it is likely that periods of low Pleistocene sea levels were more significant in terms of affecting species distributions. For example, the coastal plain south of the Breede River on the West Coast, was widened from 50m to 200km during the regression of the last glacial maximum when sea level dropped 120m below its present level (Dingle & Rogers, 1972). This would have created numerous opportunities and alternative routes for dispersal and migration. Bathymetry maps of the coastal reaches around the Cape shores reveal a deep canyon, reflecting an old drainage line, on the Atlantic side of the Peninsula.

The distribution of haplotypes from the ten sampled populations, represented by the two clades, roughly correspond to streams arising from mountains with their source above the 900ft contour line and those below. While there is no association between geographic and genetic distance, the two clades include east flowing and west flowing rivers. The Klaasjagers, Schusters and Diep rivers all drain to the west. While Glencairn drains to the east, it arises at a low altitude nick in the mountain fold with an intermittent river course, possibly representing an ancient river course, draining to the west. The Lourens, Eerste and Silverstroom rivers all flow into False Bay. Initially running off Table Mountain in an easterly direction from Skeleton Gorge, the river then drains onto the Cape Flats where it now flows to the north before moving east out into Table Bay. Prior to the erosion of the land bridge connection the Hottentot's Holland and Table Mountain it is likely that this stream drained east and into False Bay. Thus, while many have invoked explanations of river capture as a way of explaining contemporary patterns of genetic structure in freshwater fishes, the patterns observed among populations of *G. zebratus* would appear to be derived from the extension of the coastal platform and of the river courses. As the coastal platform has extended during low sea level periods, these rivers have joined together on the low-lying plains providing free exchange of individuals. As climates changed and sea levels rose, these streams were shortened in length, with the effect of decreasing the effective population size, thereby potentially eroding genetic variability, and isolating individual rivers.

While there is little variation to facilitate examination of population processes, the distribution of haplotypes and monophyletic nature of the catchment units indicates there is no movement beyond the catchment unit. Furthermore, the high levels of divergence displayed over small geographic scales within both the allozymes and mitochondrial DNA suggest the presence of a possible species complex. Climatic oscillations, in association with drainage extensions, rather than re-arrangements, sea level changes and erosional processes set against a back drop of relative habitat stability would appear to have provided for the evolution of extreme levels of genetic divergence among populations of the Cape galaxiid. The biological relevance of the magnitude of these genetic differences remains to be assessed. Detailed morphological and osteological examinations should be carried out, preferably in association with mate choice experiments (see Chapter 6), in order to ascertain and

resolve the relationships between the various different forms across the entire species range.

Chapter 5

The Net-Winged Midge
Elporia barnardi
(Diptera: Blephariceridae)

Introduction

The Blephariceridae, commonly referred to as the net-winged midges, constitute one of the most distinct and isolated groups within the Diptera (Figure 5.1). Both the adults and larvae have extremely high habitat specificity, inhabiting only cold mountain streams where they are restricted to fast flowing, highly oxygenated habitats. Adaptations to these environments include a series of six mid-ventral suckers and the cephalothorax (fused head, thorax and first abdominal segment) that enable the larvae to maintain and ascend against velocities of more than 240 cm sec^{-1} (Dorier & Vaillant, 1964). Similarly, three pairs of suction pads anchor the pupae to rocks while the adult flies, having emerged from the pupae under water, are on the wing for only one or two weeks. Susceptible to low levels of humidity, the adult flies are also largely confined to the highly humid splash zones within the stream margins. Thus, despite a winged adult stage, the highly specific habitat requirements and morphological characteristics of the net-winged midges result in a very poor potential for dispersal.

Figure 5.1. The net-winged midge (Diptera: Blephariceridae).

Small to medium-length nematocerous flies, the adults are long-legged and slender with elongate, multi-segmented antennae lacking conspicuous pilosity. They are easily recognized by the numerous delicate cracks and folds that form a network in the wing membrane. Size varies from small to moderate, with the wing lengths ranging from *ca.* 4.0 to 12.5 mm in some species of Australian Blephariceridae. In comparison, the fully-grown larvae of *Elporia barnardi* are between 2 and 4mm. While male adults have reduced mouthparts and do not generally feed (except in some groups consuming flower secretions, e.g., Apistomyiini), female mouthparts are typically well-developed, with many observed feeding on smaller insects that are caught with specialized hind tarsi (Zwick, 1977). The larvae are typically scrapers, grazing on microscopic growths, diatoms and algae (Wishart, unpublished data). Although life cycles may vary from semivoltine to plurivoltine, most of the net-winged midges, including *E. barnardi*, have a univoltine life cycle (Wishart, unpublished data). Life histories are usually asynchronous with egg-hatching and adult emergence occurring over 2-3 month period. Unlike many Diptera though, the Blephariceridae are unusual in that they over winter as eggs, rather than larvae or pupae.

While generally absent from oceanic islands, the 300 or so species, from *ca.* 30 genera, are of considerable age and widely distributed around the world, known from all of the continents. Although there are no known fossil records for the family (Alexander, 1958) the blephariceridids are thought to date back to the mid-Mesozoic, perhaps 150 MyBP. Two genera are known from sub-Saharan Africa, Edwardsininae (*Edwardsiniinae*), from a single record in West Africa, and *Elporia*. Although it remains to be shown cladistically, the southern African blephariceridid fauna evidently form a monophyletic group belonging to the Paltostomatini, related to some Palaearctic and Neotropical genera (B. Stuckenberg, pers. Comm.). Thought to have evolved from a generalised Paltostomine (Stuckenberg, 1962), Edwards (1915) erected the genus *Elporia* for the southern African species, which had been previously ascribed in to the South American genus *Kelloggina* Williston. Edwards (1929) later altered the status of the genus to a subgenus within the South American genus *Curupira* Ostem-Sacken, however, Barnard (1947) subsequently restored the status of *Elporia* Edwards back to that of genus.

Most of the suitable habitat types to the north of South Africa are too low in altitude relative to the hot climate and the rather sparse rainfall to allow the presence of Blephariceridae. Despite extensive surveys and searching (A. Harrison and B. Stuckenberg, pers comm; M. Wishart, pers obs) the only other species to have been collected from the African continent include those from two sites, in Nigeria and Cameroon (B. Stuckenberg, pers comm). Prominent tropical mountains with apparently suitable streams, such as the Ruwenzori and the volcanoes of East Africa, are too young or too isolated to have acquired a blephariceridae fauna. The escarpments fringing the rift valleys provide suitable environments (in Malawi, for example) but the Blephariceridae are not known from anywhere in the East African volcanic belts. Its possible the escarpments are too young, or perhaps the immense amount of volcanic activity that accompanied the formation of the rift valleys simply exterminated any aquatic fauna in the older mountains of East Africa. The Ethiopian Highlands are a huge blanket of lava and are not thought to have any old endemics. It is possible that the peneplanation of much of tropical Africa could have eliminated the Blephariceridae from vast areas now occupied by savannah. The ancient escarpment in South Africa, and the Cape Fold Mountains, has provided a refuge for an aquatic fauna dating back to early Cretaceous times.

Endemic to Southern Africa, the genus *Elporia* Edwards currently comprises 19 species, all of which have been described from within South Africa (Barnard, 1947). Species within the genus constitute a very heterogeneous group and display considerable variation in the structure of the compound eyes, a relatively constant character in other Blephariceridae genera (Stuckenberg, 1955). It was the structure of the compound eyes, along with the number of segments in the maxillary palps and antennae and the nature of the wing membrane, that led Edwards (1932, 1933) to divide the species of *Elporia* into two groups. A third group, consisting of two species, *E. saltatrix* and *E. armata*, was subsequently described by Barnard (1947).

Species of the genus *Elporia* are widely distributed throughout the Cape Fold Mountains of the south-western Cape (Figure 5.2). Barnard (1947) reported species of this genus extending throughout the Cape mountains from Garcias Pass (Riversdale) in the Langeburg Range, and Meiring's Poort in the Zwartberg Range, north to the Cedar Mts (Clanwilliam) and the Kamiesberg (Namaqualand). Despite the wide

spread distribution, *E. barnardi* is as yet the only species to be found on the Cape Peninsula. It is possible therefore that this reflects a phyletically older species dating back to before the time of the Tertiary marine transgression across the Cape Flats.

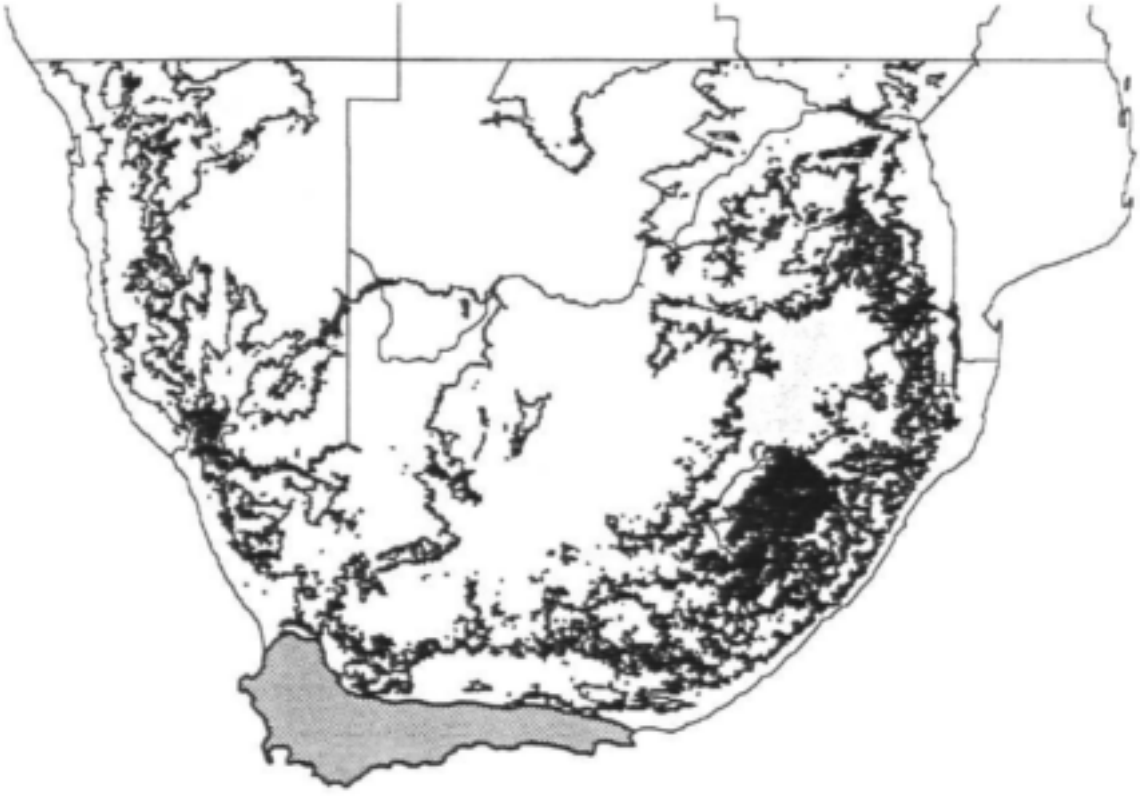

Figure 5.2. Distribution of the net-winged midge, *Elporia barnardi* (Diptera: Blephariceridae) in the southwestern Cape, South Africa.

The distribution of genetic variation and levels of sub-division among populations of *E. barnardi* were examined using a hierarchical sampling design. Three sites were sampled within each of a number of streams in two discontinuous mountain ranges to examine the effect of stream structure as well as catchment units on patterns of genetic variation in mitochondrial and nuclear DNA using allozymes and direct sequencing of the COI region of the mtDNA.

Methods

Collection

Despite their small size (< 10mm length), the Blephariceridae are an obvious component of the stream fauna by virtue of their habitat specificity, distinct shape and characteristic movement. Between 30 and 50 final instar larvae of *Elporia barnardi* were collected by hand from three sites in Kasteelspoort, Platteklip, Skeleton Gorge streams on Table Mountain (Figure 5.3a). These represent the upper and lower limits of the species range, along with an intermediate site. The species range was determined by entering the stream at low altitude and walking upstream until the first individuals were observed. The upper most sites typically coincided with the stream source with intermediate sites based on the mid-point between these. Individuals were also collected from a single site in Nursery Ravine adjacent to Skeleton Gorge on Table Mountain. Samples from the Hottentot's Holland Mountains were collected from four sites within the Jonkershoek catchment. Three of these were situated along the Eerste River with the fourth within a first order tributary, Langrivier (Figure 5.3b). Sites within each of these streams were typically no more than 750m stream length apart, with *E. barnardi* only found above ~200 mASL. Individuals were placed into sampling containers and returned to the laboratory alive where any algae or silk threads from simuliid larvae were removed. *Elporia barnardi* is the only blepharicerid species recorded from Table Mountain, and while four species have been recorded from the Jonkershoek catchment all species identifications were confirmed in the laboratory using Barnard (1947) and all individuals frozen at -80°C.

Allozymes

Cellulose acetate electrophoresis was used to screen 17 different enzymes. Problems with clarity and interpretation left only seven enzymes that stained reliably and consistently (Table 5.1). Of those enzymes that gave consistent and reliable stains, HK (Hexokinase) and ADK (Adenylate Kinase) were not used due to insufficient variability, with a single allele occurring with a frequency greater than 0.95. Aspartate Aminotransferase (AAT) was found to be polymorphic and scored at two separate loci, giving six diagnostic polymorphic loci (Appendix 2).

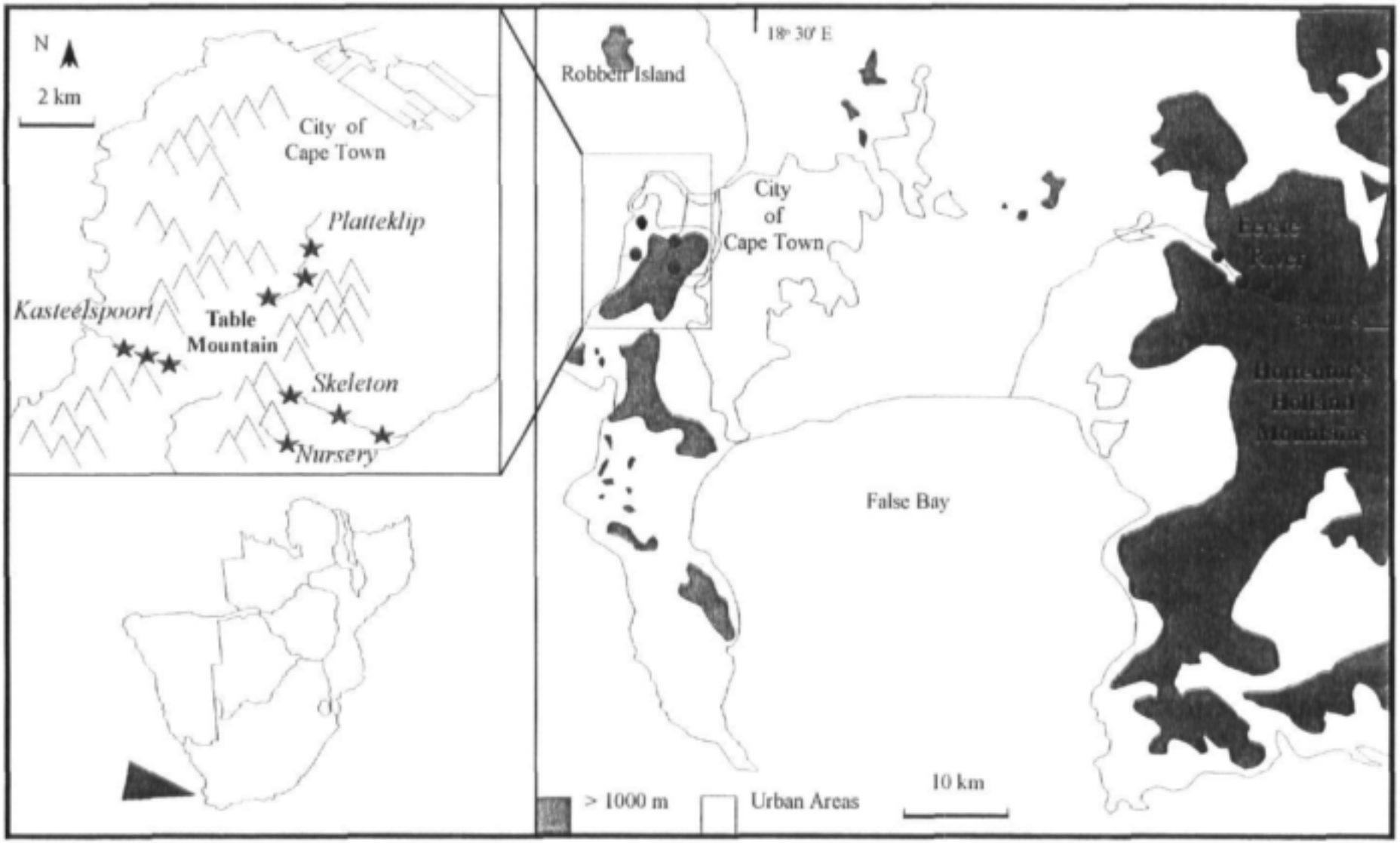

Figure 5.3. Location of sampling sites for *Elporia barnardi* (Diptera: Blephariceridae) among streams of the southwestern Cape, South Africa.

Table 5.1. Enzymes and running conditions used in the allozyme electrophoretic analysis of *Elporia barnardi* (Diptera: Blephariceridae).

Enzyme		E.C.Number	Run Buffer	Run Time (min)
Amylase	*AMY*	3.2.1.1	Tris-glycine	60
Glucose Phosphate Isomerase	*PGI*	5.3.1.9	Tris-citrate	120
6-Phosphogluconate Dehydrogenase	*PGD*	1.1.1.49	CAEA	120
Malate Dehydrogenase	*MDH*	1.1.1.37	CAEA	120
Aspartate Aminotransferase	*AAT*	2.6.1.1	CAEA	120

MtDNA

Sequence data for the COI region of the mtDNA was examined in between three and five individuals from each site, to give data available from a total of between ten and 15 individuals in each stream.

Results

Allozyme Electrophoresis

The *Mdh* and *Aat-1* loci were monomorphic in all Hottentot's Holland populations, and *Pgi* was monomorphic among all Table Mountain populations. Allele frequencies of polymorphic loci and sample sizes for each of the sites are given in Appendix 2.

Deviations from Hardy-Weinberg equilibrium

Genotypic frequencies deviated significantly from Hardy-Weinberg equilibrium in 17 of the 57 individual comparisons made (29.82 %: Table 5.2). The observed deviations occur across all loci, displaying no clear or obvious patterns and are more than would be expected due to chance alone (Expected = 2.85). All deviations from Hardy-Weinberg equilibrium were positive, indicating a deficiency of heterozygotes. Of the 210 pairwise comparisons carried out to determine the presence of linkage disequilibrium only seven were significant ($P < 0.05$), a result that could be expected through chance alone.

Table 5.2. The fixation index (F_{is}) for each of the populations of *Elporia barnardi* (Diptera: Blephariceridae).

	Locus					
Site	**Aat-1**	**Aat-2**	**Amy**	**Mdh**	**Pgd**	Pgi
Kasteelspoort 1	-0.10	0.52*	0.06*	0.44**	-	-
Kasteelspoort 2	-0.09	0.38	0.67***	0.25	-	-
Kasteelspoort 3	-0.23	0.27	0.31	0.68***	-	-
Platteklip 1	0.489**	0.70***	-0.08	-	0.352*	-0.037
Platteklip 2	0.41**	-	-0.08	-0.032	-	-
Platteklip 3	0.14	1***	-0.19	-0.020	-0.010	-
Nursery	-0.12	0.31*	-0.28	0.222	-0.062	-
Skeleton 1	0.22	-0.29	0.49	-0.246	-	-
Skeleton 2	0.08	-0.14	-0.15	-0.127	-0.041	-
Skeleton 3	-0.22	-0.26	0.18	-0.207	-0.091	-
Eerste 1	-	0.90***	-	-	0.2246	0.022*
Eerste 2	-	0.80***	-0.02	-	0.329*	-0.131
Eerste 3	-	1*	-	-	0.187	-0.030
Langrivier	-	1***	-0.01	-	0.051	0.044

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Differentiation between Sites

The magnitude of the F_{ST} value between mountain ranges indicates significant differentiation between populations sampled on Table Mountain and those from the Jonkershoek catchment across all loci (0.39±0.10; $P < 0.001$: Table 5.3). All loci displayed highly significant levels of differentiation. The presence of a single allele at the *Pgi* locus in Table Mountain populations and a single allele at the *Mdh* and *Aat-1* loci in populations from the Hottentot's Holland suggests an absence or extremely low frequency of exchange between the two ranges.

Levels of differentiation observed between streams located on Table Mountain were similar to those between ranges, with highly significant F_{ST} values observed across all loci ($p < 0.001$: Table 5.3). Populations from the Hottentot's Holland all come from within the same catchment and display lower levels of differentiation than those between streams on Table Mountain (Table 5.3). The *Aat-2* and *Pgd* loci both show significant levels of differentiation, although almost an order of magnitude less in relative strength than those differences observed between streams on Table Mountain. Differences between populations within the Hottentot's Holland are similar to the

levels of genetic differentiation calculated for populations taken from within the same stream on Table Mountain. All three streams on Table Mountain, for which three sites were sampled, showed significant levels of differentiation (Table 5.4). All of the Table Mountain streams display a similar pattern, with the *Pgd* locus showing the strongest degree of differentiation. Kasteelspoort populations exhibit only one of the two alleles present at the *Pgi* locus in other Table Mountain streams. The lowermost population sampled in the Skeleton Gorge stream (Site 3) displayed a very different allele frequency to the upper two sites, resulting in a relatively high F_{ST} value suggesting relatively low gene flow among Skeleton Gorge sites. This was primarily due to differences at the *Pgd* locus. With the exception of the *Pgd* locus there were no clear patterns in the differentiation of individual loci across streams.

Genetic Identities and Distances

Using allele frequencies, unbiased estimates of genetic identities (Nei, 1978) were calculated between each pair of sampling sites (Table 5.5), and a dendrogram constructed (Figure 5.4). The dendrogram shows a clear division into two groups, separating sites located on Table Mountain from those located in the Hottentot's Holland (Average I = 0.725, Range 0.862 to 0.605). Within each of these two broad groups there is obvious structuring with sites from within the same stream tending to be grouped together. Values of genetic identity among sites in streams on Table Mountain show little differentiation (Average I = 0.909). The exception to this is downstream from the Skeleton Gorge stream site (S3), which clusters last with other stream sites on Table Mountain with a mean genetic identity of 0.849. Sites within streams have pairwise identity values ≤ 0.942. Streams on Table Mountain exhibit levels of identity (0.909) similar to those between sites within streams.

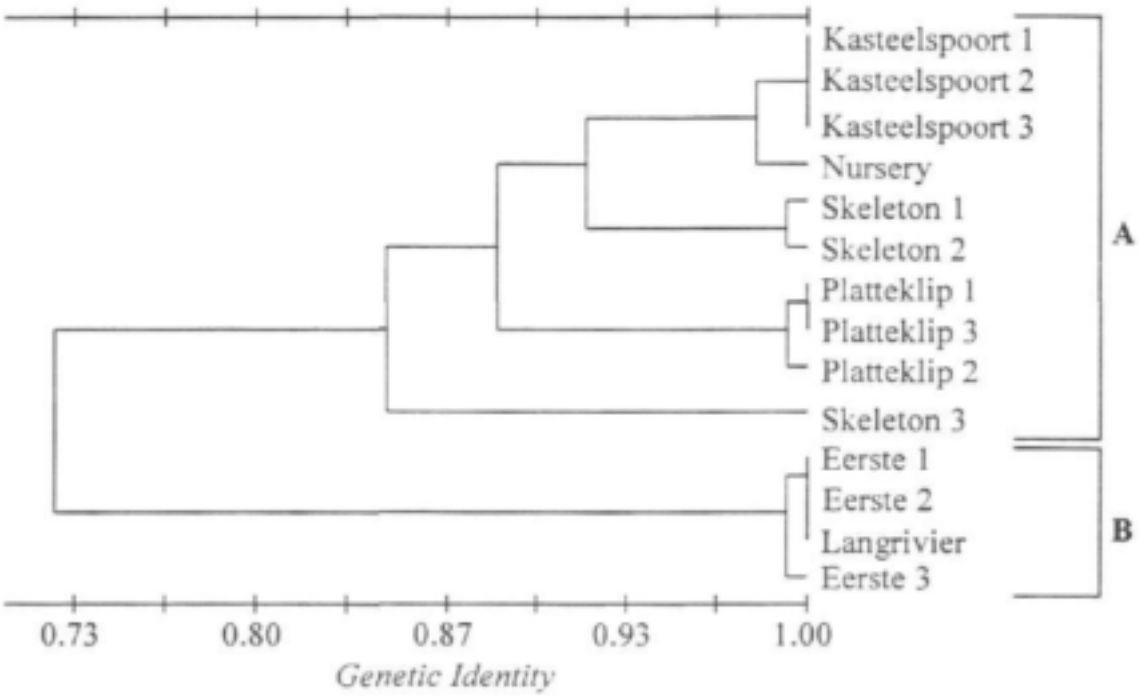

Figure 5.4. Phenogram showing the results of UPGMA analysis based on Nei's genetic identity for *Elporia barnardi* (Diptera: Blephariceridae).
(A: sites sampled from Table Mountain; B: sites sampled in the Hottentot's Holland).

Table 5.3. Jackknifed estimates of F_{ST} values for within and between each of the two ranges (Table Mountain and Hottentot's Holland) at each locus for populations of *Elporia barnardi* (Diptera: Blephariceridae).

	Locus						
F-statistic	**Aat-1**	**Aat-2**	**Amy**	**Mdh**	**Pgd**	**Pgi**	**All Loci**
Between#	0.57*	0.14*	0.14*	0.25*	0.32*	0.62*	0.39±0.10*
Table Mountain	0.17±0.05*	0.25±0.09*	0.13±0.04*	0.29±0.09*	0.60±0.33*	-	0.23±0.04*
Jonkershoek	-	0.03±0.03*	-0.00± 0.00	-	0.035±0.034**	-0.00±0.01	0.02±0.02**

*** $p < 0.05$, ** $p < 0.01$, * $p < 0.001$
#(pooled all TblMtn Sites & run against Eerste sites)

Table 5.4. Jackknifed estimates of F_{ST} values for sites within rivers at each locus for populations of *Elporia barnardi* (Diptera: Blephariceridae)

	Locus						
Site	**Aat-1**	**Aat-2**	**Amy**	**Mdh**	**Pgd**	**Pgi**	**All Loci**
Platteklip	0.04±0.04*	0.02±0.05	0.01±0.02	0.00±0.03	0.12±0.06***	-	0.03±0.01**
Skeleton	-0.00±0.01	-0.01±0.00	0.06±0.06***	0.03±0.04*	0.83±0.35*	-	0.11±0.11***
Kasteelspoort	0.02±0.03*	-0.02±0.00	-0.02±0.01	-0.12±0.00**	-	-	-0.01±0.01**
Eerste	-	0.03±0.03*	-0.00± 0.00	-	0.04±0.03**	-0.00±0.00	0.02±0.02**

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 5.5. Unbiased estimates of Nei's (1978) genetic identity calculated between populations of *Elporia barnardi* (Diptera: Blephariceridae)

		Kasteelspoort			Platteklip			Skeleton			Nursery	Eerste			Langrivier
		1	2	3	1	2	3	1	2	3		1	2	3	
	1	-													
Kasteelspoort	2	1.00	-												
	3	1.00	1.00	-											
	1	0.91	0.91	0.89	-										
Platteklip	2	0.88	0.88	0.88	0.99	-									
	3	0.92	0.92	0.91	0.99	0.99	-								
	1	0.91	0.92	0.92	0.89	0.90	0.89	-							
Skeleton	2	0.89	0.89	0.91	0.84	0.86	0.85	0.99	-						
	3	0.83	0.83	0.84	0.82	0.81	0.80	0.91	0.92	-					
Nursery		0.98	0.98	0.99	0.89	0.88	0.90	0.97	0.96	0.89	-				
	1	0.62	0.62	0.63	0.79	0.82	0.80	0.77	0.74	0.79	0.66	-			
Eerste	2	0.62	0.62	0.63	0.79	0.82	0.77	0.76	0.73	0.82	0.66	1.00	-		
	3	0.64	0.64	0.65	0.82	0.86	0.82	0.79	0.76	0.78	0.68	1.00	0.99	-	
Langrivier		0.61	0.61	0.61	0.80	0.83	0.78	0.75	0.72	0.81	0.65	1.00	1.00	0.99	-

MtDNA

A 641-bp fragment of the cytochrome *c* oxidase subunit I region of the mtDNA (TC content = 55.3%) was obtained from 93 individuals, revealing a total of 25 putative haplotypes. These were differentiated by 64 variable sites (Table 5.6). Of these, 59 were 3rd codon position changes, along with a single 2nd position and four 1st codon position changes. The transition (TS):tranvserion (TV) bias was relatively low at 8.7:1, with 15 pairwise substitutions transitions and two transversional pairs. One of these transitional pairs was at the 1st position with the other 14 at the 3rd position and both transversional pairs in the 3rd position. These changes resulted in six (of 213) amino acid changes, three of which were parsimoniously informative.

The NJ tree (Figure 5.5) reveals two very divergent clades supported by high bootstrap values. These two clades, separated by roughly 5% sequence divergence, correspond to the separation between the Hottentot's Holland range and Table Mountain (Tables 4.7 and 4.8). Based on an estimated 2.3% sequence divergence per million years, the level of divergence between these two clades suggests streams between these ranges have been isolated for around 2 MYrs. Clade A includes 13 haplotypes found only in streams on Table Mountain, while Clade B contains 11 haplotypes confined to the Hottentot's Holland range. Short internodal branch lengths within each of these clades are consistent with a star phylogeny. The distribution of haplotypes in Clade A shows an association with streams. For example, haplotypes B1 to B5 are found only in Nursery and Skeleton Gorges, which confluence at roughly the position of the intermediate site on Skeleton Gorge. Similarly, B6 to B9 are only found in Kasteelspoort and B10 to B13 only in Platteklip. The levels of divergence among the different streams on Table Mountain are in all instances equal to or less than 0.01±0.004 (Table 5.8). Among sites within the Jonkershoek catchment of the Hottentot's Holland range the levels of divergence. In comparison, average divergence among sites within the same stream was typically zero, and never more than 0.002±0.001.

Table 5.6. Variable nucleotide positions in the 25 *Elporia barnardi* haplotypes of a 641bp fragment of the mtDNA CO1 gene identified by DNA sequencing. Dots indicate identity to haplotype B1. Numbers refer to position of base pairs from the start of the fragment. Entire sequences can be retrieved from the GenBank database at accession numbers as indicated in the column to the far right of the table.

	1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 3 3 3 3 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6	GenBank Accession Numbers
	9 5 1 5 8 6 2 1 8 1 3 5 0 9 2 7 3 0 5 7 0 8 8 4 0 3 1 2 8 6 6 5 7 6 5 8 9 2 5 8 1 5 1 4 2 7 6 5 8 1 4 0 7 9	
B1	T G A C C G A A C C C T C A C G T T T C G C G C C C C T T C C A G G A C A C T T C T C C T C C C A G A A T T	AF427014
B2	. . . . . A	AF427015
B3	. . . . . G . . . . . A	AF427016
B4	. . G . . . . . A	AF427017
B5	. . . . . A . . . . . G	AF427018
B6	. . . . . A G . . . . . A . . . . . G	AF427019
B7	. . . . . T . . G . . C C . . . A . . . T C . . . . . T . . . . G . . .	AF427020
B8	. . . . . G . . . C . . . . A . . . . . C T . . . . G . . .	AF427021
B9	. . . . . G . . . C . . . . A . . . . . A . . . . . T . . . . T . . . . G . . .	AF427022
B10	. . . . . G . . . C . . . . A . . . . . A . . . . . T . . . . G . . .	AF427023
B11	C . . . . A C . . . . . G . . . C . . . . A . . . . . G . . . . . T . . . . G . . .	AF427024
B12	C T . . . A . . . . . G . . . C . . . T A . . . . . G . . . . . T . . . . G . . .	AF427025
B13	C . . . . A . . . . . G . . . C . . . . A . . . . . G . . . . . T . . . . G . . .	AF427026
B14	C . . . . A . . . . . G . . . C . . . . A . . . . . G . . . . . T . . . . G . . .	AF427027
B15	. ? . T T A G G T . . C . G T A . . C A A T A . T T T . C . . . C A . T . . . C . C T T . T T . . . G . . C	AF427028
B16	. T . T T A G G T . . C T . T A . . C A A T A . T T T . C . G G C . T T G T . C . C T T . T T . . . . . G C	AF427029
B17	. T . T T A G G T . . C T . T A . . C A A T A . T T T . C . G G C . T T G T . C . C T T . T T C	AF427030
B18	. T . T T A G G T . . C T . T A . . C A A T A . T T T . C . G G C . T T . T . C . C T T . T T C	AF427031
B19	. T . T T A G G T . . C . . . A . . C A A T A . T T T . C . . G C C . T . . . C . C T T . T T C	AF427032
B20	. T . T T A G G T . . C . . . A . . C A A T A . T T T . C . . G C A . T . . . C . C T T . T T C	AF427033
B21	. T . T T A G G T . . C . . . A . . C A A T A . T T T . C . . G C A . T . . C C . C T T . T T C	AF427034
B22	. T . T T A G G T . . C . . . A . . C A A T A . T T T . C . . G C A . T . . . C . C T T . T T . . . . G . C	AF427035
B23	. T . T T A G G T . . C . . . A . . C A A T A T T T T . C . . G C A . T . . . C . C T T . T T . . . . G . C	AF427036
B24	. T . T T A G G T . . C . . . A . . C A A T A T T T T . C T . G C A . T . . . C . C T T . T T G . . . G . C	AF427037
B25	. T . T T A G G T . . C . . . A . . C A A T A T T T T . C . . G C A . T . . . C . C T T . T T . . A . G . C	AF427038

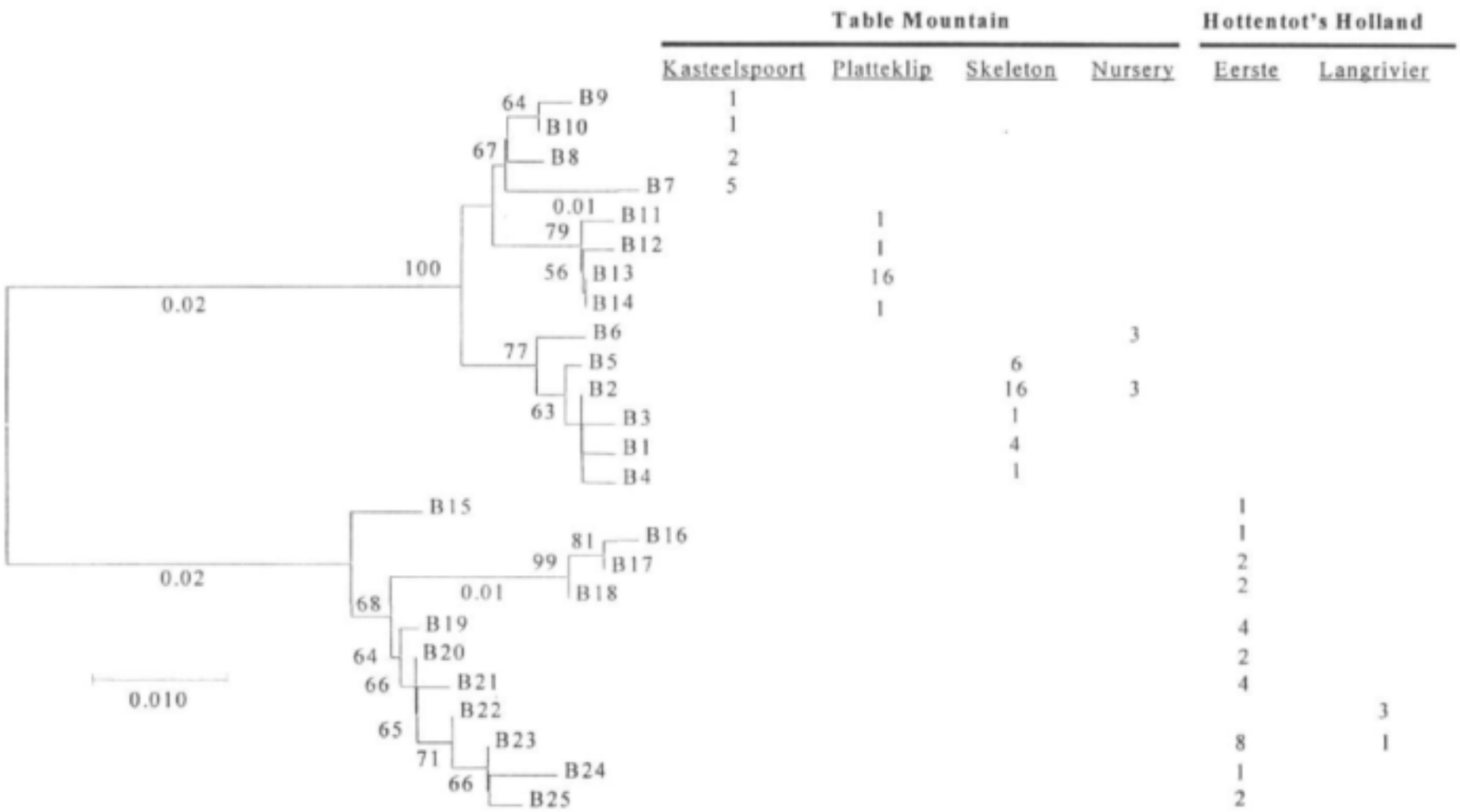

Figure 5.5. A mid-point rooted neighbour-joining tree, constructed using the Kimura-2-parameter (1980) model of substitution, for all COI haplotypes for *Elporia barnardi* (Diptera: Blephariceridae).

Table 5.7. Net nucleotide divergence (below diagonal) and standard error (top diagonal) among groups. Genetic distance among haplotypes was estimated using Kimura-2 parameter model (1980). See Materials and Methods section for further details.

		Kasteelspoort			Platteklip			Skeleton			Nursery	Eerste			Langrivier
		1	2	3	1	2	3	1	2	3		1	2	3	
Kasteelsport	1		0.001	0.001	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.009	0.009	0.009	0.009
	2	0.002		0.001	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.009	0.009	0.009	0.009
	3	0.000	0.002		0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.009	0.009	0.009	0.009
Platteklip	1	0.005	0.011	0.005		0.000	0.000	0.004	0.004	0.004	0.004	0.009	0.009	0.009	0.009
	2	0.005	0.011	0.005	0.000		0.000	0.004	0.004	0.004	0.004	0.009	0.009	0.008	0.009
	3	0.005	0.011	0.005	0.000	0.000		0.004	0.004	0.004	0.004	0.009	0.009	0.008	0.009
Skeleton	1	0.006	0.013	0.006	0.011	0.011	0.011		0.000	0.000	0.000	0.009	0.009	0.008	0.009
	2	0.006	0.013	0.006	0.011	0.011	0.011	0.000		0.000	0.000	0.009	0.009	0.008	0.009
	3	0.006	0.013	0.006	0.011	0.011	0.011	0.000	0.000		0.001	0.009	0.009	0.008	0.009
Nursery		0.007	0.014	0.007	0.010	0.010	0.010	0.001	0.001	0.001		0.008	0.008	0.008	0.008
Eerste	1	0.047	0.052	0.047	0.047	0.046	0.046	0.045	0.045	0.045	0.043		0.000	0.000	0.000
	2	0.046	0.051	0.046	0.046	0.046	0.046	0.045	0.045	0.045	0.042	0.000		0.001	0.000
	3	0.044	0.049	0.044	0.044	0.044	0.044	0.043	0.043	0.043	0.041	0.000	0.001		0.001
Langrivier		0.047	0.053	0.047	0.047	0.047	0.047	0.046	0.046	0.046	0.043	0.001	0.000	0.002	

Table 5.8. Net nucleotide divergence (below diagonal) and standard error (top diagonal) among groups and the within group divergence (on diagonal in bold).

	Kasteelspoort	Platteklip	Skeleton	Nursery	Eerste	Langrivier
Kasteelspoort	**0.005**	0.003	0.003	0.003	0.008	0.009
Platteklip	0.007	**0.000**	0.004	0.004	0.008	0.008
Skeleton	0.008	0.011	**0.001**	0.000	0.008	0.008
Nursery	0.009	0.010	0.001	**0.000**	0.008	0.008
Eerste	0.047	0.045	0.044	0.042	**0.010**	0.001
Langrivier	0.049	0.047	0.046	0.043	0.001	**0.001**

While unable to resolve the relationships between the two mountain ranges, the TCS haplotype network shows the distribution and relationship of the haplotypes among sites and streams within each of the ranges (Figure 5.6). From this it is evident that each stream is represented by its own unique set of haplotypes not shared between catchments. While the Hottentot's Holland network suggests that the sample sizes are insufficient with a number of missing haplotypes within the network, the Table Mountain haplotype network shows three distinct groups corresponding to catchment units. While the Kasteelspoort group reveals some missing internal haplotypes this could be due to small sample size. The most common haplotypes in both Platteklip and Skeleton Gorge streams are distributed across all three instream sites. These most common haplotypes (B2 and B13) are located internally (ancestral haplotypes) to a number of other haplotypes found in low numbers.

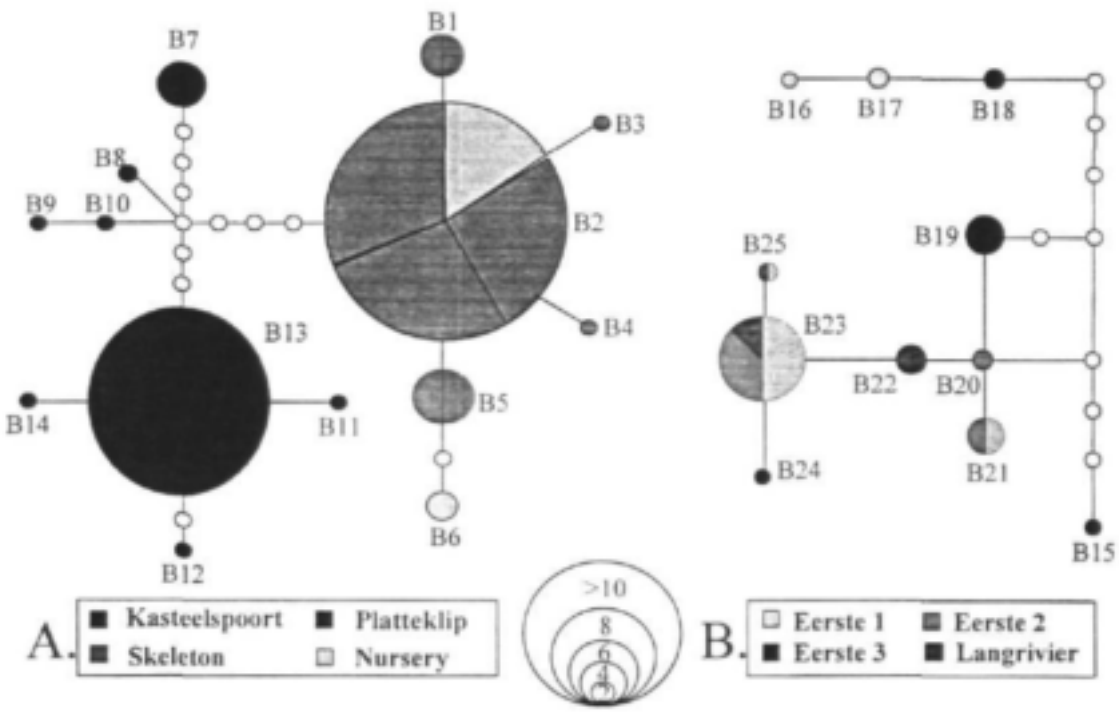

Figure 5.6. A haplotype network showing the relative frequencies and relationships between haplotype for *Elporia barnardi* (Diptera: Blephariceridae). The open circles represent missing haplotypes.

Analysis of molecular variance (AMOVA) was used to examine two different hierarchical models of variation. The first examined the distribution of genetic variation i) among ranges; ii) among streams within ranges and iii) within streams. The distribution of haplotypes among the two ranges explained nearly 80% of the variation (Table 5.9) with differentiation among stream populations, as indicated by the F_{ST} value, being extremely high (F_{ST}=0.94; P<0.001). The second hierarchical model examined variation i) among streams on Table Mountain; ii) among sites within these Table Mountain streams and iii) within each of these sites. Looking within a range the distribution of variation is again explained largely by differences among individual streams, more than 80% of the variation being between the streams on Table Mountain (Table 5.10). Again there is a large degree of population sub-structuring as indicated by a large, and significant, F_{ST} value (F_{ST}=0.88; P<0.001).

Table 5.9. Comparative results of a hierarchical Analysis of Molecular Variance (AMOVA), using Kimura-2 parameter model of substitution (1980) for determining genetic distances, examining the distribution of genetic variation among populations of the net-winged midge, *Elporia barnardi*, in the south-western Cape, South Africa.

Source of variation	***% Total Variation***	**Φ**	***P***
Among ranges	79.14	F_{CT} = 0.79	0.000
Among streams within ranges	14.68	F_{SC} = 0.70	< 0.001
Within streams	6.18	F_{ST} = 0.94	< 0.001

Table 5.10. Comparative results of a hierarchical Analysis of Molecular Variance (AMOVA), using Kimura-2 parameter model of substitution (1980) for determining genetic distances, examining the distribution of genetic variation among populations of the net-winged midge, *Elporia barnardi*, among streams on Table Mountain.

Source of variation	***% Total Variation***	**Φ**	***P***
Among streams on Table Mountain	85.67	F_{CT} = 0.86	0.00±0.00
Among sites within TMtn streams	2.17	F_{SC} = 0.15	0.24±0.01
Within sites	12.16	F_{ST} = 0.88	< 0.001

There is a significant relationship between genetic divergence and geographic distance (P<0.001) supporting a stepping stone model of isolation by distance. This is perhaps not unexpected given the large geographic distance between the two ranges and the effects of outliers on regression statistics. However, analysis of the relationship between genetic divergence and geographic distance among populations within each of the ranges supports this relationship ($P < 0.001$ in both instances).

Discussion

The Blephariceridae generally exhibit specialised habitat requirements and a high degree of habitat fidelity. Furthermore, Craig (1969) found the flight muscles in some New Zealand blepharicerids were degenerate and apparently unable to support sustained flight. All of this would appear to be reflected in the distribution of genetic variation observed among populations of *Elporia barnardi*, with both the allozyme and the mtDNA data exhibiting a high degree of structure. The hierarchical distribution of this genetic variation, the pattern of isolation by distance and structure reflected within the phenograms suggest a extremely limited capacity for dispersal beyond the catchment unit, with the possibility of restricted instream movement. The high degree of structure and patterns of observed variation reflect those expected for many obligate, non-aerial freshwater species.

Hardy-Weinberg equilibrium

Departures from Hardy-Weinberg equilibrium similar to those found in *Elporia barnardi* have been observed in a number of other aquatic insects (Snyder & Linton, 1984; Sweeney *et al.*, 1987; Robinson *et al.*, 1992), including populations of diving beetles in western Europe (Bilton, 1992) and baetids in south-east Queensland (Schmidt *et al.*, 1995). Hardy-Weinberg equilibrium assumes that samples are drawn from a very large population of randomly mating individuals (Hartl & Clarke, 1989). Departures from Hardy-Weinberg equilibrium can be indicative of a non-randomly mating population, result from inbreeding (Wright, 1978) or from the Wahlund effect, the statistical pooling of subpopulations which differ in allele frequency (Hartl & Clarke, 1989; Spiess, 1989). Many aquatic insects have non-random mating behaviours due to life history traits or demographics with sex ratios significantly different from 1:1 (Hayashi, 1988; Freilich, 1991; Waringer, 1991). Although there is no data available for the Blephariceridae, the closely related mountain midges (Diptera: Deuterophlebiidae: see Wood & Borkent, 1989; Courtney 1990, 1991) often display highly skewed sex ratios in favour of males (Kennedy 1958, 1960; Turner *et al.*, 1986). It is possible that natural selection acting upon a particular locus in favour of heterozygotes could lead to a similar departure from Hardy-Weinberg equilibrium (i.e. Watt *et al.*, 1996). However, the observed deviations from Hardy-Weinberg

equilibrium do not show a uniform pattern across particular loci as would be expected if they were due to selection.

It has been proposed that departures from HW observed in many aquatic insects are due to sampling biases, with the individuals sampled being from a limited number of matings. If this were the case, then the maternal mode of mtDNA inheritance would result in all individuals from a particular site having a shared haplotype. Given that individuals were collected from three sites spanning the species range you would also expect there to be strong, monophyletic relationship with the individual sites, with each represented by a unique haplotype. While each of the catchments displayed a unique set of haplotypes, each site was represented by more than a single haplotype. Furthermore, if such patterns were due to a limited number of females giving rise to the larvae sampled, and not as a result of restricted movement, it is unlikely that the catchment units would be monophyletic.

Geographic Population Structuring

The observed F_{ST} values among sites of *E. barnardi* in different streams and mountain ranges are only slightly less, and sometimes greater, than those observed at much wider geographic scales for some other lotic species. For example, Jackson & Resh (1992) found an F_{ST} value of 0.524 between caddisfly populations in California and the eastern part of America, while populations of the diving beetle, *Hydroporus glabriusculus*, across northern Europe displayed an F_{ST} of 0.199 (Bilton, 1992). Indeed, these values represent some of the highest recorded for aquatic invertebrates, especially those with a winged adult stage.

The phylogenetic relationships among mtDNA haplotypes shown here confirm a high degree of population sub-structuring over small spatial scales with the observed pattern of genetic variation similar to that of many obligate freshwater species. Although originally proposed for freshwater fish (Meffe & Vrijenhoek, 1988) the results of the AMOVA conform to the predictions of the hierarchical model proposed for non-diadromous freshwater fish species. Most of the variation can be explained by differences between the ranges, then by differences among streams within a range and a small part of the variation explained by differences within streams. This pattern was

repeated when the pattern of genetic variation was examined among Table Mountain streams, although little of the variation was explained by differences between the sites within a stream. Furthermore, the positive association between genetic divergence and geographic distance supports a model of isolation by distance (Slatkin, 1993).

Between Catchments

The similarity between F_{ST} values calculated between ranges and those between streams on Table Mountain suggest that the catchment is the primary unit within which movement of *E. barnardi* takes place. If individuals were moving freely beyond the catchment unit this would be reflected by low F_{ST} values. Similarly, the NJ tree of mtDNA haplotypes and the gene genealogy shows the larger Table Mountain clade includes a number of smaller, monophyletic clades corresponding to individual catchment units. With each catchment represented by a unique array of haplotypes not found elsewhere, it would appear that individuals are confined to within, and do not move between, the catchment units. The relatively small degree of divergence between these catchment clades (~1% or less) suggests a relatively short period of isolation around 400 to 700 ky.

Large differences in F_{ST} values have been observed between other geographically proximate populations of aquatic invertebrates. F_{ST} values for caddisfly populations in three California catchments separated by roughly 150km showed significant levels of genetic differentiation (F_{ST} = 0.425: Jackson & Resh, 1992). This decreased to 0.044 with removal of one of the three catchments. Levels of differentiation between stonefly populations in the northern Rocky Mountains reflect the isolating nature of the steep canyon catchments (F_{ST} = 0.156; Hughes *et al.*, 1999). In contrast, Hughes *et al.* (1998) found relatively little genetic differentiation in caddisfly populations in streams in northern Australia, which they attributed to adult dispersal. Patterns in the genetic structuring of *E. barnardi* populations are more similar to those observed for fully aquatic species. For example, the fully aquatic atyid species in northern Australia, *Paratya australiensis* and *Caridina zebra*, both show significant levels of differentiation between catchments (F_{ST} = 0.570 and 0.318, respectively) (Hughes *et al.*, 1995, 1996) similar to those of *E. barnardi* (F_{ST} = 0.39) in streams of the south-western Cape.

Within Stream

Significant differences in the genetic structure of populations within the streams on Table Mountain could be a reflection of the limited potential for dispersal of *E. barnardi*. Jackson and Resh (1992) found very small F_{ST} values between sites within three rivers in California. Values between three sites in the Eel River, Big Sulphur Creek and Alameda Creek (0.034, 0.008 and 0.015, respectively) are similar to those observed between sites within the Table Mountain streams. The large F_{ST} value recorded for Skeleton Gorge is dominated by the *Pgd* locus. Allele frequencies for this locus at the most downstream site (S3) are very different from those observed upstream. It is possible that the presence of a waterfall (10-15 m high) above this site provides an effective barrier to dispersal. The number of individuals for which mtDNA is available is insufficient to facilitate an examination of mtDNA structure.

Taxonomic Issues

The Peninsula area, including Table Mountain, is about 470km^2 and has long been renowned as a global hotspot of biodiversity within the Cape Floristic Kingdom for its diversity and endemism of higher plants (Trinder-Smith *et al.*, 1996). The first examination of the Table Mountain fauna failed to identify any endemic invertebrate species (Harrison & Barnard, 1971), leading the authors to conclude that there must be at least some active or passive dispersal to and from the mainland. Subsequent analyses based on improved taxonomic resolution have revealed a large number of endemic forms (Picker & Samways, 1996), such that the Peninsula has arguably one of the richest concentrations of endemic invertebrate species for any comparably small area in the world (Picker & Samways, 1996). Most of the 112 endemic species, from 14 invertebrate groups for which full species lists are available, are centred on Table Mountain. Levels of endemism within these 14 groups range from 5.3 to 67% (Picker & Samways, 1996). In all, there are 23 (including the two amphibian species) aquatic species endemic to the Peninsula mountain chain of which 12 are insects. These 12 aquatic insect species represent half of the endemic insects found on Table Mountain. The majority of these endemic species typically inhabit palaeogenic zones such as upper-reach forest streams, riverine forest and caves. Found at relatively high elevations and confined to the fast-flowing, high-gradient streams *Elporia barnardi*

represents part of this paleao-endemic fauna that is typical of much of the CFK and that has Gondwanaland affinities (Harrison, 1965).

The presence of a single allele at the *Aat-1* and *Mdh* loci in the Hottentot's Holland populations indicates negligible gene flow from Table Mountain to populations in the Jonkershoek catchment. The same is true for the single allele at the *Pgi* locus in all of the Table Mountain populations, which would also indicate negligible gene flow in the reverse direction from Jonkershoek populations to Table Mountain. Further evidence for allopatric speciation of the Table Mountain populations can be obtained from the estimates of genetic distance. The degree of genetic divergence as estimated by Nei's (1978) genetic identity (*I*) can be related to taxonomic level. Reviewing the relationship between genetic identity and taxonomic separation Thorpe (1982) concluded that about 97% of *I* values between species were less than 0.85. About 85% of *I* values between con-generic species exceed 0.35, suggesting that genetic identity for species within the same genera fall between 0.35 and 0.85. About 98% of conspecific *I* values exceed 0.85 (e.g. Brussard *et al*., 1985; Funk & Sweeney, 1990). While the genetic identity separating populations on Table Mountain from those in the Hottentot's Holland averaged 0.73, suggesting the existence of two possible taxa, such comparisons should only be based on a random selection of loci.

The level of sequence divergence between streams in the Hottentot's Holland and on Table Mountain shown in the NJ tree reflects a long period of isolation and would appear to support the idea that these two mountain ranges are inhabited by genetically distinct forms. Indeed, the degree of sequence divergence observed between these two ranges is within the range of COI values observed among a number of recognised con-generic species. In the absence of other available data, the level of genetic divergence among these populations suggests that isolation of this mountain massif has been important in speciation within the paleao-endemic, Gondwanan element of the fauna. For example, levels of divergence within the COI region among various sea urchin species ranged from 4.97% to 24.28% (McCartney *et al*., 2000) while among various species of aquatic dytiscids inter-specific values ranged from between 0.4 and 16.4% in the genus *Deronectes* and 0.97 and 15.8% in *Ilybius* (Ribera *et al*., 2001). Concordance between results obtained for mitochondrial alleles and nuclear loci would suggest that the catchment unit, in providing an effective barrier to dispersal of

E. barnardi, represents the primary unit within which micro-evolutionary processes are played out.

While rates of sequence divergence for the COI region have typically been based on Brower's (1994) survey of arthropod mtDNA and estimated at 2.3% per MY, more recent estimates from comparisons of sibling taxa across the Isthmus of Panama provide an estimate of 1.4% per MY (Knowlton & Weigt, 1998). This suggests that streams within these two ranges have been isolated for roughly two to three and half million years, around the end of the Tertiary (~2-65 MY). This was a geologically stable period in the Cape region, during which it is thought the land bridge connecting the Peninsula range, which includes Table Mountain, to the closest fold of the Hottentot's Holland was eroded (Walker, 1952; Deacon, 1983; Theron *et al.*, 1992). Persisting in a relatively stable environment for such extended periods, in the absence of gene flow, provides the basis for vicariant speciation. Such a model of isolation has been invoked as a model for the speciation of some taxa in the *Aphanicerca capensis* (Plecoptera: Notonemouridae) species complex. Within this group, levels of genetic divergence between Table Mountain and Hottentot's Holland species are similar to those observed in *E. barnardi*, at around 6% (see Chapter 5).

Ignoring the issues surrounding the degree of congruence between genetic data and true taxonomic units, the significant differentiation of allele frequencies at nuclear loci and the reciprocally monophyletic nature of mtDNA alleles for populations within individual catchments fulfils the criteria set out by Moritz (1994) for identifying evolutionarily significant units (ESUs). While the validity and definition of ESUs remains contentious (see Crandall *et al.*, 2000), units such as those identified here represent a potentially important component in the evolutionary legacy of a species, if not only in population processes. Such limited dispersal among adjacent catchments, and possibly even within, has important implications for the recovery of lotic systems following disturbance. Furthermore, given that it remains difficult to define or determine what is evolutionarily important, the development of inter-basin water transfers connecting historically isolated and genetically distinct populations poses potential threats to the evolutionary legacy of species such as *E. barnardi*. Given the increasing recognition accorded to the precautionary principle, with its inclusion now underpinning many international and national treaties on the protection

of biodiversity, it would seem prudent to incorporate such genetically distinct and potentially important units into the development of a conservation framework for lotic organisms.

In all, these results would seem to reflect the highly specialised habitat requirements exhibited by members of the Blephariceridae. While the significant deviations from Hardy-Weinberg equilibrium may be indicative of non-random mating or biased sampling of larvae that represent the product of only a few mating events, the distribution of mtDNA haplotypes would appear to suggest otherwise. Although little information is known of the mating behaviour other species of the group swarm and the adults are known to exhibit high habitat fidelity. The high F_{ST} values and patterns of mtDNA variation show a high degree of differentiation among populations in adjacent streams and between mountain ranges similar to those observed in obligate freshwater species. Although a non-random selection of nuclear loci was examined, the level of mtDNA sequence divergence and long period of separation would support the idea of two genetically distinct taxa. The observed structure and magnitude of differences observed at both nuclear and mtDNA loci strongly suggest that dispersal is limited to within the catchment unit within which movement takes place, with no movement of individuals between catchments.

Chapter 6

The Common Cape Stonefly
Aphanicerca capensis
(Plecoptera: Notonemouridae)

Introduction

The Plecoptera represent a primitive order of winged insects whose eggs and larval stages are entirely aquatic. Often one of the most important and dominant insect components of the stream fauna, nymphs typically require cool, well-aerated waters. Thus their distribution is limited to the fast-flowing reaches of high altitude streams, where the larvae are usually found on the underside of rocks in the main channel. The adults, while typically found on rocks projecting from or bordering the stream, are also common among the marginal vegetation. Sluggish insects with irregular, slow flights of short distance they are usually reluctant to take to the wing, typically eluding capture by running instead of flying.

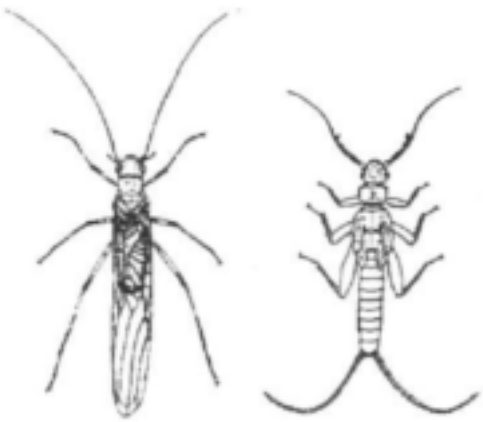

Figure 6.1. The stonefly, *Aphanicerca capensis*, (Plecoptera: Notonemouridae).

The adults are slender, elongate and generally short lived, typically emerging during winter and early spring and surviving for only a few days or a week or so. Specifically, *Aphanicerca capensis* exhibits a fairly asynchronous life history with a succession of broods that can be found more or less throughout the year, with the exception of a short period in mid-winter and late summer. While some stoneflies can reach sizes of up to 40mm (such as some of the large Eustheniidae) (Riek, 1973),

adults of the southern Africa Notonemouridae are typically much smaller, varying in size between *ca.* 5-8mm (Picker, 1996). In comparison to the adults, the larvae live for between one or two years, during which time they go through between 20 and 30 different instar stages. While the feeding habits of the southern African stoneflies are not well known, they are assumed to be similar to those of other species, with the nymphs feeding on plants and detritus and the adults on lichens and algae (Picker, 1996).

While the Plecoptera contains more than 1000 species world wide, the Notonemouridae are considered endemic to the Southern Hemisphere. With representatives found in Australia, New Zealand, South America, Madagascar and southern Africa they display a 'southern' distribution considered typical of elements of the palaeogenic fauna (Stuckenberg, 1962). The southern African distribution of the Notonemouridae is similar to that observed in other palaeogenic invertebrates, such as the Blephariceridae, following closely the boundaries of the escarpment and the Cape fold mountains. While both of these regions are characterised by a distinct fauna, the south-western region of the Western Cape Province is considered the distribution centre of the southern African Notonemouridae (Stevens & Picker, 1995), being far more speciose and supporting a larger number of genera and endemic species.

Specific identification of larval Plecoptera is generally difficult (Hynes, 1976; Picker & Stevens, 1997), with the Notonemouridae being no exception (Picker & Stevens, 1997). While acknowledging this, 31 species in six genera have been described from southern Africa (Stevens & Picker, in press). Barnard (1934) noted slight variations in the male and female genitalia from specimen localities off the Cape Peninsula but concluded them insufficient to justify varietal names. More recent work by Stevens and Picker (1999; unpublished data) has revealed a number of possible species complexes within the Cape Notonemouridae. For example, previously considered a single species, recent morphological revisions of *Aphanicercella barnardi* by Stevens and Picker (1999) identified five different, geographically discrete forms differentiated on the basis of morphological variation in the genitalia. Mate choice experiments subsequently confirmed the biological species status of each of these morphologically discrete forms.

Currently the genus *Aphanicerca* comprises six species. Of these *A. tereta* Barnard, *A. uncinata* Barnard and *A. bovina* Barnard are considered rare in comparison to the more abundant and widely distributed *A. capensis* Barnard, *A. bicornis* Barnard and *A. lyrata* Barnard (Picker & Stevens, 1997). Restricted to the southwestern Cape region, Stevens and Picker (pers. comm.) have, however, recently identified a number of consistent morphological differences in specimens of *Aphanicerca capensis* between discrete geographic localities (Figure 6.2). Thus, in addition to examining the hierarchical relationships and patterns of genetic variation among catchments common to the other taxa surveyed in this work, direct sequencing of the COI fragment of the mtDNA was used to examine relationships and levels of divergence between a three of these morphologically discrete forms. The observed levels of sequence divergence were subsequently examined in relation to data available from mate recognition experiments and detailed morphological examinations of the genitalia (Rous, 1999; Stevens, unpublished data).

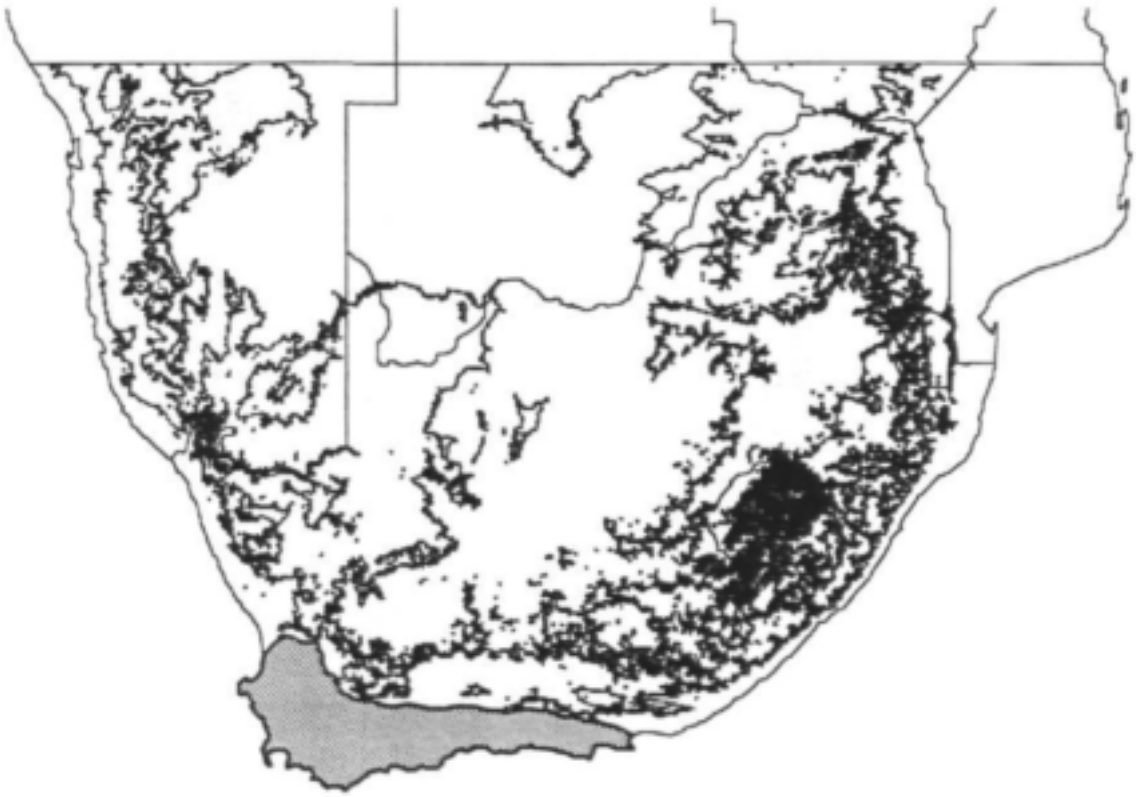

Figure 6.2. Approximate distribution of *Aphanicerca capensis* (Plecoptera: Notonemouridae) in the southwestern Cape, South Africa.

Methods

Collection

Fifty or more individuals were sampled from three sites in the Kasteelspoort, Platteklip and Skeleton Gorge streams on Table Mountain and three sites along the Eerste River in the Jonkershoek catchment (Figure 6.3a). Individuals were also collected from a single site in Swartboskloof, Assgaibos and Langrivier, all first order tributaries in the Jonkershoek catchment (Figure 6.3b). In addition, sequence variation was examined in a further five individuals from three streams representing a third morphologically discrete form identified by Stevens and Picker (pers comm) (Figure 6.3c).

Allozyme Electrophoresis

Starch gel electrophoresis was used to examine genetic population structure in fifty individuals from each of the three sites in Kasteelspoort, Platteklip and Skeleton Gorge on Table Mountain and all six sites within the Jonkershoek catchment. Whole individuals of *A. capensis* were homogenised and a total of 39 enzymes screened initially. Eight of these yielded interpretable results.

MtDNA

Approximately five individuals were sequenced from each of the sites in streams common to all taxa on Table Mountain and in the Eerste River. This gave data on mtDNA sequence variation for roughly fifteen individuals from each of these streams. In addition, mtDNA sequence data was obtained from five individuals from a number of other streams. These included Assgaibos and Swartboskloof in the Eerste River catchment and Bainskloof, representing the second morphological form. Individuals from two streams in the Garcias Pass and a single stream in the Marloth Nature Reserve were also sequenced, representing the third morphologically discrete form.

Results

Allozyme Electrophoresis

Allozyme electrophoresis revealed no variation among the roughly 700 individuals and 13 sites surveyed. Of the 39 enzymes initially screened all eight of those resolved for populations of *A. capensis* were monomorphic, fixed for the same allele among each of the sites across both mountain ranges.

MtDNA

Nucleotide Composition

A 630-bp fragment of the cytochrome *c* oxidase subunit I (COI) region of the mtDNA was sequenced from a total of 51 individuals taken from 19 sites among 10 different streams. From these individuals a total of nine putative haplotypes were identified (Table 6.1). These sequences have been deposited in the GenBank Data Library with the Accession Numbers given in Table 6.2. There were a total of 51 (8.10%) variable sites with an observed T/C content of 53.3%. Of these variable sites, 47 (92.16%) were in the third codon position and four (7.84%) were in the first position. There was a transition:transverion bias of 18:6, with two first position and 16 third position transitional pairs and six third position transversional pairs. These changes resulted in three amino acid changes. Diversity within each of the groups was very low, 0.001 or less in all instances. Four of the five groups showed no within group variation at all being represented by a single haplotype. Despite this low variability within groups, there were large levels of divergences recorded between some groups with estimates of the Kimura-2 parameter distances ranging from 0.071±0.01 to zero divergence between groups within the same catchment or range (Table 6.2).

Table 6.1 Variable nucleotide positions in the nine *Aphanicerca capensis* haplotypes of a 630-bp fragment of the mtDNA cytochrome oxidase *c* subunit 1 gene identified by DNA sequencing. Dots indicate identity to haplotype A1. Numbers refer to position of base pairs from the start of the fragment. Entire sequences can be retrieved from the GenBank database at accession numbers as indicated in the column to the far right of the table.

	1 1 1 1 1 1 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 6 6 6 6	Gene Bank Accession No[1]
	2 3 4 4 7 1 3 5 7 8 9 0 1 2 2 3 4 8 0 1 1 2 3 3 4 6 7 7 8 8 9 2 2 3 4 5 6 7 0 2 2 4 5 5 5 6 0 0 0 1	
	9 1 6 5 8 5 7 5 9 7 4 8 1 3 5 8 4 9 2 6 2 8 1 3 9 2 0 2 8 7 8 4 0 3 8 4 0 8 4 7 2 5 3 2 5 8 8 3 6 9 8	
A1	G C G C G T A T G T A G C C C T G C A T G A T A G C T A G T T A C A A T G A A A A G G A C C C T A G	AF429296
A2	. . T	AF429297
A3	. A	AF429298
A4	A G A A A . G . A C G A T . A . A T G A A T . . A T C C . C C G A C . C A G G G C A A C T T T . G A	AF429299
A5	A G A A A . G . A C G A T . A . A T G A A T C . A T C C . C C G A C . C A G G G C A A C T T T . G A	AF429300
A6	A G A A A . G C A C G A T . A . A T G A A T . . A T C C . C C G A C . C A . G G C A A C T T T . G A	AF429301
A7	A G A A A . G C A C G A T A A . A . G A A T . C A T C . A C C G A C . C A . G G C A A C T T T C . A	AY058902
A8	A G A A A . G C A C G A T A A C A . G A A T . C A T C . A C C G A C C C A . G G C A A C T T T C . A	AY058903
A9	A G A A A C G C A C G A T A A . A . G A A T . C A T C . A C C G A C C C A . G G C A A C T T T C . A	AY058904

Table 6.2. Nucleotide diversity within groups (on diagonal) and net nucleotide divergence (below diagonal) and standard error (top diagonal) among groups which were identified by the diversity of mtDNA haplotypes and their distribution. Genetic distance among haplotypes was estimated using Kimura-2 parameter model (1980). See Materials and Methods section for further details.

	Langrivier	Assegai	Eerste	Kasteelspoort	Platteklip	Skeleton
Langrivier	**0.000**	0.00	0.00	0.01	0.01	0.01
Assegai	0.000	**0.000**	0.00	0.01	0.01	0.01
Eerste	0.000	0.000	**0.001**	0.01	0.01	0.01
Kasteelspoort	0.071	0.071	0.071	**0.001**	0.00	0.00
Platteklip	0.070	0.070	0.071	0.001	**0.000**	0.00
Skeleton	0.071	0.071	0.071	0.000	0.000	**0.000**

Three distinct clades, each well supported by high bootstrap values, are evident from the NJ tree for *Aphanicerca capensis* (Figure 6.4). This pattern reflects the pattern of diversity and degree of sequence divergence, with the large divide corresponding to divergence between the individuals from the Hottentot's Holland mountain range and Table Mountain. The same pattern is reflected in the haplotype networks for *A. capensis*. Due to the high degree of sequence divergence, the TCS programme was unable to resolve the relationships between the three clades (Figure 6.5).

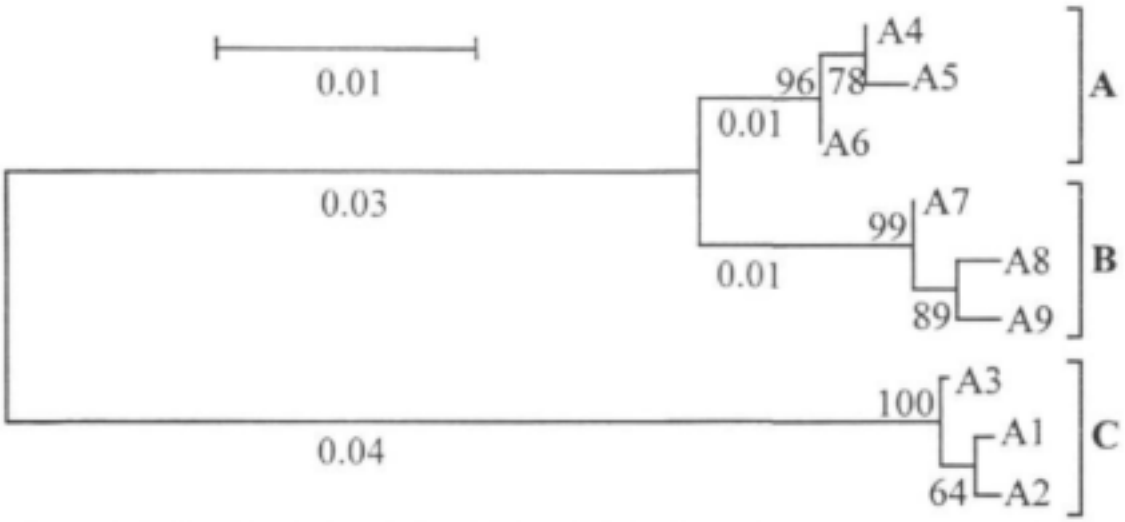

Figure 6.4. A mid-point rooted neighbour-joining haplotype tree, constructed using the Kimura-2 parameter (1980) model of substitution, for all COI haplotypes for *Aphanicerca capensis* (Plecoptera: Notonemouridae). The reconstructed phylogeny indicates three major clades: A. representing the first morphological form identified from streams in the Jonkershoek and Bainskloof catchments; B. haplotypes from the second morphological form sampled from Garcia's Pass and Marloth Nature Reserve; and C. haplotypes from streams on table Mountain representing the third morphological form.

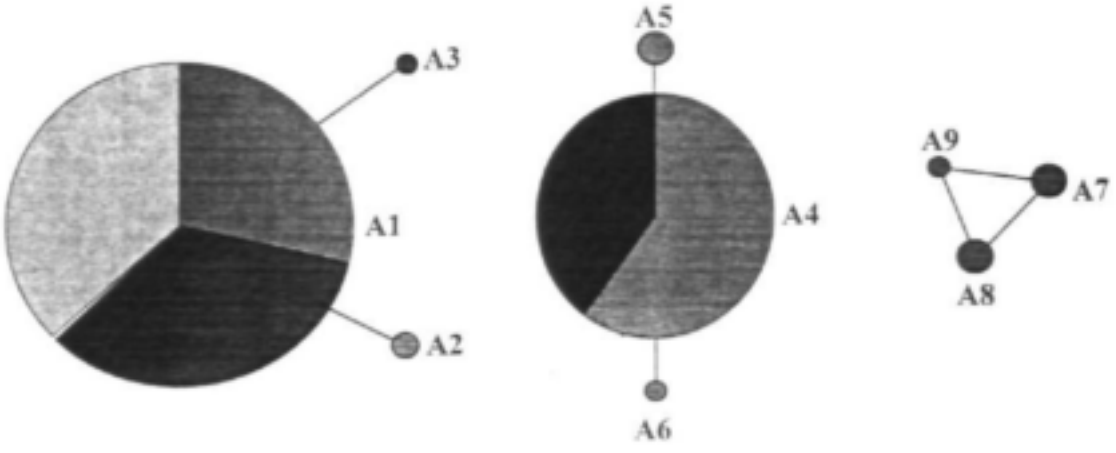

Figure 6.5. A haplotype network showing the relative frequencies and relationships between haplotypes of *Aphanicerca capensis*. The open circles represent missing haplotypes.

Examination of the distribution of the genetic variation using Analysis of Molecular Variance (AMOVA) shows that for *Aphanicerca capensis* 99.35% of the variation is among the Hottentot's Holland and Table Mountain ranges (Table 6.3).

Table 6.3a. Comparative results of a hierarchical Analysis of Molecular Variance (AMOVA), using Kimura-2 parameter model of substitution (1980) for determining genetic distances, examining the distribution of genetic variation in the stonefly, *Aphanicerca capensis*, among populations in the south-western Cape, South Africa.

Source of Variation	*% Total Variation*	**Φ**	***P***
Among ranges	99.34	F_{CT}=0.99	0.09±0.01
Among streams within ranges	0.03	F_{SC}=0.05	0.16±0.01
Within streams	0.62	F_{ST}=0.99	< 0.001

Table 6.3b. Comparative results of a hierarchical Analysis of Molecular Variance (AMOVA), using Kimura-2 parameter model of substitution (1980) for determining genetic distances, examining the distribution of genetic variation in the stonefly, *Aphanicerca capensis*, among streams on Table Mountain in the south-western Cape, South Africa.

Source of Variation	*% Total Variation*	**Φ**	P
Among streams on Table Mountain	11.12	F_{CT}=0.11	0.21±0.01
Among sites within Table Mountain streams	-11.11	F_{SC}=-0.13	1.00±0.00
Within sites	99.99	F_{ST}=0.00	1.62±0.01

Discussion

Geographic Population Structuring

Due to the isolating nature of catchment units and the hierarchical stream structure, lotic organisms are typically assumed to exhibit a high degree of genetic structure (Meffe & Vrijenhoek, 1988; Hughes *et al.*, 1995; Wishart & Hughes, 2001). However, while contemporary patterns of genetic variation reflect the influence of demographic and ecological processes they are also constrained by the effects of biogeographic processes that have operated throughout a species history. As such, determining the influence of contemporary dispersal on the observed patterns of genetic population structure in *Aphanicerca capensis* would appear to be limited by historical events that have largely eroded any genetic variation among these populations. Indeed, the lack of variation among populations of *A. capensis* makes it impossible to make any inferences about the effects of contemporary processes, such as dispersal, on genetic population structure.

It is evident from the monophyletic nature of the three primary clades and level of divergence reflected in the NJ tree for the COI region of the mtDNA that there is no gene flow between the three groups. The level of sequence divergence would suggest that individuals on Table Mountain have been isolated for roughly 4 MYrsBP, while the two groups in the Hottentot's Holland range have been isolated for in the order of a million years. Furthermore, in the absence of significant variation at the population level it could be hypothesised that the lack of congruence between monophyletic clades and catchment units could be indicative of dispersal among streams within a range. If dispersal were limited to within a catchment unit as has been observed in other stonefly species (Hughes *et al.*, 1999), then over time processes such as random mutation, genetic drift and lineage sorting would result in unique genetic signals associated with the catchment unit. Support for such an hypothesis can be derived from a comparative phylogeographic approach, as undertaken in Chapter 8.

Variability

The low level of genetic variability within and between ranges suggests historical factors have been important in eroding levels of genetic variation. The lack of variability in both nuclear and mtDNA in *A. capensis* could arise from founder effects or recent expansion events. Given the ancient Gondwanaland origins of the Cape fauna and the level of mtDNA sequence divergence between the two ranges it is likely that these patterns are not due to founder effects, but rather successive bottlenecks that have operated throughout the species history. Given that all nuclear loci were fixed for the same allele it is likely that the population experienced a bottleneck prior to separation of the two ranges. The deep divergence, lack of shared haplotypes and absence of any variability within mtDNA in either of the ranges would further suggest that these populations have experienced subsequent bottlenecks after erosion of the land bridge between the two ranges. The level of sequence divergence between the two clades would suggest that this has taken place over the last three to four million years, most probably during climatically induced sea level changes during the past 400 ky. While the mountains of the south-western Cape were low enough to escape the effects of glaciation, the low elevation would have meant that sea level rises would have severely reduced total stream lengths thus effectively reducing population sizes.

Taxonomic Issues

Recent revisions of the stonefly genus *Aphanicercella* have identified a number of morphologically discrete forms of *A. barnardi* that differ from one another and from other known species of *Aphanicercella* (Stevens & Picker, 1999). These forms display minor yet consistent differences in male and female genitalia, with no evidence of intermediate forms, and have smaller geographical ranges than other more morphologically diverse species. Stevens and Picker (1999) consider these forms to be five valid new and closely related species within the *A. barnardi* species-complex. Clear positive assortative mating within forms, indicative of reproductive isolation, was used to confirm biological species status. Similar morphological variation among *A. capensis* was at first not considered sufficient to warrant varietal names (Barnard, 1934). More detailed morphological examinations have subsequently identified ten

morphologically discrete forms among *A. capensis* (Stevens & Picker, unpublished data). Consideration of these differences in relation to genetic divergence and mate choice experiments reveals some interesting considerations in delineating species or functional units.

Concepts defining segments of biological diversity, such as species and evolutionarily significant units, are paramount to the timely and effective conservation of biodiversity. Such segments can be conceptually regarded as cohesive groups of individuals or organisms through time and over space that posses their own independent evolutionary fate and historical tendencies (Wiley & Mayden, 1997). However, the question of delineating and defining such units has been debated for centuries (Darwin, 1859; Mayr, 1957; Avise & Walker, 1999; Hendry *et al.*, 2000). Ideally, a species concept should provide a set of universally valid criteria that are easily applicable and yet theoretically significant such that they resulting in biologically meaningful groupings (Mallet, 1995; Hull, 1997). Mayden (1997) identified no less than 25 different species concepts, each derived from a different set of assumptions about descent with modification.

The lack of congruence between discrete morphological and phylogenetic units and the pattern of mate recognition in *A. capensis* highlights some of the problems associated with species delineations. Classic considerations of species units recognise that each is usually morphologically distinguishable from its closest relatives, with morphological distinctiveness assumed to be constant and heritable, thus serving as a surrogate for lineage independence. While such morphological considerations provide an operational framework for separating taxa, they are focussed primarily on pattern and lack the necessary theoretical foundation incorporating the processes involved. With the recent advances in gene technologies, more recent approaches have focussed on phylogenetic differences between different units. Such approaches provide a fairly pragmatic approach, and allow an assessment of gene flow, often used as a surrogate for reproductive isolation. However, unless such phylogenetic comparisons are conducted on sympatrically occurring taxa interpretation is ambiguous. Despite this, there have been many attempts to equate levels of genetic divergence with taxonomic status (Thorpe, 1982; McCartney *et al.*, 2000; Ribera *et al.*, 2001).

Many of the problems surrounding the use of genetic criteria to define species units arises from disagreement on the degree of convergence between mtDNA discontinuities and other species attributes, such as morphology and mate recognition. For example, Avise and Walker (1999) concluded from their study of mtDNA diversity within 252 taxonomic species of vertebrates that "mtDNA data and traditional taxonomic assignments tend to converge on what therefore may be real biotic units in nature". However, re-analysing their data, Hendry *et al.* (2000) came to the conclusion that mtDNA discontinuities do not match those of recognised taxonomic species and advocate abandoning the concept of species in favour of grouping organisms at any level by specifying the amount of difference in various traits. Both groups acknowledge that there are many problems associated with such comparisons. For example, the non-equivalence of taxonomic rank among different types of organisms (Avise & Johns, 1999), the subset of species chosen, their ranges and the subset of samples within these, along with the use of different genetic markers (Avise & Walker, 1999). As such, they advocate further efforts to extend tests of species realities to larger data sets, other taxa, and to the use of additional analytical techniques. While it may not facilitate such broad scale comparisons and generalisations, many of these problems can be overcome by looking within a particular group or species complex.

The consistent morphological differences among the genitalia in the three forms of *A. capensis* presented here, as identified by Stevens (Department of Zoology, University of Cape Town), would suggest the presence of three separate species under a morphological species definition. Congruence between these morphological forms and the three monophyletic clades in the NJ tree would further support such delineations. The levels of sequence divergence would also appear to correspond with various geological events of importance in terms of isolating populations. However, considering the pattern of assortative mating provides some interesting considerations. Mate choice experiments have been carried out in various combinations among the ten identified morphological forms (Stevens, unpublished data) and phylogenetic studies are continuing (Wishart, unpublished data). Of the three forms represented here mate choice experiments were carried out between the Jonkershoek and Table Mountain forms. Males from Table Mountain streams placed with females from both Table Mountain and Jonkershoek show positive assortative mating with females from

Table Mountain. This would suggest the evolution of unique mate recognition system and supporting separate species status. In comparison, when males from the Jonkershoek catchment are presented with a choice of females they show a random pattern of mating, suggesting that males of the Jonkershoek form are unable to distinguish between females of different forms.

While genetic distance is increasingly being used to differentiate species, evolutionary units and other biological entities, these results show that while there may be a correlation between morphological and genetic differentiation, they do not necessarily reflect biological species status. Indeed, from the data available for *A. capensis* there appears to be no relationship between morphological, phylogenetic and biological species. It should be noted however, as a word of caution in assigning species status, that the viability and relative fitness of subsequent generations has not been assessed. Furthermore, while mtDNA is increasingly being used to address inter- and intra-specific questions the COI fragment of the mtDNA, indeed the mitochondria itself, effectively represents a single character. It is possible that other genetic markers may reveal a different pattern. Despite such considerations, the relative comparisons between the different criteria do not display a consistent pattern of differentiation. While more detailed morphological, phylogenetic and mate choice experiments are being carried out these preliminary results highlight some important theoretical considerations. According to a biological species definition species status is only accorded to those reproductively isolated species. As such, the potential for interbreeding among different forms, even though only in one direction, highlights the need to identify other important components of biodiversity, such as evolutionarily significant units. Ignoring the current debate surrounding the definition of such units, the presence of morphologically and genetically discrete units along with the partial evolution of a unique mate recognition system in one form would provide support for delineating such units.

Chapter 7

The South African Stream Hawker
Aeshna subpupillata
(Odonata: Aeshnidae)

Introduction

Renowned for their diversity, widespread distribution and highly developed powers of flight the dragonflies represent one of the most characteristic components of the aquatic fauna. An ancient group, the dragonflies have remained relatively morphologically unchanged since the Permian period, some 290 MyBP. The adults are elongate and robust in both body form and wing structure with a high degree of secondary reticulation, with the larvae exhibiting a similarly robust form. While some adults can exceed more than 150mm in length, the largest of the African dragonflies are the Aeshnidae, which have a wingspan ranging between 90 and 140mm, abdomen length of 40-80 mm (Pinhey, 1996) and larvae that can grow to around 100 mm in length.

Figure 7.1. Adult and larval diagrams of the dragonfly, *Aeshna subpupillata* (Odonata: Aeshnidae).

Possessing highly developed powers of flight, there is evidence to suggest that some dragonfly species are capable of flying many hundreds of kilometres. Locally, some southern African species, such as the aeshnid *Anax tristic* and the libellulid *Tholymis tillarga*, have been recorded more than 60km off the Angolan mainland in the open Atlantic Ocean (Schneider, 1991). Other dragonfly species, such as the African aeshnid *Anax imperator*, undertake annual migrations. While common across Africa the distribution of *A. imperator* extends across Europe and western Asia (Pihney,

1996). While possessing the potential for rapid, widespread movement many of these fast flying adults often also exhibit complex behaviours related, but not limited, to territories and reproduction. Such territorial behaviours can result in a high habitat or site fidelity for mating pairs. However, dragonflies will typically fly away from the water after emergence (Corbet, 1980) and adults caught at a site are usually emigrants (Michiels & Dhondt, 1991). While the adults are responsible for movement across large geographic distances between catchments the larvae, which by virtue of the fact they are voracious predators constitute a dominant part of the aquatic community, are capable of rapid movement either by crawling or expelling water from the anus. Furthermore they do not exhibit any specific habitat requirements.

At present around 6000 species of dragonflies and damselflies have been identified from all parts of the world. Continental Africa is home to about 1000 species of odonate, many of which are cosmopolitan species found throughout Africa from the Mediterranean to the southern Cape regions (Skaife, 1994). In South Africa, 155 species have been recorded, of which 29 (18.7%) are endemic (Samways, 1999). The family Aeshnidae, with its Gondwanaland origins, is represented by five cosmopolitan genera that includes roughly 15 or more species (Pinhey, 1996). The genus *Aeshna* includes 3 species in sub-Saharan Africa, with *A. subpupillata* and *A. minuscula* the only representatives of this genus found in the south-western Cape region. Known as the South African Stream Hawker, *Aeshna subpupillata* McLachlan 1896, is especially common in montane bush or forest streams, all the way from the Cape, throughout South Africa and into parts of Mashonaland in Zimbabwe (Figure 7.2).

Given the highly developed powers of flight, the robust morphology and characteristics of *Aeshna subpupillata* it was hypothesised that samples from the different catchments would show a homogeneous genetic structure reflecting a widespread, interbreeding and panmictic population. In order to examine the extent of gene flow among this widely dispersed, highly mobile species a hierarchical sampling design was used to examine the distribution of genetic variation and degree of population sub-structuring. Patterns of genetic variation among individuals collected from sites common to other sampled species on Table Mountain and in the Hottentot's Holland were examined using allozyme electrophoresis and direct sequencing of the mtDNA as outlined in Chapter 3.

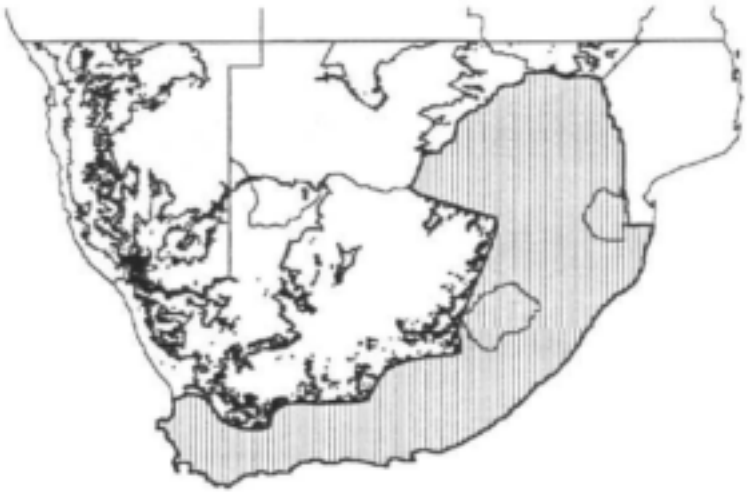

Figure 7.2. Approximate distribution of the dragonfly, *Aeshna subpupillata* (Odonata: Aeshnidae).

Methods

Collection

Due to the longevity and relatively low densities of the larvae, individuals were collected over a relatively wider range and from a single locality within each stream. A minimum of fifty individuals were collected using a kick net or by hand. Four streams on Table Mountain were sampled, these being the Disa, Kasteelspoort, Platteklip and Skeleton Gorge. In addition, another two sites were sampled in the Jonkershoek catchment. Individuals were collected from within the Eerste River and Langrivier, a first order tributary to the Eerste River (Figure 7.3). Individuals were identified using Wilmot (1960) and stored at -80°C.

Allozyme Electrophoresis

Starch gel electrophoresis was used to examine genetic population structure, with small samples of tissue removed from inside the abdomen. A total of 39 enzymes were initially screened of which 15 yielded consistently reliably and interpretable results (Table 7.1). More detail on the methods used are given in Chapter 3.

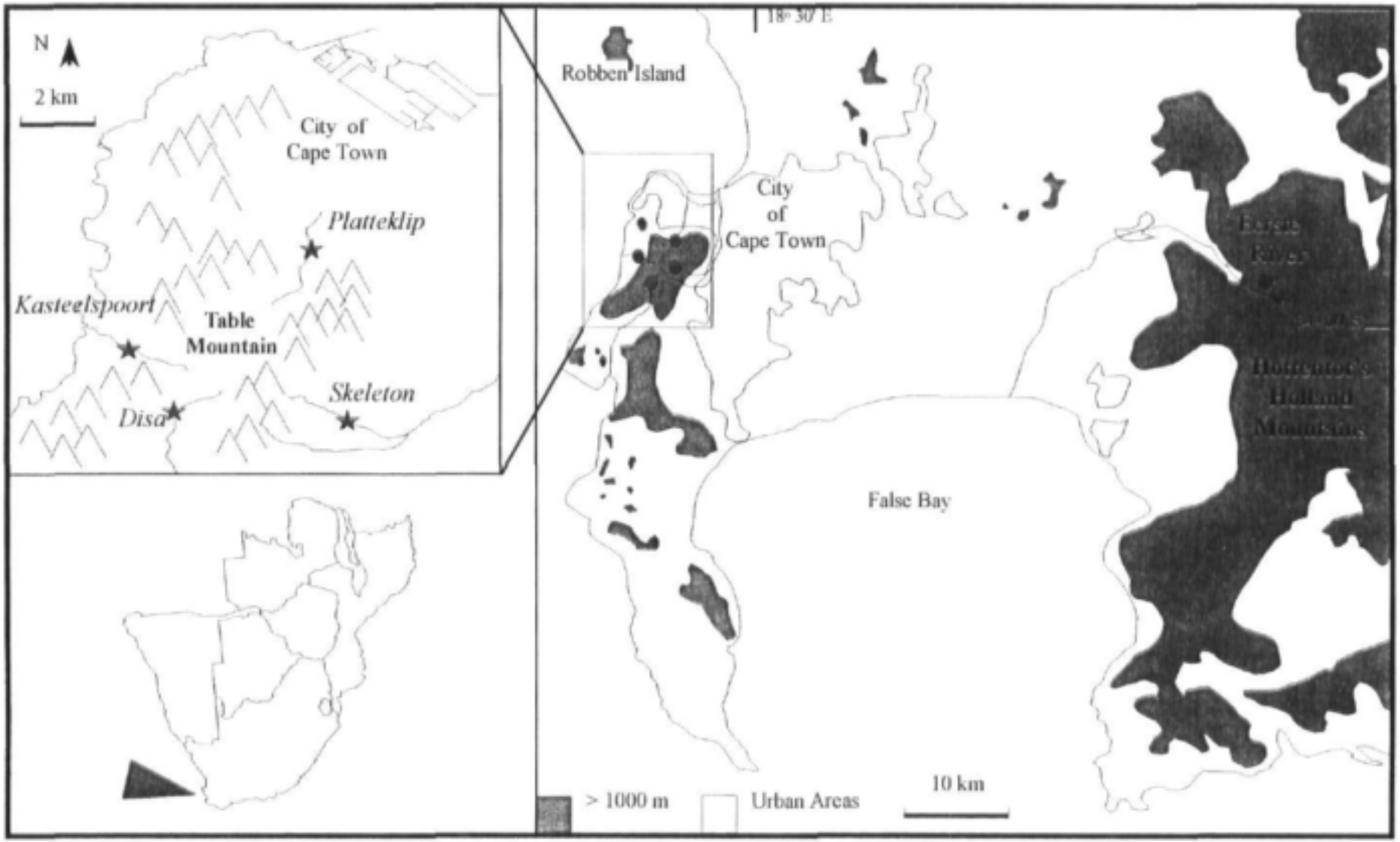

Figure 7.3. Location of sampling sites for *Aeshna subpupillata* (Odonata: Aeshnidae) among streams of the southwestern Cape, South Africa.

Table 7.1. Enzymes and running conditions used in the allozyme electrophoretic analysis of *Aeshna subpupillata* (Odonata: Aeshnidae). Buffer systems used were: MF: Tris-Borate-EDTA (Markert & Faulhaber, 1965); RW: Lithium Hydroxide-Borate (Ridgway *et al.*, 1970) and TC: Tris citrate (Whitt, 1970).

Protein	Abbr.	E.C. #	Run Buffer
Lactate dehydrogenase	*LDH*	1.1.1.27	RW
Malate dehydrogenase	*MDH*	1.1.1.37	TC
Malic enzyme	*ME*	1.1.1.40	MF
Iso-citrate dehydrogenase	*IDH*	1.1.1.42	TC
Glucose dehydrogenase	*GDH*	1.1.1.47	TC
6-Phosphogluconate Dehydrogenase	*PGD*	1.1.1.49	TC
Glyceraldehyde-3-phosphate dehydrogenase	*GAP/ G3pdh*	1.2.1.12	TC
Diaphorase	*DIA*	1.6.2.2	RW
Aspartate Aminotransferase	*AAT*	2.6.1.1	TC
Hexokinase	*HK*	2.7.1.1	TC
Creatine kinase	*CK*	2.7.4.2	RW
Adenylate kinase	*AK*	2.7.4.3	RW
Phosphoglucomutase	*PGM*	2.7.5.1	TC
Peptidase Glycyl-leucine	*PepGL*	3.4.11.-	MF
Peptidase Leucyl-Tyrosine	*PepLT*	3.4.11.-	MF
Peptidase LGG	*PepLGG*	3.4.11.-	MF
Peptidase PHP	*PepPHP*	3.4.11.-	MF
Fumerase	*FUM*	4.2.1.2	TC
Mannose-6-phosphate isomerase	*MPI*	5.3.1.8	TC
Glucose Phosphate Isomerase	*PGI*	5.3.1.9	RW
Phosphoglucomutase	*PGM*	5.4.2.2	MF

MtDNA

Sequence variation in the COI region of the mtDNA was examined among a minimum of ten individuals per stream according to the protocols outlined in Chapter 3. A 648-bp fragment was resolved for 55 individuals of *A. subpupillata* and analysed according to the methods outlined in Chapter 3.

Results

Allozyme Electrophoresis

Allozyme electrophoresis revealed little variation among populations. Of the 39 enzymes initially screened 13 produced interpretable data revealing a total of 20 loci. While seven loci displayed polymorphism among all sites suitable for the calculation

of *F* statistics, only four provided sufficient within site variation for calculation of Hardy-Weinberg estimates. Allele frequencies of polymorphic loci and sample sizes for each of the sites are given in Appendix 3.

Deviations from Hardy-Weinberg equilibrium

Genotypic frequencies deviated significantly from Hardy-Weinberg equilibrium in 11 of the 26 loci examined (42.31%: Table 7.2). The observed deviations are more than would be expected due to chance alone (1.3). With the exception of the DIA3 locus at Platteklip, all Pgm and DIA3 loci show deviations from the expected Hardy-Weinberg frequencies. All deviations, aside from the MDH2 locus, from Hardy-Weinberg equilibrium were positive indicating a deficiency of heterozygotes. Of the pairwise comparisons carried out to determine the presence of linkage disequilibrium only 12 were significant (1.04 %), a result that could be expected through chance alone.

Table 7.2. Estimates of the fixation index (F_{IS}: Weir & Cockerham, 1984) for each of the samples of *Aeshna subpupillata* (Odonata: Aeshnidae) from streams in the south-western Cape, South Africa. Bold indicate significance.

	Pgm	GPI	DIA3	MDH1	MDH2	PepLGG
Disa	**0.33**	0.23	**0.26**		-0.17	
Skeleton	**0.42**	**0.65**	**0.60**	**1.00**	-0.09	
Kasteelspoort	**0.48**	0.32	**0.48**		-0.51	
Platteklip	**0.50**	-0.23	0.21		0.09	
Eerste	**0.44**	0.30	**0.44**		-0.19	-0.03

Differentiation between Sites

The value of F_{ST} provides a relative measure of the level of genetic differentiation, or population sub-structuring, from which inferences can be drawn as to the relative degree of movement between geographically separate populations. The magnitude of the F_{ST} value over all populations for *A. subpupillata* indicates a low, yet significant, level of population sub-structuring (Table 7.3: $F_{ST} = 0.03 \pm 0.01$ $P < 0.001$). Observed

F_{ST} values, calculated when sampling sites are partitioned according to mountain ranges, suggest significant differentiation between *A. subpupillata* populations sampled on Table Mountain and those from the Jonkershoek catchment (Table 7.3; F_{ST} = 0.06±0.03; P < 0.001). Two of the six individual loci examined displayed significant F_{ST} values. Levels of differentiation between streams located on Table Mountain, as reflected by the calculated F_{ST} values, were small and non-significant (Table 7.3). This pattern was reflected across all the loci and suggests that individuals sampled from the different sites, representing four streams on Table Mountain, comprise a single panmictic population.

Table 7.3. Jackknifed estimates of F_{st} values for streams on Table Mountain and between each of the two ranges (Table Mountain and Hottentot's Holland) at each locus for populations of *Aeshna subpupillata* (Odonata: Aeshnidae).

F-statistic	All Pop^{n}s	Between Ranges#	Table Mountain
Pgm	0.04±0.03**	0.05**	0.01±0.02
GPI	0.01±0.01	0.01	0.02±0.02
DIA3	0.06±0.06*	0.13*	-0.01±0.01
MDH2	0.01±0.02	0.02	0.00±0.01
PepLTT	-0.01±0.01	0.00	-0.01±0.01
PepGL	-0.01±0.00***	0.00	-0.01±0.00
Total	0.03±0.01*	0.06±0.03*	0.00±0.00

p < 0.05, ** p < 0.01, *** p< 0.001
can not calculated SE between two samples. Overall SE calculated over all loci not populations

Genetic Identities and Distances

Using allele frequencies, unbiased estimates of genetic identities (Nei, 1978) were calculated between each pair of populations for *A. subpupillata* (Table 7.4), and a dendrogram constructed (Figure 7.4). The dendrogram reflects the low levels of population sub-division as suggested in the F_{ST} values. The dendrogram highlights the panmictic nature of those sites on Table Mountain, with all four streams grouping together with similar identity values (Table 7.4).

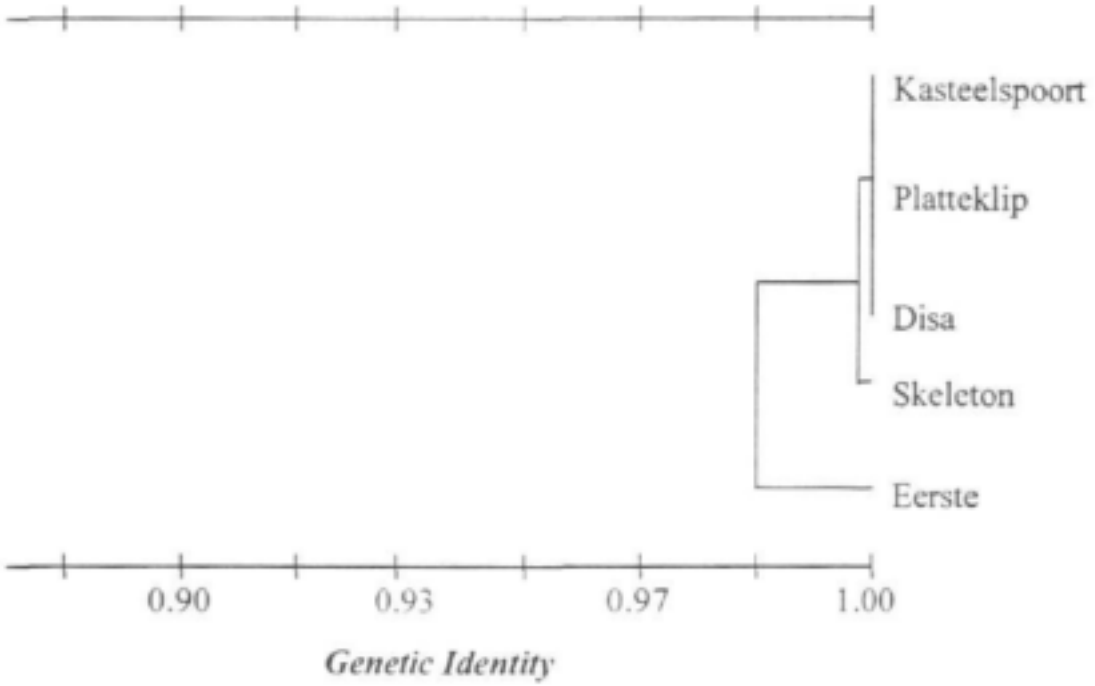

Figure 7.4. Phenogram showing the results of UPGMA analysis based on Nei's genetic identity for *Aeshna subpupillata* (Odonata: Aeshnidae).

Table 7.4. Unbiased estimates of Nei's (1978) genetic identity calculated between populations of *Aeshna subpupillata* (Odonata: Aeshnidae).

Population	Kasteelspoort	Platteklip	Skeleton	Disa	Eerste
Kasteelspoort	***				
Platteklip	1.000	***			
Skeleton	1.000	0.998	***		
Disa	1.000	0.999	0.999	***	
Eerste	0.995	0.996	0.989	0.993	***

MtDNA

Nucleotide Composition

The fifty-five, 648 base pair sequences of the cytochrome oxidase *c* sub unit 1 region of the mitochondrial DNA obtained for *Aeshnae subpupillata* revealed 12 putative haplotypes differentiated by eight variable nucleotide sites (Table 7.5). The overall nucleotide composition included a T/C content of 52%. All eight of these variable sites were 3rd codon position changes with 2 transitional pairs. Levels of nucleotide diversity within groups ranged between 0.001 and 0.003, with low levels of divergence between groups averaging 0.01±0.00 (Table 7.6)

Table 7.5. Variable nucleotide positions in the 12 *Aeshna subpupillata* haplotypes of a 648-bp fragment of the mtDNA cytochrome oxidase *c* subunit 1 gene identified by DNA sequencing. Dots indicate identity to haplotype A1. Numbers refer to position of base pairs from the start of the fragment. Entire sequences can be retrieved from the GenBank database at accession numbers as indicated in the column to the far right of the table.

	225	258	336	408	525	624	627	630	GenBank Accession No's
A1	G	A	T	T	T	A	A	C	AF429284
A2	.	.	.	.	.	G	.	.	AF429285
A3	.	.	.	C	.	.	.	.	AF429286
A4	.	.	.	C	C	.	.	.	AF429287
A5	.	.	.	C	.	G	.	T	AF429288
A6	.	.	.	C	.	.	.	T	AF429289
A7	.	.	.	C	.	G	.	.	AF429290
A8	A	.	.	C	.	G	.	.	AF429291
A9	.	G	.	C	.	G	.	.	AF429292
A10	.	.	C	C	.	.	.	.	AF429293
A11	.	.	C	C	.	G	.	.	AF429294
A12	.	.	C	C	.	G	G	.	AF429295

Table 7.6. Nucleotide diversity within groups (on diagonal) and net nucleotide divergence (below diagonal) and standard error (top diagonal) among groups which were identified by the diversity of mtDNA haplotypes and their distribution. Genetic distance among haplotypes was estimated using Kimura-2 parameter model (1980). See Materials and Methods section for further details.

	Eerste	Kasteelspoort	Platteklip	Skeleton
Eerste	0.003	0.000	0.000	0.001
Kasteelspoort	0.001	0.002	0.000	0.000
Platteklip	0.001	0.000	0.002	0.001
Skeleton	0.001	0.000	0.001	0.001

These patterns of diversity and divergence are reflected in the NJ trees for both taxa (Figure 7.5). The NJ tree for *Aeshna subpupillata* shows no clear structuring with poor resolution of the phylogenetic relationships between individuals, as evidenced by the low bootstrap confidence levels. A similar pattern is reflected in the haplotype network, with the TCS program unable to resolve the relationship between many of the haplotypes (Figure 7.6). While the most common haplotype appears central to the network, the position and relationships of the less frequent haplotypes are unclear, most likely due to inadequate sampling.

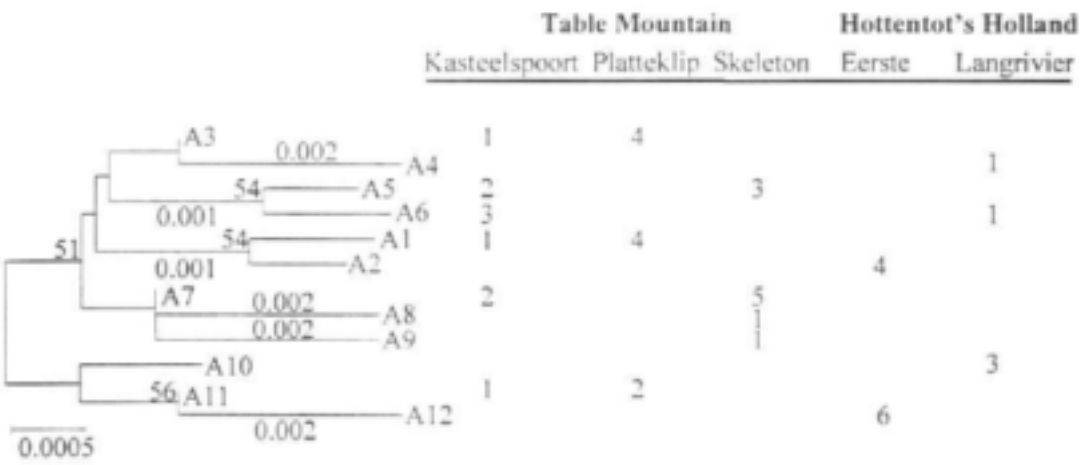

Figure 7.5. A mid-point rooted neighbour-joining tree, constructed using the Kimura-2-parameter (1980) model of substitution, for all COI haplotypes for *Aeshna subpupillata* (Odonata: Aeshnidae).

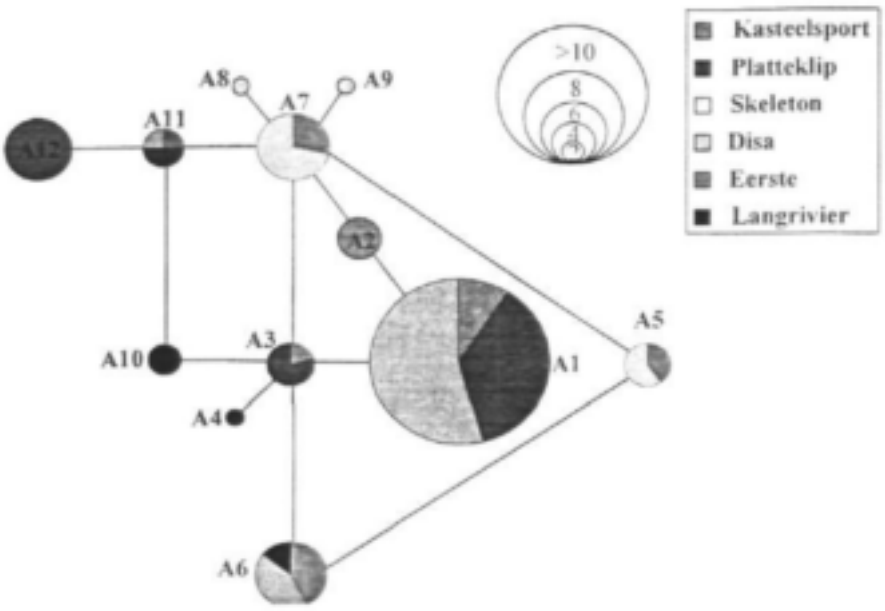

Figure 7.6. A haplotype network showing the relative frequencies and relationships between haplotypes for *Aeshnae subpupillata* (Odonata: Aeshnidae). The open circles represent missing haplotypes.

Examination of the distribution of the genetic variation was carried out using Analysis of Molecular Variance (AMOVA) based on haplotype frequency and levels of sequence divergence. A hierarchical model was used examining the distribution of genetic variation among ranges and among streams within ranges. Most of the genetic variation among populations of *A. subpupillata* is distributed within streams (61.87%), with differences between the two ranges accounting for less than 10% of the total variation.

Table 7.7. Comparative results of a hierarchical Analysis of Molecular Variance (AMOVA), using Kimura-2 parameter model of substitution (1980) for determining genetic distances, examining the distribution of genetic variation in the dragonfly, *Aeshna subpupillata* among populations in the south-western Cape, South Africa.

Source of Variation	*% Total Variation*	**Φ**	***P***
Among ranges	9.00	F_{CT}=0.09	0.14±0.01
Among streams within ranges	29.13	F_{SC}=0.32	< 0.001
Within streams	61.87	F_{ST}=0.38	< 0.001

Discussion

As a group, the dragonflies possess a number of morphological and behavioural adaptations for which they are renowned. Their highly developed powers of adult flight and larval anal propulsion enable movement across spatial scales including both within streams and landscapes. Such widespread and rapid powers of locomotion also facilitate the predatory behaviour exhibited by the larvae. Some species, such as *Anax*, even undertake annual migrations, while others, including some *Aeshna* species have been observed miles out to sea (Schneider, 1991). The characteristic robust morphological and behavioural attributes of *A. subpupillata* would appear to be reflected in the distribution of genetic variation observed among populations in the southwestern Cape. There is a lack of structure evident among the populations sampled in both allozyme and the mtDNA data. Most of the variation can be explained by the within stream variation, with populations exhibiting a high degree of genetic similarity and no obvious structure reflected in the NJ tree of mtDNA haplotypes.

Geographic Structure

While there is an increasing interest in the patterns of genetic population structure in lotic insects (for example see Bunn & Hughes, 1997), there have been few studies that have examined patterns of genetic variation within the dragonflies (although see Greenenl *et al.*, in press; McPeek & Brown, in press; Brown *et al.*, in press). While there have been several taxonomic studies examining speciation of the damselflies of North America, and a few population studies on some of the endangered or behaviourally habitat specific damselflies (i.e. McPeek & Brown, in press; Brown *et al.*, in press) there haven't been any published accounts of genetic population structure among Odonate populations. This is probably a reflection of the fact that as a study species capable of such wide dispersal they are assumed not to exhibit much structure and therefore have been largely ignored.

Neglect of the more mobile lotic taxa, such as the dragonflies, has in part led to the assumption that lotic insects typically exhibit a high degree of genetic structure (Hughes *et al.*, 1995, 1996; Wishart, 2001; Wishart & Hughes, 2001). The geological

nature of the catchment and the dendritic and hierarchical structure of the rivers that drain them have been shown to impose formidable barriers to the dispersal of many lotic species and influence genetic population structure (Meffe & Vrijenhoek, 1988). However, the genetic homogeneity exhibited among populations of *A. subpupillata* shows that for at least some lotic species catchment units do not provide an effective barrier to dispersal. The low, although significant, level of differentiation among nuclear loci in populations of the dragonfly, *A. subpupillata*, would suggest that individuals are freely moving among catchment units. This idea of a widely dispersed, panmictic population would appear to be further supported by the lack of structure evident in the NJ tree of mtDNA haplotypes and the relationship between these as indicated in the haplotype network. In support, the pattern of variation reflected among streams on Table Mountain shows complete homogeneity, with no differences in allele frequencies between any of the streams sampled and the results of the AMOVA partitioning most of the variation among individuals within streams.

The haplotype network is largely unresolved, suggesting that sampling may have been insufficient. The large number of haplotypes and the relatively low number of individuals sampled may have been insufficient to resolve the relationships. A;ternatively, it is more likely that sampling was not carried out at a sufficient spatial scale. The widespread distribution of this species means there are a likely to be a number of other haplotypes across the species range, many of which may be ancestral or more common than those sampled. If *A. subpupillata* were limited in its dispersal then the probability of sampling all available haplotypes would presumably be greater and the network therefore resolved.

While departures from Hardy-Weinberg equilibrium have been observed in a number of aquatic insects (Snyder & Linton, 1984; Sweeney *et al.*, 1987; Robinson *et al.*, 1992; Wishart & Hughes, 2001), all deviations from equilibrium observed in *A. subpupillata* were positive, indicating a deficiency in heterozygotes, and associated with the *Pgm* and *DIA3* loci. Heterozygote deficiencies in natural populations can be caused by several factors, including the presence of null alleles, selection against heterozygotes, inbreeding (Wright, 1978), assortative mating, and from the Wahlund effect, the pooling of sub-populations which differ in allele frequency (Hartl & Clarke, 1989; Spiess, 1989). While many aquatic insects have non-random mating

behaviours due to life history traits or demographics with sex rations significantly different from 1:1 (Hayashi, 1988; Freilich, 1991; Waringer, 1991), the strong uniform pattern associated with only two loci could indicate possible selection acting against heterozygotes for these enzymes.

The waters of the southwestern Cape region are characterised by low pH and other chemical factors that are thought to have been important in the evolution of regions endemic fauna (Wishart & Day, 2001). As the Karoo sediments eroded to give way to the Table Mountain group pH decreased as a result of the composition. Presumably this imposed a strong selective pressure upon the fauna driving speciation through adaptation. The low pH of the regions waters would also have prevented other species, components of a northward, Ethiopian fauna common to much of Africa from penetrating. It is possible that these conditions invoke a selective pressure against heterozygotes resulting in an overall deficiency. Most of the patterns are driven by differences in the Disa River population. While the Disa River is part of a protected area, the upper reaches of this system are feed with Alum, which could also influence the success and viability of heterozygotes.

While patterns of genetic variation observed among populations of *A. subpupillata* reflect the dispersal potential of this species, the levels of genetic variability within the species was very low. Such effects can be due to population bottlenecks or founder effects and can provide clues to historical influences having acted upon natural populations. Climatically induced sea level changes during the ?? would have reduced the stream length of streams draining the low elevation of the mountains of the southwestern Cape. This would have severely reduced the total stream length, availability of suitable habitat and thus had implications for the population size of many low-density, lotic invertebrates.

Chapter 8

A comparative phylogenetic approach toward defining functional units for the conservation of biodiversity in lotic ecosystems

Introduction

Efforts directed toward river conservation have primarily focused on maintaining the physical and structural processes and properties of a system. Increasingly, such efforts are being implemented at catchment scales, which are now being defined as the primary unit for the development and implementation of strategies aimed at protecting and providing safe, potable water supplies for human consumption within an environmentally integrated and sustainable framework. Given the inextricable linkages between catchment processes and the instream environment it makes sense to define units at this scale for management of the physical and chemical condition of the stream. The conservation of biodiversity, however, requires approaches that go beyond simple considerations of the physical and structural nature of the stream environment. Congruence between processes of biotic importance and catchment units is of considerable management importance if conservation is to be fully integrated into future catchment management and water resources planning. The aim of this thesis has been to assess the correlation between the genetic structure of natural populations in a number of different lotic organisms and catchment units. In this chapter a comparative phylogeographic approach is used to examine the correlation between genetic structure and catchment units.

Phylogeography is concerned with the principles and processes governing the geographic distributions of genealogical lineages (Avise, 2000). One of the central tenants of phylogeography is that different species should have similar patterns of genetic variation across biogeographic boundaries and at barriers which restrict gene flow (Avise, 1992). Such phylogeographic concordances (and dis-concordances) have a number of specific implications and ramifications for the pragmatic efforts of conservation as well as evolutionary theory and taxonomy (Walker & Avise, 1998). A comparative phylogeographic approach using taxa with different dispersal characteristics sampled from the same localities allows an assessment of the extent to

which catchments impose barriers or restrictions to the movement of individuals. The Stream Hierarchy Model (Meffe & Vrijenhoek, 1988) predicts that for freshwater species the dendritic and hierarchical nature of stream systems within the landscape will variously restrict gene flow through barriers to dispersal and thus be primarily responsible for determining patterns of genetic variation. As a result, for a system in equilibrium the patterns of genetic variation and the degree of differentiation between populations will be related to the level of isolation. The level of isolation is in turn determined by the probability of connectivity between populations as measured by the position in the stream hierarchy.

While several methods exist to facilitate direct comparisons between phylogeographic structure among independent taxa (Page, 1994; Avise, 2000), this study was carried out over a relatively small geographic area and a qualitative approach was used to compare patterns of genetic structure. A correlation between the genetic population structure and catchment scale would re-affirm the suitability of integrated catchment scale management as appropriate for the conservation of biodiversity. It would also provide some interesting challenges to managers and conservation planners in light of the increasing development and reliance upon IBTs for the redistribution of water, and the inadvertent re-distribution of organisms between otherwise historically isolated populations. Alternatively, a lack of any correlation between genetic structure and catchment units would suggest populations operating at scales larger than the catchment. In order to ensure protection of these populations, conservation and management strategies would have to consider protected areas that incorporate a number of adjacent catchments spread across a species range.

Contemporary patterns of genetic variation observed among natural populations are determined by often complex interactions between demographic, genetic and historical processes (Slatkin, 1987; Bohonak, 1999; Avise, 2000). For lotic populations, the isolating effects of catchment units and the relative dispersal potential of the individual species will play a crucial role in determining the patterns of genetic population structure. These patterns will obviously be set within the constraints imposed by historical biogeographic influences. Furthermore, the advent and development of human technologies aimed at ensuring long-term sustainable potable water supplies are also likely to have significant implications for the genetic structure

of natural populations, both now and for the future. The isolating effects of dams, transfer of individuals between otherwise historically isolated catchments through IBTs and the introduction of alien species all have the potential to significantly effect natural populations of lotic organisms.

The aim of this chapter is to bring together congruent data available on patterns of genetic variation for the four different lotic organisms examined previously in order to assess the suitability of catchment units as functional units for the conservation of lotic biodiversity. It is hypothesised that species with higher dispersal capabilities should exhibit relatively shallower population histories, with lower levels of genetic differentiation and population structure than those species with lesser potential for dispersal (see e.g. Waples, 1987; Bohonak, 1999). If catchments are the appropriate units for defining conservation units for the management of biodiversity in lotic systems, then given sufficient time and restrictions on dispersal, it could be hypothesised that lineage sorting will lead to monophyletic clades of mtDNA haplotypes in different catchment units.

Methods

MtDNA and allozyme electrophoresis data were used from four different species that reflected differences in dispersal traits. These included the three insect species with different powers of dispersal and a fish, the Cape galaxias, (*Galaxias zebratus*). The insects included the net-winged midge, *Elporia barnardi* (Diptera: Blephariceridae) very limited in its dispersal: the stonefly *Aphanicerca capensis* (Plecoptera: Notonemouridae), intermediate in its dispersal potential: and a dragonfly, *Aeshna subpupillata* (Odonata: Aeshnidae) capable of wide dispersal. A comparative approach was employed in which only data available from streams in which all four species were found and sampled were included, or in the case of the fish, *G. zebratus*, sites that were in close geographic proximity (Figure 8.1). These included three streams on Table Mountain and the Eerste River in the Jonkershoek catchment of the Hottentot's Holland mountain range. Table Mountain streams in which all three of the insect species were found included Kasteelspoort, Platteklip and Skeleton Gorge. While the fish were found in the lower parts of the Skeleton Gorge, they were absent from the relatively short, steep gradient exposed coastal streams of Kasteelspoort and

Platteklip. As a result, data for *G. zebratus* were included from two other streams on the Table Mountain range, Disa and Silvermine streams.

Collection

A variety of collection methods were used. More detailed descriptions for the individual taxa are provided in the respective chapters and general methods (Chapter 3). In all, between thirty and fifty individuals were collected from within each of the streams. For the midges and the stoneflies three sites were sampled within each stream, allowing for an examination of possible instream structure. Due to lower densities and the numbers required for sound statistical analyses 30 individuals of the dragonfly, *A. subpupillata*, and the fish, *G. zebratus*, were collected from over a relatively longer length of stream within each streams.

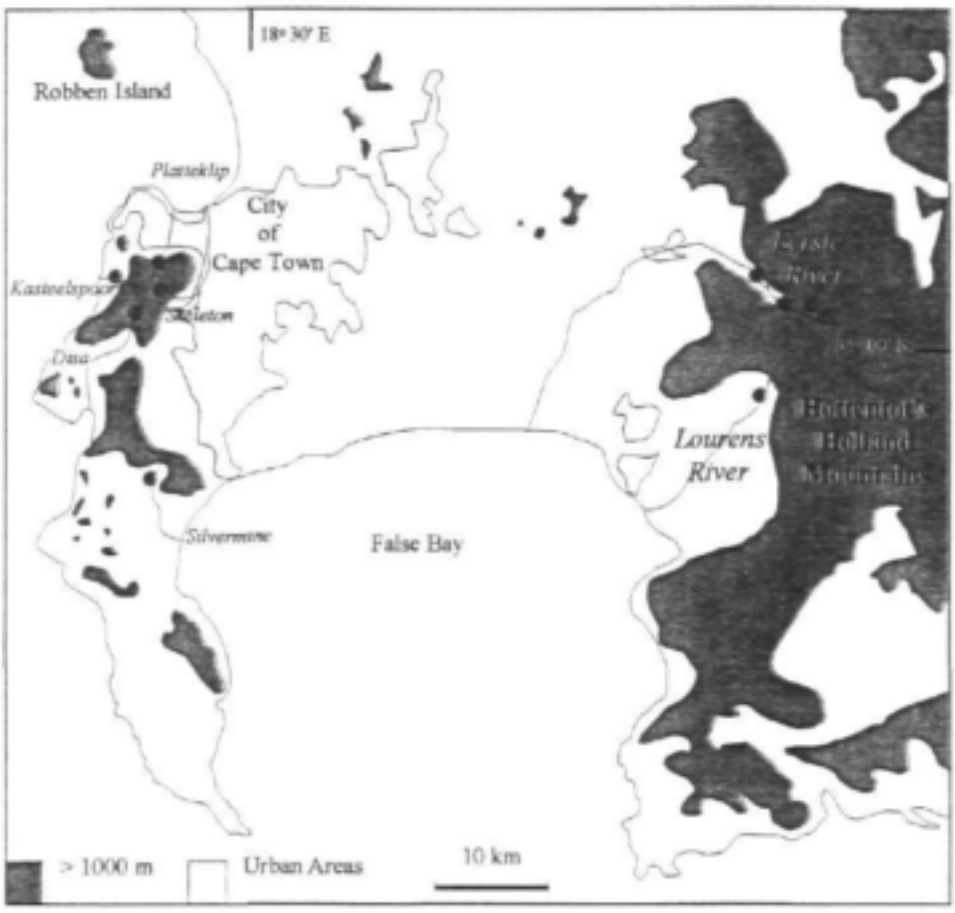

Figure 8.1. Streams from which lotic organisms with different dispersal traits were sampled for comparative phylogeographic analyses.

DNA

The various different techniques used for examining the nuclear and mitochondrial DNA are outlined in the respective chapters dealing with individual species and the general methods section (Chapter 3).

Analysis

Specific methods used are outlined in the general methods and individual species chapters. In order to examine the relationship between dispersal and patterns of genetic population structure in relation to catchment units qualitative comparisons were made for various measures. Based on allozyme data F_{ST} values were calculated at a variety of scales and compared across taxa. Levels of nucleotide variability and net nucleotide divergence among mtDNA sequences were also compared among taxa. The degree to which each of the taxa conformed to models of contemporary gene flow were then examined by contrasting the results from an Analysis of Molecular Variance and Mantel's test for each taxa.

Results

Allozymes

All four taxa displayed low levels of variability among allozyme loci. In the case of the stonefly, *A. capensis*, all loci resolved were fixed for the same allele across both ranges. Values of F_{ST} show that there is no relationship between the level of population sub-division and inferred dispersal ability across discontinuous mountain ranges (Figure 8.2). In comparison, F_{ST} values among streams on Table Mountain show there to be a positive correlation between the inferred dispersal potential of each species and the level of observed population sub-division (Figure 8.3). It should be noted, however, that the pattern of variability and population sub-division observed in the freshwater fish, *G. zebratus*, is slightly erroneous due to the levels of variability and distribution of this variation. For a more detailed explanation see Chapter 3.

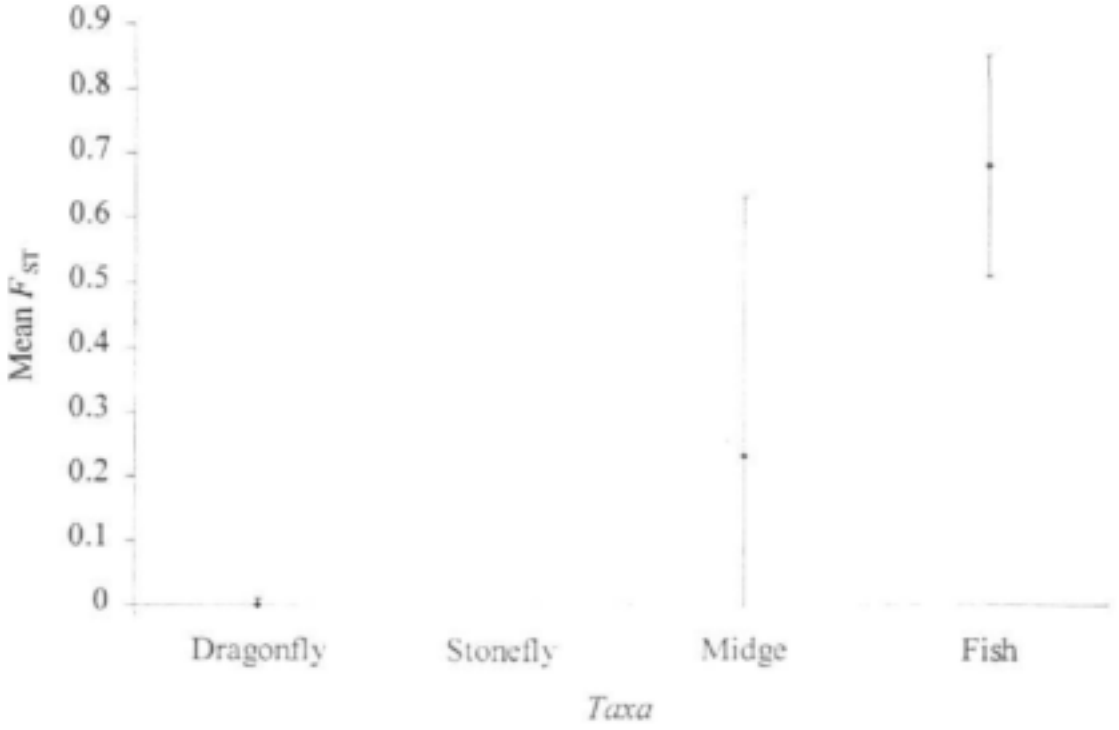

Figure 8.2. Comparative F_{ST} values for various aquatic organisms with different dispersal traits. These included the dragonfly, *Aeshna subpupillata*; the stonefly, *Aphanicerca capensis*, the net-winged midge, *Elporia barnardi*, and the fish, *Galaxias zebratus*. All enzymes examined among populations of *A. capensis*, were monomorphic and fixed for the same loci. See text for more detail explanation.

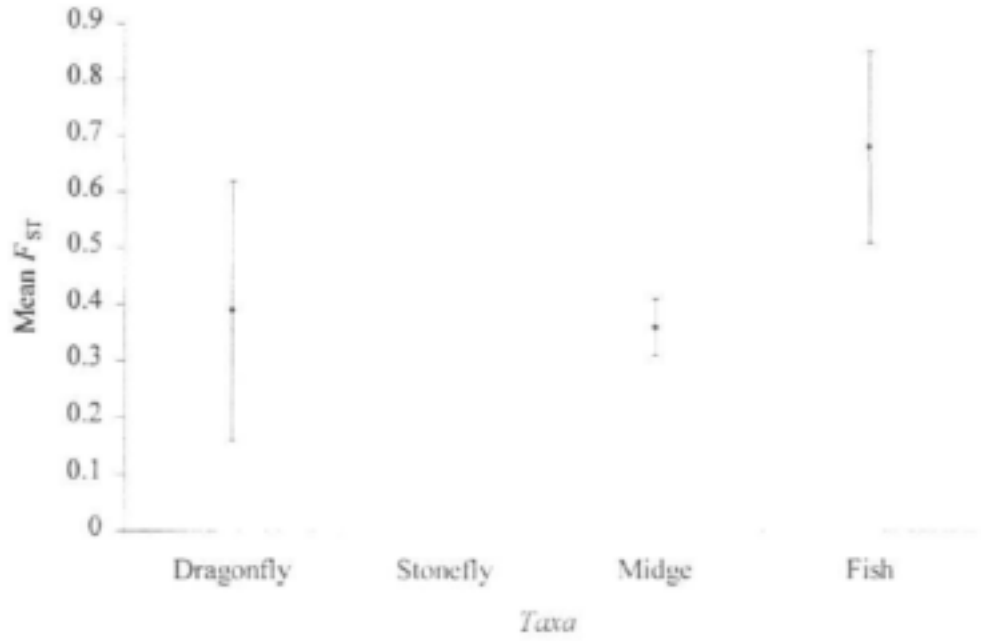

Figure 8.3. Comparative F_{ST} values for various aquatic organisms with different dispersal traits among streams on Table Mountain, southwestern Cape, South Africa. All enzymes examined among populations of the stonefly, *Aphanicerca capensis*, were monomorphic and fixed for the same loci. See text for more detail explanation.

MtDNA

It is interesting to note that with the exception of the fish, *G. zebratus*, the levels of nucleotide variability and the number of haplotypes roughly corresponds to the dispersal potential of each species (Table 8.1). As previously noted, the observed distribution and degree of genetic variation in *G. zebratus* is derived from different historical influences as outlined in Chapter 4.

Table 8.1. The number of variable nucleotide sites and haplotypes observed in a roughly 600-bp fragment of the COI region of the mtDNA in four taxa with different dispersal capabilities.

Taxa	# Variable Sites	# Haplotypes
Fish (*G. zebratus*)	11	9
Midge (*E. barnardi*)	64	24
Stonefly (*A. capensis*)	43	6
Dragonfly (*A. subpupillata*)	8	12

The dispersal potential of each of these species is further reflected in part in the distribution of variation in the mtDNA (Figure 8.3). As is to be expected for a species with a high potential for dispersal the NJ tree for the dragonfly, *A. subpupillata*, does not reflect any structural patterns that correspond with catchment units. In comparison, both the midge, *E. barnardi*, and the Cape galaxiid, *G. zebratus*, exhibit patterns of genetic variation typical of obligate freshwater species, with each catchment having its own unique complement of mtDNA haplotypes. As such, the NJ trees for both these species show monophyletic clades of mtDNA haplotypes corresponding to individual catchment units. The pattern of genetic variation displayed by the stonefly, *A. capensis*, is somewhat different due to the low number of haplotypes and lack of variability. This winged insect of intermediate dispersal potential does, however, show two well-supported clades corresponding to the Hottentots Holland and Table Mountain ranges, separated by roughly 8%. The level of sequence divergence between these two clades is similar to that observed in the midge, *E. barnardi*. Such congruence between population trees would suggest a vicariant explanation for the observed patterns of isolation.

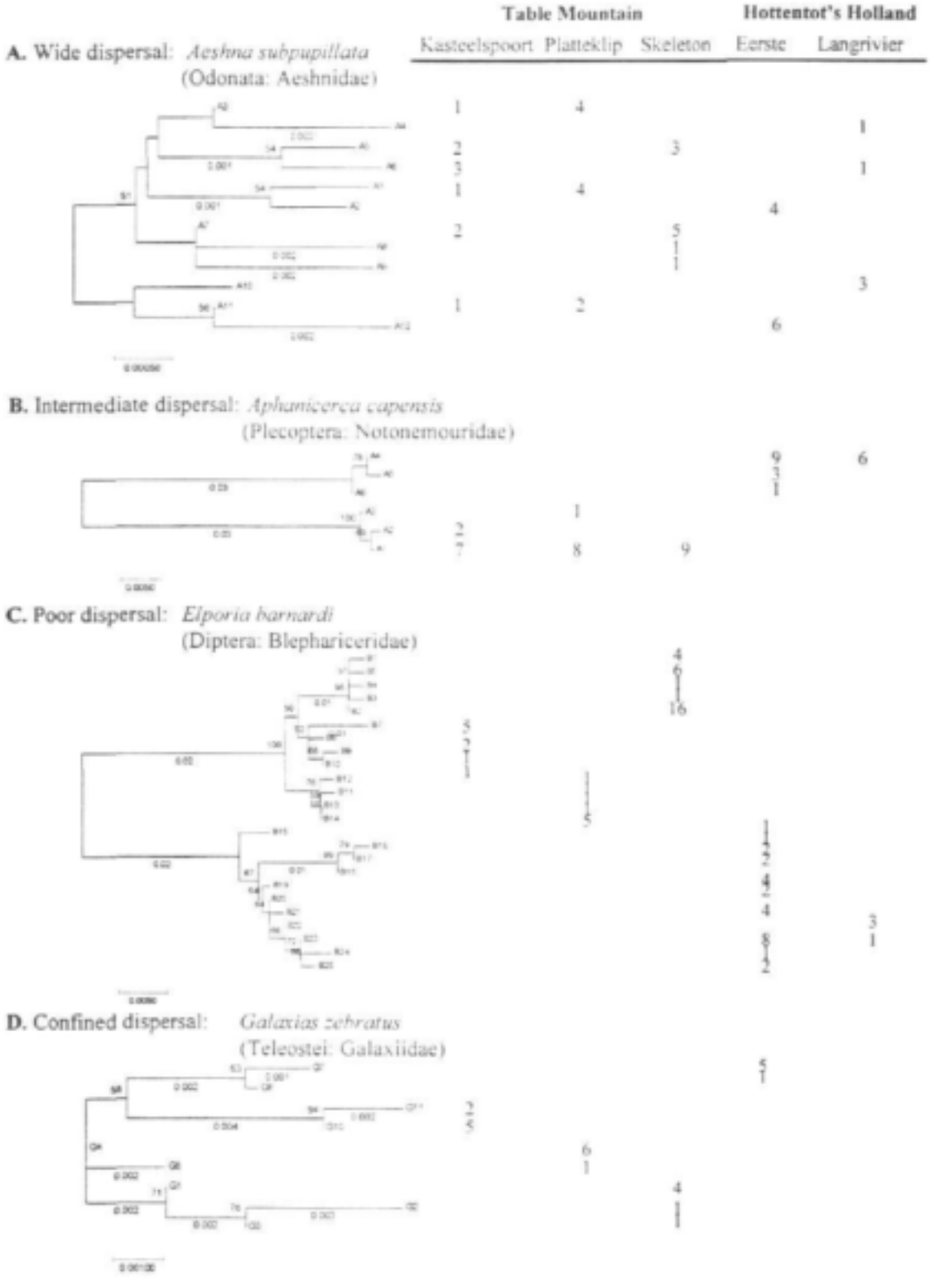

Figure 8.4. Mid-point rooted neighbour-joining trees, constructed using the Kimura-2-parameter (1980) model of substitution, for all COI haplotypes from four lotic species found in streams among two mountain ranges within the southwestern Cape region. Species differ in their ability to disperse, from the wide dispersal of the dragonfly, *Aeshna subpupillata*, to the more restricted dispersal of the fish, *Galaxias zebratus*.

Analysis of Molecular Variance

Comparative results from analysis of molecular variance shows a general agreement with a hierarchical structure (Table 8.2). Again, the pattern of variation observed in *G. zebratus* does not conform with the general pattern. The pattern of variation among sampled populations of *G. zebratus* reflects some of the problems of looking at patterns in simply a contemporary context. Processes, such as those hypothesised in Chapter 4, relating not so much to contemporary dispersal but historical factors, such as climatic and ensuing sea level changes would appear to have been important in determining these patterns. In comparison, the pattern of variation among the insects, all of which share similar habitat requirements and have presumably experienced similar history offer a better basis for a comparative analysis, with the pattern and distribution of genetic variation reflecting the dispersal potential of each species. Both the midge, *E. barnardi*, and the stonefly, *A. capensis*, show most variation among mountain ranges, reflecting an absence of gene flow between these discontinuous ranges. The pattern of variation among the widely dispersing dragonfly is explained by differences within streams, with little variation explained by differences between streams or mountain ranges.

Table 8.2. Results of a hierarchical Analysis of Molecular Variance (AMOVA) examining the distribution of genetic variation among populations of various lotic organisms with different dispersal potential among streams in the southwestern Cape. See text for more detailed explanation.

a.

Source of variation	Taxa	% Variation	ϕ Statistics	P
Among ranges	*G. zebratus*	-23.31	F_{CT} = -0.23	1.000
	E. barnardi	79.14	F_{CT} = 0.79	0.000
	A. capensis	99.34	F_{CT} = 0.99	0.09±0.01
	A. subpupillata	9.00	F_{CT} = 0.09	0.14±0.01

b.

Source of variation	Taxa	% Variation	ϕ Statistics	P
Among streams within ranges	*G. zebratus*	98.19	F_{SC} = 0.80	0.000
	E. barnardi	14.68	F_{SC} = 0.70	< 0.001
	A. capensis	0.03	F_{SC} = 0.05	0.16±0.01
	A. subpupillata	29.13	F_{SC} = 0.32	< 0.001

c.

Source of variation	Taxa	% Variation	ϕ Statistics	P
Among all sites	*G. zebratus*	25.12	F_{ST} = 0.75	0.000
	E. barnardi	6.18	F_{ST} = 0.94	< 0.001
	A. capensis	0.62	F_{ST} = 0.99	< 0.001
	A. subpupillata	61.87	F_{ST} = 0.38	< 0.001

This relationship between dispersal and the distribution of genetic variation is re-iterated when we examine the distribution of genetic variation among streams on Table Mountain (Table 8.3). Due to the density and abundance of dragonflies and galaxiids, samples were collected from a single site in streams on Table Mountain, thus precluding analysis of molecular variance. However, in midge, *Elporia barnardi* the distribution of genetic variation again adheres to a hierarchical structure, with most of the variation explained by differences among streams. In comparison, the distribution of genetic variation among samples of the stonefly, *Aphanicerca capensis*, shows a general lack of agreement with any hierarchical structure, with most of the variation located among individuals within a site.

Table 8.3. Results of a hierarchical Analysis of Molecular Variance (AMOVA), using Kimura-2 parameter model of substitution (1980) for determining genetic distances, examining the distribution of genetic variation among populations of two lotic organisms with different dispersal potential among streams on Table Mountain. See text for more detailed explanation.

a.

Source of variation	Taxa	% Variation	ϕ Statistics	P
Among streams on Table Mountain	*E. barnardi*	85.67	$F_{CT} = 0.86$	0.00±0.00
	A. capensis	11.12	$F_{CT} = 0.11$	0.21±0.01

b.

Source of variation	Taxa	% Variation	ϕ Statistics	P
Among sites within Table Mountain streams	*E. barnardi*	2.17	$F_{SC} = 0.15$	0.24±0.01
	A. capensis	-11.11	$F_{SC} = -0.13$	1.00±0.00

c.

Source of variation	Taxa	% Variation	ϕ Statistics	P
Within sites	*E. barnardi*	12.16	$F_{ST} = 0.88$	< 0.001
	A. capensis	99.99	$F_{ST} = 0.00$	1.62±0.01

Discussion

It is apparent from the results presented in the preceding chapter and the comparative analyses that there is considerable variation in the genetic population structure of the selected taxa. In general these differences correspond to differences in the dispersal traits of the respective taxa. Some of the apparent anomalies can be attributed to the fact that while phylogeographic patterns typically reflect contemporary demographic and ecological processes, they are ultimately set within an historical framework of biogeographic factors that have operated throughout a species evolutionary history. Such anomalies can be seen in the distribution and patterns of variation in the galaxiid, *G. zebratus*, and the stonefly, *A. capensis*.

The stream hierarchy model was proposed for obligate freshwater species, based upon the premise that gene flow is a rare event between drainages and that equilibrium, populations in each river system will eventually become reciprocally monophyletic (Meffe & Vrijenhoek, 1988). Such reciprocal monophyly can be seen in the population trees for the obligate freshwater species, *G. zebratus*. Interestingly, despite having an aerial adult phase, the net-winged midge, *E. barnardi*, displays a pattern of genetic variation similar to that expected for an obligate freshwater species. This is further reflected by the reciprocally monophyletic nature of the clades in the population tree. In both these species dispersal would appear to be confined to within the catchment unit.

While the distribution of genetic variation and pattern of the population tree for the dragonfly reflects that of a highly mobile and widely dispersed population, the lack of variation makes it difficult to draw conclusions as to the effect of dispersal in the stonefly, *A. capensis*. However, comparing the diversity and pattern observed in *A. capensis* with that of *A. subpupillata* and *E. barnardi* does allow some interesting insights and inferences to be made relating to the potential movement and history of populations of *A. capensis*. For example, while populations of *E. barnardi* displayed low haplotype diversity, each catchment on Table Mountain contained a unique complement of haplotypes, indicating there to be no movement outside of the catchment unit. Similar conclusions of limited dispersal, confined to within the catchment unit, have been made for various stonefly species based on nuclear loci using allozymes (Hughes *et al.*, 1999). If dispersal of *A. capensis* were similarly

limited within the catchment unit, then it might be assumed that mutations and drift would produce a similar pattern of reciprocal monophyly to that observed in *E. barnardi*, with each catchment represented by its own unique array of haplotypes. If we assume the low levels of variation are due to historical bottlenecks, the presence of a single dominant haplotype among all Table Mountain streams suggests that there is, or at least until very recently has been, movement of individuals between Table Mountain streams. As new haplotypes arise through drift or mutation they are effectively drowned out by the dominant haplotype.

Despite the lack of genetic variability, such a comparative phylogenetic approach, given certain assumptions and constraints, allows inferences to be made about the relationship between dispersal and genetic structure. If we accept the processes involved in the formation and maintenance of low variability in *A. capensis*, the results suggest an association between an organisms dispersal capability and genetic population structure. For those species capable of wide dispersal, such as *A. subpupillata*, natural barriers and discontinuities between mountain ranges or catchment units do not appear to limit dispersal or influence genetic population structure. In comparison, while discontinuities between mountain ranges provide an effective barrier to dispersal for those species with more limited powers of dispersal, such as *A. capensis*, catchment units do not. For those species with highly specific habitat requirements and poor powers of dispersal, such as *E. barnardi*, dispersal would appear to be limited to within the catchment unit.

Such comparisons also provide valuable insights into historical processes having acted upon a regional fauna. The magnitude and congruence between the sequence divergence for both *A. capensis* and *E. barnardi*, for example, suggests a vicariant event around 3-4MyBP. Such congruence between phylogenetically independent taxa would suggest a vicariant explanation for this sequence divergence (Avise *et al.*, 1987). Interestingly, the land bridge connecting Table Mountain and the Peninsula range with the Hottentot's Holland mountain range is thought to have commenced during the late Cretaceous (~65MyBP) and completed in the late Tertiary (~2MyBP). Thus the levels of divergence and separation suggested by dating of the mtDNA would indicate this to be roughly the time at which populations within these two ranges were isolated.

Chapter 9

Conclusions, Recommendations and Future Research

Given the historical focus of river conservation and the increasing shift toward integrated approaches to catchment management, the central aim of this project has been to assess the suitability of catchments as functional units for the conservation of lotic biodiversity. Inevitably however, observed patterns of genetic variation reflect the effects of contemporary population and ecological processes as well as the culmination of biogeographic processes that have operated throughout the history of the species. As a result, contemporary patterns of genetic variation are often complex and difficult to interpret. A comparative phylogeographic approach, in which the same fragment of DNA is examined in a number of phylogenetically independent species sampled from geographically identical localities, enables not only direct observation of patterns of genetic variation but, in comparison, can alleviate some of the problems inherent in single species studies.

These results should be interpreted in light of the fact that both the landscapes and the fauna of the south-western Cape comprise some of the countrys oldest and most ancient forms. Accordingly, other less ancient landscapes and faunas might be expected to show very different patterns to those described in this report. With this in mind, the results of the research reported here reveal the following:

- Low levels of genetic variability were observed in all of the populations, giving insights into the demographic history of the organisms over the past millions of years.

This suggests that historical processes, such as climate changes or shifts in available habitat, have reduced population sizes and eroded genetic variability through repreated inbreeding depression. It has been suggested that low genetic variability can impede an organisms ability to respond to environmental fluctuations. This has wide ranging serious implications for almost any changein the environment from the advent

of disease, changes in global climates, through to human manipulation of the environment by IBTs.

- Movement of individuals as reflected by the degree of genetic population structure mirrored the dispersal characteristics of each species; viz: robust dispersers displayed little genetic structure, whilst poor dispersers displayed relatively high degrees of population structure in terms of their genetics.

For some species, such as the net-winged midges and the Cape galaxiid, catchment units pose an effective barrier to dispersal creating effective islands of aquatic habitat within a terrestrial landscape. Such isolation, given sufficient time, will result in reciprocally monophyletic units, or unique genetic signatures associated with individual catchment units. For such species, individual catchment management strategies would appear to represent an appropriate scale of management. However, the development of IBTs between such historically isolated units will provide a conduit for the transfer of individuals allowing gene flow between genetically discrete populations. Thus the development of IBTs has the potential to undermine the evolutionary processes important in species formation, and thus generation, of biodiversity.

For other more mobile species, such as the South African stream hawker, the distribution of genetic variation and the pattern of population sub-division suggests an effective population covering a wide geographic range. For such wide spread populations, a conduit for gene flow through development of IBTs between adjacent catchments would have no negative impacts on genetic population structure. However, it should be noted that there are a number of extant IBTs that transfer water over huge distances, from catchments far removed from each other. While beyond the scope of this project, such IBTs may have conservation implications, facilitating gene flow among geographically isolated populations and across different biogeographical zones. Such patterns of genetic population structure do have, however, other conservation and management implications. For example, a homogeneous, panmictic (widespread) population across a wide geographic area would suggest that catchment units may not represent the appropriate scale for conservation of the aquatic fauna.

- The long period of geological and climatic stability and the isolation of individual catchments has resulted in a unique assemblage of aquatic organisms such that individual catchments seem to be exhibiting unique signatures in terms of their species complements and genetic structure.

The implications of these patterns in terms of the design and designation of protected areas has been discussed above. However, when one considers catchment-by-catchment comparisons there are further implications when considered in conjunction with ecological surveys and classification schemes. These patterns provide the underlying explanations for the observed contemporary distribution of organisms and may be useful in explaining the apparent unique "catchment signatures" (e.g. WRC Project K5754: Linking abiotic and biotic data on South African rivers).

- The levels of genetic divergence observed *within* three of the target species were equivalent to those reported *between* other clearly defined species indicating that our target species probably comprise several new species that have hitherto not been described.

The synthesis of this and other information is resulting in the Cape fynbos region being recognized not only as a region of botanical importance, but as a region with a unique and highly endemic aquatic fauna. Thus, while it has long been recognized as an area of outstanding floral diversity and endemism, it is becoming increasingly apparent that the fauna of the aquatic systems of the Cape also harbour a unique degree of largely as yet unrecognised diversity and a degree of endemism comparable, if not greater, than that of the flora of the region.

These results suggest that there remains a large amount of as yet undescribed biodiversity. This has a number of important implications for the recognition and development of conservation areas and strategies. For example, the Water Research Commission is currently funding the development of a series of invertebrate field guides for southern Africa's aquatic fauna (WRC Project K5/916/0/01'). While this represents a significant step toward the documentation and description of the aquatic fauna of the region, our research indicates that there remains a wealth of genetically

discrete and presently undescribed species. The WRC should take cognisance of this fact and recognise that completion of these field guides will not draw the description of the regions fauna to a close. In this regard, whilst the country nor the Water Research Commission are in a position to fund such research, it is our opinion that the WRC should motivate the Department of Environmental Affairs and Tourism (DEAT) to develop, with internationally available funding through organisations such as the Global Environment Fund (GEF), nationally co-ordinated projects to undertake revisions of the countrys' freshwater biodiversity.

Given South Africa's national and international obligations, the results presented in the preceding chapters have a number of important implications for the development of conservation strategies for riverine biodiversity and specific consideration of the implications of inter-basin water transfers. As such we recommend that:

- all future developments which involve the planning and construction of IBTs should take cognisance of the potential for genetic mixing of riverine biotas in receiving systems;
- the genetic structure of target organisms *such as those identified in this report* be included in all impact assessments associated with IBTs bearing in mind South Africa's commitments to maintain organismal diversity in a sustainable manner as a signatory to the international Convention on Biological Diversity; and
- information generated by the research reported here should be incorporated into the design and development of future protected areas for the conservation of riverine organisms as opposed to catchment-based management for water supply.

Further to this, the report serves as a statement of consequence, providing an iteration of the facts and the implications of certain actions, or non-actions, on future conservation strategies. Responsibility for incorporating such considerations into practical management decisions is beyond the scope and mandate of this report. Indeed, the onus of responsibility lies with the decision makers, such as the ministers and departments of Water Affairs and Forestry, Environmental Affairs and Tourism

and Mineral and Energy Affairs, and any approaches toward conservation will need to take place within the practical constraints of meeting the water supply requirements.

The precautionary principle is being afforded increasing recognition, having already been incorporated into many international conventions and national legislations. It would therefor seem prudent to incorporate such considerations into the development of conservation strategies. From the distribution and patterns of genetic variation reflected among the taxa examined, efforts at conserving the fauna of riverine ecosystems should move beyond individual catchment considerations to incorporate protected areas and management strategies that cover and incorporate a number of adjacent catchments. Such areas or management plans should be replicated and distributed across identified biogeographic regions. Further consideration should be afforded to the influence of water resources developments on the genetic population structure of aquatic organisms and on the long-term sustainable conservation of aquatic ecosystems and processes therein. As a signatory to a number of international treaties and conventions addressing the conservation and management of biodiversity, South Africa has an obligation to take necessary mitagatory measure to ensure the protection of such components of biodiversity for the long-term sustainable utilisation and protection of the countries natural heritage.

Given the age and antiquity of the south-western Cape region, where the research presented here was undertaken, there is a clear need to expand this type of research to other distinct biogeographical regions. For instance, we regard it as an imperative that similar research be undertaken on Mpumalanga tropical low-veld rivers, high-veld rivers and systems within ranges such as the Drakensberg in KwaZulu/Natal. This in order to establish whether or not there are similar patterns of genetic distinction elsewhere in the country and, hence, the implications for future IBTs in those regions.

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List of Technology Transfer Actions

Scientific Papers

A. Published Papers

Wishart, M.J. & Day, J.A. (in press) Endemism in the freshwater fauna of the South-Western Cape, South Africa. *Verhandlungen Internationale Vereinigung Limnologie*

Wishart, M.J. & Davies, B.R. (in press) Considerations of scale for conserving river basin integrity in relation to inter-basin water transfers. *Verhandlungen Internationale Vereinigung Limnologie*

Wishart, M.J. & Hughes, J.M. (2001) Exploring patterns of population sub-division in the net-winged midge, *Elporia barnardi* (Diptera: Blephariceridae), in mountain streams of the south-western Cape, South Africa. *Freshwater Biology* 46: 479-490

Wishart, M.J. (2000) Catchments as Conservation Units for Riverine Biodiversity. *African Journal of Aquatic Sciences* 25: 169-174

Snaddon, C.D., Wishart, M.J., Davies, B.R. (1998) Some implications of inter-basin water transfers for river ecosystem functioning and water resources management in southern Africa. *Journal of Aquatic Ecosystem Health*, 1: 159-182.

B. Manuscripts Under Review

Wishart, M.J. & Hughes, J.M. (in review) Effects of stream structure and dispersal on the genetic population structure of the net-winged midge, *Elporia barnardi* (Diptera: Blephariceridae), in streams of the south-western Cape, South Africa. *Freshwater Biology*

Wishart, M.J., Stewart, B.A. & Hughes, J.M. (in review) Effects of dispersal on genetic population structure of two lotic insects in streams of the south-western Cape, South Africa. *Marine and Freshwater*

Wishart, M.J. & Davies, B.R. (in review) Beyond catchment considerations in the conservation of lotic biodiversity. *Aquatic Conservation: Marine and freshwater Ecosystems*

C. Completed Manuscripts to be Submitted Soon– draft copies are available

Wishart, M.J., Hughes, J.M., Stewart, B.A., Davies, B.R. & Bunn, S. (completed MS) A comparative phylogeographic approach toward defining functional units for the conservation of biodiversity in lotic ecosystems. *Conservation Biology*

Wishart, M.J., Hughes, J.M., Cook, B.A. & Impson, D. (completed MS). Extreme levels of intra-specific divergence among the Cape galaxiid, *Galaxias zebratus* Castelnau 1861 reveals a possible species complex. *Journal of Fish Biology*

Wishart, M.J., Cook, B.A., Hughes, J.M. & Impson, D. (completed MS). Genetic population structure in the Cape galaxiid, *Galaxias zebratus*, South Africa. *Heredity*

Wishart, M.J., Stevens, D., Rivera, M.A. & Picker, M.D. (completed MS) Examination of morphological, phylogenetic and biological species delineations in the *Aphanicerca* complex. *Evolution*

Wishart, M.J., Adams, S. & Davies, B.R. (completed MS) Long-term temporal and short-term spatial co-existence in the Blephariceridae (Diptera) of a mountain stream in the south-western Cape, South Africa. *Aquatic Insects*

Chapters in Books

Davies, B.R. & Wishart, M.J. (2000) River conservation in the countries of the Southern African Development Community (SADC). In: P. Boon & B.R. Davies (Eds.) *Global perspectives in river conservation: science, policy and management.* John Wiley & Sons, England. Pp. 179-204.

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Conference Presentations

Wishart, M.J. & Day, J.A. (2001) An endemic isle on Africa's southern tip: the aquatic fauna of the Cape Floristic Region. *15th Annual Meeting of the Society for Conservation Biology, Hawaii, U.S.A.*

Wishart, M.J. & Hughes, J.M. (2001) Examining the effects of catchments on the genetic structure of lotic organisms and their role in defining units for conservation. *15th Annual Meeting of the Society for Conservation Biology, Hawaii, U.S.A.*

Wishart, M.J. (2001) Conservation of South Africa's riverine biodiversity: areas of importance and the identification of functional units. Annual Evolution and Ecology Postgraduate Symposium, Griffith University, Brisbane, Australia.

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Wishart, M.J. (1999). Catchments as Conservation Units for Riverine Biodiversity. Meeting of the Southern Africa Society of Aquatic Scientists, Swakopmund, Namibia.

Student Theses

Wishart, M.J. (submitted) A phylogeographic approach toward defining functional units for the conservation of biodiversity in riverine ecosystems. CRC Freshwater Ecology, Griffith University and Freshwater Research Unit, University of Cape Town.

Roos, C. (1999) Mate recognition and sexual behaviour in the stonefly *Aphanicerca capensis* Barnard (Plecoptera: Notonemouridae): a species complex ? Unpublished Honours Thesis. Zoology Department, University of Cape Town.

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Appendix 1. Allele frequencies at various enzyme loci for *Galaxias zebratus* (Teleostei: Galaxiidae) populations in the south-western Cape, South Africa. (N = sample size)

	Population										
Locus	**Klaasjagers**	**Schusters**	**Diep**	**Silverstroom**	**Glencairn**	**Silvermine**	**Disa**	**Liesbeek**	**Eerste**	**Lourens**	**All**
AK											
(N)	30	30	30	30	6	30	30	30	30	30	
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
CK											
(N)	30	30	30	30	6	30	30	30	30	30	
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
EST											
(N)	30	30	30	30	6	30	30	30	30	30	
1	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.46
2	0.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00	0.54
GAP											
(N)	30	30	30	30	6	30	30	30	30	30	
1	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.46
2	0.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00	0.54
GPI											
(N)	30	30	30	30	6	30	30	30	30	30	
1	0.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00	0.54
2	0.95	0.83	0.93	0.92	1.00	0.00	0.00	0.00	0.00	0.00	0.42
3	0.05	0.17	0.07	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.04
GDH											
(N)	30	30	30	30	6	30	30	30	30	30	
1	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.46
2	0.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00	0.54

Appendix 1. continued....

IDH											
(N)	30	30	30	30	6	30	30	30	30	30	
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
LAP											
(N)	30	30	30	30	6	30	30	30	30	30	
1	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.46
2	0.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00	0.54
LDH											
(N)	30	30	30	30	6	30	30	30	30	30	
1	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.46
2	0.00	0.00	0.00	0.00	.00	1.00	1.00	1.00	1.00	1.00	0.54
MDH 1											
(N)	30	30	30	30	6	30	30	30	30	30	
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MDH 2											
(N)	30	30	30	30	6	30	30	30	30	30	
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
MDH 3											
(N)	30	30	30	30	6	30	25	30	30	30	
1	1.00	1.00	0.48	1.00	1.00	1.00	0.48	1.00	1.00	1.00	0.90
2	0.00	0.00	0.52	0.00	0.00	0.00	0.52	0.00	0.00	0.00	0.10

Appendix 1. continued....

ME											
(N)	30		30	30	30	6	30	29	30	30	30	
1	1.00		1.00	1.00	1.00	1.00	0.00	0.45	0.00	0.00	.00	0.51
2	0.00		0.00	0.00	0.00	0.00	1.00	0.55	1.00	1.00	1.00	0.49
PepL T1											
(N)	30		30	30	30	6	30	30	30	30	30	
1	0.00		0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00	0.54
2	1.00		1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.46
PepL T2											
(N)	30		30	30	30	6	30	30	30	30	30	
1	1.00		1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.46
2	0.00		0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00	0.54
PGD											
(N)	30		30	30	30	6	30	30	30	30	30	
1	1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
PGM											
(N)	24		25	30	30	6	30	30	30	30	30	
1	0.75		0.88	0.00	0.00	0.00	1.00	0.18	1.00	1.00	1.00	0.63
2	0.10		0.08	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.27
3	0.15		0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
4	0.00		0.00	0.00	0.00	0.00	0.00	0.82	0.00	0.00	0.00	0.09
SDH											
(N)	30		30	30	30	6	30	30	30	30	30	
1	1.00		1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.46
2	0.00		0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00	0.54

Appendix 2. Allele frequencies at enzyme loci for *Elporia barnardi* (Diptera: Blephariceridae) populations in the south-western Cape, South Africa. (N = sample size)

Locus	Kasteelspoort			Platteklip			Nursery	Skeleton			Eerste			Langrivier	All
Allele	1	2	3	1	2	3		1	2	3	1	2	3		
Aat-1															
(N)	33	35	36	57	50	52	46	51	47	53	40	55	40	45	
3	0.91	0.89	0.80	0.79	0.62	0.769	0.74	0.45	0.40	0.36	-	-	-	-	0.47
5	0.09	0.11	0.20	0.21	0.38	0.231	0.26	0.55	0.61	0.64	1	1	1	1	0.53
Aat-2															
(N)	33	35	36	57	50	52	46	51	47	46	40	55	40	45	
3	0.61	0.58	0.58	0.10	-	0.058	0.52	0.50	0.5	0.53	0.16	0.173	0.025	0.067	0.29
5	0.39	0.42	0.42	0.90	1	0.942	0.48	0.50	0.5	0.47	0.84	0.827	0.975	0.933	0.71
Amy															
(N)	31	31	33	56	49	52	46	51	47	53	50	65	50	50	
2	-	-	-	-	-	-	-	-	-	-	0.01	0.008	-	0.01	-
3	0.60	0.57	0.50	0.91	0.92	0.84	0.67	0.96	0.86	0.78	0.99	0.969	0.99	0.98	0.85
5	0.40	0.44	0.50	0.08	0.08	0.16	0.33	0.04	0.14	0.22	0	0.023	0.01	0.01	0.15
6	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-
Mdh															
(N)	33	35	36	57	50	52	46	51	52	46	50	65	50	50	
3	0.73	0.70	0.65	1	0.96	0.97	0.46	0.49	0.33	0.5	1	1	1	1	0.79
5	0.27	0.30	0.35	-	0.04	0.03	0.54	0.51	0.67	0.5	-	-	-	-	0.21
Pgd															
(N)	37	35	37	54	50	52	44	51	52	51	46	65	41	42	
3	-	-	-	0.13	0.01	0.02	0.07	-	0.05	0.60	0.41	0.523	0.293	0.548	0.20
5	1.00	1.00	1.00	0.87	0.99	0.98	0.93	1	0.95	0.40	0.59	0.477	0.707	0.452	0.80
Pgi															
(N)	37	36	38	57	50	52	46	51	52	55	50	65	50	50	
3	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.43	0.5	0.5	0.54	0.84
5	-	-	-	-	-	-	-	-	-	-	0.57	0.5	0.5	0.46	0.16

Appendix 3. Allele frequencies at enzyme loci for *Aeshna subpupillata* (Odonata: Aeshnidae) populations in the south-western Cape, South Africa. (N = sample size)

Locus / Allele	Kasteelspoort	Platteklip	Skeleton	Disa	Eerste	All
Pgm						
N	32	40	30	40	40	
1	0.453	0.425	0.500	0.613	0.325	0.462
2	0.516	0.575	0.433	0.388	0.675	0.522
3	0.031	0.000	0.067	0.000	0.000	0.016
GPI						
N	32	40	30	40	40	
1	0.844	0.750	0.900	0.850	0.775	0.819
2	0.141	0.225	0.067	0.150	0.225	0.168
3	0.016	0.025	0.033	0.000	0.000	0.014
DIA1						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
DIA2						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
DIA3						
N	30	40	30	40	40	
1	0.500	0.500	0.567	0.413	0.225	0.431
2	0.500	0.500	0.433	0.513	0.775	0.553
3	0.000	0.000	0.000	0.075	0.000	0.017
MDH1						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000

Appendix 3. continued….

MDH2						
N	32	40	30	40	40	
1	0.625	0.575	0.700	0.588	0.500	0.591
2	0.375	0.425	0.300	0.413	0.500	0.409
LAP1						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
LAP2						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
LAP3						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
PepLTT						
N	32	40	30	40	40	
1	0.984	1.000	1.000	0.988	1.000	0.995
2	0.016	0.000	0.000	0.013	0.000	0.005
PepLGG						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
PepPHP						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
PepGL						
N	32	40	30	40	40	
1	0.984	0.988	1.000	0.988	1.000	0.992
2	0.016	0.013	0.000	0.013	0.000	0.008

Appendix 3. continued....

MPI						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
LDH						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
IDH1						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
IDH2						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
HEX						
N	32	40	30	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000
ARK						
N	32	40	26	40	40	
1	1.000	1.000	1.000	1.000	1.000	1.000

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CD Snaddon and BR Davies

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