The invasive guttural toad, *Amietophrynus gutturalis*

Nicolas S. Telford

A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the Department of Biodiversity and Conservation Biology, University of the Western Cape.

Funding provided by the National Research Foundation (NRF)

Ethics clearance

Ethics clearance for this project was granted by the UWC Ethics Committee 11 September 2013, ref 04/4/10

‘They have a predilection for macadamised roads at night and thus are squashed by passing motorists. On a 2 km long road that I traverse there may be 20 to 30 dead toads most mornings for months on end – the supply seems limitless – they must die in this way throughout the country in countless thousands.’ Wager, 1986

DECLARATION

I declare that, **The invasive guttural toad, *Amietophrynus gutturalis***, is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Nicolas Simon Telford

Date:

Signed: .....

ABSTRACT

The invasive guttural toad, *Amietophrynus gutturalis*

N. S. Telford

MSc Thesis, Department of Biodiversity and Conservation Biology, University of the Western Cape

The guttural toad, *Amietophrynus gutturalis,* Power 1927, is a common toad with a broad geographic range through much of temperate, sub-tropical and tropical southern and central Africa. Introduced to the islands of Mauritius and Reunion in the 1960's, and subsequently to Cape Town in the 1990's, the species has become invasive in its extra-limital ranges. Determining the invasion history of a species provides valuable information for conservation biologists and managers and it is fundamentally important for improving our understanding of the underlying processes of biological invasions. This study aimed to determine the source populations of the extra-limital populations from Mauritius and Cape Town. Furthermore, studies investigating genetic diversity and demographics of African Bufonidae are largely absent from the literature. Understanding the evolutionary history of the species may also assist with determining their invasive ability and identifying similar features in other bufonids such as *Amietophrynus regularis* and *A. xeros.* Using mtDNA sequence data from the 16S and ND2 markers four geographically distinct clades were identified through Bayesian phylogenies and haplotype networks. However, a spatial analysis of molecular variance (SAMOVA) indicated a grouping structure of three clades. A total of 16 haplotypes were identified from 53 samples for the 16S marker and 22 haplotypes were identified from 43 samples for the ND2 marker. Both the Mauritius and Cape Town invasive populations were found to have originated from the eastern clade. However, they matched the common haplotype from this region which was found across a vast area that spans the KwaZulu-Natal province and into the Mpumulanga and Limpopo provinces. This did not allow for identifying a more precise region for the origin of the founder populations. The presence of haplotypes unique to the Cape Town invasive population, which group with the eastern clade, indicates that there has potentially been more than one introduction event. Demographic analysis revealed a recent population expansion in both the northern (Fs = -2.92) and the eastern clades (Fs = -5.03). Significant genetic variation was found among groups (93.92%), with low variation among populations and among populations within groups. Population pairwise differences were found to be significantly different between all clades except between the central and the southern clade. There was a negligible difference in the genetic

diversity of the invasive populations when compared to the eastern clade. The eastern clades' genetic diversity was low compared to the two other clades and demographic analysis revealed that this region has undergone the most recent population expansion. The negligible difference between the eastern clades' genetic diversity and both invasive populations indicate that founder effects and genetic bottlenecking should have no impact on the invasive populations.

ACKNOWLEDGEMENTS

The completion of any major project can never be accomplished without the assistance and guidance of various people. There are many people and institutions I would like to thank and acknowledge for providing assistance, samples and funding, without which this research would not have been possible.

My sincere gratitude must be conveyed firstly to my supervisors Dr Vanessa Couldridge and Professor Alan Channing for their invaluable guidance, assistance, insight, funding assistance, provision of tissue samples and patience through the course of this project. Without their assistance this project would never have come to fruition. I would also like to thank Professor John Measey from the Centre for Invasion Biology at Stellenbosch University, for providing the opportunity to use his research topic, for providing tissues from his own collection and the tissues from the toads collected from the Cape Town invasive population as well as for his assistance and guidance on various topics of the project. I would also like to thank Professor Krystal Tolley from the South African National Biodiversity Institute for assisting me with various aspects surrounding my biogeographical analysis and Dr Claudia Bader and Dr Vincent Florens from the University of Mauritius for providing invaluable samples from the invasive population of Mauritius. I would also like to thank Atherton de Villiers for valuable information provided regarding the invasive toad population in Cape Town.

Furthermore, the study would not have been possible without the bursary monies I received from the National Research Foundation and the University of the Western Cape, of which I am deeply grateful. I would also like to thank the Ezemvelo KZN Wildlife, Cape Nature, Eastern Cape Department of Environmental Affairs, and the Gauteng Directorate of Nature Conservation for the provision of permits and I am deeply grateful to all the private land owners that allowed me to collect samples on their land.

Last, but not least, I would like to express my deepest thanks to my family and close friends for their help, love and guidance over the duration of this study.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

TABLES

FIGURES

haplotype. The colours represented in the legend correspond with those represented by the haplotype network. Numbers in the haplotypes are represented in Table 3.2.

Figure 3.5: Graph indicating the distribution of F_{CT} values with an increase in the amount of groups (*K* groups). The maximum variance indicated is at the point when *K*=3 groups.

Figure 3.6: Mantel test for IBD indicating the relationship between pairwise Φ_{ST} values and geographic distance for *Amietophrynus gutturalis*.

AIMS AND OBJECTIVES

The focus of this project was to investigate the genetic variation, demographics and population genetics of the invasive and natural populations of the guttural toad, *Amietophrynus gutturalis*, using mitochondrial DNA sequence data. In order to accomplish this, the following questions were asked:

- What is the phylogenetic composition of *A. gutturalis* across their natural range?
- What is the demographic history of *A. gutturalis*?
- What is the genetic diversity of the invasive population and would this influence the species ability to persist?
- What is the genetic variation of the natural population and how could this influence the invasiveness of the species?
- Where did the Cape Town and Maurtius invasive populations originate from?

CHAPTER 1
GENERAL INTRODUCTION

The Bufonidae are well researched and there is a thorough understanding of most species life histories. Bufonidae systematics have been recently revised (Frost, 2015) and there has been a renewed interest in biogeographical studies (Froufe *et al*., 2009; Vasconcellos *et al*., 2010; Portik & Papenfuss, 2015). Although there was a focus on bufonid evolution during the 1960's and 1970's (Tihen, 1962; Blaire, 1972; Tandy, 1972), there remain major gaps in the understanding of their historical biogeography. With the advent of modern molecular techniques it is now possible to investigate this at a deeper level.

Figure 1.1: Photograph of the guttural toad, *Amietophrynus gutturalis*, Power 1927.

The *Amietophrynus* genus comprises 44 currently recognized species (Frost, 2015). They are widely distributed across Africa and parts of the Middle East (Frost, 2015). Many species within the genus are common, have a broad geographic distribution and exhibit a generalist life history strategy. Even though much is known about the guttural toad, *Amietophrynus gutturalis* (Fig. 1.1), there has been renewed interest in the species due to the successful establishment of invasive populations outside of their natural range. Furthermore, no research has been conducted on the population genetics of the species. In order to manage invasive species adequately, it is important to have a clear understanding of their life history, their abilities to hybridize and their evolutionary history. Although a multi-species molecular study (Cunningham & Cherry, 2004), which included *A. gutturalis*, has already been conducted, a species specific investigation of the natural and invasive populations is yet to be completed.

Background on *Amietophrynus gutturalis*

The guttural toad, *Amietophrynus gutturalis* (Fig. 1.1), is a large (up to 140 mm SVL) common and widespread species which garners its name from its loud guttural advertisement call (Channing, 2001). The species wide distribution includes Angola, Botswana, The

Democratic Republic of Congo, Kenya, Lesotho, Malawi, Mozambique, Namibia, Somalia, South Africa, Swaziland, Tanzania, Zambia and Zimbabwe (Channing 2001; du Preez *et al*., 2004; IUCN, 2013), but is absent from the arid regions of western Botswana, southern Namibia and southern South Africa (Fig. 1.2) (Channing, 2001). This generalist toad is found in a wide variety of habitats from sea level to ~1 900 m (Channing, 2001; du Preez *et al*., 2004). It is a highly adaptable species and can be found in an assortment of savannahs, grasslands, thickets, agricultural lands and it readily adjusts to urban areas where it often inhabits garden ponds (Channing, 2001; du Preez *et al*., 2004).

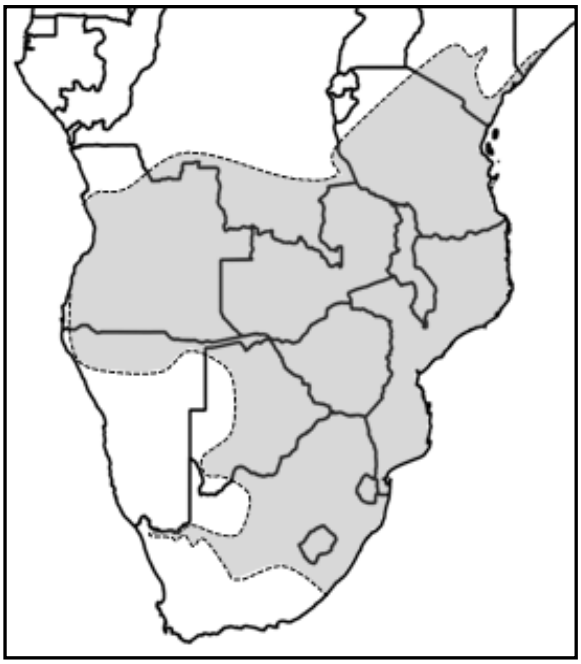

Figure 1.2: Distribution map of *A. gutturalis* across Africa (adapted from the IUCN Red List).

They are active nocturnally and take refuge during the day under logs, rocks, in gutters and drain-pipes, and burrows or holes that they excavate in soft ground (du Preez *et al*., 2004). Guttural toads are prolific breeders and a single pair is able to deposit between 15 000 and 25 000 eggs in a single clutch (Wager, 1986; Channing, 2001; du Preez *et al*., 2004). Two gelatinous strings of eggs are laid in shallow water at the edge of pools, and are often wound in and around vegetation (Channing, 2001). In the tropical and sub-tropical regions of the species range, they are able to breed year round and females will often produce two clutches. However, in the more temperate southern regions of their range, they reproduce seasonally (Channing, 2001; du Preez *et al*., 2004), during the warm, wet summer months.

Guttural toads are voracious feeders that prey on a plethora of invertebrates. They have been known to eat lizards as well as other frogs such as the common squeaker, *Arthroleptis stenodactylus* and tree frogs from the *Leptopelis* genus (Wager, 1986; Channing, 2001; du Preez *et al*., 2004). Adult and juvenile *A. gutturalis* are often preyed upon by snakes such as the rhombic night adder, *Causus rhombeatus*, forest cobra, *Naja melanoleuca*, black-necked spitting cobra, *Naja nigricollis* and the Angola green snake, *Philothamnus angolensis* amongst others. They are also prey to predators such as the African Civet, *Viverra civetta* and the Serrated Hinged Terrapin, *Pelusios sinuatus* (Channing, 2001; du Preez *et al*., 2004). Tadpoles are preyed upon by a variety of birds, water insects, fish such as the dwarf bream,

Pseudocrenilabrus philander and the common platanna, *Xenopus laevis* (Channing, 2001; du Preez *et al*., 2004).

These widely distributed toads appear to be expanding their range along the southern limits of their natural distribution. Surveys conducted during the data collection period for the Atlas and Red Data Book of Frogs of South Africa, Lesotho and Swaziland (Minter *et al*., 2004) found populations at the Hluhleka and Cwebe Nature Reserves, Amalinda Fish Station and the Cintsa district in the Eastern Cape province of South Africa. Previous surveys (e.g. Hewitt, 1935, 1937; Poynton, 1964; Guttman, 1967; Tandy, 1972) found the most southerly populations roughly 190 km north in Port St Johns (du Preez *et al*., 2004). This could either indicate a range expansion or, alternatively, may be the result of a more systematic survey than those conducted in the past (du Preez *et al*., 2004). Another potential explanation for this observed range expansion is the colonisation of new farm dams along the southern edge of the *A. gutturalis* distribution. This could be as a result of the advent of modern peri-urban and agricultural development which would facilitate this expansion (Cunningham, 2004).

Hybridisation

There has been consistent evidence of hybridisation between *A. gutturalis* and the ranger's toad, *Amietophrynus rangeri*, at various sites in the Eastern Cape and KwaZulu-Natal provinces as well as Swaziland. Hybrid toads can be identified in the field and in the laboratory through their aberrant morphology and the intermediate structure of their calls as well as through genetic analysis (du Preez *et al*., 2004). *Amietophrynus rangeri* is endemic to South Africa, Lesotho and Swaziland (Cunningham, 2004). They can be found in every province, but are restricted to the corridors created by the Vaal and Gariep rivers in the arid North West and Northern Cape provinces (Cunningham, 2004). Much of the *A. rangeri* distribution is partially overlapping with the *A. gutturalis* range. However, *Amietophrynus rangeri* is usually restricted to altitudes below 1000 m and *A. gutturalis* is absent from the Western Cape Province and the southern half of the Eastern Cape (Cunningham, 2004; du Preez *et al*., 2004).

Hybridisation between these two species has been observed along their shared eastern distributions at the following sites: Groenkloof, Port St. Johns, Weza, Harding, Pietermaritzburg and Jamestown in South Africa as well as Lubaye Falls in Swaziland (Cunningham, 2004; du Preez *et al*., 2004). The extent of introgression between these two species is largely unknown and very little research has been conducted. Guttman (1967) was

the first to investigate hybridisation between the two species at Port St Johns. Further work by Tandy and Keith (Chapter 9), Bogart (Chapter 10) and Blaire (Chapter 11) in Blaire's (1972) compendium on the evolution of the genus Bufo, examined this Port St Johns 'hybrid swarm' further and found that although the two species are hybridising, their progeny show evidence of considerable genetic blockage which is exhibited by hybrid sterility and polymorphism in chromosome numbers.

Tandy and Keith (1972) suggested that only closely related species within the genus are able to hybridise as there are only a few and imperfect barriers to heterospecific mating (Cunningham & Cherry, 2004). However both these species exhibit significantly different behavioural systems regarding mate choice as well as notably different calls (Telford & Van Sickle, 1989; Cherry, 1993). Work done by Cunningham and Cherry (2004) examined this hybrid conundrum further when examining the phylogenetics of the African 20-chromosome toads. Through their genetic analysis they found that the 'gutturalis' and 'rangeri' clades separated early in the evolution of 20-chromosome toads as well as that both lineages exhibited a range of call types and morphotypes. Therefore, the hybridisation between the two species does not imply a close relationship or recent origin of phenotypic differences between the species (Cunningham & Cherry, 2004).

Invasion history

Little research has been conducted on the three known *A. gutturalis* invasions. The first introductions of the species outside of their natural range were to the Mascarene Islands of Mauritius and Reunion, with a more recent introduction to Cape Town, South Africa. The toads were first introduced to Mauritius in 1922 and five years later to Reunion Island, both as an attempted bio-control for mosquitoes (Cheke & Hume, 2008; Kraus, 2009; Dervin *et al*., 2014). The species has established itself successfully and is widespread across both islands (Chuttoo, 2006; Cheke & Hume, 2008; Florens, pers. comm., 2014). Very little research on the impact of this species on the native biota of the Mascarene Islands has been conducted, nor has there been any indication of where the invasive population originated from. Cunningham and Cherry (2004) indicated that the Mauritius population likely originated from Albert Falls in KwaZulu-Natal or Malalotja in Swaziland. This however stems from only a single sample of matched 16S mtDNA haplotypes. These findings indicate a possible origin of the Mauritius invasive population but are not conclusive.

The establishment of the *A. gutturalis* population in Cape Town occurred more recently and is thought to have been an accidental introduction through a landscaping project (de Villiers, 2006). It is not known when exactly the introduction may have occurred, but toads were first heard calling from a house in the Cape Town suburb of Constantia in January 2000 (de Villiers, 2006). The introduction of this extra-limital population likely occurred a few years prior. By 2007, the *A. gutturalis* population was observed to be expanding and during the 2008/2009 breeding season, the City of Cape Town mapped their extent of occurrence. They found that guttural toads were present across an area of approximately 5 km^2 in Constantia (Richardson, 2014). This expanding *A. gutturalis* population was found across an area where the IUCN listed Endangered western leopard toad, *Amietophrynus pantherinus*, breeds (SA-FRoG, 2010a). This caused the City of Cape Town to contract the Nature Conservation Corporation (NCC) to initiate an eradication programme.

The eradication programme was started at the beginning of the *A. gutturalis* breeding season, from October 2010, and fieldwork continued for six months until the end of March 2011. This programme has continued through the 2011/2012, 2012/2013 and 2013/2014 breeding seasons and a total of 6 014 adults, juveniles and tadpoles have been removed since the project was initiated (Richardson, 2014). It is not known if the breeding population is being significantly affected by the eradication efforts. The differences in the number of removals (males, females, tadpoles, juveniles and the combined total thereof) through the four breeding seasons does not indicate a pattern of population decline. However, the capture rate of juvenile toads during the past four seasons may indicate a demographic shift from an 'older' population to a 'newer' population (Richardson, 2014).

During the 2013/2014 season a number of new breeding sites were found and toads were recorded calling at various new sites in the Bishopscourt suburb (Richardson, 2014). Furthermore, an individual toad was found in Kirstenbosch National Botanical Gardens (see http://www.ispot.org.za/node/225675). Examination of the locality data from the eradication program indicates that the *A. gutturalis* range seems to have expanded significantly over the past two breeding seasons. However, the range data should be viewed as questionable due to different survey methods being applied by different employees during the various breeding seasons as well as knowledge regarding their range increasing (Richardson, 2014).

The project appears to be running relatively successfully, but numerous hurdles have had to be overcome and many still pose a threat to the overall success of the endeavour. A number

of further studies are necessary in order to determine the impact this invasive species is having on the natural biota within the invasion area. It is also imperative to assess the potential impact that the species may have if it were to invade nearby natural areas that are home to Critically Endangered species (e.g. the micro frog, *Microbatrachella capensis* and the Table Mountain ghost frog, *Heleophryne rosei*).

Amphibian conservation and the threat of invasive species

The threat of invasive species has been well researched and has been repeatedly highlighted as one of the most pressing conservation concerns of the new century (Wilcove *et al*., 1998; Dukes & Mooney, 1999; Pimental *et al*., 2000; Kraus, 2009). Species are being moved across regions that have been disconnected for millennia and have now been spread across the globe through a variety of human activities. Since the advent and increase in modes of travel over the last few hundred years, there has been a dramatic increase in the rate and extent of biological introductions. Species have been introduced through numerous vectors, some accidental and many intentional, with almost all a result of human mediated dispersal (Kraus, 2009). There are numerous examples of biological invasions with disastrous consequences.

The introduction of Nile perch, *Lates niloticus*, to Lake Victoria in 1954 caused the extinction of more than 200 endemic fish species (Lowe *et al*., 2000; Goudswaard *et al*., 2008). The introduction of Australia's brown tree snake, *Boiga irregularis*, to Guam during the late 1940's caused major power outages across the island and has been responsible for the almost complete extermination of the islands native forest birds (Lowe *et al*., 2000). The Miconia tree, *Miconia calvescens*, introduced to the Tahitian islands in 1937, caused major ecological and economic damage as a result of landslides because of its superficial and tentacular root system. Furthermore, the species dominated the landscape causing major habitat loss in the region (Lowe *et al*., 2000). The water hyacinth, *Eichhornia crassipes*, has been introduced to more than 50 countries across the world. It has caused severe economic damage by blocking waterways and its dense growth prevents sunlight and oxygen from reaching the water column which dramatically reduces biodiversity in aquatic ecosystems (Lowe *et al*., 2000). These few examples, emanating from a list of many, illustrate clearly the extent of damage that invasive species can have on ecological and economic systems across the world.

Although the anthropogenic (whether accidental or deliberate) dispersal of vertebrate fauna contains a vast list of species, only relatively few amphibians have managed to establish successful invasive populations in their novel habitats. The probability of a successful

invasion is largely dependent on the suitability of the novel habitat, the prevailing climate and the ability of the introduced species to withstand these changes. As such, invasive species can frequently be characterised by a general set of traits, and this is certainly evident in the predominant invasive amphibian species across the world (see Van Bocxlaer *et al*., 2010). Often characterized as generalists, these species exhibit many similarities in that they have a high reproductive rate which allows for rapid population growth and the ability to withstand stochastic events. They are often small and secretive which allows them to remain undetected until a population has been established, and they have a generalized diet which allows them to utilize the resources available in the novel habitat (Pitt *et al*., 2005).

Species that exhibit these traits are often the most successful invaders, even though the probability of establishing a successful invasion is often reliant on the suitability of both climate and habitat (Simberloff & Von Halle, 1999). In a global context, amphibians have been considerably translocated, and as a result a small number have become major problems both ecologically and economically (Kraus, 2009). Many of these introductions have caused detrimental effects on native biota. It has become increasingly clear that the disruptions caused by introduced species rivals the threats posed by better known ecological problems such as habitat loss, pollution and climate change (Kraus, 2009). This can be attributed largely to the fact that many alien invasions are often irreversible and less prone to correction, unlike various other ecological problems.

The effects of biological invasions are vast and can cause significant ecological and economic damage. The ecological impacts from such invasions may cause an alteration in community structure and convert the ecosystem from one state to another. Furthermore, they may cause a disruption to ecosystems food-webs and ultimately may cause species to go extinct. Major economic impacts through vectors such as watershed degradation, building damage, disease epidemics, fisheries collapse and the resultant management costs have the potential to cause major damage (Mooney, 2005; Kraus, 2009; Simberloff *et al*., 2013).

A compendium of anuran introductions by Kraus (2009) indicates that numerous amphibian introductions have occurred across the globe. A rough total of some 81 amphibian species have proven to be successful invaders. Of these, certain species have been particularly damaging. They include toads such as the American bullfrog, *Lithobates catesbeianus*, the cane toad, *Rhinella marina* and the guttural toad, *Amietophrynus gutturalis*, as well as other anurans such as the African clawed frog, *Xenopus laevis*, the common coqui,

Eleutherodactylus coqui, the greenhouse frog, *Eleutherodactylus planirostris*, and the Cuban treefrog, *Osteopilus septentrionalis*. Of these *L. catesbeianus*, *R. marina* and *E. coqui* have been listed by the Global Invasive Species Database as part of the top 100 of the world's worst invasive species (Lowe *et al*, 2000).

One of the most broadly researched and commonly known invasive amphibians is the cane toad, *Rhinella marina*. The species has a broad natural distribution which ranges from southern Texas, USA, and extends south through tropical Mexico, Central America, as well as northern South America where its range extends as far south as northern Bolivia and central Brazil (Zug & Zug, 1979; Solis *et al*., 2009; Slade & Moritz, 2013). Kraus (2009) indicates that it has been introduced to numerous regions and has established successful populations in American Samoa, Antigua and Barbados, Aruba, Australia, Bermuda, British Virgin Islands, Canary Islands Canouan, Carriacou, Cayman Islands, Chagos Archipelago, Northern Mariana Islands, Dominican Republic, Fiji, Federated States of Micronesia, Grenada, Gaudeloupe, Guam, Haiti, Hawaii, Jamaica, Japan (Ogasawara and Ryukyu Islands), Martinique, Montserrat, Mustique, Nevis, Palau, Papua New Guinea, Philippines, Puerto Rico, Solomon Islands, St. Kitts, St. Lucia, St. Vincent, Tuvalu, U.S. Virgin Islands, and southern Florida in the United States.

Rhinella marina is a widely researched species. The effects on the Australian biota have been particularly dire and thus the greatest amount of research on this invasive amphibian has stemmed from this region. It was introduced as a bio-control in 1935 to the Cairns-Innisfail area of northern tropical Queensland, Australia, in order to control the grey-backed beetle, *Dermolepida albohirtun*, and the Frenchi beetle, *Lepidiota frenchi* (Slade & Moritz, 1998; Clarke *et al*., 2000). The inability of these toads to effectively control the sugar cane pests was not perceived. These cane beetles spend their time eating the leaves of the sugar cane and their larvae feed on the roots. These factors and the dry nature of cane fields ensured that the use of *R. marina* in this manner failed. The toads flourished through exploiting other niches and they continue to expand rapidly throughout much of Australia (Lampo & de Leo, 1998). *Rhinella marina* has proven to be a potential ecological disaster. But, through this invasion we have gained insight into the mechanisms and processes of anuran invasions.

The effects and invasion dynamics of this species have been widely documented (see Lampo & de Leo, 1998; Slade & Moritz, 1998; Crossland, 2000; Greenlees *et al*., 2006; Phillips *et al*., 2006; Shine, 2010; amongst others). The ecological effects within Australia indicate that

the species can severely alter communities and have a significant impact on ecosystem dynamics. Crossland (2000) used pond experiments to examine the direct and indirect effects of *R. marina* on native anurans and found that the toxicity of *R. marina* tadpoles resulted in a decline of *Limnodynastes ornatus* as a result of these predatory tadpoles dying when feeding on *R. marina* tadpoles. Consequently, the population of a sympatric species, *Litoria rubella,* increased as a result of decreased predation by *L. ornatus.* A later study by Greenlees *et al*., (2006) examined the ecological effect of *R. marina* on invertebrate communities in Darwin, Australia. Their results indicated that *R. marina* have a major negative effect on both invertebrate abundance and species richness and thus act as a massive nutrient sink in the floodplains of Australia's Northern Territory. A more recent study by Shine (2010) investigated the ecological impact of *R. marina* further in terms of both direct and indirect effects. Many predatory species (varanid and scincid lizards, snakes, birds, freshwater crocodiles and dasyurid marsupials) have been affected as a result of predation on these toads. The impacts within and between species have varied spatially and in many cases where species were predicted to be severely affected, there has been no considerable effect.

The impacts of this invasive amphibian on Australia's native biota are vast and particularly concerning as the region is home to numerous endemic, rare and endangered species. These few studies from a list of many highlight the impacts that a toad with generalist life history traits may have when introduced outside of their natural range.

Invasive amphibians of the Western Cape

The Western Cape plays host to one of the 35 globally recognized biodiversity hotspots and contains high and largely endemic amphibian diversity due to the topographical heterogeneity and hydrological stability of the Cape Fold Mountains (Poynton, 1964; Measey & Davies, 2011). Three species, native to other regions of South Africa, have managed to establish populations within the Western Cape and pose a threat to the native anuran fauna and other vertebrates and invertebrates of the region. Concerns surrounding these domestic exotics include hybridization, potential trophic cascades, competition with indigenous species, as well as the transmission of novel or existing pathogens (Van Rensburg *et al*., 2011 from Measey & Davies, 2011).

The guttural toad, *Amietophrynus gutturalis*¸ the painted reed frog, *Hyperolius marmoratus,* and the African clawed frog, *Xenopus laevis,* have established successful and problematic populations in the Western Cape and pose a variety of threats to the native biota. *Hyperolius*

marmoratus was first detected in 2001, and by 2006 it was widespread across the province. It can be found in garden ponds and farm dams across all but the driest and most mountainous parts of the province (Measey & Davies, 2011). Research conducted by Tolley *et al*. (2008) investigated the species range expansion and found that the invasive populations were established as a result of multiple human-mediated jump dispersals from their ancestral ranges in northern and central KwaZulu-Natal, the Eastern Cape and the Southern Cape. Similar to the translocation hypothesis for *A. gutturalis*, it is thought that these frogs were accidentally introduced through vectors such as landscaping and the moving of nursery plants, or hitchhiking on cars, caravans, boats or when moving building materials (Measey & Davies, 2011).

The rapid spread of these frogs and their widespread distribution means that their control or eradication is unlikely to be feasible. The sympatric arum lily frog, *Hyperolius horstockii*, a fynbos endemic, may potentially be threatened by the closely related *H. marmoratus* as they share a similar feeding niche and there is potential for hybridization. However, the impacts of this are currently unknown. Similarly, the impact of sharing a similar feeding niche between the introduced guttural toad and the Endangered western leopard toad, *Amietophrynus pantherinus*, (also a fynbos endemic) is concerning and needs to be investigated. This may cause a shift in niche dynamics and a reduction in *A. pantherinus* niche size may be detrimental to this endangered amphibian.

The African clawed frog, *Xenopus laevis*, also poses a threat to another congener, the Endangered Cape platanna, *Xenopus gilli*. The invasion history of *X. laevis* is somewhat complex due to the unknown natural distribution of the species and a lack of a specific type locality (Measey & Davies, 2011; Frost, 2015). *Xenopus laevis* has a broad distribution. It is quick to colonise new and disturbed water bodies, is able to spread quickly over land, prefers eutrophic water bodies and is able to build up large population densities over a short space of time (Van Dijk, 1977; Measey & Channing, 2003; SA-FRoG, 2010c). In contrast, *X. gilli* has a limited distribution across a small area of the southwestern Cape and is adapted to only inhabit acidic black water streams and pools (Picker, 1993). The often highly transformed acid fynbos vegetation where this species occurs is under ongoing threat (Driver *et al*., 2005) and the invasion of the disturbed *X. gilli* habitat has initiated conservation actions in order to prevent further hybridisation between the two species (Picker, 1985; Picker & De Villiers, 1989; Measey & Davies, 2011).

Currently these three amphibians are the only successfully established invasive anurans in the Western Cape. However, it is clear that translocations, whether deliberate or accidental, have the potential to seriously alter ecosystem function and community dynamics. Furthermore, national legislation that covers the translocation of species across provincial borders is not sufficient for stopping the movement of other species within the country. It is thus imperative that an early detection and rapid response (EDRR) protocol should be followed when exotic species are located in a novel region (Measey & Davies, 2011).

Effects of invasive species on evolution

Since humans started travelling to different regions across the globe biotic translocations have occurred, breaching the biogeographic barriers that have kept species isolated for millions of years. This has resulted in the successful establishment of numerous species outside of their natural range. Once a population has become established, many of these introduced species become invasive. The effects of these invasive species have had broad reaching ecological and economic effects (see Wilcove *et al*., 1998; Dukes & Mooney, 1999; Pimental *et al*., 2000; Pimental *et al*., 2005; Kraus, 2009; Simberloff *et al*., 2013).

As a result there are often major effects on the evolution of both the invasive species as well as the affected native species. For instance, there are examples of introduced species influencing the evolutionary pathway of native species through niche displacement, introgression through hybridization, competitive exclusion, predation and extinction, as well as invaders evolving through their interactions with the new novel environment and their interactions with the species therein (Mooney & Cleland, 2001).

Rapid evolutionary change is often experienced during invasions (Reznick & Ghalambor, 2001), and is facilitated through epistasis, hybridization, additive genetic variance and the action of a small number of genes and genomic rearrangements (Lee, 2002). Many different species have been used to explore the evolutionary effects of biological invasions, whilst only a few successful invasive amphibians have been widely researched. The cane toad, *Rhinella marina*, has been the subject of a range of research and various studies have investigated evolutionary changes in cane toads and species that have been affected by the invasion. Predators are especially vulnerable and many die as a result of preying on these toxic toads.

A study by Phillips and Shine (2004) examined whether there have been morphological changes in snakes that are vulnerable to dying from ingesting cane toads. They hypothesised that the arrival of the toads would exert selective pressures on vulnerable species. Because

snakes are limited by gape size and have a strong negative allometry for head size, it is likely that the maximum relative prey mass would decrease with an increase in snake body size. The arrival of the toads would therefore affect snake morphology through selective pressures which would favour an increase in mean body size and a decrease in relative head size. They investigated this by examining if there was an increase in mean body size and a decrease in relative head size of two toad vulnerable species (*Pseudechis porphyriacus* and *Dendrelaphis punctulatus*) and two low risk species (*Hemiaspis signata* and *Tropidonophis mairii*), from specimens collected over the past 80 years. As they hypothesized, there was a continued increase in mean body length and a reduction in gape size with an increase in time since exposure in the two vulnerable species, while the two low risk species showed no consistent changes in these morphological traits. The results of this study provide strong evidence for the evolution of adaptive changes in native predators in response to the introduction of a toxic invasive species.

Another study by Phillips *et al*. (2006) examined morphological changes in the cane toad after being introduced to Australia. By investigating differences in leg length between toads at the invasion front and toads in longer-established populations they found that toads at the invasion front had developed longer legs and were able to move faster than those in older populations. These studies provide a greater insight into the evolutionary dynamics of introduced species and the species they affect. With a plethora of introduced vertebrates across the globe, research such as this will greatly assist in understanding the array of impacts that invasive species have on natural systems and it is hoped to lead to a more rapid response protocol when newly introduced species are discovered.

Background on toad biogeography

The Bufonidae, one of the best studied anuran families, have served as a useful test-case in anuran systematics and biogeography (e.g. Tihen, 1962; Blair, 1972; Tandy, 1972; Maxson, 1984; Pramuk *et al*., 2008 amongst others). The systematic resolution of toads is of global biogeographic interest due to their long history in Eurasia, the Americas and Africa, as well as their limited capacity for dispersal across ocean barriers (Cunningham & Cherry, 2004). Distributed across all six of Wallace's (1876) biogeographic regions, the Bufonidae are an ideal vertebrate group for reconstructing phylogenetic relations and their evolutionary history (Blair, 1972a).

There have been various contrasting hypotheses regarding the radiation of toads across the globe. Using osteological characters Tihen (1962) hypothesized that the Bufonidae is a polyphyletic assemblage resulting from multiple colonizations from Africa. Blair (1972a) argued for a paraphyletic assemblage resulting from a South American origin using primarily osteological characters (R. F. Martin, 1972). However, vocal, morphological, cytological, genetic and biochemical characters were also considered (Blair, 1972b,c; Cei *et al*., 1972; Bogart, 1972; Guttman, 1972; Low, 1972; R. F. Martin, 1972; W. F. Martin, 1972; Szarski, 1972). Maxson (1984), using studies of albumin evolution, hypothesized that Nearctic bufonids are a monophyletic assemblage resulting from a single northward radiation from the neotropics (Pauly *et al*., 2004).

Since then a more thorough phylogenetic investigation by Pramuk *et al*., (2008) has provided greater insight into the global radiation of toads. The Bufonidae were initially thought to be of Gondwanan origin, (~105 Ma) (Savage 1973; Maxson, 1984; Pramuk, 2006). However, the study by Pramuk *et al*., (2008) indicates a much later vicariance during the Cenozoic period (78–98 Ma) with the entire radiation of the major lineages of extant bufonids taking place during the Eocene (33.9–56 Ma), subsequent to their dispersal out of South America. Climatic fluctuations during the Eocene played a major role in shaping the current distributions of the Bufonidae. The research by Pramuk and her colleagues has provided a greater understanding towards the global vicariance of the Bufonidae. Nonetheless, much of the research has focused on the neotropical regions and there remains a major gap in the understanding of the evolutionary history of African Bufonidae and how it relates to global patterns.

Research conducted by Mills Tandy (1972) provided a firm base from which to investigate evolutionary patterns in African Bufonidae. There are few broad scale phylogeographic studies of species within this genus and there are numerous gaps to fill in order to attain a greater understanding of biogeographical patterns within the family.

Many of the African bufonids have broad geographical ranges (e.g. *Amietophrynus gutturalis*; *A. regularis*; *A.s garmani*; *A. maculatus*; *A. xeros*) and uncertain taxonomy (e.g. *Amietophrynus garmani*; *Amietophrynus regularis*; *Amietophrynus superciliaris*) (IUCN, 2015). Recent phylogeographic work on African bufonids has started to provide a more comprehensive understanding of the evolutionary history and biogeographic patterns of species within this diverse genus. Papers by Froufe *et al*. (2009) and Vasconcelos *et al*.

(2010) examined the phylogeography of a North African speciess, *Amietophrynus xeros* and a Central African species, *Amietophrynus regularis* respectively.

Amietophrynus xeros is distributed across much of northern Africa, from Mauritania in the extreme west and east through Niger, Mali and Senegal through to Tanzania (Froufe *et al*., 2009). The study by Froufe *et al*. (2009) aimed to determine genetic diversity across their broad range and to determine if there are any undiscovered cryptic taxa. Interestingly, it was found that there was low genetic diversity across the Sahel region which provided an indication that *A. xeros* expanded into this region relatively recently and therefore geographically dispersed and isolated populations are genetically similar. Like other species in Africa (Tolley *et al*., 2008), this vicariance is thought to have occurred after the last glacial maximum. Furthermore, Froufe *et al.* (2009) found significant errors in Genbank sequences with respect to *Amietophrynus regularis*, *A. garmani* and *A. gutturalis*. These findings are important because of the implications for past and future studies.

A more recent paper by Vasconcelos *et al*. (2010) aimed to elucidate the genetic variation of *A. regularis* which, similar to *A. xeros*, has a broad north and central African distribution. Furthermore, Vasconcelos *et al.* (2010) also attempted to resolve the previously mentioned discrepancies in sequence data from Genbank and identify the origin of an introduced population on the Cape Verde Islands. Two distinct lineages were found, one in the west and one in the eastern regions of the *A. xeros* range. Some clarity on the discrepancies in the Genbank database was provided, but the discrepancies remain unresolved.

These two papers are useful contributions to the phylogeography and the systematic resolution of the African Bufonidae. Nevertheless, major gaps in the literature remain. In order to attain a more concise picture on African bufonid biogeography it is necessary to identify patterns of speciation, determine the presence of any cryptic taxa, investigate the biogeography of individual species and work towards a greater understanding of the historical biogeography of the genus. A recent paper by Portik and Papenfuss (2015) contributes a useful piece of information with the finding that the formation of the Red Sea was likely to have driven simultaneous divergences between *Amietophrynus tihamicus* and *A. arabicus* and their closest mainland African relatives during the early Miocene. Furthermore, both Arabian species (*A. tihamicus* and *A. arabicus*) are likely to represent true African relicts which resulted from vicariance associated with the formation of the Red Sea.

Reasoning behind study

The reconstruction of invasion history is fundamentally important for improving our understanding of the processes of biological invasions (Davies, *et al*., 2013). It is of particular importance to be able to identify the source populations and their entry points (Rollins *et al*., 2011; Ruiz *et al*., 2011), the causes of range expansion (Didham *et al*., 2005; Parmesan, 2006), form hypotheses on how they spread and design models that assist with predicting the impacts of invasion (Kulhanek *et al*., 2011). Therefore it is necessary to reconstruct invasion histories as a precursor to studies of the mechanisms and limits of invasion and of invasion dynamics and their causes (Andow *et al*., 1990).

This study aims to address the invasion history of *Amietophrynus gutturalis* by determining the biogeographical history and demographics of the natural population and to identify the regions of origin of the Cape Town and Mauritius invasive populations. These are important questions because without information on invasion history, the recognition of the invasiveness of a species may be delayed which would hamper the appropriate response and measures for its control (Le Maitre *et al*., 2004; McGeoch *et al*., 2012; Davies *et al*., 2013).

Furthermore, there has been very little recent biogeographical research on the African Bufonidae. Two recent papers (Froufe *et al*., 2009 and Vasconcelos *et al*., 2010) examine phylogeographic patterns in two species that are closely related to *A. gutturalis* and share many traits. These studies are slowly assisting with understanding the historical biogeography of African Bufonidae. By investigating the population genetics of *A. gutturalis*, it will be possible to investigate historical demographics which may shed more light on the biogeographic patterns of the genus across Africa and provide a precursor to a thorough biogeographical investigation of closely related *Amietophrynus* species.

CHAPTER 2
MATERIALS AND METHODS

Sampling

Samples of *A. gutturalis* were collected in three ways. Firstly, samples from the invasive Cape Town population were provided by the Nature Conservation Corporation (NCC). The NCC has been contracted on an annual basis to eradicate toads within the Cape Town population. Tissue samples in the form of thigh muscle from 26 of the 1 787 eradicated toads (adults, juveniles and tadpoles) from the 2012/2013 breeding season were used. Samples were selected so as to attain a representative sample set across the invasive population's geographical range. Furthermore, 12 sequences that were obtained during the 2013 pilot study were included. Samples from the Mauritius invasive population were provided by C. Baider.

Secondly, tissue samples from the species natural range within South Africa were collected in the field between January and March 2014 (Fig. 2.1). The KwaZulu-Natal province was indicated as the most likely region where the invasive population originated from (de Villiers pers. comm. 2014). It is likely that the introduction was as a result of human-mediated jump dispersal which may have been as a result of a deliberate introduction or the movement of nursery plants between provinces. *Amietophrynus gutturalis* is common and widespread species and is often associated with urban environments (Channing, 2001; du Preez *et al*., 2004). Due to the species predilection for urban environments and the source population hypothesis, sampling was focused on urban areas across the eastern coast of South Africa.

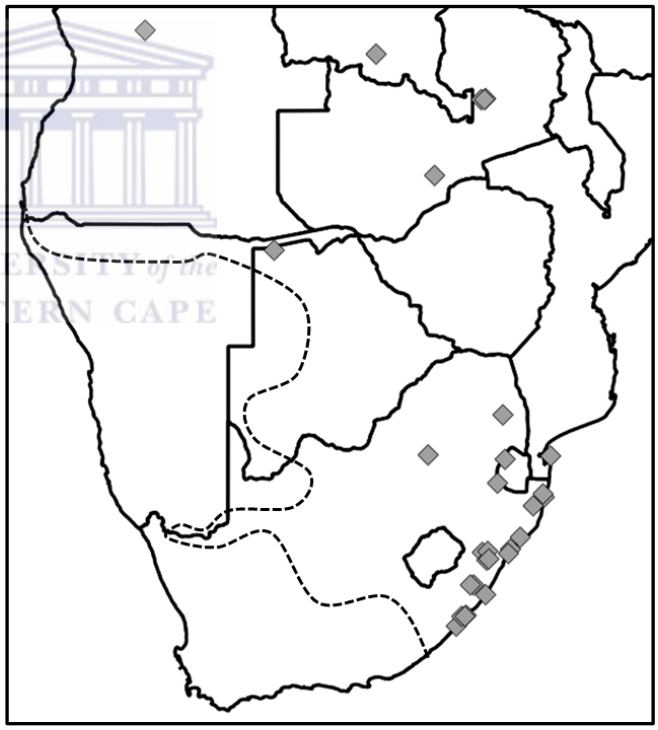

Figure 2.1: Map of all *Amietophrynus gutturalis* samples across their natural range. The southern and western boundary of their natural range is indicated by the dotted

A guideline of between 50 and 100 km was used for identifying sample sites. In most cases the distance between sample sites was approximately 100 km. Distributional data from the Atlas and Red Data Book of frogs of South Africa, Lesotho and Swaziland (Minter *et al*., 2004) was used as the primary parameter for locating sample sites. Recent surveys during the collection of atlas data (du Preez *et al*., 2004) located new populations in the Cintsa region of the Eastern Cape Province of South Africa. This was identified as the first sample site and suitable localities were chosen in a progressively northward direction. Potential sites were identified during the day and visited after sunset. The presence of toads was determined by listening for the calls of breeding males. If a breeding site wasn't located, sampling was conducted by road running. A minimum of one and maximum of five samples were collected from each sample site. All tissue samples taken from individuals were in the form of toe clippings. Each sample was recorded and stored in 99% ethanol.

Thirdly, tissue samples from countries outside of South Africa were provided by A. Channing and G. J. Measey. These samples were either in the form of thigh muscle, toe clippings or tail clippings from tadpoles. Most samples were stored in 99 % ethanol. The few that were stored in dimethyl sulfoxide (DMSO) were washed with molecular grade distilled water prior to extraction. In addition, all sequences of the appropriate genetic markers available on the Genbank database were used. All samples and relevant information are indicated in Table 2.1.

The large species range, funding and time available for field work were constraints that affected the ability to attain a thoroughly representative sample set. As a result there are various sampling gaps and the dataset does not comprehensively cover the species range.

All tissue samples (~10 mg) were digested using standard proteinase K/SDS procedures (Palumbi 1996) and total DNA was extracted with the standard phenol/chloroform method (Palumbi 1996). Total genomic DNA concentrations from each sample were obtained using a fluorometer (Qubit). Working solutions with a 2 ng/µl concentration were made for each sample.

Table 2.1: Sample population, location, country, GPS co-ordinates (in decimal degrees), Genbank accession number, site description, number of samples collected at each site (*n*) and the number of successful polymerase chain reactions (PCRs) from the ND2 and 16S genetic markers for all *Amietophrynus gutturalis* samples used in this study.

Sample Population	Location	Country	Latitude	Longitude	Accession Number	Site description	Number of Samples (*n*)	Successful PCR ND2	Successful PCR 16S
L01	Coffee Bay	South Africa	-31.98	29.14	N/A	Stagnant pool	6	3	1
L02	Port St Johns	South Africa	-31.67	29.38	N/A	Under a bridge	8	2	1
L03	Port St Johns	South Africa	-31.62	29.53	N/A	Stagnant pool	2	2	1
L04	Harding	South Africa	-30.57	29.87	N/A	Forestry road	1	1	1
L05	Southbroom	South Africa	-30.91	30.32	N/A	Road running	1	1	1
L06	Southbroom	South Africa	-30.92	30.31	N/A	Golf Course	4	2	2
L07	Howick	South Africa	-29.46	30.19	N/A	Farm dam	5	4	5
L08	Pietermaritzburg	South Africa	-29.70	30.39	N/A	Garden pond	10	2	3
L09	Stanger	South Africa	-29.32	31.34	N/A	Road running	1	0	1
L10	Salt Rock	South Africa	-29.50	31.23	N/A	Road running	5	3	3
L11	Mtunzini	South Africa	-28.93	31.73	N/A	Garden	7	2	3
L12	Sodwana Bay	South Africa	-27.51	32.65	N/A	Garden	1	1	1
L13	Kube Yini	South Africa	-27.80	32.22	N/A	Garden pond	5	3	3
L14	Pongola	South Africa	-27.38	32.63	N/A	Pond	2	2	2
L15	Piet Retief	South Africa	-27.01	30.80	N/A	Dam edge	6	3	2
L16	Johannesburg	South Africa	-25.99	28.00	N/A	Garden pond	4	3	3
L17	Klaserie	South Africa	-24.54	31.02	N/A	Garden	2	2	1
L18	Cape Town	South Africa	-34.00	18.43	N/A	Constantia	39	31	27
L19	Kangandala	Angola	-9.81	16.65	N/A	Not Available	1	0	1
L20	Kisanfu	DRC	-10.76	25.95	N/A	Mining concession	1	1	1
L21	Vacoas	Mauritius	-20.29	57.48	N/A	Not Available	12	2	3
L22	Le Pouce	Mauritius	-20.20	57.52	N/A	Not Available	2	1	1
L23	Le Pouce	Mauritius	-20.19	57.52	N/A	Not Available	2	1	1
L24	Inhaca	Mozambique	-26.02	32.96	N/A	Not Available	3	2	2
L25	Lusaka	Zambia	-15.50	28.27	N/A	Eureka Camp	2	2	2
L26	Kasanka	Zambia	-12.55	30.16	N/A	Chikufwe	1	1	1
L27	Kasanka	Zambia	-12.55	30.30	N/A	Wasa Lake	1	1	1
L28	Kasanka	Zambia	-12.52	30.33	N/A	Road	1	1	1
L29	Weza	South Africa	-30.57	29.70	AF220875 AF463777	Not Available	1	1	1
L30	Weza	South Africa	-30.57	29.70	AF220878 AF463778	Not Available	1	1	1
L31	Ashburton	South Africa	-29.67	30.46	AF220875	Not Available	1	0	1
L32	Malalotja	Swaziland	-26.13	31.12	AF220875	Malalotja NR	1	0	1
L33	Not Available	Mauritius	Not Available		AF220875	Not Available	1	0	1
L34	Shakawe	Botswana	-18.38	21.85	AF220876	Not Available	1	0	1
L37	Albert Falls	South Africa	-29.43	30.42	AF220877	Not Available	1	0	1
L38	Port St Johns	South Africa	-31.65	29.49	AF463779	Silaka NR	1	1	0

PCR amplification

The Polymerase Chain Reaction (PCR) was used to amplify segments of the 16S and NADH2 (ND2) mtDNA gene fragments from 51 samples obtained from 24 localities. The number of samples from each locality and the sequences obtained from the two different markers are detailed in tables 2.1 and 2.2. The 16SaR (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16SbR (5'-CCG GTC TGA ACT CAG ATC ACG T-3') (Palumbi *et al*., 2002) and vMet2 (5'-GCT AAA CAA GCT TTC GGG CCC ATA CC-3') and vTrp (5'- CTC CTG CTT AGG GCT TTG AAG GC-3') (Cunningham & Cherry, 2004) primer pairs were used to amplify the mitochondrial 16S rRNA and ND2 mtDNA gene fragments respectively.

PCR reaction mixes of 25µl total volume were prepared using a mix of 4µl DNA template at 2 ng/µl concentration, 1µl of each forward and reverse primer, 6.5 µl distilled water and 12.5 µl of FastTaq polymerase ready mix (Kapa Biosystems) with an $MgCl_2$ concentration of 1.5 mM/µl. PCR for the 16S gene fragment was conducted using a Techne TC–512 Thermal Cycler with a 51ºC annealing temperature for 35 cycles and a 1.5 mM/µl $MgCl_2$ concentration. Using the same thermal cycler for ND2, PCR was conducted with a 57°C annealing temperature for 35 cycles and a 1.5 mM/µl MgCl concentration. Negative controls were used for all reactions. The amplification products were examined under ultra-violet light on 0.7% agarose gels stained with ethidium bromide. All successful amplifications were sent to the Central Analytical Facility at Stellenbosch University for sequencing.

DNA sequencing and alignment

All 16S and ND2 sequences were checked to ascertain that the sequences represented the correct species through the NCBI BLAST function on Genbank (Altschul *et al*., 1990). Sequences were aligned in Sequencher v5.2.4 and were checked against the chromatograms for reading errors. Sequences of 519 bp for the 16S marker and 708 bp for the ND2 marker were recovered. The ND2 data set was aligned to the third codon position which was the correct reading frame. A data set containing concatenated sequences from samples where both 16S and ND2 markers were successfully amplified was created and aligned using Sequencher v5.2.4. All haplotypes identified from this study for both markers will be deposited in the Genbank database (http://www.ncbi.nlm.nih.gov) prior to publication of the research.

Phylogenetics

In order to obtain a broad scale phylogenetic pattern, Bayesian analyses were performed on the two *Amietophrynus gutturalis* data sets, using the default settings in MrBayes 3.2.4 (Ronquist & Huelsenbeck, 2003). Phylogenies for both the 16S rRNA and ND2 mtDNA markers were constructed using the GTR+I+G model for 2 x 10^6 generations with six rate categories and uniform priors for the gamma distribution and invariable sites in MrBayes 3.2.4 (Ronquist & Huelsenbeck, 2003). Garli 2.0 (Zwickl, 2006) was used to create a maximum likelihood tree with 1000 bootstrap repetitions. A consensus tree with maximum likelihood bootstrap values and Bayesian posterior probabilities was created using DendroPy 3.12.0 (Sukumaran & Holder, 2010). Trees were rooted with sequences of the closely related sister taxon, *Amietophrynus kisoloensis* (see Onadeko *et al*., 2014) (Genbank accession numbers DQ15864 for 16S and AF463788 for ND2). PAUP* 4.0b10 (Swofford, 2003) was used to determine uncorrected p-distributions. This resulted in the removal of six sequences that were erroneously (p-value > 3%) represented as *Amietophrynus gutturalis.* The misidentified sequences were: DQ283436 from Frost *et al*., 2006; GQ183567 from Siow *et al*., 2009; FJ882851 from Van Bocxlaer *et al*., 2009; and tissue samples from Tatanda and Utengule in Tanzania and Taita Taveta in Kenya.

The resulting phylogenies for *Amietophrynus gutturalis* were viewed in FigTree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and trees with bootstrap and posterior probability values (Fig. 3.1 and Fig. 3.2) were redrawn by hand in Microsoft Office Powerpoint.

Genetic diversity

Haplotype (*h*) and nucleotide diversity (π) were estimated for the Cape Town and Mauritius invasive populations (Table 3.3). The software Arlequin 3.5 (Excoffier *et al*., 2005) was used to calculate nucleotide diversity (π), the probability that two randomly chosen homologous nucleotides are different (Tajima, 1983; Nei, 1987) and haplotype diversity (*h*), the probability that two randomly chosen haplotypes are different (Nei, 1987).

Population genetic analyses

All samples were used for the construction of the 16S (Fig. 3.3) and ND2 (Fig. 3.4) haplotype networks in order to assist with determining the origin of the invasive populations. For the remaining population genetic analyses the samples from the Cape Town and Mauritius invasive populations were removed.

Haplotype networks were created to examine population structure at a finer scale and to identify the region where the invasive populations originated from. Previous studies have indicated that temporal and fine-scale population structure can be better estimated using haplotype networks rather than tree based methods (Bermingham & Moritz, 1998; Goldstein *et al*., 2000; Posada & Crandall, 2001; Holland *et. al*., 2004). This is because networks assess the distribution and connection of haplotypes among the localities without assuming divergence events by allowing several haplotypes to be joined by a single node. Thus, a better relatedness among maternal lineages at the population level is reflected. Using the software PopArt 1.6 (http://popart.otago.ac.nz.) with the TCS network option (Clement *et al*., 2002), haplotype networks were created for each of the maternally derived 16S rRNA and ND2 mtDNA datasets. This process of inferring haplotype networks uses the method defined by Templeton *et al*. (1992) which calculates the number of mutational steps by which each pair of haplotypes differ and determines the probability of parsimony for pairwise differences until the probability exceeds 0.95. Clades were designated according to the results obtained from the phylogenies for the 16S and ND2 markers outlined previously. Figures 3.3 and 3.4 were drawn in Adobe® Photoshop® CS5®, using the haplotype network generated from PopArt v1.6 and the maps that were created in QGIS v2.6.1. All samples from the natural population and both invasive populations were included in the construction of the networks. This was done to assist with defining the origin of the Cape Town and Mauritius invasive populations.

Concatenated sequences for all samples where PCRs were successful for both the 16S and ND2 markers were used for the spatial analysis of molecular variance (SAMOVA), analysis of molecular variance (AMOVA), and the Mantel Test for Isolation by Distance (IBD). The concatenated data set consisted of 36 samples from 21 localities and contained 23 haplotypes. A SAMOVA was conducted using the SAMOVA 2.0 software on the concatenated data set to determine the degree of differentiation among adjacent sampling sites (Dupanloup *et al*., 2002). This method uses sequence data and their corresponding geographical co-ordinates in order to assign sampling sites *a posteriori* to groups that presumably represent historically interconnected populations (Dupanloup *et al*., 2002). The SAMOVA procedure uses a simulated annealing process in order to maximise the proportion of total genetic variation between groups of sample sites using F statistics. The three F statistics indicate the proportion of total genetic variance due to differences between the groups (F_{CT}), the variation between

sample sites within groups (F_{SC}), and the genetic variation between sample sites relative to the total sample (F_{ST}) (Excoffier *et al*., 1992; Dupanloup *et al*., 2002).

jModelTest (Guindon & Gascuel, 2003; Posada, 2008) was used to determine the correct evolutionary model and the gamma distribution for the data set. The closest fit model selected by jModelTest was the Tamura and Nei model with a gamma *a* value of 0.018. The SAMOVA was run with two to ten group structures (*K* groups) in order to determine the optimal value for F_{CT}. The best grouping structure for the SAMOVA is selected when the F_{CT} value reaches a plateau and F_{CT} increases negligibly when *K* groups increases (Fig. 3.5).

The structure of the groups, as defined by the SAMOVA indicated a population structure that divided the sample set into three groups. This population structure of three groups was used for the AMOVA.

In order to further examine patterns of gene flow across the whole population of *A. gutturalis*, a Mantel Test was used to determine if there is a correlation between genetic differences and geographic distance. This can be examined through IBD, which, as defined by Wright (1943), is the accumulation of genetic differentiation with an increase in geographical distance which results from restricted dispersal when compared with the geographical range. To test this, the concatenated data set was used to investigate IBD with a Mantel Test (Mantel, 1967) using the Alleles in Space (AIS 1.0) software (Miller, 2005). A matrix of geographic distances between sample localities does not need to be created for the Mantel Test in AIS v1.0.

For this study, latitude and longitude co-ordinates in decimal degrees were used to create the input files and AIS 1.0 applies a standard 'Great Circle Distance' formula to calculate the distance between points (Miller, 2005). Standard settings were used and significance was tested with a 1000 replications. The correlation coefficient (r) indicates the degree of correlation between genetic and geographic distances, whilst the probability value of $P<0.001$ indicates that the correlation coefficients are significantly different from zero.

Demographics

Over time there are episodes where populations increase and decline and leave characteristic signatures in the distribution of pairwise differences between populations (Rogers & Harpending, 1992). Standard measures of genetic variation were used to investigate intra-clade diversity for *Amietophrynus gutturalis*. The software Arlequin 3.5 (Excoffier *et al*., 2005) was used to calculate nucleotide diversity (π), the probability that two randomly chosen

homologous nucleotides are different (Tajima, 1983; Nei, 1987) and haplotype diversity (*h*), the probability that two randomly chosen haplotypes are different (Nei, 1987) (Table 3.6).

Various statistical tests have been devised to investigate past demographic changes. Methods such as Tajima's *D* test for selective neutrality (Tajima, 1989a, 1989b), the mismatch distribution (Rogers & Harpending, 1992), the raggedness statistic *rg* (Harpending *et al*., 1993) and Fu's Fs test have been broadly used. However, Fu's Fs test has been shown to be the most powerful statistical test for detecting population growth (Ramos-Onsins & Rozas, 2002). Fu's Fs (Fu, 1997) test of selective neutrality was performed in Arlequin v3.5 (Excoffier *et al*., 2005) in order to determine the potential departure from neutrality for the population as a whole and for each defined clade. A population in a state of neutrality, where a null value (Fs = 0) accepts the null hypothesis of neutrality, indicates that different populations have remained similar in size and stable. A significantly negative Fs-value provides evidence for an excess number of alleles and indicates a recent increase in population size (Mahoney, 2004). A significantly positive Fs-value provides evidence for a deficiency of alleles which indicates that a population has either undergone a recent population bottleneck or overdominant selection. These measures of intra-clade diversity were calculated for both the 16S rRNA and ND2 mtDNA datasets and not on the concatenated dataset.

CHAPTER 3
RESULTS

Phylogenetics

The Metropolis Coupled Markov Chain Monte Carlo (Metropolis Coupled MCMC) method of Bayesian inference was used to infer a phylogenetic tree for both the 16S rRNA (Fig. 3.1) and ND2 mtDNA (Fig. 3.2) markers. Complete datasets, including all samples from both invasive populations were used. The Bayesian Metropolis Coupled MCMC returned the same tree as that of the Maximum Likelihood (ML) method for both markers. The phylogenetic analysis for the 16S rRNA and ND2 mtDNA markers returned two similar phylogenies.

A single tree with nodal support in the form of posterior probabilities and ML bootstrap values was drawn for each dataset (Figs. 3.1, 3.2). Nodal support was weaker for the tree derived from the 16S rRNA marker. The ND2 mtDNA phylogeny indicated that *A. gutturalis* can be divided into four well supported clades (Fig. 3.2). The 16S rRNA phylogeny returned a phylogeny of three well supported clades, with a fourth nested clade. For both trees, each clade conforms to a distinct geographical region.

Geographically, the four clades separate into a northern, eastern, central and southern region. The northern clade covers a broad geographical range. Samples from this clade originated from Mozambique, Botswana, Zambia and the Democratic Republic of the Congo. The eastern clade is widely distributed throughout the KwaZulu-Natal Province and into the Mpumulanga and Limpopo Province's. The central clade, is located in the Johannesburg region of the Gauteng Province and the southern clade is limited to the Eastern Cape Province. The clades identified by the 16S tree (Fig. 3.1) indicate the same geographical structure as those identified by the ND2 tree (Fig. 3.2). The primary difference between the two phylogenies is that the central clade is nested within the southern clade in the 16S tree. Furthermore, the ND2 tree indicates that there is greater genetic structuring within each of the defined clades.

The sequences from both of the invasive populations were included in the phylogenetic analysis in order to infer the source or sources of the invasive populations. Figures 3.1 and 3.2 clearly indicate that all invasive samples from both the Cape Town and Mauritius populations are most closely related to the samples collected from the eastern clade. This identifies the geographical region that includes the KwaZulu-Natal, Mpumulanga and Limpopo Province's as the source of both invasive populations.

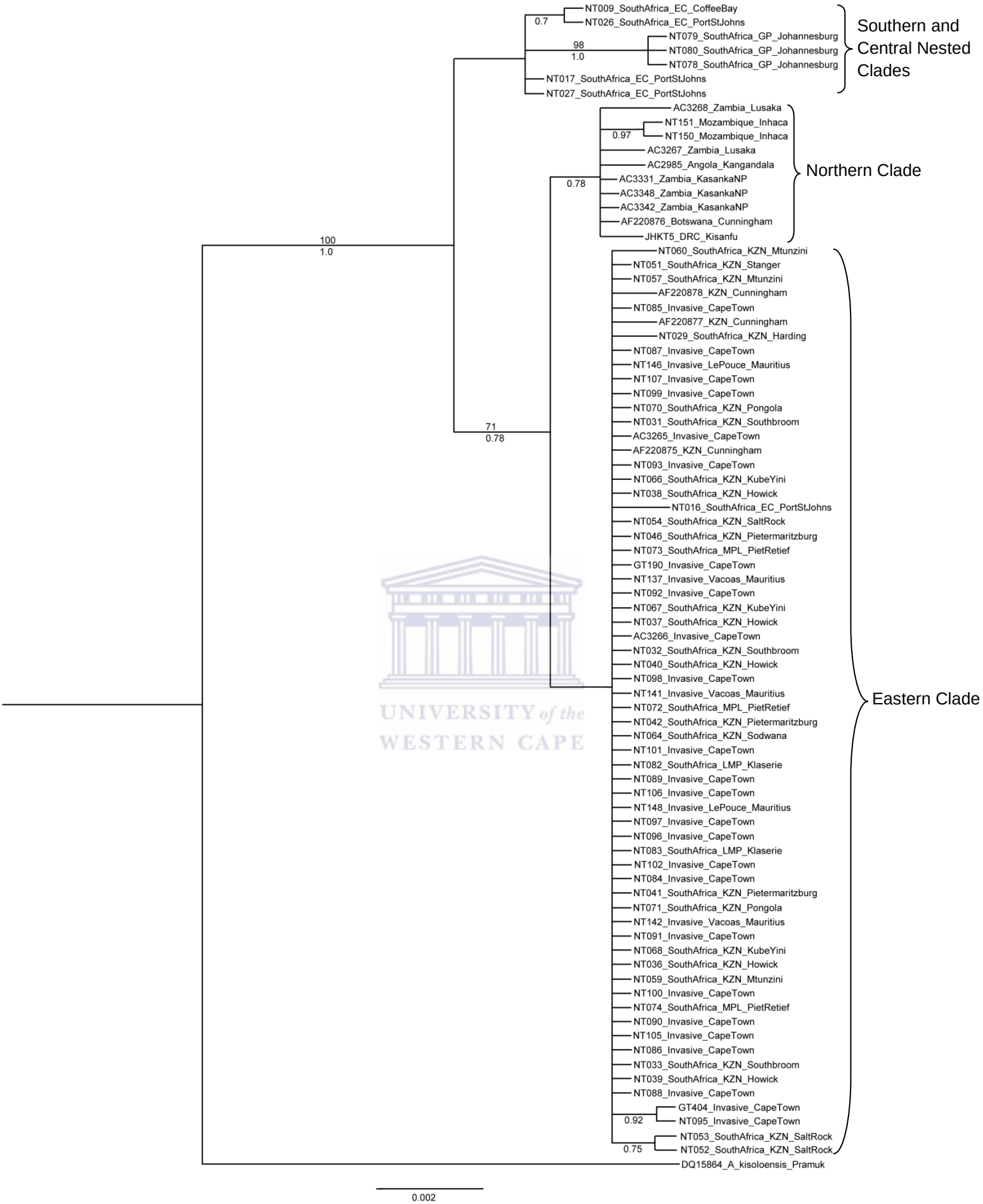

Figure 3.1: Metropolis Coupled MCMC Bayesian inference phylogeny of *Amietophrynus gutturalis* derived from the 16S rRNA marker. Maximum Likelihood bootstrap values greater than 70% are shown above and Bayesian posterior probabilities greater than 0.7 are shown below branches at terminal nodes.

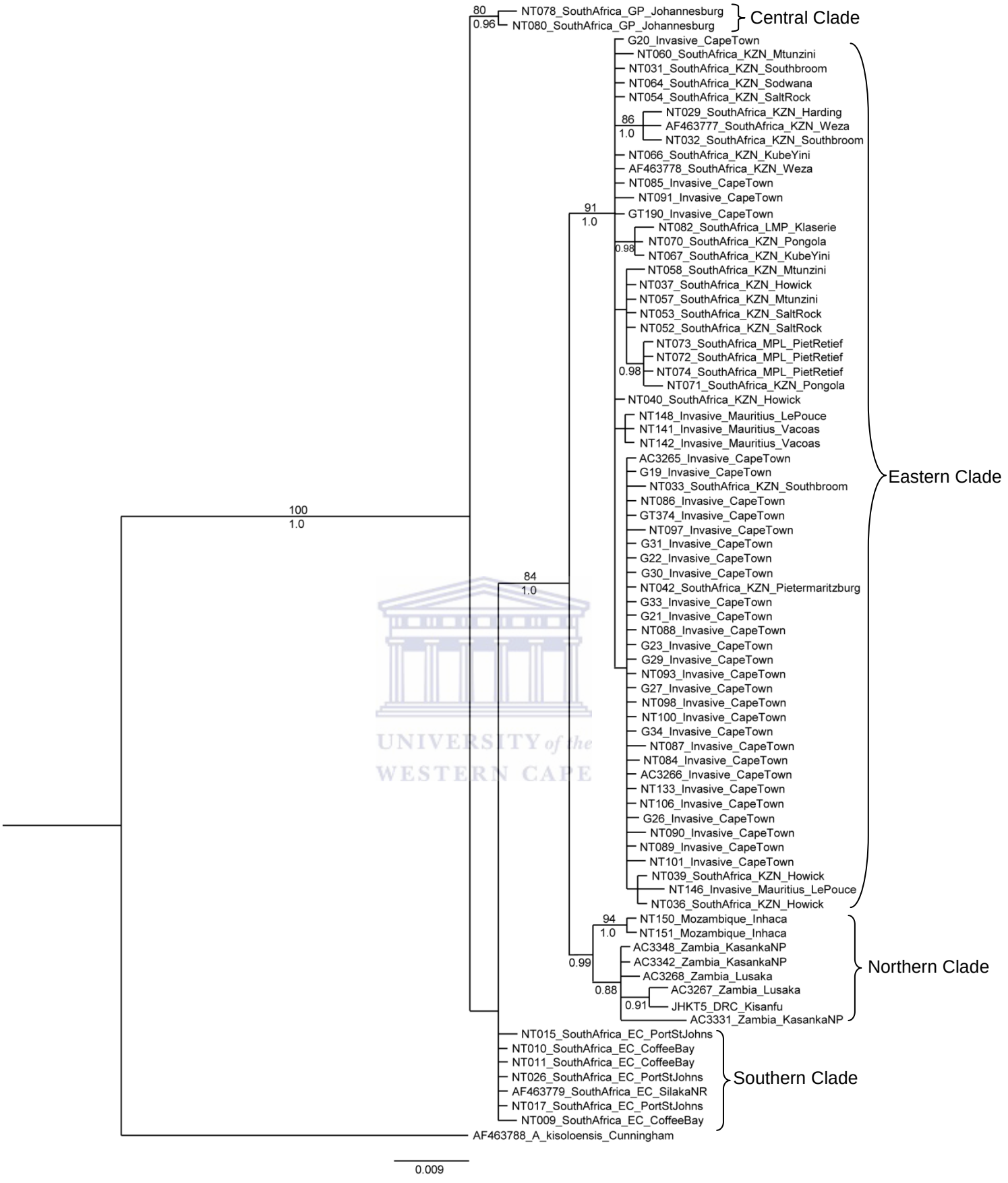

Figure 3.2: Metropolis Coupled MCMC Bayesian inference phylogeny of *Amietophrynus gutturalis* derived from the ND2 mtDNA marker. Maximum Likelihood bootstrap values greater than 70% are shown above and Bayesian posterior probabilities greater than 0.7 are shown below branches at terminal nodes.

Haplotype networks

The analysis of deeper phylogenetic relationships is allowed for by studying the non-coding 16S rRNA marker. This is because it has been shown that 16S region is conserved and evolves at a slower rate than other mitochondrial genes (Macey *et al*., 2001; Ashton & de Queiroz, 2001). Therefore, it would be expected that diversity and genetic structure within clades would be low for this marker. Standard genetic diversity within species for the 16S marker has been shown to be between 1–3% (Vences *et al*., 2005). The 16S rRNA network and uncorrectecd p-distances for *A. gutturalis* conform to this pattern.

In this study, a total of 17 haplotypes from 83 samples were identified for the 16S rRNA marker (Table 3.1). The TCS network (Fig. 3.3) revealed a common haplotype found broadly across the KwaZulu-Natal coast (Southbroom in the south, Howick and Pietermaritzburg in the centre, and Stanger, Mtunzini, Sodwana Bay, Kube Yini Nature Reserve and Pongola in the north) that extends north-west into the Mpumulanga (Piet Retief) and Limpopo (Klaserie) provinces of South Africa. This common haplotype (haplotype 4 in the network: Fig. 3.3; Table 3.1) is represented by a total of 57 samples (69% of all the retrieved haplotypes).

The diversity of the eastern clade is low. As can be seen in the network (Fig. 3.3), a star shaped pattern is evident in this clade. This is indicated by six of the seven haplotypes differing from the common haplotype by a single base change. This pattern has been shown to indicate a recent population expansion (Teixeira *et al*., 2011).

An inferred haplotype connecting haplotypes 4, 15 and 16 (Fig.3.3) indicates that the geographic boundary of this clade has not been fully determined. Haplotype 15, which falls within the eastern clade, is separated from the common haplotype by three base changes. This haplotype, represented by one sample from Port St Johns, does not conform to the geographic distribution of the clades because it was found at the same locality as samples from the southern clade. Two inferred haplotypes represented in the network (Fig. 3.3) indicates that further sampling should be conducted in the geographic regions that are void of samples between the four clades.

Figure 3.3 indicates that both the Cape Town and Mauritius invasive populations originate within this common haplotype. Of the 29 invasive samples (24 from Cape Town and five from Mauritius), 22 from Cape Town (92%) and all five from Mauritius (100%) fall within the common haplotype. The remaining two invasive samples from Cape Town (haplotype 3) share a different haplotype with a single base change from the common haplotype. The two

samples represented as haplotype three (Fig. 3.3) do not share a haplotype from the natural range. This conforms to the same patterns identified in the phylogenetic analysis, but does not identify a more specific geographic area for the origin of the invasive populations.

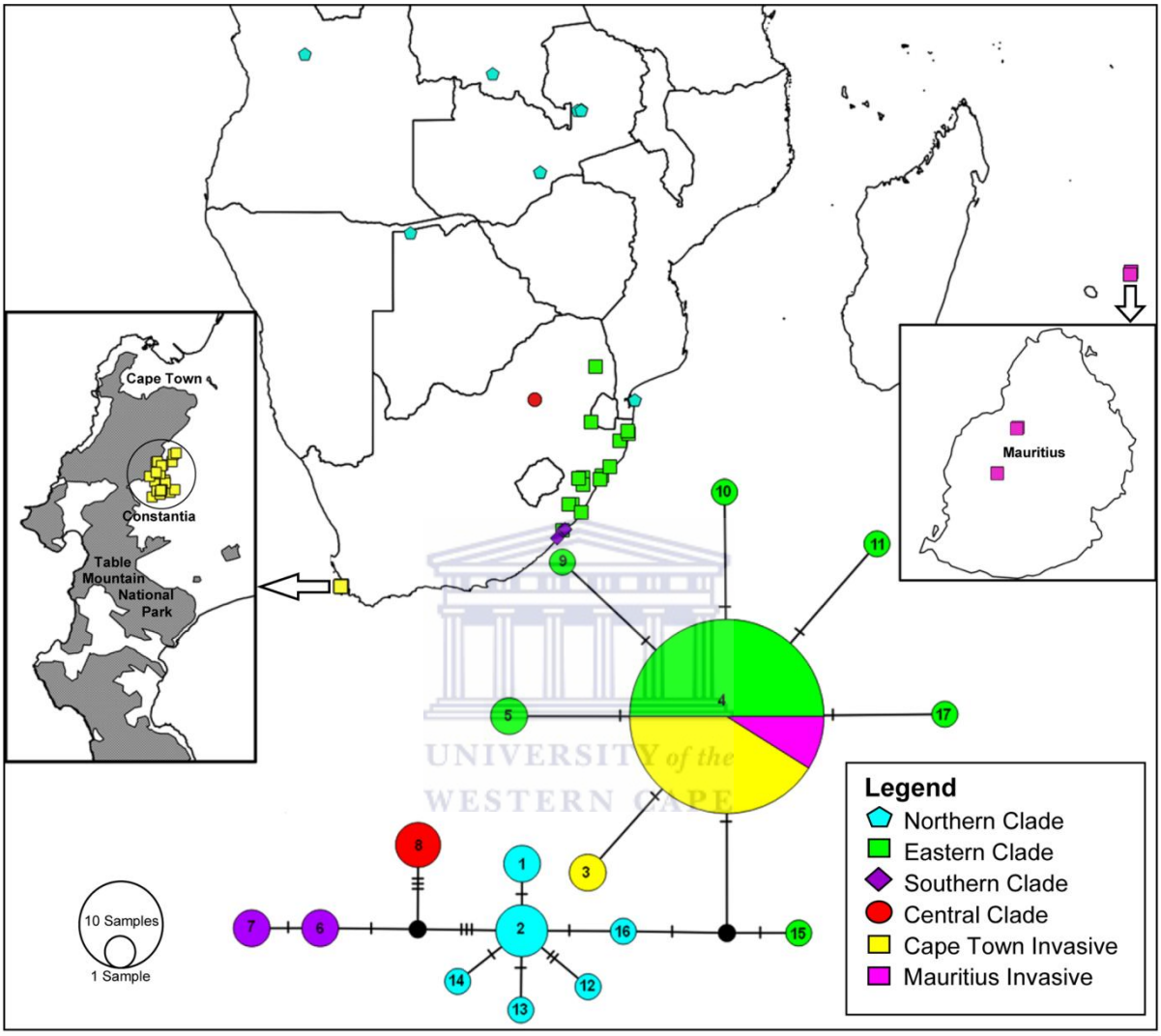

Figure 3.3: TCS haplotype network from the 16S rRNA marker for *Amietophrynus gutturalis*. Dashes on the network indicate single nucleotide polymorphisms and the nodes represented by black circles indicate inferred missing haplotypes. The circles are proportional to the amount of samples represented for each haplotype. All invasive samples from the Cape Town and Mauritius populations and the natural population are represented on the inset maps with the legend indicating which clade they represent. The colours represented in the legend correspond with those represented by the haplotype network. Numbers in the haplotypes are represented in Table 3.1.

Table 3.1: Haplotype list for the 16S rRNA genetic marker showing the localities, clade and number of samples from each locality for each haplotype. Haplotypes are numbered according to those represented in the haplotype network (Fig. 3.3).

Haplotype	Clade*	Invasive/ Natural	Location	Samples (*n*)
1	NC	Natural	Inhaca Island, Mozambique	2
2	NC	Natural	Wasa Lake, Kasanka National Park, Zambia	1
		Natural	Chikufwe, Kasanka National Park, Zambia	1
		Natural	Road next to Kasanka National Park	1
		Natural	Shakawe, Botswana**	1
3	EC	Invasive	Constantia, Cape Town, Western Cape, South Africa	2
4	EC	Natural	Weza Nature Reserve, KwaZulu-Natal, South Africa**	1
		Natural	Southbroom, KwaZulu-Natal, South Africa	3
		Natural	Howick, KwaZulu-Natal, South Africa	5
		Natural	Pietermaritzburg, KwaZulu-Natal, South Africa	3
		Natural	Ashburton, KwaZulu-Natal, South Africa**	1
		Natural	Stanger, KwaZulu-Natal, South Africa	1
		Natural	Salt Rock, KwaZulu-Natal, South Africa	1
		Natural	Mtunzini, KwaZulu-Natal, South Africa	2
		Natural	Sodwana Bay, KwaZulu-Natal, South Africa	1
		Natural	Kube Yini, KwaZulu-Natal, South Africa	3
		Natural	Pongola, KwaZulu-Natal, South Africa	2
		Natural	Piet Retief, Mpumalanga, South Africa	3
		Natural	Klaserie, Limpopo, South Africa	2
		Natural	Malalotja, Swaziland**	1
		Invasive	Mauritius**	1
		Invasive	Vacoas, Mauritius	2
		Invasive	Le Pouce, Mauritius	2
		Invasive	Constantia, Cape Town, Western Cape, South Africa	23
5	EC	Natural	Salt Rock, KwaZulu-Natal, South Africa	2
6	SC	Natural	Port St Johns, Eastern Cape, South Africa	2
7	SC	Natural	Coffee Bay, Eastern Cape, South Africa	1
		Natural	Port St Johns, Eastern Cape, South Africa	1
8	CC	Natural	Johannesburg, Gauteng Province, South Africa	3
9	EC	Natural	Mtunzini, KwaZulu-Natal, South Africa	1
10	EC	Natural	Albert Falls, KwaZulu-Natal, South Africa**	1
11	EC	Natural	Harding, KwaZulu-Natal, South Africa	1
12	NC	Natural	Lusaka, Zambia	1
13	NC	Natural	Lusaka, Zambia	1
14	NC	Natural	Kangandala, Angola	1
15	EC	Natural	Port St Johns, Eastern Cape, South Africa	1
16	NC	Natural	Kisanfu Mining Concession, Democratic Republic of the Congo	1
17	EC	Natural	Weza Nature Reserve, KwaZulu-Natal, South Africa**	1

*Key to clades NC=Northern Clade, CC=Central Clade, EC=Eastern Clade, SC=Southern Clade

**Genbank samples Haplotype 2 AF220876; Haplotype 4 AF220875; Haplotype 10 AF220877; Haplotype 17 AF220878

The ND2 mtDNA gene region, typically useful for investigating relationships at the population level (Macey *et al*., 2001; Ashton & de Quieroz, 2001), evolves at a faster rate than the 16S rRNA region. The structure of the ND2 network indicates greater genetic structure than that of the 16S rRNA network (Fig. 3.3, 3.4). Twenty nine haplotypes were identified from the 78 samples, of which two were located in the central clade, four in the southern clade, 17 from the eastern clade and six from the northern clade.

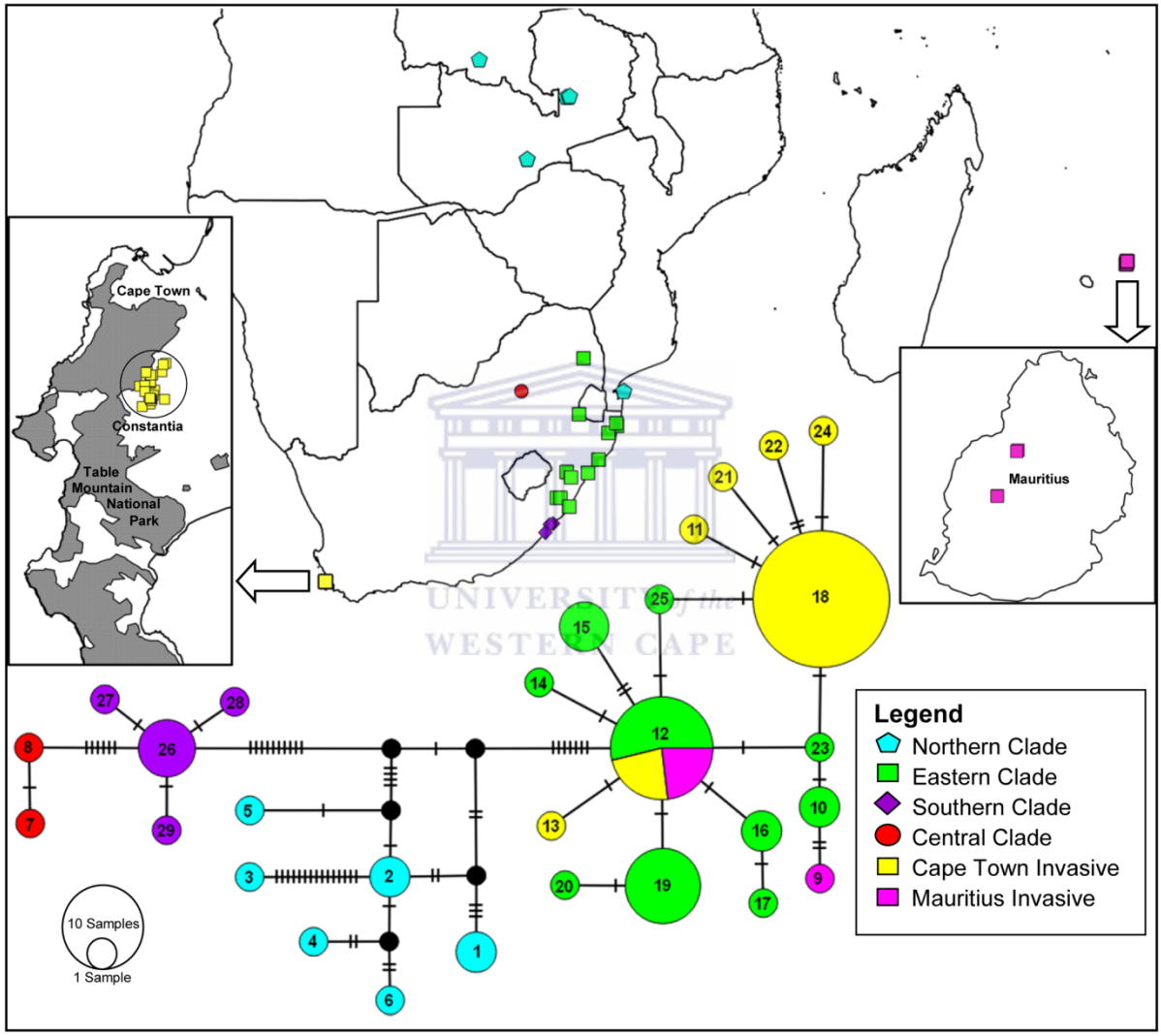

Figure 3.4: TCS haplotype network from the ND2 mtDNA marker for *Amietophrynus gutturalis.* All samples from the natural range and both the Cape Town and Mauritius invasive populations are included and indicated on the inset maps. Dashes in the network represent single nucleotide polymorphisms between haplotypes and black circles represent inferred missing haplotypes. The circles in the networks are proportional to the number of samples representing each haplotype. The colours represented in the legend correspond with those represented by the haplotype network. Numbers in the haplotypes are represented in Table 3.2.

The central clade, represented by two samples from the same locality contains two haplotypes and is separated from the southern clade by a maximum of seven base changes, the northern clade by 37 base changes and the eastern clade by 26 base changes. The northern clade is represented by eight samples from six localities and contains six haplotypes (Table 3.2; Fig. 3.4).

The northern clade shows greater genetic structure than the other three clades. Haplotypes 2 and 3 contain three samples from the Kasanka National Park region in Zambia. Both samples from inside the reserve (Chikufwe and Wasa Lake) have identical sequences for both the 16S and ND2 markers, whereas the sample collected from the road adjacent to the reserve shares the same 16S sequence, but differs by 15 base changes for ND2. The presence of five inferred nodes falling within the northern clade indicates the possibility of a further five unidentified haplotypes within the network.

The eastern clade, represented by 17 haplotypes, shows the least genetic structure even though it contains the most representative haplotypes. A maximum of six base changes separates the most variable haplotypes within this clade (haplotype 20 and haplotype 22: Fig. 3.4), with most being separated by either one or two changes. This indicates that there has been little genetic divergence within the region. As seen in the 16S rRNA network (Fig. 3.3), all the invasive samples from both the Cape Town and Mauritius populations in the ND2 network (Fig. 3.4) originate from within the eastern clade. Across the natural range of *A. gutturalis*, the most common haplotype (Haplotype 12) was found in Weza Nature Reserve and Southbroom in southern KZN, Howick in central KZN, Salt Rock, Mtunzini, Sodwana Bay, Kube Yini Nature Reserve and Pongola in northern KZN.

A slightly different pattern emerges within the ND2 network. Haplotype 12 (Fig. 3.4), which is the shared haplotype between samples from the natural population and the two invasive populations, contains 13 samples (Table 3.2). Of the 35 invasive samples (31 from Cape Town and four from Mauritius), six samples (three from Cape Town and three from Mauritius) fall within the most common haplotype (Haplotype 12; Fig. 3.4). The remaining 28 samples from the invasive populations (27 from Cape Town and one from Mauritius) are represented by seven haplotypes (Fig. 3.4). None of these are represented in the natural population and comprise only of individuals from the invasive populations. They differ from the common haplotype by between one and four base changes.

Table 3.2: Haplotype list for the ND2 mtDNA genetic marker showing the localities, clade and number of samples from each locality for each haplotype. Haplotypes are numbered according to those represented in the haplotype network (Fig. 3.4).

Haplotype	Clade*	Invasive/ Natural	Location	Samples (*n*)
1	NC	Natural	Inhaca Island, Mozambique	2
2	NC	Natural	Chikufwe, Kasanka National Park, Zambia	1
		Natural	Wasa Lake, Kasanka National Park, Zambia	1
3	NC	Natural	Road next to Kasanka National Park, Zambia	1
4	NC	Natural	Kisanfu Mining Concession, Democratic Republic of the Congo	1
5	NC	Natural	Lusaka, Zambia	1
6	NC	Natural	Lusaka, Zambia	1
7	CC	Natural	Johannesburg,Gauteng Province, South Africa	1
8	CC	Natural	Johannesburg, Gauteng Province, South Africa	1
9	EC	Invasive	Le Pouce, Mauritius	1
10	EC	Natural	Howick, South Africa	2
11	EC	Invasive	Constantia, Cape Town, Western Cape, South Africa	1
12	EC	Natural	Weza Nature Reserve, KwaZulu-Natal, South Africa**	1
		Natural	Southbroom, KwaZulu-Natal, South Africa	1
		Natural	Howick, KwaZulu-Natal, South Africa	1
		Natural	Salt Rock, KwaZulu-Natal, South Africa	1
		Natural	Sodwana Bay, KwaZulu-Natal, South Africa	1
		Natural	Kube Yini Nature Reserve, KwaZulu-Natal, South Africa	1
		Natural	Pongola, KwaZulu-Natal, South Africa	1
		Invasive	Constantia, Cape Town, Western Cape, South Africa	3
		Invasive	Vacoas, Mauritius	2
		Invasive	Le Pouce, Mauritius	1
13	EC	Invasive	Constantia, Cape Town, WC, South Africa	1
14	EC	Natural	Mtunzini, KwaZulu-Natal, South Africa	1
15	EC	Natural	Weza Nature Reserve, KwaZulu-Natal, South Africa**	1
		Natural	Harding, KwaZulu-Natal, South Africa	1
		Natural	Southbroom, KwaZulu-Natal, South Africa	1
16	EC	Natural	Kube Yini Nature Reserve, KwaZulu-Natal, South Africa	1
		Natural	Pongola, KwaZulu-Natal, South Africa	1
17	EC	Natural	Klaserie, Limpopo, South Africa	1
18	EC	Invasive	Constantia, Cape Town, Western Cape, South Africa	23
19	EC	Natural	Howick, KwaZulu-Natal, South Africa	1
		Natural	Salt Rock, KwaZulu-Natal, South Africa	2
		Natural	Mtunzini, KwaZulu-Natal, South Africa	1
		Natural	Piet Retief, Mpumalanga, South Africa	3
20	EC	Natural	Mtunzini, KwaZulu-Natal, South Africa	1
21	EC	Invasive	Constantia, Cape Town, Western Cape, South Africa	1
22	EC	Invasive	Constantia, Cape Town, Western Cape, South Africa	1
23	EC	Natural	Pietermaritzburg, KwaZulu-Natal, South Africa	1
24	EC	Invasive	Constantia, Cape Town, Western Cape, South Africa	1
25	EC	Natural	Southbroom, KwaZulu-Natal, South Africa	1
26	SC	Natural	Coffee Bay, Eastern Cape, South Africa	2
		Natural	Port St Johns, Eastern Cape, South Africa	2
27	SC	Natural	Silaka Nature Reserve, Eastern Cape, South Africa**	1
28	SC	Natural	Port St Johns, Eastern Cape, South Africa	1
29	SC	Natural	Coffee Bay, Eastern Cape, South Africa	1

*Key to clades NC=Northern Clade, CC=Central Clade, EC=Eastern Clade, SC=Southern Clade

**Genbank samples Haplotype 12 AF463778; Haplotype 15 AF463777; Haplotype 27 AF463779

The only shared haplotype between the invasive and natural populations is haplotype 12 (Fig. 3.4; Table 3.2). The remaining seven identified haplotypes are only separated by a maximum of six base changes from samples found across the species natural range. Both the phylogentic analysis and the haplotype networks indicate that these seven haplotypes are most closely related to samples derived from the eastern clade.

Genetic diversity of invasive populations

Haplotype (*h*) and nucleotide (π) diversity was calculated for the Cape Town and Mauritius invasive populations and the eastern clade (Table 3.3). The presence of overlap in values between the upper and lower confidence intervals was used to determine with 95% confidence if these measures of genetic diversity between the invasive populations and the source population are significantly different. For *h* diversity there was no overlap in values between both the invasive populations and the eastern clade for the ND2 marker (Table 3.3). This indicates that both invasive populations have lower *h* diversity than the source population. For the 16S marker no *h* or π diversity was recovered from samples collected in Mauritius (Table 3.3), as only one haplotype was recovered. There was overlap in observed *h* and π diversity values between the Cape Town invasive population and the eastern clade for the 16S marker (Table 3.3). This indicates that the *h* and π diversity of the Cape Town invasive population is not significantly lower than the source population.

Table 3.3: Standard measures of genetic diversity of the source population compared to the Cape Town and Mauritius invasive populations of *Amietophrynus gutturalis*. Sample size (*n*), nucleotide diversity (π) and haplotype diversity (*h*) shown with 95% confidence intervals (CI) in brackets.

	16S			ND2		
Population	*n*	π (95% CI)	*h* (95% CI)	*n*	π (95% CI)	*h* (95% CI)
Cape Town	25	0.0003 (±0.0005)	0.153 (±0.092)	31	0.0015 (±0.0011)	0.45 (±0.109)
Mauritius	5	0 (±0)	0 (±0)	4	0.0028 (±0.0024)	0.5 (±0.265)
Eastern Clade	33	0.0007 (±0.001)	0.33 (±0.11)	27	0.0049 (±0.003)	0.87 (±0.04)

Population genetics of *Amietophrynus gutturalis*

Only the sequences derived from samples collected from across the *A. gutturalis* natural range were included in the population genetics analyses. The nucleotide composition of the 16S (A:G:C:T = 30.73%; 20.68%; 23.41%; 25.18%) and ND2 (A:G:C:T = 30.62%; 11.32%; 27.64%; 30.42%) genes for *A. gutturalis* corresponds with values found in other amphibians (e.g. *Xenopus laevis* and *Rana nigromaculata*) (Roe *et al*., 1985; Sumida *et al*., 2001). A total of 16 haplotypes were identified from 53 samples for the 16S marker and 22 haplotypes were identified from 43 samples from the ND2 marker.

The phylogentic tree for the 16S marker (Fig. 3.1) indicated a population structure of three geographically separated clades, with the central clade nested in the southern clade. The same pattern was observed in the ND2 phylogenetic tree (Fig. 3.2), except that the central clade was not nested in the southern clade. The spatial analysis of molecular variance (SAMOVA) indicated that most plausible grouping structure was when the sample sites were separated into three groups (F_{CT} = 0.98).

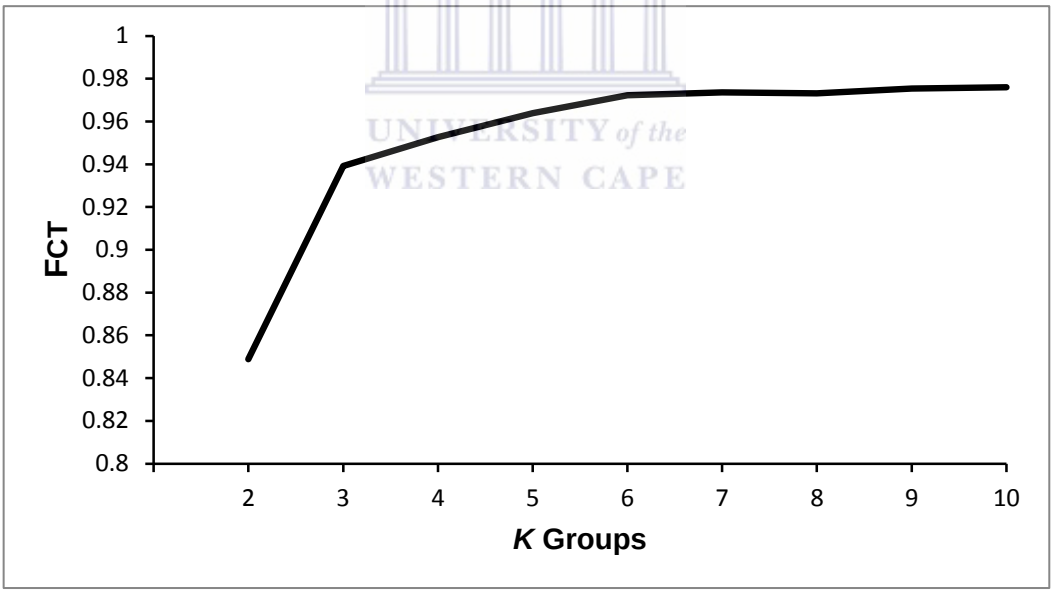

Figure 3.5: Graph indicating the distribution of F_{CT} values with an increase in the amount of groups (*K* groups). The maximum variance indicated is at the point when *K*=3 groups.

The maximum variance between populations is indicated by the F_{CT} values reaching a plateau (Fig. 3.5). When the *K* number of groups was larger than three, F_{CT} values increased at a negligible rate as a result of the continued decrease in F_{SC}. This would continue until all

sampling sites were separate and therefore the most plausible structure indicated by the SAMOVA is a northern group, an eastern group and southern group.

This grouping structure represents the same population structure as that identified by the 16S phylogeny, with the central and southern clades grouped together (Fig. 3.1).

Table 3.4: Results from the analysis of molecular variance (AMOVA) showing the percentage of variation among groups, among populations within groups and within populations as well as the associated F-statistics.

Source of Variation	d.f.	Sum of Squares	Variance	Percentage of variation
Among Groups	2	2694.59	151.09 Va	98.06
Among Populations Within Groups	18	77.93	1.92 Vb	1.25
Within Populations	15	15.96	1.06 Vc	0.69
Total	35	2788.48	154.08	100.00
Fixation Indices (Φ)				
F_{SC}	0.64			
F_{ST}	0.99			
F_{CT}	0.98			

When the three groups were specified in the AMOVA the variation among groups was shown to be the greatest source of genetic variation (98.06%), whilst both the variation among populations within groups (1.25%) and the variation within populations (0.69%) were low in comparison (Table 3.4). The fixation indices for the AMOVA (Table 3.4) indicate that there is a high amount of genetic variation among populations relative to the total variance as well as among groups relative to the total variance and that there is little variance among subpopulations within the groups.

The tests comparing the variance (10 100 permutations) were significant ($P<0.001$) for Φ_{ST} and Φ_{CT} but were not significant for Φ_{SC} ($P=0.0029$). The matrix of population pairwise Φ_{ST} values between the three groups (Table 3.5) supports the AMOVA by indicating that there is a large amount of genetic variation among groups where all groups were found to be significantly different from one another.

Table 3.5: Population pairwise Φ_{ST} values for the four geographic groups defined by SAMOVA. Significant Φ_{ST} values ($p < 0.05$) indicated by a * and highlighted in bold.

Group	Eastern	Northern
Northern	**0.89***	
Southern	**0.76***	**0.71***

The Mantel Test for IBD revealed that there is a no correlation between (P=0.0019; r^2 = 0.2083) genetic and geographic distances.

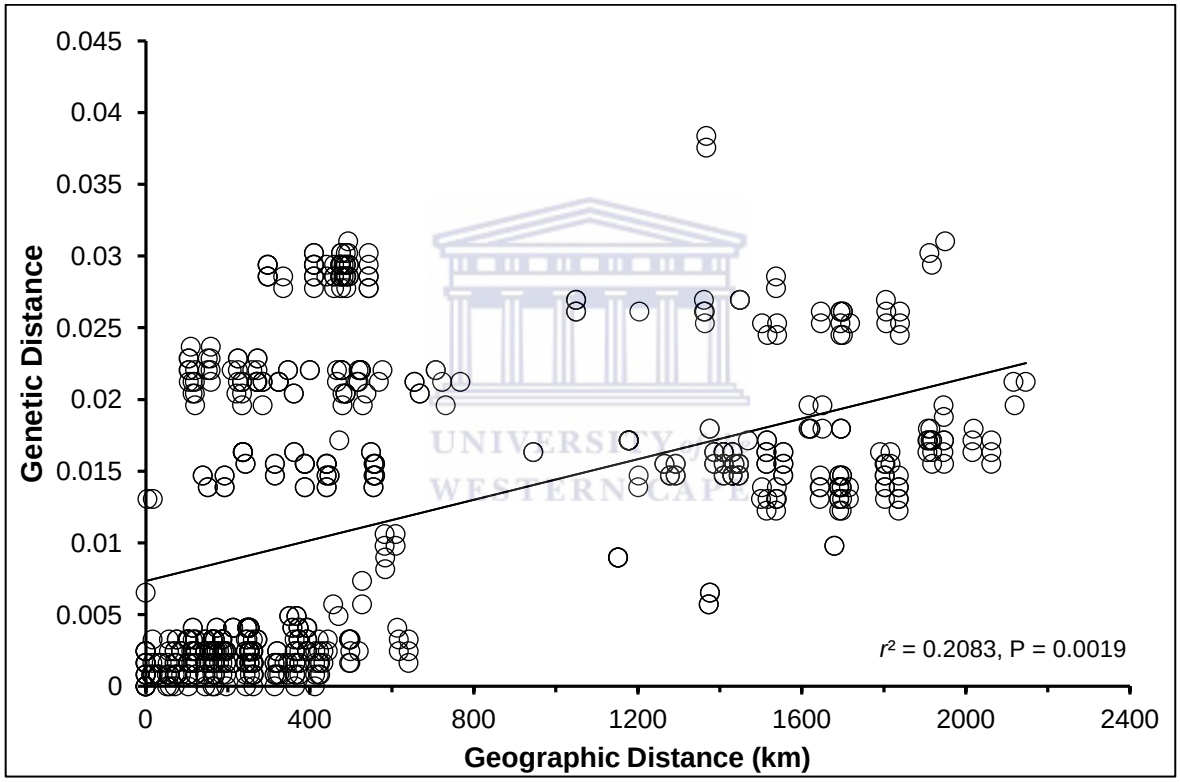

Figure 3.6: Mantel Test for IBD indicating the relationship between pairwise Φ_{ST} values and geographic distance for *Amietophrynus gutturalis*.

Demographics

Haplotype diversity (*h*), nucleotide diversity (π) and Fu's Fs test for selective neutrality were used to examine population demographics for *A. gutturalis*. High haplotype diversity accompanied by low nucleotide diversity and a significantly negative Fs-value indicate a historical population expansion (Russell *et al.*, 2005). Fu's Fs test for selective neutrality was used to investigate if *A. gutturalis* has undergone any significant demographic changes in the recent past. A significantly negative Fs-value (P<0.02), which indicates that there has been a

recent increase in population size, was found for both the eastern and the northern clades in the 16S marker. No significant results for any of the clades were obtained for the ND2 marker (Table 3.6).

Table 3.6: Standard genetic diversity indices and neutrality tests for the four geographical regions indicated by the phylogenetic analysis of *Amietophrynus gutturalis*. Sample size (*n*), nucleotide diversity (π) and haplotype diversity (*h*) shown with 95% confidence intervals (CI) in brackets. Fu's Fs (*Fs*) test for selective neutrality shown with probability values (significant when P<0.02; significant values highlighted in bold) in brackets.

	16S				ND2			
Clade	*n*	π (95% CI)	*h* (95% CI)	F_S	*n*	π (95% CI)	*h* (95% CI)	F_S
Northern	10	0.0027 (±0.002)	0.84 (±0.1)	**-2.92 (0.005)**	8	0.012 (±0.007)	0.93 (±0.084)	0.55 (0.55)
Eastern	33	0.00071 (±0.001)	0.33 (±0.11)	**-5.03 (0.0)**	27	0.0049 (±0.003)	0.87 (±0.04)	-2.4 (0.11)
Southern	8	0.0052 (±0.004)	0.8 (±0.16)	1.55 (0.79)	6	0.00096 (±0.001)	0.6 (±0.22)	-0.07 (0.46)
All Samples	51	0.006 (±0.004)	0.72 (±0.069)	-2.14 (0.26)	43	0.019 (±0.01)	0.94 (±0.02)	-0.64 (0.38)

The significantly negative Fs-value for the Eastern clade is supported by very low π. The *h* value for this clade is however low. Both a low π and high *h* support the significant negative Fs-value for the northern clade (Table 3.6).

CHAPTER 4
DISCUSSION

Population genetics

Demographics and biogeography

Significant genetic structuring was found across the broad natural distribution of *Amietophrynus gutturalis*. For both the 16S and ND2 mtDNA markers, four geographically distinct clades were identified by the ND2 phylogeny (Figs. 3.2) and the haplotype networks (Figs. 3.3, 3.4). However, the SAMOVA (Fig. 3.5) indicated a grouping structure of three geographically distinct clades in the north, the east and a combined central and southern clade. The population structure observed in the SAMOVA is consistent with that identified by the 16S phylogeny (Fig. 3.1), which indicates that the central clade is nested within the southern clade. This structure was supported by the AMOVA, which indicated that the greatest genetic variation was between groups (Table 3.4), with all groups being significantly different from one another (Table 3.5).

The contrasting results identified by the phylogenies, networks and the SAMOVA do not provide a clear indication of population structure across the range of *A. gutturalis*. The results indicate that there are either three or four geographically distinct clades. The uncertainty lies between the separation of the southern and central populations. It is likely that greater sampling resolution in this region will provide a much clearer picture of the population structure. The results will therefore be discussed for a population structure of three distinct clades which are separated into northern, eastern and southern populations. The southern clade is comprised of samples from the Eastern Cape and Gauteng provinces. The eastern clade from samples collected in KwaZulu-Natal, Mpumulanga and Limpopo provinces and the northern clade is represented by all samples collected from countries north of South Africa.

The northern clade spans across a very large geographic range through Zambia, Angola, Botswana and Mozambique and likely includes southern DRC, Zimbabwe, Malawi and Tanzania. The eastern clade is restricted to South Africa and is distributed along the east of the country from the Limpopo and Mpumulanga provinces in the north, and throughout the KwaZulu-Natal province until the border of the Eastern Cape Province. It is unclear how far this population goes inland before it diverges. The southern clade is restricted to the Eastern Cape and Gauteng provinces of South Africa. Further sampling in the region between these

two provinces may indicate further population structure and separate the southern clade into central and southern clades. This is supported by the population structure identified by the ND2 phylogeny (Fig. 3.2) and haplotype network (Fig. 3.4).

Available samples across the northern clade were sparse and the resolution of this clade is therefore unclear. The five inferred haplotypes identified by the ND2 haplotype network (Fig. 3.4) all fall within the northern clade. It is thus likely that the northern clade ranges throughout the large geographic regions between sample localities (e.g. Zimbabwe, Malawi, and Tanzania). The northern clade spans the entire region which Poynton and Broadley (1985) identified as Amphibia Zambesiaca. *Amietophrynus gutturalis* is the only bufonid distributed across the entire Zambesiaca region and there is no correlation between the Zambesiaca vegetation types and the distribution of the northern clade.

The Zambesiaca region is bordered by the Kalahari Desert in the west which forms the westward dispersal barrier and the limits of the *A. gutturalis* western range through Botswana and into most of Namibia (Channing, 2001). As is often the case with regards to species that have large geographic ranges, there are large distances between samples collected from the northern clade. This is problematic because it does not adequately define how far south the clade extends. Therefore the geographic boundaries between the northern clade and the central and eastern clades remains to be further examined. There may also be further genetic structure which has not been identified due to the broad range of the species and the large gaps between sample sites. Further sampling could possibly identify additional clades within this region.

It is widely regarded that geographic barriers such as mountain ranges (Smissen *et al*., 2013), rivers (Gascon *et al*., 2000; Li *et al*., 2008), changes in altitude (Li *et al*., 2008) and changes in vegetation types (McRae *et al*., 2005) have influenced the evolution of species. The historical formation of these barriers has influenced speciation and has also affected within species variation. The analysis of genetic variation in DNA markers has regularly been used to investigate current or historical patterns of gene flow in species (Bossart & Prowell, 1998; Avise, 2000). This is possible because historical geographic processes have influenced population division, long distance colonization and range expansion. Therefore distinct patterns in the distribution of alleles in species and the relationships between them can be expected (Templeton *et al*., 1995). It is therefore plausible that those processes can be inferred from examining patterns of genetic variation.

Maternally inherited mitochondrial DNA does not undergo recombination which allows for the reconstruction of matrilineal genealogies. These are useful because they are hierarchical and exhibit a clear relationship among individuals (Irwin, 2002). Furthermore they often consist of geographically separated clades that often come into contact in narrow regions. These phylogeographic breaks are in most cases thought to be a result of long-term barriers to gene flow. However the presence of distinct geographic barriers is not always present at phylogeographic breaks (Irwin, 2002).

The phylogeographic breaks identified for *A. gutturalis* consist of regions where there is no distinct geographic barrier and regions where there are distinct possible geographic barriers. Two possible geographic barriers are apparent for the phylogeographic breaks between the eastern and southern clades and between the eastern and northern clades. There appears to be no distinct geographic barrier between the eastern and southern clades, between the northern and southern clades and the narrow coastal strip between the northern and eastern clades.

The eastern clade appears to follow the coastal strip between the Drakensberg mountain range and the coast. It extends throughout the KwaZulu-Natal province and into the Mpumulanga and Limpopo provinces. The Drakensberg mountain range forms the eastern range of the escarpment and is a potential barrier to gene flow between the eastern clade and the Gauteng Province samples of the southern clade. The pattern observed is likely to be a reflection of sampling effort and further samples from the inland regions of the *A. gutturalis* range in South Africa would provide a clearer pattern.

The Lebombo mountain range further north is a plausible barrier between the northern and the eastern clade. The coastal strip is the only region where no barrier is present between the northern clade and the eastern and southern clades. The southernmost sample from the northern clade is from Inhaca Island in southern Mozambique. This locality is relatively close to the sample collected from Sodwana Bay in northern KwaZulu-Natal. The Sodwana Bay sample groups with the eastern clade.

There is no apparent north-south geographic barrier between the Inhaca Island and Sodwana Bay samples. However, as one moves from Sodwana Bay northwards towards Maputo in Mozambique the prevailing climate changes from a sub-tropical to a tropical climate. A similar scenario is evident between the southern and the eastern clades where there is a transition from a warm temperate climate to a sub-tropical climate, with no major geographic barriers between the two clades.

It has been shown that historical changes in climate have affected the speciation and radiation of species (Tolley *et al*., 2008; Portik & Papenfuss, 2015). Research by Tolley *et al*. (2008) investigated the influence of historical climate changes on the diversity, distribution and radiation of dwarf chameleons (*Bradypodium spp.*) through the Maputuland-Pondoland-Albany hotspot in South Africa. They found that across the complete phylogeny of South Africa's dwarf chameleons, the timing and the mode of diversification exhibits spatio-temporal patterns that are linked to the evolution of the regions climate through the past 14 million years (Tolley *et al*., 2008).

It appears that the phylogeographic patterns observed in the geographic distribution of clades and the radiation of *A. gutturalis* may be linked to historical changes in the region's climate. Each clade can be predominantly found in specific climatic regions. The northern clade is distributed through tropical southern Africa into central and eastern Africa. The eastern clade is distributed through the sub-tropical climatic region of South Africa and the southern clade appears when the climate changes from a sub-tropical to a temperate climate.

Demographic analysis for the three defined clades indicated that both the eastern and northern clades have recently undergone a population expansion (Table 3.6). The southern clade was shown to not have undergone a recent population expansion. Further samples from new localities between the already defined clades should be collected. To achieve this it would be useful to attain a greater resolution in samples from the inland region between the KwaZulu-Natal coast and the samples from Gauteng Province as well as various regions of the species northern distribution. This will greatly assist in further defining the clades geographical boundaries, as well as gauging a better picture on their historical biogeography.

The eastern clade was shown to have undergone the most recent population expansion as indicated by the significant Fs-value for the 16S marker. This is supported by the clades low genetic diversity, wide distribution of a common haplotype, large negative Fs-value and the star shaped pattern of the haplotype networks (Fig. 3.3; 3.4). The northern clade has also undergone a recent population expansion. However, there is no star-shaped pattern in the haplotype network and there is higher nucleotide (π) and haplotype diversity (h) (Table 3.6). Furthermore, the negative Fs-value is greater than the Fs-value of the eastern clade and there is no broad distribution of a common haplotype (Table 3.6). This indicates that the eastern clade diverged and expanded more recently than the northern clade.

Biogeographical patterns in the region are coupled with lineage turnover (Lawes *et al*., 2007; Tolley *et al*., 2008), because radiations occurred in species that were able to adapt to the increase in C_4 habitats whereas extinctions are identified in lineages confined to the shrinking of C_3 habitats (Tolley *et al*., 2008). The patterns observed in the radiation of *A. gutturalis* appear to track historical climatic changes during the last glacial maximum. This is most evident along the eastern coastal strip of the *A. gutturalis* range and is apparent at the north-south phylogeographic breaks between the eastern clade and the northern clade and between the eastern clade and the southern clade. The Maputaland-Pondoland-Albany biodiversity hotspot extends through both of these phylogeographic breaks. The generalist nature of *A. gutturalis* likely favoured the species expansion through tropical and temperate southern and central Africa. It would be particularly useful to apply dating and divergence time analyses to test if the *A. gutturalis* radiation was influenced by historical changes in climate during the last glacial maximum.

Amietophrynus gutturalis occurs naturally across a large geographic range through much of southern and central Africa. With such a large natural distribution it was not possible to collect samples in many regions. These sampling gaps were evident when implementing various analyses. The samples attained were sufficient for gaining a greater understanding of the population genetics of *A. gutturalis*, identifying the source population of the invasive populations and for identifying geographic regions where further samples should be collected. These gaps are most evident when examining the haplotype networks (Fig. 3.3, 3.4). The networks identified various inferred nodes which indicate the presence of unidentified haplotypes. Two inferred nodes were identified by the 16S network (Fig. 3.3) and five by the ND2 network (Fig. 3.4). In the 16S network, an inferred haplotype connecting haplotypes 4, 15 and 16 (Fig. 3.3) indicates that the geographic boundaries between the eastern and southern clades and the southern and northern clades have not been identified. The five inferred haplotypes identified by the ND2 network (Fig. 3.4) all fall within the northern clade. However, similar to the 16S network, these inferred nodes indicate that further sampling is necessary in the same regions identified between the clades.

Invasive *Amietophrynus gutturalis*

The guttural toad has already proven to be a successful invasive in the Mascarene Islands of Mauritius and Reunion (Chuttoo, 2006; Cheke & Hume, 2008; Florens, pers. comm., 2014) however no attempt at their control has been initiated. Nor has any work been conducted on identifying the impacts of this species on the local biota or their invasion history.

Furthermore, there has been no genetic study on the species and no attempt has been made to identify the origin or origins of the three known invasive populations. In order to implement adequate control measures for invasive species it is important to understand their evolutionary history (Leblois *et al*., 2000; Kraus, 2009). Understanding an invasive species' evolutionary history and using information on the species life history traits allows one to construct predictive models to examine possible future spread and therefore apply adequate control measures (Kulhanek *et al*., 2011). Investigating the biological impact of *A. gutturalis* on native biota is beyond the scope of this study. However, the results of this study provide a basis for further research and an impetus for the continued control of the species within its invasive range in Cape Town.

Reconstructing the invasion history of *A. gutturalis* is the first step towards understanding the invasion dynamics of the species. This information provides a precursor to studies on the mechanisms and limits of the invasion and to the species invasion dynamics and their causes (Andow *et al*., 1990). Furthermore, the information attained regarding their invasion history assists with identifying appropriate responses and implementing adequate control measures (Le Maitre *et al*., 2004; McGeoch *et al*., 2012).

Where did the invasive populations originate from?

Various studies have used mtDNA to investigate the origins of invasive amphibians (e.g. Lampo & de Leo, 1998; Kuraishi *et al*., 2009). These studies were not able to provide a precise location of the origin of the invasive species. They were however able to narrow the origin down to a broad geographic region. Similarly, the results of this study do not provide a clear or a precise location for the origin of the invasive populations but do narrow the origin down to a broad geographic region.

Both the Cape Town and Mauritius invasive populations originate from the eastern clade which has a broad distribution from southern KwaZulu-Natal northwards into the Limpopo and Mpumulanga provinces (Figs. 3.3, 3.4). This is apparent when examining both the phylogenies (Figs. 3.1, 3.2) and the haplotype networks (Figs. 3.3, 3.4). For the 16S marker, all but two of the invasive samples (haplotype 3, Table 3.1) match the common haplotype from the natural population. Due to this common haplotype occurring across a large region (some 700 km from the southernmost point to the northernmost point), it is not possible to determine a precise region for the origin of these animals.

Examination of the ND2 marker indicates a slightly different scenario. The bulk of samples from the invasive population do not match the haplotypes identified from the natural population (Table 3.2). However they only differ from the common haplotype by between two and four base changes. These results indicate that both the Mauritius and Cape Town invasive populations originated from the eastern clade. Due to both invasive populations sharing haplotypes with the common haplotype from the eastern clade it is not possible to make a more specific inference on the origin of the invasive populations.

It is therefore not possible to pin point a more precise origin of the invasive population. However, as it is plausible that the invasive population originated from the KwaZulu-Natal province through the horticultural trade it would be pertinent to implement stricter cross border controls between the Western Cape and KwaZulu-Natal.

Genetic diversity

Many human mediated introductions of non-native species across the globe have resulted in the establishment of new founder populations (Tsutsui *et al*., 2000). Theoretically, these founder populations should only establish with a fraction of the amount of genetic variation than that of the source population (Nei *et al*., 1975; Barrett & Husband, 1990). The loss of genetic diversity in founder populations is determined by the effective minimum population (*Ne*) and the rate of population growth, where a lower *Ne* will lead to the loss of alleles (especially those that are rare) (Nei *et al*., 1975). Various experimental and observational studies support this theory (see McCommas & Bryant, 1990; Leberg, 1992; England *et al*., 2003; Eldridge *et al*., 2004). Low genetic diversity is therefore expected in introduced populations that originate from a small founder population, whilst populations that have established from multiple introductions from different geographic regions would augment Mendelian trait diversity by raising population growth rate and the effective founder population size (Ellstrand & Schierenbeck, 2000).

Nucleotide (π) and haplotype (h) diversity in *A. gutturalis* for both genetic markers used in this study (Table 3.3) indicate that both the Mauritius and Cape Town invasive populations stem from small founder populations. When compared to the diversity of the origin population (Table 3.3), nucleotide diversity is marginally lower for both genetic markers in the Cape Town invasive population, whilst the same is true for the ND2 marker in the Mauritius population.

No genetic diversity was found in the Mauritius invasive population for the 16S marker because only one haplotype was recovered. As there is only a negligible difference in nucleotide diversity between the invasive population and the eastern clade it would be unlikely for there to be any deleterious genetic effects such as founder effects and genetic bottlenecking in the invasive population. This implies that low genetic diversity in the invasive population is likely to have a limited impact on their ability to continue to expand and for the species to persist. Suitable habitat, or the lack thereof, will likely have a greater impact on the expansion of the Cape Town population than the lack of genetic diversity.

Many introduced species are negatively impacted as a result of reduced genetic variation through genetic drift and founder effects (Frankham & Ralls, 1998; Allendorf & Lundquist, 2003). However many species that experience similar conditions when introduced manage to persist, evolve rapidly, exhibit rapid range expansion and become invasive (Novak & Mack, 1993; Reznick & Ghalambor, 2001; Sakai *et al*., 2001; Lee, 2002; Phillips & Shine, 2004; Phillips *et al*., 2006). These findings suggest that species that become invasive are able to circumvent the loss of genetic variation associated with bottlenecks (Kolbe *et al*., 2004).

On the other side of the spectrum, it has been shown that the effects of bottlenecking and the resultant loss of genetic diversity in the invasive Argentine ant, *Linepithema humile*, lead to its widespread ecological success (Tsutsui *et al*., 2000). This was as a result of the loss of certain alleles that caused the ants to be less aggressive between colonies which allowed for the formation of super-colonies (Tsutsui *et al*., 2000).

Although there is low genetic diversity in the introduced populations of *A. gutturalis*, there should be minimal deleterious genetic effects. Reduced gene flow and genetic bottlenecking are likely to not provide any natural assistance with the management of this invasive species.

Furthermore, the results indicate that both the Cape Town and Mauritius invasive populations originate from a single introduction event. All recovered haplotypes originate from within the eastern clade (Figs. 3.1–3.4), and none of the haplotypes recovered from the invasive samples fall within any of the other geographically separated clades.

Research conducted on the introduced European populations of the American bullfrog, *Lithobates catesbeianus*, by Ficetola *et al*. (2008) used simulations to determine the size of founder populations. They found that most non-native populations from their study area originated from less than six females. Although a more robust estimate of the Cape Town and

Mauritius invasive *A. gutturalis* populations would be useful, it is possible to indicate that the Cape Town population originated from a minimum of seven females (seven ND2 haplotypes recovered) and the Mauritius population from a minimum of two females (two ND2 haplotypes recovered). The capability of an introduced species to persist from such a small founder population is concerning and challenges usual management strategies. It is therefore important that species that are able to persist from such small founder populations be identified at an early stage of introduction and relevant management strategies implemented at the soonest possible time.

Biotic implications

It is widely accepted that there are numerous ecological and economic impacts as a direct result of the establishment of invasive species in novel regions. Once a population has established and becomes invasive it is extremely difficult to eradicate and requires intensive management to control. The extent of conservation management implemented is linked to the known and projected impacts of each invasive species. However, conservation management responses are often too late because they are initiated as a response to an observed rather than a projected impact. As a result management priorities are often skewed in favour of controlling an already problematic invasive species rather than eradicating an early detected species whose impacts are unknown. This type of management is problematic and a more pragmatic approach has surfaced over recent times where an early detection and rapid response framework has been suggested (Chornesky & Randall, 2003; Westbrooks, 2004; Britton *et al*., 2010; Kaiser & Burnett, 2010).

There was never an interest in controlling or attempting to eradicate *A. gutturalis* in Mauritius and it is unlikely to be prioritised in the near future. The introduced population in Cape Town was detected relatively early but was only responded to a few years later when an eradication program was initiated (de Villiers, 2006). The main concern for conservationists and the impetus for initiating eradication efforts were the effects that *A. gutturalis* could have on the Endangered western leopard toad, *Amietophrynus pantherinus* (Measey & Davies, 2011; Richardson, 2014).

Listed as Endangered on the IUCN Red List, *A. pantherinus* has seen major reductions in the quantity and quality of suitable habitat throughout its localised distribution (de Villiers, 2004a, 2006; Measey & Tolley, 2009). They are explosive breeders associated with specific breeding sites and these toads face numerous challenges during their short annual breeding

season. Many individuals are killed crossing roads and are subjected to a wide variety of barriers when migrating or dispersing (Measey & Tolley, 2009). The challenges facing this endangered species are exacerbated by the introduction of *A. gutturalis* into a region that comprises some of their primary breeding habitat. The effects of the *A. gutturalis* introduction are potentially extensive with increased competition for resources, predation and reproductive interference further hampering the western leopard toads' ability to persist. Potential indirect effects such as trophic cascades and changing ecosystem processes may also influence *A. pantherinus* in the species affected areas (Crossland, 2000).

The ecological effects that *A. gutturalis* may have on this sympatric species are yet to be investigated. Because *A. pantherinus* is the most directly affected species, it should be a priority to determine if there are any critical ecological impacts. Research into these impacts would allow for conservation managers to implement control or eradication measures more effectively.

Amietophrynus pantherinus will not be affected genetically by the introduced *A. gutturalis* population. The two species cannot interbreed as they are distantly related and most importantly because they have different numbers of chromosomes (Cunningham & Cherry, 2004). Although *A. gutturalis* has low genetic diversity across its invasive range it is unlikely to be impacted by founder effects or genetic bottlenecking.

The biotic impacts that *A. gutturalis* could have if it were to expand further from its limited range in Cape Town are a further cause for concern. Two Critically Endangered amphibian species, the table mountain ghost frog, *Heleophryne rosei*, (SA-FroG, 2010b) and the micro frog, *Microbatrachella capensis*, (SA-FRoG, 2011) are found in their limited range some 4 km away from the centre of the *A. gutturalis* invasive range. It is unlikely that *A. gutturalis* will expand into the *H. rosei* range as the fast flowing mountain stream habitat that they can be found in is not the preferred *A. gutturalis* habitat (Channing, 2001; de Villiers, 2004b). In spite of this it is important to monitor the northern expansion of the species.

The case of *M. capensis* is a greater cause for concern as the habitat of the species is more suitable for the establishment of an *A. gutturalis* population. But even more concerning is that *M. capensis* is restricted to two small disjunct regions and four sub-populations (de Villiers, 2004c). There are numerous urban barriers that could hamper the *A. gutturalis* expansion which should stem the species range extension. Nevertheless the eastern boundary of the guttural toad range should be monitored for expansion.

The Mauritius *A. gutturalis* population has already expanded across the entire island and it has been suggested that they are having an effect on native invertebrate fauna. This is indicated by the discovery of a previously thought extinct land snail, *Omphalotropis plicosa* Pfeiffer, 1852, in the stomach contents of an adult *A. gutturalis* on Mauritius (Chuttoo, 2006; Florens & Baider, 2007). Further investigations into the impacts of this invasion on the islands biota are required and a robust study on the impacts that *A. gutturalis* is having on the islands invertebrate community is recommended. .

Implications for conservation management

The Western Cape province of South Africa is an extremely bio-diverse region, is home to numerous endemic range-restricted flora and fauna and is one of the hotspots of conservation concern (Myers *et al*., 2000; Goldblatt & Manning, 2002). The region is also plagued by a wide variety of invasive species which threaten the regions ecosystems and species (Measey & Davies, 2011; South Africa, 2014). The control and removal of invasive species is mandated in the National Environmental Management: Biodiversity Act of 2004 (NEMBA, 2004). In spite of strict regulations surrounding invasive species there is limited legislation concerned with the movement of indigenous species within the country. In this respect there have been three cases of indigenous amphibian relocations in South Africa and the resultant establishment of domestic exotic populations (Measey & Davies, 2011).

Of these three introductions, the *A. gutturalis* introduction in Cape Town has had an eradication program in place for the past five years. This is mandated by the National Environmental Management: Biodiversity Act of 2004 (NEMBA, 2004) because the guttural toad is listed as a category 1b invasive species in the Western Cape (NEMBA, 2014). This legislation requires that the *A. gutturalis* population in Cape Town is required by law to be contained. However, the eradication program faces numerous hurdles and therefore provides conservation management with logistical issues that hamper eradication efforts. It is difficult to evaluate the success of the eradication efforts or to provide an indication of the success of the project.

The genetic analysis of this study shows that the *A. gutturalis* invasive population will likely experience no negative effects as a function of reduced genetic variation and lack of gene flow. More importantly the source of the invasive population stems from a single region and likely from a single introduction. Furthermore, concerns regarding hybridization between *A. gutturalis* and *A. pantherinus* have been addressed and are not concerning.

Although this study does not address the ecological impacts that *A. gutturalis* is having on native biota, it is important to address this issue. The effects that this introduction is having on community system dynamics should be investigated. Research investigating these ecological impacts will provide conservation management with important information that will assist with defining appropriate management strategies.

Recommendations for further research

It has been shown that other successful invasive amphibians have been able to rapidly adapt when introduced to a novel region. For instance, Phillips *et al.* (2006) investigated morphological changes in the cane toad, *Rhinella marina*, along the invasion front in Australia. They found that toads on the invasion front developed longer legs than the conspecifics that arrived later and that the toads with longer legs were able to move faster and thus disperse further and at a faster rate. The study highlights the importance for conservation biologists and managers to consider the possibility of rapid adaptive change in invasive organisms. Because, if there is no disadvantage in the fitness of individuals at the invasion front, evolutionary forces would likely facilitate the fine tuning of organismal traits that would allow for a more rapid expansion of the invading population. Therefore, it is of utmost importance for control efforts to be launched as soon as possible, before the invader has had sufficient time to evolve into a better adapted adversary (Phillips *et al*., 2006).

In this regard it would be useful to investigate if there has been any morphological change that may have occurred in the invasive *A. gutturalis* populations. The three populations of invasive *A. gutturalis* have been established in their respective introduced ranges for varying amounts of time. If any rapid adaptive change can be identified in these populations, it would be possible to gauge an estimate of how quickly these toads are able to evolve and adapt to their new environments. This would assist with the broader picture of understanding the evolutionary responses toads may have when introduced to a novel environment.

As is often the case, invasive species are common through their natural range and have large natural distributions. It is important for conservation managers to rapidly identify whether an introduced species has the potential for becoming invasive. With regard to anurans, life history traits would provide clues for the invasive potential of different species. In the case of bufonids, the study by Van Bocxlaer *et al*. (2010) which investigated the global radiation of toads by examining particular traits, would also serve as a useful proxy for determining the invasive ability of toads. Both the guttural toad and the cane toad exhibit most of these traits,

which indicates that the traits associated with range expansion may serve as useful indicators of invasive abilities for bufonids and other anurans.

The Western Cape has the greatest problem with amphibian domestic exotics (Measey & Davies, 2011) within South Africa and is home to a diverse and often endemic herpetofauna. It would be valuable to investigate which of the anuran species that do not naturally occur in the Western Cape have the potential to become invasive if they were to be introduced. It is widely regarded that in order to attempt to successfully stop a biotic invasion, the introduced species needs to be detected early and responded to rapidly (Westbrooks, 2004; Kaiser & Burnett, 2010). A predictive model which assigns or ranks the invasive ability of other amphibian species would better equip conservation managers to make decisions rapidly once an extra-limital species has been detected. This would be particularly helpful to conservation managers when responding to the introduction of a new species.

REFERENCES

Allendorf, F. W. and L. L. Lundquist. 2003. Introduction: population biology, evolution, and control of invasive species. *Conservation Biology*, 17: 24–30.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman. 1990. Basic local alignment search tool. *Journal of Molecular Biology*, 215: 403–410. Available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi

Andow, D. A., P. M. Kareiva, S. A. Levin and A. Okubo. 1990. Spread of invading organisms. *Landscape ecology*, 4: 177–188.

Ashton, K. G. and A. de Queiroz. 2001. Molecular systematic of the western rattlesnake, *Crotalus viridis* (Viperidae), with comments on the utility of the D-Loop in phylogenetic studies of snakes. *Molecular Systematics and Evolution*, 21 (2): 176–189.

Avise, J. C. 2000. Phylogeography: the history and formation of species. Harvard University Press, Cambridge, MA.

Baard, E. H. W., W. R. Branch, A. Channing, A. De Villiers, A. Le Roux and L. F. N. P. Mouton. 1999. A review of the amphibians and reptiles of the Cape Floristic Region as indicators of centres of biodiversity, sensitive habitats and sites of special interest. CapeNature, Cape Town.

Bermingham, E. and C. Moritz. 1998. Comparative phylogeography: concepts and applications. *Molecular Ecology*. **7:** 367 – 369.

Blair, W. F. 1972. Evolution in the genus *Bufo*. University of Texas Press, Austin and London.

Blair, W. F. 1972a. Summary. Pp. 329–343. In Blair, W. F. (Ed.), Evolution in the genus *Bufo*. University of Texas Press, Austin and London.

Blair, W. F. 1972b. Characteristics of the testes. Pp. 324–328. In Blair, W. F. (Ed.), Evolution in the genus *Bufo*. University of Texas Press, Austin and London.

Blair, W. F. 1972c. Pp. 196–232. Evidence from hybridization. In Blair, W. F. (Ed.), Evolution in the genus *Bufo*. University of Texas Press, Austin and London.

Bogart, J. P. 1972. Karyotypes. In Blair, W. F. (Ed.), Evolution in the genus *Bufo*. University of Texas Press, Austin and London.

Bossart, J. L. and D. P. Prowell. 1998. Genetic estimates of population structure and gene flow: limitations, lessons and new directions. *Trends in Ecology and Evolution*, 13: 202–206.

Britton, K. O., P. White, A. Kramer and G. Hudler. 2010. A new approach to stopping the spread of invasive insects and pathogens: early detection and rapid response via a global network of sentinel plantings. *New Zealand Journal of Forestry Science*, 40: 109–114.

Cei, J. M., V. Erspamer and M. Roseghini. 1972. Biogenic amines. Pp. 233–243. In Blair, W. F. (Ed.), Evolution in the genus *Bufo*. University of Texas Press, Austin and London.

Channing, A. 2001. Amphibians of central and southern Africa. Cornell University Press, Ithaca.

Cheke, A. and J. Hume. 2008. Lost land of the Dodo – An ecological history of Mauritius, Réunion and Rodrigues. T & Ad Poyser, Ed., London.

Cherry, M. I. 1993. Sexual selection in the raucous toad, *Bufo rangeri*. *Animal Behaviour* 45: 359–373.

Chornesky, E. A. and J. M. Randall. 2003. The threat of invasive alien species to biological diversity: setting a future course. *Annals of the Missouri Botanical Garden*, 90 (1): 67–76.

Chuttoo, Y. D. 2006. The diet of the introduced toad (*Bufo gutturalis*) and implications for the conservation of native invertebrates. BSc dissertation, University of Mauritius, Mauritius.

Clarke, G. M., S. Gross, M. Matthews, P. C. Catling, B. Baker, C. L. Hewitt, D. Crowther and S. R. Saddler. 2000. State of the Environment Indicators for Exotic Environmental Pest Species. State of the Environment, Second Technical Paper Series (Biodiversity), Department of the Environment and Heritage, Canberra.

Clement, M., Q. Snell, P. Walke, D. Posada and K. Crandall. 2002. TCS: estimating gene genealogies. Proceedings of the 16th International Parallel and Distributed Processing Symposium 2: 184.

Crossland, M. R. 2000. Direct and indirect effects of the introduced toad *Bufo marinus* (Anura: Bufonidae) on populations of native anuran larvae in Australia. *Ecography* 23: 283–290.

Cunningham, M. J. 2004. *Bufo rangeri* Hewitt, 1935. Pp. 67–69. In: Minter, L. R., Burger, M., Harrison, J. A., Braack, H. H., Bishop, P. J. and Kloepfer, D. (Eds.), Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland. SI/MAB Series #9. Smithsonian Institution, Washington, DC.

Cunningham, M. and M. I. Cherry. 2004. Molecular systematic of African 20-chromosome toads (Anura: Bufonidae). *Molecular Phylogenetics and Evolution* 32: 671–685.

Davies, S. J., S. Clusella-Trullas, C. Hui and M. A. McGeoch. 2013. Farm dams facilitate amphibian invasion: Extra-limital range expansion of the painted reed frog in South Africa. *Austral Ecology*, 38: 851–863.

Dervin, S., M. Sanchez, C. R. Bursey and S. R. Goldberg. 2014. First record of *Raillietiella affinis* Bovien, 1927 (Arthropoda: Raellietiellidae) as an endoparasite of the Madagascan giant day gecko, *Phelsuma grandis* Gray, 1870 introduced at Reunion Island. *Herpetology Notes* 7: 779–781.

de Villiers, A. 2004a. *Bufo pantherinus* Smith, 1828. Pp. 71–74. In: L. R. Minter, M. Burger, J. A. Harrison, H. H. Braack, P. J. Bishop & D. Kloepfer (Eds.), Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland. SI/MAB Series #9. Smithsonian Institution, Washington, DC.

de Villiers, A. 2004b. *Heleophryne rosei* Hewitt, 1925. Pp. 108–111. In: L. R. Minter, M. Burger, J. A. Harrison, H. H. Braack, P. J. Bishop & D. Kloepfer (Eds.), Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland. SI/MAB Series #9. Smithsonian Institution, Washington, DC.

de Villiers, A. 2004c. *Microbatrachella capensis*, Boulenger, 1910. Pp. 241–244. In: L. R. Minter, M. Burger, J. A. Harrison, H. H. Braack, P. J. Bishop & D. Kloepfer (Eds.),

Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland. SI/MAB Series #9. Smithsonian Institution, Washington, DC.

de Villiers, A. 2006. *Bufo gutturalis* Power, 1927. Guttural Toad. Introduced population. *African Herp News* 40: 28–29.

Didham, R. K., J. M. Tylianakis, M. A. Hutchinson, R. M. Ewers and N. J. Gemmell. 2005. Are invasive species the drivers of ecological change? *Trends in Ecology and Evolution*, 20: 470–474.

Driver, A., K. Maze, M. Rouget, A. T. Lombard, J. Nel, J. K. Turpie, R. M. Cowling, P. Desmet, P. Goodman, J. Harris, Z. Jonas, B. Reyers, K. Sink and T. Strauss. 2005. *National Spatial Biodiversity Assessment 2004: Priorities for biodiversity conservation in South Africa*. Pretoria: South African National Biodiversity Institute.

Dukes, J. S. and H. A. Mooney. 1999. Does global change increase the success of biological invaders. *Trends in Ecology and Evolution*, 14 (4): 135–139.

Dupanloup, I., S. Schneider and L. Excoffier. 2002. A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology*, 11: 2571–2581.

Du Preez, L. H., C. Weldon, M. J. Cunningham, and A. A. Turner. 2004. *Bufo gutturalis* Power, 1927. Pp. 67–69. In: Minter, L. R., Burger, M., Harrison, J. A., Braack, H.H., Bishop, P.J. and Kloepfer, D. (Eds.), Atlas and Red Data Book of the Frogs of South Africa, Lesotho and Swaziland. SI/MAB Series #9. Smithsonian Institution, Washington, DC.

Eldridge, M. D. B., C. Rummery, C. Bray, K. R. Zenger, T. L. Browning and R. L. Close. 2004. Genetic analysis of a population crash in brush-tailed rock-wallabies (*Petrogale penicillata*) from Jenolan Caves, south-eastern Australia. *Wildlife Research*, 31: 229–240.

Ellstrand, N. C. and K. A. Schierenbeck. 2000. Hybridization as a stimulus for the invasiveness in plants. *Proceedings of the National Academy of Sciences, USA*, 97: 7043–7050.

England, P. R., G. H. R. Osler, L. M. Woodworth, M. E. Montgomery, D. A. Briscoe and R. Frankham. 2003. Effects of intense versus diffuse population bottlenecks on

microsatellite genetic diversity and evolutionary potential. *Conservation Genetics*, 4: 595–604.

Excoffier, L., P. Smouse and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, 131: 479–491.

Ficetola, G. F., A. Bonin and C. Miaud. 2008. Population genetics reveals origin and number of founders in a biological invasion. *Molecular Ecology*, 17: 773–782.

FigTree 1.4.2. Available at: http://tree.bio.ed.ac.uk/software/figtree/.

Florens, F. B. V. and C. Baider. 2007. Relocation of *Omphalotropis plicosa* (Pfeiffer, 1852), a Mauritian landsnail believed extinct. *Journal of Molluscan Studies* 73: 205–206.

Frankham, R. and K. Ralls. Conservation biology: inbreeding leads to extinction. *Nature*, 392: 441–442.

Frost, D. R. 2015. Amphibian Species of the World: an Online Reference. Version 6.0 (*29 September 2015*). Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/index.html. American Museum of Natural History, New York, USA.

Frost, D.R., T. Grant, J. Faivovich, R.H. Bain, A. Haas, C.F.B. Haddad, R.O. de Sa´ , A. Channing, M. Wilkinson, S.C. Donnellan, C.J. Raxworthy, J.A. Campbell, B.L. Blotto, P. Moler, R.C. Drewes, R.A. Nussbaum, J.D. Lynch, D.M. Green and W.C. Wheeler. 2006. The amphibian tree of life. *Bulletin of the American Museum of Natural History*, 297: 1–370.

Froufe, E., J. C. Brito and D. J. Harris. 2009. Phylogeography of North African *Amietophrynus xeros* estimated from mitochondrial DNA sequences. *African Zoology*, 44 (2): 208–215.

Fu, Y. X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, 147: 915–925.

Gascon, C., J. R. Malcolm, J. L. Patton, M. N. F. da Silva, J. P. Bogart, S. C. Lougheed, C. A. Peres, S. Neckel and P. T. Boag. 2000. Riverine barriers and the geographic

distribution of Amazonian species. *Proceedings of the National Academy of Sciences USA*, 97 (25): 13672–13677.

Goldblatt, P. and J. C. Manning. 2002. Plant diversity of the Cape region of southern Africa. *Annals of the Missouri Botanical Garden*, 89 (2): 281–302.

Goldstein, P. Z., R. DeSalle, G. Amato and A. P. Vogler. 2000. Conservation genetics at the species boundary. *Conservation Biology,* 14: 120–131.

Goudswaard, K. P. C., F. Witte and E. F. B. Katunzi. 2008. The invasion of an introduced predator, Nile perch (*Lates niloticus*, L.) in Lake Victoria (East Africa): chronology and causes. *Environmental Biology of Fishes*, 81: 127–139.

Greenlees, M. J., G. P. Brown, J. K. Webb, B. L. Phillips and R. Shine. 2006. Effects of an invasive anuran [the cane toad (*Bufo marinus*)] on the invertebrate fauna of a tropical Australian floodplain.

Guindon, S. and O. Gascuel. 2003. A simple, fast and accurate method to estimate large phylogenies by maximum-likelihood. *Systematic Biology*, 52: 696–704.

Guttman, S. I. 1967. Transferrin and haemoglobin polymorphism, hybridization and introgression in two African toads, Bufo regularis and Bufo rangeri. *Comparative Biochemistry and Physiology*, 23: 871–877.

Guttman, S. I. 1972. Blood proteins. Pp. 265–278. In Blair, W. F. (Ed.), Evolution in the genus *Bufo*. University of Texas Press, Austin and London.

Harpending, H. C., M. A. Batzer, M. Gurven, L. B. Jorbe, A. R. Rogers and S. T. Sherry. 1998. Genetic traces of ancient demography. *Proceedings of the National Academy of Sciences, USA*, 95: 1961–1967.

Hewitt, J. 1935. Some new forms of batrachians and reptiles from South Africa. *Records of the Albany Museum* 4: 283–357.

Hewitt, J. 1937. A guide to the vertebrate fauna of the Eastern Cape province South Africa. Part II. Reptiles, amphibians and freshwater fishes. Albany Museum, Grahamstown.

Hillis, D.M., Mable, B.K., Larson, A., Davis, S.K. and Zimmer, E.A. 1996. Nucleic acids IV: Sequencing and cloning IN Hillis, D.M., Moritz, C. and Mable, B.K. Molecular Systematics 2nd Ed. Sinauer Associates, Sunderland, Massachusetts.

Holland, B.R., K.T. Huber, V. Moulton and P. Lockhart. 2004. Using consensus networks to visualise contradictory evidence for species phylogeny. *Molecular Biology and Evolution*, 21: 1459–1461.

Hoffman, M., *et al.* 2010. The impact of conservation on the status of the World's vertebrates. *Science*, 330: 1503–1509.

Hoffmeister, T. S., L. E. M. Vet, A. Biere, K. Holsinger and J. Filser. 2005. Ecological and evolutionary consequences of biological invasion and habitat fragmentation. *Ecosystems*, 8: 657-667.

Irwin, D. E. 2002. Phylogeographic breaks without geographic barriers to gene flow. *Evolution*, 56 (12): 2383–2394.

Jacobs, J. F. and W. R. Heyer. 1994. Collecting tissue for biochemical analysis. In Heyer, W.R. *et al.* Measuring and monitoring biological diversity. Standard methods for amphibians. Smithsonian Institution Press.

Kaiser, B. A. and K. M. Burnett. 2010. Spatial economic analysis of early detection and rapid response strategies for an invasive species. *Resource and Energy Economics*, 32: 566–585.

Kolbe, J. J., R. E. Glor, L. R. Schettino, A. C. Lara, A. Larson and J. B. Losos. 2004. Genetic variation increases during biological invasion by a Cuban lizard. *Nature*, 431: 177–181.

Komaki, S., A. Kurabayashi, M. M. Islam, K. Tojo, and M. Sumida. 2012. Distributional change and epidemic introgression in overlapping areas of Japanese pond frog species over 30 years. *Zoological Science*, 29: 351-358.

Kraus, F. 2009. Alien reptiles and amphibians: a scientific compendium and analysis – Springer Series in Invasion Ecology 4. Springer Science and Business Media B.V., Netherlands.

Kulhanek, S. A., A. Ricciardi and B. Leung. 2011. Is invasion history a useful tool for predicting the impacts of the world's worst aquatic invasive species? *Ecological Applications*, 21: 189–202.

Kuraishi, N., M. Matsui and H. Ota. 2009. Estimation of the origin of *Polypedates leucomystax* (Amphibia: Anura: Rhacophoridae) introduced to the Ryukyu Archipelago, Japan. *Pacific Science*, 63 (3): 317–325.

Lampo, M. and G. A. de Leo. 1998. The invasion ecology of the toad *Bufo marinus*: From South America to Australia.*cological Applications* 8 (2): 388–396.

Lawes, M. J., H. A. C Eeley, N. J. Findlay and D. Forbes. 2007. Resilient forest faunal communities in South Africa: a legacy of palaeoclimatic change and extinction filtering? *Journal of Biogeography*, 34: 1246–1264.

Leberg, P. L. 1992. Effects of population bottlenecks on genetic diversity as measured by allozyme electrophoresis. *Evolution*, 46: 477–494.

Leblois, R., F. Rousset, D. Tikel, C. Moritz and A. Estoup. 2000. Absence of evidence for isolation by distance in an expanding cane toad (*Bufo marinus*) population: an individual-based analysis of microsatellite genotypes. *Molecular Ecology*, 2000 (9): 1905–1909.

Lee, C. E. 2002. Evolutionary genetics of invasive species. *Trends in Ecology and Evolution*, 17 (8): 386–391.

Le Maitre, D. C., D. M. Richardson and R. A. Chapman. 2004. Alien plant invasions in South Africa: Driving forces and the human dimension. *South African Journal of Science*, 100: 103–112.

Li, R., W. Chen, L. Tu and J. Fu. 2008. Rivers as barriers for high elevation amphibians: a phylogeographic analysis of the alpine stream frog of the Hengduan Mountains. *Journal of Zoology*, 277: 309–316.

Low, B. S. 1972. Evidence from parotoid-gland secretions. Pp. 244–264. In Blair, W. F. (Ed.), Evolution in the genus *Bufo*. University of Texas Press, Austin and London.

Lowe, S., M. Browne, S. Boudjelas, and M. De Poorter. 2000. 100 of the World's Worst Invasive Alien Species A selection from the Global Invasive Species Database. The

Invasive Species Specialist Group (ISSG**)** a specialist group of the Species Survival Commission (SSC) of the World Conservation Union (IUCN), 12pp. First published as special lift-out in Aliens 12, December 2000. Updated and reprinted version: November 2004.

Macey, J. R., J. L. Strasburg, J. A. Brisson, V. T. Vredenburg, M. Jennings and A. Larson. 2001. Molecular phylogenetics of western North American frogs of the *Rana boylii* species group. *Molecular Phylogenetics and Evolution*, 19 (1): 131–143.

Mahoney, M. J. 2004. Molecular systematic and phylogeography of the *Plethodon elongates* species group: combining phylogenetic and population genetic methods to investigate species history. *Molecular Ecology*, 13: 149–166.

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research*, 27: 209–220.

Martin, R. F. 1972. Evidence from osteology. Pp. 37–70. In Blair, W. F. (Ed.), Evolution in the genus *Bufo*. University of Texas Press, Austin and London.

Martin, W. F. 1972. Evolution of vocalization in the genus *Bufo*. Pp. 279–309. In Blair, W. F. (Ed.), Evolution in the genus *Bufo*. University of Texas Press, Austin and London.

Maxson, L. R. 1984. Molecular probes of phylogeny and biogeography in toads of the widespread genus *Bufo*. *Molecular Biology and Evolution*, 1: 324–356.

McCommas, S. A. and E. H. Bryant. 1990. Loss of electrophoretic variation in serially bottlenecked populations. *Heredity*, 64: 315–321.

McGeoch, M. A., D. Spear, E. J. Kleynhans and E. Marais. 2012. Uncertainty in invasive alien species listing. *Ecological Applications*, 22: 959–971.

McRae, B. H., P. Beier, L. Y. Huynh and P. Keim. 2005. Habitat barriers limit gene flow and illuminate historical events in a wide-ranging carnivore, the American puma. *Molecular Ecology*, 14: 1965–1977.

Measey, G. J. (Ed.). 2011. Ensuring a future for South Africa's frogs: a strategy for conservation research. SANBI Biodiversity Series 19. South African National Biodiversity Institute, Pretoria.

Measey, G. J. and A. Channing. 2003. Phylogeography of the genus *Xenopus* in southern Africa. *Amphibia-Reptilia*, 24: 321–330.

Measey, G. J., and S. J. Davies. 2011. Struggling against domestic exotics at the southern end of Africa. *FrogLog*, 97: 28–30.

Measey, G. J., and K. A. Tolley. 2009. Investigating the cause of the disjunct distribution of *Amietophrynus pantherinus*, the Endangered South African western leopard toad. *Conservation Genetics*, 12: 61-70.

Millenium Ecosystem Assessment. 2005. Ecosystems and human well-being: Biodiversity synthesis. World Resources Institute, Washington, DC.

Miller, M. P. 2005. Alleles in Space (AIS): Computer software for the joint analysis of interindividual spatial and genetic information. *Journal of Heredity*, 96 (6): 722–724.

Minter, L. R., M. Burger, J. A. Harrison, H. H. Braack, P. J. Bishop and D. Kloepfer. 2004. Atlas and red data book of the frogs of South Africa, Lesotho and Swaziland. SI/MAB Series #9. Smithsonian Institution, Washington, DC.

Mooney, H. A. and E. E. Cleland. 2001. The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences, USA*, 98: 5446–5451.

Mooney, H. A. 2005. Invasive alien species: the nature of the problem. Island Press, Washington DC, USA.

Myers, N., R. A. Mittermeier, C. G. Mittermeier, G. A. B da Fonseca and J. Kent. 2000. Biodiversity hotspots for conservation priorities. *Nature*, 403: 853–858.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Nei, M., T. Maruyama and R. Chakraborty. 1975. The bottleneck effect and genetic variability in populations. *Evolution*, 29: 1–10.

NEMBA. 2004. National Environmental Management: Biodiversity Act, 2004. Government Printer, Republic of South Africa.

Novak, S. J. and R. N. Mack. 1993. Genetic variation in *Bromus tectorum* (Poaceae): comparison between native and introduced populations. *Heredity*, 71: 167–176.

Onadeko, A. B., M–O. Rödel, H. O. Liedtke and M. F. Barej. 2014. The rediscovery of Perret's toad, *Amietophrynus perreti* (Schiøtz, 1963) after more than 40 years, with comments on the species' phylogenetic placement and conservation status. *Zoosystematics and Evolution*, 90 (2): 113–119.

Palumbi, S. R. 1996. Nucleic acids II: The polymerase chain reaction. Pp. 205–247. In: D. M. Hillis, C. Moritz & B. K. Mable (Eds.), Molecular Systematics. Sinnauer Associates Inc., Sunderland.

Palumbi, S., A. Martin, S. Romano, W. O. McMillan, L. Stice and G. Grabowski. 2002. Simple Fool's Guide to PCR, Version 2.0. University of Hawaii.

Parmesan, C. 2006. Ecological and evolutionary responses to recent climate change. *Annual Review of Ecology, Evolution and Systematics*, 37: 637–639.

Passmore, N. I. 1972. Intergrading between members of the "regularis group" of toads in South Africa. *Journal of Zoology, London*, 167: 143–151.

Pauly, G. B., D. M. Hillis, and D. C. Cannatella. 2004. The history of a Nearctic colonization: Molecular phylogenetics and biogeography of the Nearctic toads (*Bufo*). *Evolution*, 58: 2517–2535.

Phillips, B. L. and R. Shine. 2004. Adapting to an invasive species: Toxic can toads induce morphological change in Australian snakes. *Proceedings of the National Academy of Sciences, USA*, 101: 17150–17155.

Phillips, B. L., G. P. Brown, J. K. Webb and R. Shine. 2006. Invasion and the evolution of speed in toads: Cane toads seem to have honed their dispersal ability to devastating effect over the generations. *Nature*, 439: 803.

Picker, M. D. 1985. Hybridization and habitat selection in *Xenopus gilli* and *Xenopus laevis* in the southwestern Cape Province. *Copeia*, 574: 574–580.

Picker, M. D., C. J. McKenzie and P. Fielding. 1993. Embryonic tolerance of *Xenopus* (Anura) to acidic blackwater. *Copeia*, 1993 (4): 1072–1081.

Picker, M. D. and A. L. De Villiers. 1989. The distribution and conservation status of *Xenopus gilli* (Anura, Pipidae). *Biological Conservation*, 49: 169–183.

Pimental, D., L. Lach, R. Zuniga and D. Morrison. 2000. Environmental and economic costs of non-indigenous species in the United States. *BioScience*, 50: 53–65.

Pimentel, D., R. Zuniga and D. Morrison. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics*, 52: 273–288.

Pitt, W., D. Vice and M. Pitzler. 2005. Challenges of invasive reptiles and amphibians. Wildlife Damage Management Conferences–Proceedings. Paper 84.

PopArt 1.6. Population Analysis with Reticulate Trees. Allan Wilson Centre, Imaging Evolution Initiative. Available at: http://popart.otago.ac.nz.

Posada, D. 2008. jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution*, 25 (7): 1253–1256.

Posada, D. and K. A. Crandall. 2001. Intraspecific phylogenetics: Trees grafting into networks. *Trends in Ecology and Evolution.* 16: 37–45.

Portik, D. M. and T. J. Papenfuss. 2015. Historical biogeography resolves the origins of endemic Arabian toad lineages (Anura: Bufonidae): Evidence for ancient vicariance and dispersal events with the Horn of Africa and South Asia. *BMC Evolutionary Biology*, 15 (152).

Poynton, J. C. 1964. The Amphibia of southern Africa: a faunal study. *Annals of the Natal Museum*, 17: 1–334.

Poynton, J. C. and D. G. Broadley. 1991. Amphibia Zambesiaca 5: Zoogeography. *Annals of the Natal Museum*, 32. 221–277.

Pramuk, J. B. 2006. Phylogeny of South American *Bufo* (Anura: Bufonidae) inferred from combined evidence. Zoological Journal of the Linnean Society 146: 407–452.

Pramuk, J. B., T. Robertson, J. W. Sites Jr and B. P. Noonan. 2008. Around the world in 10 million years: biogeography of the nearly cosmopolitan true toads (Anura: Bufonidae). *Global Ecology and Biogeography*, 17: 72–83.

Ramos-Onsins, S. E. and J. Rozas. 2002. Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution*, 19 (12): 2092–2100.

Reznick, D. N. and C. K. Ghalambor. 2001. The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. *Genetica*, 112: 183–198.

Richardson, S. 2014. Final report – Guttural toad *Amietophrynus gutturalis* control programme. NCC Environmental Services.

Rochat, J. 2008. Terrestrial invertebrate biodiveristy of Reunion Island. Insectarium de La Réunion. Available at: www.regionreunion.com/fr/spip/IMG/pdf/insectarium_ in_english.pdf

Roe, B. A., D-P. Ma, R. K. Wilson and J. F-H. Wong. 1985. The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. *The Journal of Biological Chemistry*, 250 (17): 9759–9774.

Rogers, A. R. and H. Harpending. 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, 9: 552–569.

Rollins, L. A., A. P. Woolnough, R. Sinclair, N. J. Mooney and W. B. Sherwin. 2011. Mitochondrial DNA offers unique insights into invasion history of the common starling. *Molecular Ecology*, 20: 2307–2317.

Ronquist, F. and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19: 1572–1574.

Ruiz, G. M., P. W. Fofonoff, B. Steves, S. F. Foss and S. N. Shib. 2011. Marine invasion history and vector analysis of California: a hotspot for western North America. *Diversity and Distributions*, 17: 362–373.

Russell, A. L., R. A. Medelín and G. F. McCracken. 2005. Genetic variation and migration in the Mexican free-tailed bat (*Tadarida brasiliensis mexicana*). *Molecular Ecology*, 14: 2207–2222.

Sakai, A. K., F. W. Allendorf, J. S. Holt, D. M. Lodge, J. Molofsky, K. A. With, S. Baughman, R. J. Cabin, J. E. Cohen, N. C. Ellstrand, D. E. McCauley, P. O'Neil, I. M. Parker, J. N. Thompson and S. G. Weller. 2001. The population biology of invasive species. *Annual Review of Ecology and Systematics*, 32: 305–332.

Savage, J. M. 1973. The geographic distribution of frogs: patterns and predictions. Pp. 351–445. In Vial, J. L. (Ed.), Evolutionary biology of the anurans: contemporary research on major problems. University of Missouri Press, Columbia.

Sequencher® version 5.2.4 sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA. Available at: http://www.genecodes.com

Shine, R. 2010. The ecological impact of invasive cane toads (*Bufo marinus*) in Australia. *Quarterly Review of Biology* 85 (3): 253–291.

Siow, M. T. M., A. Channing,S. Kigoolo, E. La Marca, S. Schick, M. Veith, K. C. Wollenberg, B. Viertel, and S. Loetters. 2009. Amphibian species richness and conservation status in the Rwenzori Mountains, Eastern Afromontane biodiversity hotspot. Unpublished submitted to genbank 2009.

Simberloff, D., J. L. Martin, P. Genovesi, V. Maris, D. A. Wardle, J. Aronson, F. Courchamp, B. Galil, E. García-Berthou, M. Pascal, P. Pyšek, R. Sousa, E. Tabacchi and M. Vila. 2013. Impacts of biological invasions: what's what and the way forward. Trends in Ecology and Evolution, 28: 58–66.

Simberloff, D. and B. Von Holle. 1999. Positive interactions of non-indigenous species: Invasional meltdown? *Biological Invasions*, 1:21–32.

Slade, R. W. and C. Moritz. 2013. Phylogeography of *Bufo marinus* from its natural and introduced ranges. *Proceedings of the Royal Society of London B,* 265: 769–777.

Smissen, P. J., J. Melville, J. Sumner and T. J. Jessop. 2013. Mountain barriers and river conduits: phylogeographical structure in a large, mobile lizard (Varanidae: *Varanus varius*) from eastern Australia. *Journal of Biogeography*, 40: 1729–1740.

Solís, R., R. Ibáñez, G. Hammerson, B. Hedges, A. Diesmos, M. Matsui, J-M. Hero, S. Richards, L. Coloma, S. Ron, E. La Marca, J. Hardy, R. Powell, F. Bolaños, G. Chaves, and P. Ponce. 2009. *Rhinella marina*. The IUCN Red List of Threatened Species. Version 2014.2. Available at: www.iucnredlist.org. Downloaded on 15 September 2014.

South Africa. 2014. National Environmental Management: Biodiversity Act (10/2004): Alien and Invasive Species List. Notice 599 of 2014. *Government Gazette*, 590 (37886): 3–75, August 1.

South African Frog Re-assessment Group (SA-FRoG), IUCN SSC Amphibian Specialist Group 2010a. *Amietophrynus pantherinus*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1. Available at: www.iucnredlist.org. Downloaded on 17 August 2013.

South African Frog Re-assessment Group (SA-FRoG), IUCN SSC Amphibian Specialist Group. 2010b. *Heleophryne rosei*. The IUCN Red List of Threatened Species 2010. Version 2015.4. Available at: www.iucnredlist.org. Downloaded on 22 February 2016.

South African Frog Re-assessment Group (SA-FRoG), IUCN SSC Amphibian Specialist Group. 2010c. *Xenopus gilli*. The IUCN Red List of Threatened Species 2010. Version 2015.4. Available at: www.iucnredlist.org. Downloaded on 22 February 2016.

South African Frog Re-assessment Group (SA-FRoG), IUCN SSC Amphibian Specialist Group. 2011. *Microbatrachella capensis*. The IUCN Red List of Threatened Species 2011. Version 2015.4. Available at: www.iucnredlist.org. Downloaded on 22 February 2016.

Stuart, S.N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and Trends of Amphibian Declines and Extinctions Worldwide. *Science*, 306: 1783–1786.

Sukumaran, J. and M. T. Holder. 2010. DendroPy: A python library for phylogenetic computing. *Bioinformatics*, 26: 1569–1571.

Sumida, M., Y. Kanamori, H. Kaneda, Y. Kato, M. Nishioka, M. Hasegawa and H. Yonekawa. 2001. Complete nucleotide sequence and gene arrangement of the mitochondrial genome of the Japanese pond frog *Rana nigromaculata*. *Genes and Genetic Systems*, 76: 311–325.

Sumida, M. and T. Ishihara. 1997. Natural hybridization and introgression between *Rana nigromaculata* and *Rana porosa porosa* in central Japan. *Amphibia-Reptilia*, 18: 249-257.

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tajima, F. 1983. Evolutionary relationship of DNA sequences in finite populations. *Genetics.* 105: 437–460.

Tajima, F. 1989a. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123: 585–595.

Tajima, F. 1989b. The effect of change in population size on DNA polymorphism. *Genetics*, 123: 597–601.

Tandy, M., J. Poynton, L. Minter and S. Lötters. 2004. *Amietophrynus gutturalis*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2014.2. Available at: www.iucnredlist.org. Downloaded on 13 August 2014.

Tandy, M. and R. Keith. 1972. *Bufo* of Africa. In Blair, W. F. (Ed.), Evolution in the genus *Bufo*. University of Texas Press, Austin and London.

Tandy, R. M. 1972. The evolution of African Bufo. Ph.D. thesis. The University of Texas at Austin.

Teixeira, S., M-A. Cambon-Bonavita, E. A. Serrão, D. Desbruyéres and S. Arnaud-Haond. 2011. Recent population expansion and connectivity in the hydrothermal shrimp *Rimicaris exoculata* along the Mid-Atlantic Ridge. *Journal of Biogeography*, 38 (3): 564–574.

Telford, S. R. and J. van Sickle. 1989. Sexual selection in an African toad (*Bufo gutturalis*): The roles of morphology, amplexus displacement and chorus participation. *Behaviour*, 110: 62–75.

Templeton, A. R., E. Routman and C. A. Phillips. 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum.* *Genetics*, 140: 767–782.

Templeton, A. R., K. A. Crandall and C. F. Sing. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, 132: 619–633.

Tihen, J. A. 1962. Osteological observations on New World *Bufo*. *American Midland Naturalist*, 67: 157–183.

Tolley, K. A., B. M. Chase and F. Forest. 2008. Speciation and radiations track climate transitions since the Miocene Climatic Optimum: a case study of southern African chameleons. *Journal of Biogeography*, 35: 1402–1414.

Tsutsui, N. D., A. V. Suarez, D. A. Holway and T. J. Case. 2000. Reduced genetic variation and the success of an invasive species. *Proceedings of the National Academy of Sciences, USA*, 97: 5948–5953.

Van Bocxlaer, I., S. P. Loader, K. Roelants, S. D. Biju, M. Menegon and F. Bossuyt. 2010. Gradual adaptation toward a range-expansion phenotype initiated the global radiation of toads. *Science* 327: 679–682.

Van Djik, D. E. 1977. Habitats and dispersal of southern African Anura. *Zoologica Africana*, 12: 169–181.

Van Rensburg, B. J., O. L. F. Weyl, S. J. Davies, N. J. van Wilgen, D. Spear, C. T. Chimimba and D. S. Peacock. 2011. Invasive vertebrates of South Africa. In: Pimental, D. (Ed.), Biological invasions: Economic and environmental costs of alien plant, animal, and microbe species, Second Edition: 325. CRC Press, Boca Raton.

Vasconcelos, R., E. Froufe, J. C. Brito, S. Carranza and D. J. Harris. 2010. Phylogeography of the African common toad, *Amietophrynus regularis*, based on mitochondrial DNA sequences: Inferences regarding the Cape Verde population and biogeographical patterns. *African Zoology*, 45 (2): 291–298.

Vences, M., M. Thomas, A. van der Meijden, Y. Chiari and D. R. Vieites. 2005. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Frontiers in Zoology*, 2: 5–17.

Wager, V. A. 1986. Frogs of South Africa, 2nd ed. Delta Books, Craighall, South Africa.

Wallace, A. R. 1876. The geographical distribution of animals: With a study of the relations of living and extinct faunas as elucidating the past changes of the Earth's surface, Volume 1. Macmillan and Company.

Westbrooks, R. G. 2004. New approaches for early detection and rapid response to invasive plants in the United States. *Weed Technology*, 18: 1468–1471.

Wilcove, D. S., D. Rothstein, J. Dubow, A. Phillips and E. Losos. 1998. Quantifying threats to imperilled species in the United States. *BioScience*, 48 (8): 607–615.

Wright, S. 1943. Isolation by distance. *Genetics*, 28 (2): 114–138.

Zug, G. R. and P. B. Zug. 1979. The marine toad, *Bufo marinus*: a natural history résumé of native populations. *Smithsonian Contributions to Zoology*, 284: 1–58.

Zwickl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation. The University of Texas at Austin.