It seems to me that if you wait until the frogs and toads

have croaked their last to take some action,

you've missed the point.

Kermit the Frog

This thesis is dedicated to my son, Adam, and his generation, that they may know the splendour of our natural heritage as we have.

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I declare that this thesis is my own work unless specifically acknowledges in the text. It has not been submitted before for any degree or examination in any other university.

STarrant

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2nd day of May 2012

THESIS SUMMARY

Amphibians are the most threatened Class of vertebrate on Earth, with 30% of all species IUCN Red Listed. This proportion is reflected in South Africa, where 29% of all species assessed in 2011 (SA-FRoG 2010; IUCN 2011; Measey 2011) fall into the threatened categories of Critically Endangered (7%), Endangered (12%) or Vulnerable (10%). This study is focussed on these species, with a particular emphasis on those that occur in the KwaZulu-Natal Province. The thesis is structured as follows:

CHAPTER 1 gives a broad introduction to the global situation with regard to amphibian declines and the threats causing them as based on the literature. Additional information pertinent to successive chapters is given, including descriptions of KwaZulu-Natal"s threatened frogs, detail on the disease chytridiomycosis and its causal agent, *Batrachochytrium dendrobatidis* (*Bd*), and the importance of the application of systematics for conservation.

CHAPTER 2 provides baseline information on a national scale regarding the occurrence and prevalence of infection with *Bd* in South Africa"s threatened frogs. This pathogen causes the disease chytridiomycosis and is responsible for amphibian declines globally. Samples were collected by means of skin swabs and analysed using quantitative PCR. Prevalence varied widely between threatened species (Avg. = 14.8%), with infection intensity being predominantly influenced by life history characteristics. The study also provides, for the first time, a distribution model for *B. dendrobatidis* occurrence in South Africa, indicating regions that are likely to harbour the pathogen. Such information is useful for application in disease prevention and control plans.

CHAPTER 3 provides threat assessments for certain of the threatened species in KwaZulu-Natal, focussing on two sites per species, and makes recommendations on additional research requirements and appropriate conservation actions for these species. Particular emphasis is placed on *Hyperolius pickersgilli*, the province"s only Critically Endangered species.

CHAPTER 4 is focussed on the distribution of *Hyperolius pickersgilli*. Ecological niche modelling using Maxent was employed to predict the distribution of this species, and high probability areas were surveyed over two breeding seasons. Known historical sites were also re-visited and assessed for species presence. As a result of surveying, 5 new localities for the species were revealed, but half of the historical sites have been transformed to such an extent that the species no longer occurs there. In total, the species currently occurs at 17 localities, the majority of which are highly fragmented and threatened by human activity. The results of the survey were used to recalculate the area of occupancy (AOO) and extent of occurrence (EOO) for *H. pickersgilli.* Based on these findings, and the level of threat at the majority of sites and degree of fragmentation between them, the Critically Endangered status of this species remains warranted.

CHAPTER 5 reviews the systematics of the *Afrixalus spinifrons* complex using morphological, call and molecular analysis. Although the first two methods have been used historically to delimit boundaries for taxa within this complex, genetic analysis has not been previously conducted. This study makes use of DNA sequencing from mitochondrial and nuclear gene markers to elucidate phylogenetic relationships within the complex. The results confirm that *A. knysnae* is part of the A. *spinifrons* clade, but is a separate species. *Afrixalus spinifrons spinifrons* and *A. s. intermedius* form distinct clusters, but are closely related confirming that the subspecies diagnosis as a representation of evolutionary divergence is accurate. The study does however differ from previous conclusions in that populations from the Eastern Cape group with *A. s. intermedius* from the KwaZulu-Natal midlands as opposed to *A. s. spinifrons* from the coast. Although these findings do not warrant designation of the subspecies to full species, they should be treated as evolutionary significant units for the purposes of conservation.

OPSOMMING

Met 30% van alle amfibieërspesies op die IUCN Rooilys word die Amfibia as die mees bedreigde Klas van die gewerwelde diere op aarde beskou. Hierdie syfers word weerspieël in Suid-Afrika, waar 29% van die spesies as bedreig geklasifiseer word. Meer spesifiek; Krities Bedreig (7%), Bedreig (12%) en Kwesbaar (10%) (SA-FRoG 2010; IUCN 2011; Measey 2011). Hierdie studie fokus op die bedreigde spesies, met 'n spesifieke klem op die wat in die KwaZulu-Natal Provinsie aangetref word. Die proefskrif word soos volg gestruktureer:

HOOFSTUK 1 gee 'n breedvoerige inleiding tot die globale tendens van amfibieër populasie-afnames en die moontlike oorsake daarvan. Bykomende inligting relevant tot die opeenvolgende hoofstukke word gegee. Dit sluit in die bekendstelling aan die bedreigde paddas van KwaZulu-Natal, besonderhede oor die siekte kitridiomikose en die fungus Batrachochytrium dendrobatidis (Bd) wat dit veroorsaak, en die belangrikheid van sistematiese hersiening vir bewaring.

HOOFSTUK 2 bevat basiese inligting ten opsigte van die voorkoms en die prevalensie van Bd in Suid-Afrika se bedreigde paddas. Hierdie patogeen veroorsaak die siekte kitridiomikose en dra by tot die afname in amfibieërgetalle. Velskraapmonsters is met behulp van steriele deppers geneem. Dit is ontleed deur gebruik te maak van kwantitatiewe "PCR". Waargenome prevalensievlakke het baie tussen spesies gevarieër (Gem. = 14.8%), en infeksie-intensiteit is grotendeels deur spesiespesifieke gedrag beïnvloed. Die studie bied ook vir die eerste keer 'n verspreidingsmodel vir die voorkoms van Bd in Suid-Afrika. Hierdie inligting is nuttig vir siektevoorkoming en die bewaring van spesies.

HOOFSTUK 3 handel oor die identifisering en evaluering van bedreigings wat nadelig mag wees vir sommige van die bedreigde spesies in KwaZulu-Natal. Besondere klem word op Hyperolius pickersgilli geplaas, die provinsie se enigste Krities Bedreigde paddaspesie.

HOOFSTUK 4 fokus op die verspreiding van *Hyperolius pickersgilli*. Ekologiese nis-modellering, is met behulp van Maxent sagteware gedoen. Habitatseienskappe en bekende geografiese verspreiding van die spesie is gebruik om potensieële nuwe lokaliteite te identifiseer. Hierdie potensiële nuwe lokaliteite asook historiese lokaliteite waar *H.*

pickersgilli al waargeneem is, is besoek om vas te stel of Pickersgill se rietpadda wel daar voorkom. Vyf nuwe lokaliteite waar die spesie voorkom is ontdek, maar daar is gevind dat by die helfte van die histories bekende lokaliteite, die spesie gladnie meer voorkom nie. Die spesie kom tans by 17 lokaliteite voor, waarvan die meeste hoogs gefragmenteerd is en bedreig word deur antropogeniese aktiwiteite. Die bevindinge van die opname word gebruik om die oppervlak van voorkoms (AOO) en die omvang van verspreiding (EOO) van *H. pickersgilli* te herbereken.

HOOFSTUK 5 gee ’n oorsig oor die sistematiek van die *Afrixalus spinifrons* kompleks deur gebruik te maak van morfometriese-, lokroep- en molekulêre analise. Alhoewel die eerste twee metodes in die verlede gebruik is om die grense binne die kompleks vir hierdie taksa te bepaal, is genetiese analise nie voorheen uitgevoer nie. Die studie maak gebruik van DNA volgordebepaling vanaf mitochondriale en nukleêre geenmerkers om die filogenetiese verwantskappe binne hierdie kompleks te bepaal. Die resultate bevestig dat *A. knysnae* deel is van die *A. spinifrons* kompleks, maar ’n afsonderlike spesie is. *Afrixalus spinifrons spinifrons* en *A. s. intermedius* vorm duidelik onderskeibare groeperings, maar is nou verwant. Hierdie resultaat bevestig dus dat die subspesie diagnose as verteenwoordiging van evolusionêre afwyking akkuraat is. Die studie verskil wel van vorige gevolgtrekkings deurdat bevind is dat die populasies van die Oos-Kaap groepeer saam met *A. s. intermedius* vanaf Kwa-Zulu Natal middelland, in teenstelling met die *A. s. spinifrons* populasies van die kus. Alhoewel hierdie bevindinge nie die aanwysing van subspesie na volle spesie regverdig nie, dui dit daarop dat die twee groepe as evolusionêr betekenisvolle entiteite hanteer moet word vir bewaringsdoeleindes.

ABBREVIATIONS USED IN THE TEXT

Bd	*Batrachochytrium dendrobatidis*
°C	Degrees Celsius
CR	Critically Endangered (IUCN Standards)
DD	Data Deficient (IUCN Standards)
E	East
EC	Eastern Cape Province
EN	Endangered (IUCN Standards)
Ha	Hectare
HW	Head Width
Hz	Frequency (Hertz)
KZN	KwaZulu-Natal Province
Lat.	Latitude
Long.	Longitude
LP	Limpopo Province
M	Metres
mm	Millimetres
m.a.s.l.	Metres above sea level
Mya	Million years ago
N	North
NC	Northern Cape Province
NT	Near Threatened (IUCN Standards)
s	Seconds
S	South
SVL	Snout-Vent Length
TL	Tibia Length
VU	Vulnerable (IUCN Standards)
W	West
WC	Western Cape Province

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CHAPTER 2: PREVALENCE AND PREDICTED DISTRIBUTION OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN THE THREATENED FROGS OF SOUTH AFRICA

CHAPTER 3: STATUS OF AND CONSERVATION RECOMMENDATIONS FOR KWAZULU-NATAL‟S THREATENED FROGS

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 The value of Amphibians

Why care about amphibians? In the light of the extinction crisis now recognised as one of the biggest challenges facing scientists and conservationists (Gascon *et al*. 2007; Stuart *et al*. 2008), this question is being asked by society. This is because public perception surrounding amphibians has oftentimes been less than positive. Changing such perceptions plays a crucial part in the overall plan to save amphibians. The reasons for conserving amphibians are as many and varied as the Families comprising this fascinating Class of vertebrates. Although amphibians are seldom seen, they are of crucial importance, both in their function within ecosystems, and with regard to their evolutionary significance (Roelants *et al*. 2007; Cox *et al*. 2008). In evolutionary terms, modern amphibians are a monophyletic group descended from a common ancestor approximately 315 million years ago and represent the all-important transition of aquatic tetrapods onto land (Carroll 2001; Cannatella 2007; Wells 2007; San Mauro 2010) (Figure 1.1). This transition is reflected today in the vast array of species of amphibians and the distinctive biphasic lifestyle with which most are associated.

Amphibia is an extremely diverse Class of vertebrates, comprised of three Orders including approximately 6771 species: *Anura* (frogs and toads), of which there are currently 5,966 known species; *Caudata* (salamanders) 619 species and; *Gymnophiona* (caecilians), 186 species (Frost 2011). The recent rate of new amphibian species descriptions has been extremely high, with an overall 60% increase in the number of recognised species since 1985 (AmphibiaWeb 2012). Indeed, amphibian species diversity now exceeds that of mammals (Glaw & Köhler 1998). Should this trend in descriptions continue, it is estimated that in the next five decades the total number of amphibian species may reach approximately 12,000 (Köhler *et al*. 2008). Amphibians are distributed globally with particularly high species richness in the tropics (Duellman 1999; Roelants *et al*. 2007). Accordingly, the bulk of new descriptions are being reported from these regions (Köhler *et al.* 2008; Funk *et al*. 2011).

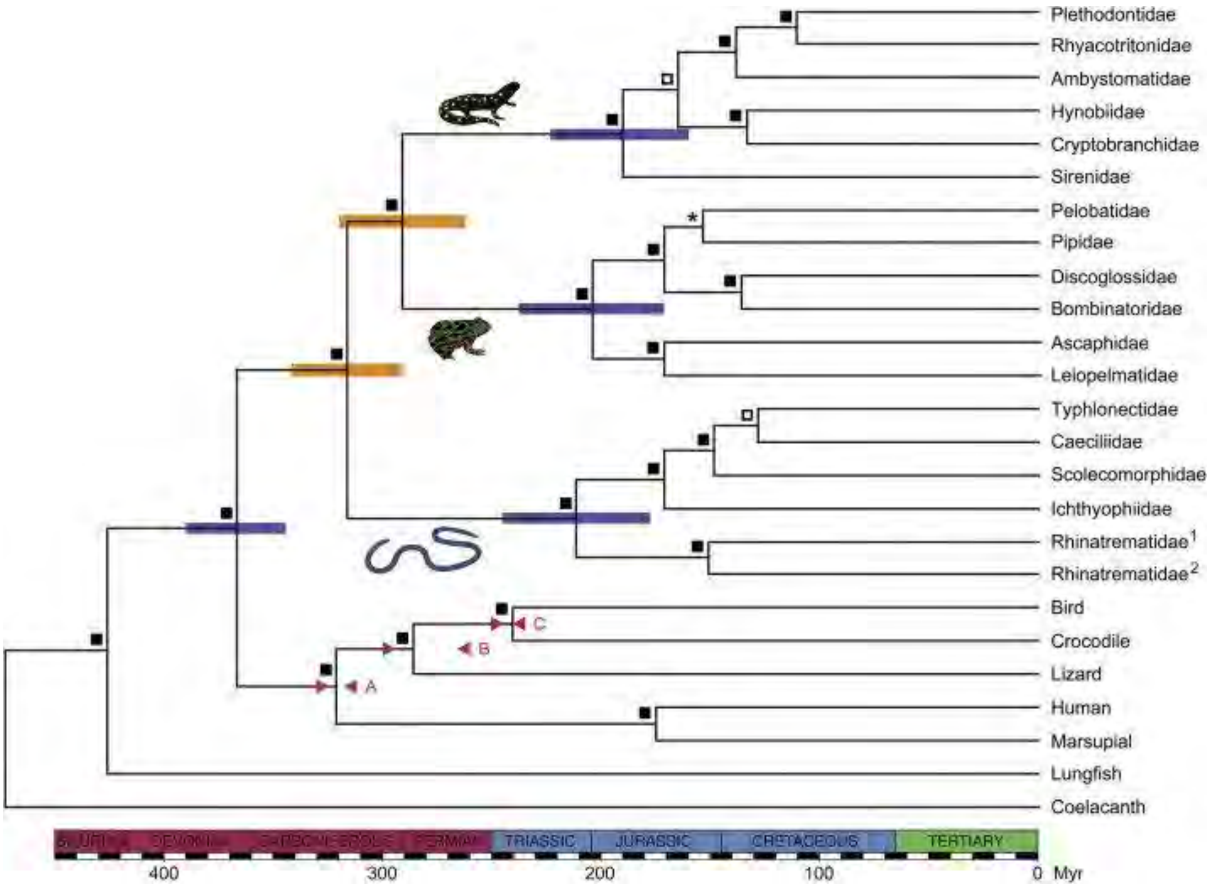

Figure 1.1: Phylogenetic tree of the origin of extant amphibians based on a multilocus molecular dataset. Divergence between caecilians and batrachians occurred in the Late Carboniferous, around 315 Mya, and the divergence between frogs and salamanders occurred in the Early Permian, around 290 Mya (Adapted from San Mauro 2010).

Of prime significance are the integral roles amphibians play in most ecosystems (Duellman & Trueb 1994; Semlitsch 2003; Wells 2007). Despite often going unnoticed, amphibians can comprise the bulk of terrestrial vertebrate biomass in temperate and tropical environments (Cox *et al*. 2008). Such abundance is probably linked to the role of both adults and larvae as primary predators in both the terrestrial and aquatic environments. Adults consume vast quantities of small invertebrates (mostly insects), many of which are not available to other vertebrate groups (Semlitsch 2003). For example, they are known to prey on hundreds of flies and mosquitoes in a single night and are accordingly vital as bio-control agents for agricultural pests and disease-carrying insects (Wager 1986; Battish *et al.* 1989; Greenlees *et al*. 2006). Amphibian larvae are usually aquatic and are consumers of primary production in the form of algae (periphyton and phytoplankton), and by doing so, assist in keeping waterways clean (Ranvestel *et al*. 2004). As prey, amphibians (both adults and larvae) are an important protein source for numerous species of invertebrates, reptiles, birds, mammals, and other amphibians (Rose 1962; Wager 1986; Wells 2007; Cox *et al*. 2008). Thus, through the processes of emigration and immigration, amphibians play an important role as nutrient vectors, connecting the aquatic and terrestrial environments (Semlitsch 2003).

Amphibians are also well recognised as important bio-indicators (Cooke 1981; Vitt *et al*. 1990; Carey and Bryant 1995; Welsh & Ollivier 1998; Hammer *et al*. 2004; Waddle 2006). This is because amphibians have a number of physiological, ecological and life-history characteristics that make them prone to changes in the environment (Blaustein & Wake 1995; Cox *et al*. 2008). Most species make use of both the aquatic and terrestrial environments during their lifecycles, and as a result, are sensitive to changes in both systems. Both habitats are also impacted by intense human use (Alford & Richards 1999). Due to this biphasic lifestyle and their sensitive semi-permeable skins, amphibians are considered good indicators of environmental health and the state of the biosphere as a whole (Vitt *et al*. 1990; Lips 1999; Blaustein *et al*. 2003a). Owing to their low vagility, they are particularly sensitive to habitat fragmentation and are vulnerable to the changes brought about through habitat transformation (Carr & Fahrig 2001; Fahrig 2003; Cushman 2006). Despite the high rate of new species descriptions for amphibians, this group is the most threatened Class of vertebrate (IUCN 2011). The high proportion of amphibian declines is an indication that ecosystems worldwide are in potential jeopardy (Gascon *et al*. 2007). Based on the percentage of species currently threatened with extinction (30 %), the expected magnitude in the loss of amphibians is significant and will undoubtedly have a multiplier effect, ultimately contributing to declines and extinctions of other species which rely on them (Collins & Storfer 2003; IUCN 2011). An interesting example of this is provided by long-term studies of the declines of mountain yellow-legged frogs (*Rana muscosa*) of the Sierra Nevada and the occurrence of bears. Brown bears (*Ursus arctos*) have been observed to feed extensively on frogs when fish are absent. The disappearance of *R. muscosa* from many sites has resulted in a decrease in bear activity around lakes, causing them to seek alternate food in campsites and towns (Knapp 2009). The consequences of amphibian declines on other species are thus potentially far-reaching.

In terms of their social, cultural and religious importance, amphibians have been viewed in a variety of roles (Hutchins *et al*. 2003). Some cultures have held them in the highest regard as keepers of rain or agents of fertility and good luck. Others have persecuted them, regarding them as evil (Hofrichter 2000). Either way, amphibians have featured large in much of society"s folklore. Amphibians have important aesthetic value and play an important role in education about biodiversity, especially in increasingly urban environments (McKinney 2008). Their fascinating life-cycle is an often-used educational tool at school level. The medicinal properties derived from amphibians have also long been recognised by humans (Marshall 1999). In many parts of the world they are important source of protein for people (Cox *et al.* 2008) and are also used extensively in traditional medicine for treatments of ailments as varied as warts and heart disease (Anderson 1993). More recently, the use of amphibian products for western medicine has gained increased attention. One of the first uses was for pregnancy testing, with the African clawed frog *Xenopus laevis* used extensively for this purpose (Hansen 1960; Shapiro & Zwarenstein 1934). Amphibian skin secretions (predominantly peptides and alkaloids) harbour a diversity of defensive biological compounds, which provide immunity against infections, viruses and bacteria (Apponyi *et al.* 2004; Melzer & Bisop 2011). Peptides isolated from amphibian skin are showing pharmacological promise as antibiotics and analgesics (Jensen & Camp 2003). Current active fields of research include the investigation of frog skin peptides to block HIV transmission (VanCompernolle *et al.* 2005) and inhibit growth of chytrid zoospores (Rollins-Smith & Conlon 2005; Melzer & Bishop 2010). Loss of species could mean the inadvertent loss of potential cures for important diseases. The loss of biodiversity in general does not bode well for human well-being considering our dependence on ecosystem processes and services such as clean water, pollination, food, medicines and material resources (Begon *et al.* 1990; Díaz *et al.* 2006).

Despite mixed social attitudes towards amphibians, the general public remain apathetic to the plight of amphibians and their importance in general (Wells 2007). This is particularly relevant in South Africa where superstitious beliefs and fears place frogs in a negative light (Tolley *et al.* 2011). Overcoming this apathy through education and raised awareness is necessary for improving the support (and hence effectiveness) of amphibian conservation efforts (Gibbons 2003).

1.2 Amphibian declines

The alarming loss of biodiversity currently taking place is commonly becoming known as the Sixth Mass Extinction (Leakey & Lewin 1995; Ehrlich 1998; Eldredge 2001). Mass extinctions are those in which 75% of species on Earth disappear in a geologically short space of time, and there is accumulating evidence to suggest that we are indeed entering such a scenario for the sixth time since complex life began some 540 million years ago (Balmford *et al*. 2003; Thomas *et al*. 2004; Barnosky *et al*. 2011). Five previous mass extinctions occurred before the present, namely the Ordovician (440 million years ago); Devonian (365 Mya); Permian (245 Mya); Triassic (210 Mya) and Cretaceous (65 Mya) (Wilson 1992). These preceding extinction events occurred as a result of natural phenomena, such as the sudden climate change brought about by a massive asteroid collision that is theorized to have caused the Cretaceous-Palaeogene extinction event, which included the sudden disappearance of the dinosaurs approximately 65 million years ago (Alvarez *et al*. 1980). Full recovery to original levels of diversity from these five major extinction events required tens of millions of years (Wilson 1992).

The Sixth Mass Extinction, also called the Holocene Extinction, is undoubtedly attributable to the overwhelming dominance of a single species, *Homo sapiens* (Anderson 1999; Wooldridge 2008; Zalasiewicz *et al*. 2008). This period of human-caused extinctions began in the late Pleistocene (12 000 years ago) with the disappearance of many species of large-bodied mammals such as mastodons (Lyons *et al*. 2004). Following the industrial revolution, massive growth in the human population and accompanying unsustainable anthropogenic activity continues to radically transform the natural world, resulting in the irretrievable loss of many species (Eldredge 1998; Hoekstra *et al*. 2005; Turner *et al*. 2007). Humans have had a direct impact on more than three-quarters of the ice-free land on earth (Walsh 2012). Changes to global biodiversity, as measured by the Living Planet Index (LPI), have been most profound over the past 50 years with an almost 30 percent reduction between 1970 and 2007 (Millennium Ecosystem Assessment 2005; Loh *et al*. 2010). Extinction rates are 100 to 1000 times faster than the background, pre-human rate of extinction (Thackery 1990; Cincotta & Engelman 2000; Baillie *et al*. 2004). Extinctions as a result of human activity have occurred in a wide range of plants, including many fynbos and orchid species, and animal taxa including reef-building corals, freshwater crustaceans, amphibians, reptiles, birds and mammals (IUCN 2011). Worryingly, many species extinctions across these groups remain undocumented, highlighting the need both to increase efforts to identify extinctions and for more targeted conservation strategies (Hanken 1999; Köhler *et al.* 2005). Ironically, this massive loss in biodiversity is co-occurring at a time when species descriptions are at an all time high (Köhler *et al.* 2008).

In terms of vertebrates, amphibians are at the forefront of this extinction event (Wake 1991, Wake & Vrendenburg 2008; Kiesecker *et al.* 2001; Stuart *et al.* 2004; Mendelson *et al.* 2006; Collen *et al.* 2011). Almost half of all known amphibian species are threatened to some extent (IUCN 2011). These unprecedented global declines in amphibians are one of the biggest challenges currently facing conservationists (Houlehan *et al.* 2000; Gascon *et al.* 2007). The challenge is that not only is one third of an entire Class potentially on the brink of being lost, but that this loss will have dire and far-reaching consequences for the multitude of other species that depend on them, including humans (Frost *et al.* 2006, Stuart *et al.* 2004).

Amphibian declines started gaining scientists" attention in the 1970s and 1980s but many of the first reports were anecdotal (Hayes & Jennings 1986; Heyer *et al.* 1988; Barinaga 1990). Much scepticism also surrounded such accounts since amphibian populations are known to fluctuate widely (Blaustein *et al.* 1994). However, by the 1990s firm evidence of declines began to accumulate in the form of publications based on long-term monitoring research (for example, Drost & Fellers 1996 and Pounds *et al.* 1997). Sudden and large declines were noted particularly in the montane regions of Central America (Crump *et al.* 1992; Pounds & Crump 1994) and in Australia (McDonald 1990). Worryingly, many of these first records of disappearances occurred in protected areas (Bradford 1991; Crump *et al.* 1992; Carey 1993; Drost & Fellers 1996; Hines *et al.* 1999). In such cases, habitat destruction could not account for the observed declines, prompting the need for more comprehensive research into population trends, distributions and threats. Today, a multitude of robust studies have convinced the scientific world that amphibian declines are indeed a global phenomenon (e.g. Lips 1999; Lips *et al.* 2005a; b; Bank *et al.* 2006; Blaustein & Dobson, 2006; Lacan *et al.* 2008).

The cumulative effort of such research was collated by the World Conservation Union Global Amphibian Assessment (GAA) to assess amphibian declines (Stuart *et al.* 2004). All known species at that time (5743) were assessed in order to gain an understanding of the crisis and to begin to devise conservation strategies. The findings showed that 32.5 % of amphibians are globally threatened according to the IUCN Red List Categories. This includes the categories Critically Endangered, Endangered or Vulnerable. This far exceeds the proportion of either threatened birds (12%) or mammals (23%). A further 31% are either Near Threatened or Data Deficient (IUCN, 2010). One hundred and sixty nine species are believed to already be extinct (39 known to be extinct or are extinct in the wild and 130 not found in recent years) (http://www.amphibianark.org).

The majority of those that have already become extinct have vanished from seemingly undisturbed environments. For example, the pristine Monteverde Cloud Forest Reserve of Costa Rica (Pounds & Crump 1994; Pounds *et al*. 2006). Today, of the 6771 presently known amphibian species, a total of 62% (including Data Deficient species) are experiencing some form of population decrease (Stuart 2008; Frost 2011). Stopping, or even slowing, the potential extinction of such a large proportion of such an important group of animals will be a monumental task, especially when considering the inertia of some governments in terms of conservation action and ignorance of the general public with regard to awareness of biodiversity loss (particularly amphibians) and the consequences thereof (Gibbons 2003).

Amphibian declines have occurred worldwide, but are most significant in Australia (Laurance *et al*. 1996; Berger *et al*. 1998), South and Central America (Burrowes *et al*. 2004; Lips *et al*. 2005a; b; Blaustein & Dobson 2006) and the high altitude regions of Western America (Carey 1993; Pounds *et al*. 1999; Bank *et al*. 2006). In addition, population crashes, in which as much as half of the population is lost, have been reported from Africa (Weldon & du Preez 2004b; Channing *et al*. 2006), Britain (Beebee 1977), Canada (Green 1997), Europe (Mutschmann *et al*. 2000; Bosch *et al*. 2001) and New Zealand (Bell *et al*. 2004). Compared with the scale of declines elsewhere on the globe, South Africa (and Africa as a whole) has not experienced severe loss of amphibian biodiversity (Measey 2011). However, populations are by no means stable and many species in the region (15% in South Africa) are categorised as threatened (IUCN 2011; Measey 2011). For the majority of these southern African species, further research is required to assess threats and provide knowledge on population sizes, species biology and ecology (Hero & Kriger 2008; Measey 2011). The implementation of long-term conservation strategies based on such research is necessary to ensure long-term survival of threatened southern African species.

Ongoing global research into amphibian declines has identified numerous threats in addition to habitat destruction, with an especially important discovery being that of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, and the devastating disease, chytridiomycosis it causes (Berger *et al.* 1999; Daszak & Cunningham 1999; Carey 2000). In general, declines are due to a complex array of causative agents, some of which are working synergistically to create amplified opportunities for species extinctions (Kiesecker *et al.* 2001; Garner *et al.* 2006). Disturbingly, a high proportion (48%) of those classified as "rapid declines" have no immediately identifiable cause and have been classed as "enigmatic" with climate change and disease being cited as the most probable causes (Stuart *et al.* 2004). Amphibians appear to be more vulnerable than other animal groups due to an array of complex factors, including their relatively low vagility; high vulnerability to death, especially when moving across transformed landscapes; narrow habitat tolerances and high vulnerability to disease, climate change and pollution (Semlitsch 2003; Cushman 2006).

The challenge of conserving amphibians is immense, but with increasing awareness of the problem, ongoing research and more funding being made available, solutions to the amphibian crisis are becoming a reality (Semlitsch & Rothermel 2003; Gascon *et al.* 2007; Measey 2011). Multiple organisations including the IUCN SSC Amphibian Specialist Group (ASG), Amphibian Ark, Amphibian Survival Alliance and more are becoming established to support amphibian conservation projects around the world. Preservation of habitats is fundamental to the conservation of amphibians and should be a priority (Beja & Alcazar 2003; Cushman 2006; Kremen *et al.* 2008). Improved knowledge of species biology, distribution and behaviour is also crucial if conservation plans are to be successful (Measey *et al.* 2011a). Treatments for chytridiomycosis are being sought (Rollins-Smith *et al.* 2003; Ramsey *et al.* 2010) and infections have been eliminated in captive animals (Boyle *et al.* 2004). This allows the creation of assurance populations for the re-introduction of severely threatened species back into the wild. Such programmes are already underway, for example the Kihansi Spray Toad, *Nectophrynoides asperginis*, from the Udzungwa Mountains, (Krajick 2006; Channing *et al.* 2006).

Because of their high vulnerability and unique life cycles, amphibians often require conservation strategies that may differ from management practices for other groups of animals (Semlitsch & Rothermel 2003). Despite their importance in ecosystem functioning, amphibians are often accorded lower priority than other wild species (Semlitsch & Rothermel

2003). These circumstances dictate that amphibians require both specific conservation actions as well as being able to benefit from existing conservation strategies for other biological taxa. Such needs require the development of a unifying framework for efforts to conserve amphibians. This has been realised in the form of the Amphibian Conservation Action Plan (ACAP), which was developed based on the Global Amphibian Assessment (Stuart *et al*. 2004; Gascon *et al*. 2007). The ACAP sets out four kinds of intervention to save amphibians:

1) To expand the understanding of the causes of declines and extinctions;

2) To continue to document amphibian diversity, and how it is changing;

3) To develop and implement long-term conservation programmes; and

4) To respond to emergencies and immediate crises (Gascon *et al*. 2007).

This global plan is filtering to country level and following the most recent re-assessment of South African frog species for Red Listing (IUCN 2011) a strategy document to guide conservation plans for these species has been compiled (Measey 2011). This study aims to provide research knowledge which can be used toward conservation actions for South African threatened frogs, with a focus on certain species in the KwaZulu-Natal province.

1.3 Threats to Amphibians

The global human population has grown exponentially over the past two millennia, and reached an unprecedented 7 billion people in October 2011 (http://www.census.gov). This recent rapid human population expansion and concomitant unsustainable use of natural resources (Meadows *et al*. 1972; Kendall & Pimentel 1994; Vitousek *et al*. 1997) – resources upon which other species also rely on - is having dire consequences for a broad range of species (Cincotta & Engelman 2000; Hero & Kriger 2008). Population growth coupled with increasing per capita consumption is creating human-dominated ecosystems in which the survival of wild species is precarious (Cincotta & Engelman 2000). Such systems result in increases in human-associated organisms including domestic species, pests, weeds and disease-causing organisms which in themselves threaten biodiversity. Moreover, ongoing population growth is making conservation efforts more difficult, expensive and more likely to conflict with human needs (Cincotta & Engelman 2000). Amphibians are particularly sensitive to changes within the environment and as a result are suffering some of the biggest losses of species.

There is no simple explanation as to what causes amphibian declines (Stuart *et al*. 2004). Combinations of various anthropogenic factors are however the likely root (Lips *et al.* 2005b). Identified threats are many and varied and can be divided into two classes (Kriger 2007). Class I causes, including habitat destruction, alien species and over-exploitation, have been negatively affecting amphibians for well over a century and the ecological mechanisms underpinning them are well understood. Class II causes include climate change, contaminants and infectious disease and are less well understood and are likely to interact both with each other and with class I threats (Collins & Storfer 2003). Improved knowledge about these causes is essential to understanding why amphibians are at risk and hence for designing amphibian conservation plans (Semlitsch & Rothermel 2003). Effective solutions to this overwhelming crisis must be implemented as soon as possible and include ecological, economic and socio-political aspects (Gascon *et al*. 2007; Hero & Kriger 2008).

1.3.1 Habitat destruction, alteration and fragmentation

Habitat loss is the most significant threat to biodiversity, affecting amphibians by a factor of four over the next largest threat, pollution (Cushman 2006; IUCN 2011). Furthermore, nine out of ten threatened species are experiencing declines because of habitat loss (Dodd & Smith 2003). Habitat loss and/or degradation occur due to a wide range of human activities. Humans are continuously altering the natural landscape for development of infrastructure or land-clearing for agriculture or harvesting of natural resources. These land use changes can directly result in local and even regional extinction of populations/species by killing organisms, removing habitat or preventing access to breeding sites (Collins & Storfer 2003).

South African species are no exception, with the vast majority (well over 50%) of threatened species being affected by habitat loss caused by agricultural activities; urban development and biological resource use (Measey *et al.* 2011a). Freshwater ecosystems, in particular, are being severely degraded, with rivers and wetlands among the most threatened systems globally (Vitousek *et al.* 1997), and particularly in South Africa (Amis *et al.* 2007). Despite being globally threatened, freshwater ecosystems have been accorded less protection than their marine and terrestrial counterparts (Amis *et al.* 2007). Due to the semi-arid nature of the country, rivers are at risk of overexploitation and modification, with 48% listed as moderately modified, 26% as critically modified and just 26% as intact (DEA 2011). This inevitably has implications for freshwater biodiversity depending on river systems. Wetlands are the third most important life support system on Earth (Higgins 2003; Amis *et al.* 2007). Through their action as natural filters they provide essential ecosystem services including moderating run-off, flood attenuation, reduce erosion, recycle nutrients and gradually release purified water back into the system. They also provide important habitat to many species, not least of all amphibians. As a result, freshwater biodiversity is under severe pressure and it is estimated that approximately 30% of freshwater vertebrate species have already become extinct or are threatened (IUCN 2011). In South Africa, 44% of major rivers are Critically Endangered, highlighting the need to include freshwater ecosystems into overall conservation planning (Amis *et al.* 2007).

Although all amphibians rely on freshwater to some extent, and conservation of fresh water resources is key to preserving amphibian biodiversity, it is also important to maintain a holistic approach and incorporate terrestrial systems. Contrary to popular perception, not all amphibians exhibit the biphasic lifestyle in which eggs are laid and developed in water with adults being mostly terrestrial (Duellman & Trueb 1994). Some species are entirely terrestrial, while others only make contact with water for a very short period in their life cycle. At the other end of the spectrum, some amphibians are confined almost entirely to water throughout their lives (Dodd & Smith 2003). Understanding the array of complex life histories of different species and identifying niche habitat usage is a further necessity for conserving amphibians.

Habitat destruction entails the complete elimination of an ecosystem resulting in a loss of biological function (Dodd & Smith 2003). Urban development is one of the main causes of habitat destruction and can have drastic effects on species richness and abundance. Such land use changes are usually irreversible. For amphibians, examples include wetland drainage and clear cutting of forest or grassland for the conversion of natural habitat to parking lots, housing developments and agricultural developments. Vast areas of wetland have been lost throughout the world and in South Africa it is estimated that at least 50% of wetlands have been destroyed (Cowan 1995; DEA 2011). A dearth in information regarding the state of wetlands in the country impedes the ability to adequately protect them; 10% of wetlands in South Africa are fully protected and another 8% are partly protected. 16% of the wetlands have no legal protection and no information is available for 66% of wetlands (DEA 2011). Wetlands are important amphibian habitat and the widespread elimination of wetlands will certainly have impacted on amphibian populations.

Slightly less devastating is habitat alteration whereby adverse changes are made to an ecosystem, but which may not be permanent (and may be restored). For example, over-grazing caused by livestock which results in trampling of vegetation and soil erosion. Concomitant to habitat destruction is habitat fragmentation, which results in the isolation of remaining populations. Habitat fragmentation, for example that which is caused by roads, is of particular concern since it decreases dispersal, thereby reducing genetic diversity and increasing extinction risk (Gibbs 1998; Cushman 2006). Maintaining habitat connectivity, and thereby the processes of juvenile dispersal and immigration, is thus key to regional persistence of amphibian populations (Marsh & Trenham 2001; Rothermel & Semlitsch 2002)

Road traffic as a threat to amphibians is well-documented (Fahrig *et al.* 1995; Eigenbrod *et al.* 2009). Roads represent a major physical barrier to amphibians both in terms of their contribution to habitat fragmentation (Forman & Alexander 1998; Hilty *et al.* 2006; Holderegger & Di Guilo 2010) and direct road mortality of amphibians as they migrate between breeding and foraging habitats (Hels & Buchweld 2001; Gibbs & Shriver 2003; Sutherland *et al.* 2010). In South Africa, there are approximately 754 600 km of roads, which bisect habitats throughout the country. Data on the impact of vehicle collisions are few, but recent studies show that road kill rates are high even within a short time-frame, with amphibian mortalities highest in the wet summer months (W. Collinson, pers. comm.). Roads directly contribute to mortality, especially of amphibians with high vagility, i.e. those that are more active in terms of movement, such as toads, since they are more likely to encounter roads (Carr & Fahrig 2001). With regard to fragmentation, roads create isolated patches and smaller populations which are more vulnerable to stochastic environmental events which may lead to local extinction (Wilcox & Murphy 1985; Lande 1988). Furthermore, road traffic noise also impacts on calling traits of anurans and thereby has an effect on breeding behaviour and, ultimately, on reproductive success (Bee & Swanson 2007: Lengagne 2008; Parris *et al.* 2009; Hoskin & Goosen 2010)

1.3.2 Invasive species

The spread of alien species around the world due to humans has occurred at an unprecedented rate in the last century. Human-introductions of alien species occur both intentionally and inadvertently through trade and travel. Although it is estimated that only one in a thousand introduced species becomes established, invasion by exotic species on an ecosystem is one of the major threats to global biodiversity (Cox 1997; Cockburn *et al.* 2008). Again, amphibians are particularly sensitive to such invasions (Garner *et al.* 2006) and there is evidence that the negative impacts of introduced species exacerbate the effects of other threats (Amphibia Web 2012). Invasive species are one of the biggest impacts on freshwater systems in most parts of the world (Kiesecker 2003). Considering amphibians" dependence on freshwater, invasive species are recognised as an important global problem contributing to amphibian declines.

Direct mechanisms whereby alien species impact negatively on native species include: predation, competition for resources, co-introduction of novel pathogens, alteration of preexisting disease dynamics and hybridisation (Collins & Storfer 2003; Cunningham *et al.* 2003). Well known examples of introduced frog species having a negative effect on native amphibian communities are the North American Bullfrog, *Rana catesbieana*, the African Clawed Frog, *Xenopus laevis*, and the Cane Toad, *Bufo marinus*. All have been able to successfully colonise ecosystems beyond their native ranges (Kiesecker 2003; Weldon & Fisher 2011). Aside from impacting directly on native amphibian communities through predation and competition, these species are thought to be effective vectors of the causal agent for the disease chytridiomycosis (Weldon *et al.* 2004; Garner *et al.* 2006; Weldon & Fisher 2011).

In South Africa the African Clawed Frog, *Xenopus laevis*, the Guttural Toad, *Amietophrynus gutturalis*, and the Painted Reed Frog, *Hyperolius marmoratus*, have moved beyond their historical ranges in the east and are invading the Western Cape (de Villiers 2006; Tolley *et al.* 2008). It is likely that these range extensions have been human-mediated. Whether these invasions are having an impact is not yet known, but each of these species has a congener species in the Western Cape (the Cape Platanna, *Xenopus gilli*, the Western Leopard Toad, *Amietophrynus pantherinus* and the Arum Lily Frog, *Hyperolius horstockii* respectively) with which the non-native species may compete directly for food and habitat (S. Davies, pers. comm.). For example, *A. gutturalis* tends to dominate breeding sites and may compete with *A. pantherinus*. Furthermore, *X. laevis* is known to hybridise with *X. gilli*, posing an additional threat to this restricted species (Picker 1985; Picker *et al.* 1996). Awareness of, and efforts to control, unintentional introductions of frogs beyond their native ranges must form part of conservation plans for South Africa"s frogs (Measey 2011).

The introduction of over 160 predatory fish species to 120 countries worldwide is a major global threat to amphibian biodiversity (Kiesecker 2003). The introduction of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) into South African rivers and water bodies for recreational fishing does appear to have an impact on frog communities. For example, the Phofung River Frog (*Amietia vertebralis*) in the Lesotho Highlands occurs only in smaller rivers and tributaries not accessible to trout, or above waterfalls, which inhibit the movement of trout (M. Cunningham, pers. comm.).

In total, 37 % of South African frogs are threatened by invasive species (Measey 2011). This proportion is much higher than the global average of 15.7%. Alien vegetation, including afforestation, is especially problematic to South African frogs affecting 40 of 43 species that are impacted by invasive or problematic species and genes (Measey 2011). A major repercussion brought about by the presence of alien vegetation is increased fire intensity, which has been shown to adversely affect frog communities (Ashton & Knipps 2011). Long-term monitoring of frogs in the fire-prone vegetation of the Cape Floral Region is revealing information on the effects of fire on frog populations; severe fires appear to eliminate populations and delay re-colonisation more than that of controlled fires (Turner & de Villiers 2011). Management of burning regimes as close to natural fire frequency and intensity as possible is necessary for maintaining species richness and abundance (Parr & Chown 2003; Ashton & Knipps 2011).

1.3.3 Commercial over-exploitation

Amphibians are harvested for a number of uses around the world including food, medical research, and the pet trade. Frog legs as food are very popular in Asia, Europe, North America and Canada (Jensen & Camp 2003), resulting in the deaths of tens of millions of frogs yearly. For example, over six million Chinese Edible Frogs (*Hoplobatrachus rugulosus*) were imported from Thailand to Hong Kong in a single year (Hero & Kriger 2008), while during the five year period, 1996 – 2001, 25 million live amphibians were imported into the United States from South America (Cunningham *et al*. 2003). Another interesting example of over-exploitation of frogs for food is that which took place in North Korea during the famine of the 1990s, with the desperation of starving people resulting in frog populations in the country being totally decimated, despite frog not previously being part of the Korean diet (Demick 2010). More often than not, harvesting and trade of frogs in developing countries is unregulated, with the vast majority of these animals being removed from the wild. Even in countries where collecting and trade are banned, poaching remains a problem (Jensen & Camp 2003).

The trade of amphibians as pets has also increased significantly in recent decades, especially for rare and brightly coloured exotic species. There are an estimated 278 amphibian species currently traded for the pet market, most of which are wild-caught (Stuart *et al*. 2008). This increase in demand has negatively affected natural populations in Madagascar, South America and Asia. Despite most of these sought-after species (*Mantella* and *Dendrobates* species in particular) being listed by CITES, trade in wild-caught animals continues to be a significant threat (Jensen & Camp 2003). The Giant Bullfrog (*Pyxicephalus adspersus*) is the most popular southern African species for the pet trade and is sold illegally in pet stores around the world, especially in Europe (C. Yetman, pers. comm.). Furthermore this large species and the closely related African Bullfrog (*P. edulis*) are harvested for food in various regions of southern Africa (Channing *et al*. 1994; Channing 2001; Minter *et al*. 2004).

Amphibians, especially frogs, are also widely used for education (in the form of biology class dissections) and medical research (Jensen & Camp 2003). *Xenopus laevis* has been exported from South Africa for use in laboratories worldwide, first for human pregnancy testing (Shapiro & Zwarenstein 1934) and subsequently for potential pharmaceutical applications using their skin peptides as antibiotics (Gibble *et al*. 2007). In the 1990s, this species became the most widely used amphibian in research, especially for molecular biology, embryology and ecotoxicology (Weldon *et al*. 2007). On average, more than 10 000 wild-caught *X. laevis* are still exported annually to over 30 countries (Weldon *et al*. 2007). This poses a real threat to native frogs in the destination countries in terms of the ability of *X. laevis* to establish feral populations and its role as a reservoir host for the amphibian chytrid fungus.

1.3.4 Pollution

Pollution includes all chemical and radioactive forms of air, soil and water contaminants. These can act on a local (e.g. pesticides and eutrophication), regional (acid rain) and global (carbon dioxide emissions and climate change) scale (Kiesecker 2003). The freshwater habitats of the earth are under severe pressure and are being contaminated by numerous forms of pollution including, human waste, chemicals and pesticides. Water quality in South Africa is rapidly declining as a result of the country already being highly water-constrained, coupled with ongoing development and the fact that large cities are located on watershed divides rather than rivers, lakes or the coast. The effluent return from these areas is so heavily polluted that it is unfit for human or industrial consumption let alone habitable for freshwater biodiversity (Turton 2008). A loss of skilled personnel and withdrawal of government funding from water quality studies is further compounding the problem (Turton 2008).

Wetlands, important amphibian habitat, are impacted by nutrient enrichment (eutrophication), sediments, acidic compounds, heavy metals and biocides (Cowan 1995). Anurans are essential in the maintenance of wetlands due to their utilisation of multiple trophic levels;

larvae are primary consumers of algae, adults are predators of invertebrates while they in turn are preyed on by a vast array of animals. Pollution of wetlands inevitably disrupts this balance, while complete wetland destruction is likely to cause local extinction of resident species due to their limited dispersal ability (Channing & Van Dijk 1995). Eutrophication is usually caused by excess nitrogen and phosphorous (from fertilizers and sewage) and results in proliferation of nuisance aquatic plants, especially blue-green algae. This over-abundance of phytoplankton interferes with nutrient cycling and leads to a break down in wetland functionality ultimately excluding original fauna and flora. Nutrient enriched water is also directly responsible for the prolific growth of invasive aquatic plants, which also threaten fresh water biodiversity (Henderson 2010). Eutrophication and acid mine drainage are two of the biggest problems threatening freshwater systems in South Africa (Turton 2008).

The vast majority of amphibians are directly dependant on fresh water for their larval development, and in some taxa, for their entire lives (Vences & Kohler 2008). This dependence on freshwater and their limited mobility and thin, semi-permeable skins make amphibians particularly prone to pollution (Hayes *et al*. 2006). Studies show that amphibians are usually more sensitive than other vertebrates to contaminants, especially metals and combinations of organic chemicals (Boone & Bridges 2003). The liberal use of pesticides (herbicides, insecticides and fungicides) throughout the world for enhancing food production has had negative impacts on “non-target” organisms (Alford & Richards 1999; Gallant *et al*. 2007; Yordanova *et al*. 2009). Experimental investigations have shown that pesticides can have profound effects on aquatic communities (Relyea 2005a). Roundup, a widely used herbicide, has been shown experimentally to cause high rates of mortality of both tadpoles (up to 100% mortality in three weeks) and post-metamorphic anurans (79% mortality in one day). The high mortality appears to be caused directly by toxicity, rather than indirectly due to reduction in algal food resources (Relyea 2005b). While the effects *in situ* have not been assessed, the outcomes of the effect of Roundup are likely to be complex depending on the combination of surfactants used, timing of application, and changes in amphibian sensitivity with development. The herbicide, atrazine, is also widely used and one of the most common contaminants of ground and surface water that is active at low, ecologically relevant concentrations (Hayes *et al*. 2006). Its effect as an endocrine disruptor in amphibians has been well-studied and has been shown to both inhibit growth and cause immune-suppression, making animals more susceptible to pathogens.

Aside from acting on organisms at the point of application, pesticides can also be transported by leaching, wind and through the food chain and are able to persist for long periods and thus may account for some of enigmatic amphibian declines in otherwise pristine areas. Pesticides can also act indirectly on amphibians, for example, herbicides may deplete algal food resources that tadpoles rely on, ultimately affecting the population (Boone & Bridges 2003). Pesticides can also affect amphibian life cycles by inhibiting metamorphosis size and growth, leading to decreased egg production. Moreover, in reality pesticides tend to have much greater effects when present in low concentrations (0.1 ppb) and in combination (as is usually the case in the wild, especially in agricultural areas) than when acting individually (Hayes *et al*. 2006). Not only is larval growth retarded, but time to metamorphosis is increased and size at metamorphosis is smaller than that of metamorphs not exposed to the chemical mixture (Hayes *et al.* 2006). Such effects appear to be the result of endocrine disruption which ultimately affects survivorship of amphibian populations by delaying metamorphosis, inhibiting growth and reducing adult recruitment and the likelihood of reproduction (Hayes *et al*. 2006; Smith 1987).

In some cases, contaminants have been linked to amphibian deformities, which appear to be increasing in incidence and becoming more widespread (Sessions 2003). These abnormalities usually include multiple or missing digits/limbs in anurans, and may be an indication of the environmental problems related to amphibian declines and may represent a bigger risk to life in general, including humans. The effects of contaminants are also compounded by multiple factors including biotic interactions (e.g. predation, competition and disease) and other environmental stressors such as UV radiation. All of these factors need to be considered when assessing the link between amphibian declines and pollutants (Boone & Bridges 2003).

1.3.5 Climate change

The mean global surface temperature has increased by 0.74 °C during the last century (1906 – 2005), with the most rapid warming occurring in the last three decades (Jones *et al.* 2001). This rate of change is greater than that of any other century over the last millennium and this decade has been the hottest on record (Hansen *et al*. 2010). Predicted increases in temperature for this century are estimated at between 1.8 °C and 4 °C, with even the lower end of this scale liable to have devastating consequences for life on Earth (Walther *et al.* 2002). This increase is the result of unprecedented amounts of greenhouse gas emissions due to anthropogenic activities, as well as destruction of forests (Solomon *et al.* 2007). Total anthropogenic greenhouse gas emissions have increased by 70% since 1970 and far exceed the natural range over the last 650 000 years (IPCC 2007). At the same time the protective ozone layer has been depleted through use of CFCs, increasing damaging UV-B radiation (Blaustein *et al.* 2003b). Considerable further increases in temperature are expected, with devastating consequences for life on Earth unless drastic changes are made to human energy-use policies (Roberts 2011). Weather patterns are predicted to become more erratic and the frequency of extreme weather events such as tornados will increase. Precipitation patterns are changing and cloud cover has changed over middle and high latitudes (IPCC 2007). Growing evidence shows that these climatic changes are affecting biological systems worldwide.

The link between amphibian declines and climate change has been a subject of recent debate amongst scientists (Pounds *et al.* 2006; Alford *et al.* 2007) and it is widely accepted that changes in climate can have multiple effects on amphibians at the individual, population and community levels (Corn 2005; Minter 2011). Amphibians are heavily reliant on precipitation and the seasonality thereof as breeding cues and as a result some amphibian communities are being directly affected by climate change (Pounds & Crump 1994; Lips *et al.* 2006). This is particularly noticeable in montane regions, where species physiological tolerances to changes in temperature and/or precipitation have resulted in mortality (Carey & Alexander 2003). Warming trends also appear to be altering the breeding behaviour of amphibians, with some species in temperate regions in the Northern hemisphere responding to climate change by breeding earlier (e.g. Beebee 1995; Gibbs & Breisch 2001). Increased UV radiation is also detrimental to amphibians, both directly (for example by decreasing hatching success) and through interacting with other stressors (Blaustein *et al.* 2003b).

In addition, there is already evidence of tropical habitats moving both laterally and to higher altitudes as temperatures increase, resulting in shifts in the spatial distribution and ranges of several species (Pounds *et al.* 1999). In the past, species could move unhindered but now these shifts are often blocked by human development through habitat removal, amplifying the effects of climate change (Dawson *et al.* 2011). Thus despite amphibians having survived previous rapid climate changes, such as the glacial-interglacial transition some 12 000 – 20

000 years ago, the present situation presents multiple challenges that may result in extinction (Corn 2005; Minter 2011). As in the case of most threats to biodiversity, the effects of climate change are compounded by synergistic factors, such as species interactions in relation to various climate variables (Lips *et al.* 2008). In particular, the relationships between pathogens and their hosts are likely to be impacted, since temperature, rainfall patterns and humidity levels influence pathogen activity (Donnelly & Crump 1998; Daszak *et al.* 2003). Increased temperatures are expected to influence disease virulence as well as accelerate spread of disease, making additional hosts susceptible (Pounds *et al.* 2006). Changes in precipitation and increased UV radiation may also affect the immune systems of amphibians (radiation is also detrimental to amphibians, both directly and through interacting with other stressors (Blaustein *et al.* 2003b). Understanding the relationship between amphibian pathogens, especially *B. dendrobatidis*, and environmental change will be essential in addressing amphibian declines (Daszak *et al.* 2003).

Although the effects of climate change on amphibians are complex, the current situation will undoubtedly have far reaching consequences for many amphibian species (Minter 2011). In South Africa, increasingly arid conditions in the western regions are predicted to result in range contractions of multiple species, while increasing temperatures and rainfall in the east are also likely to result in altitudinal shifts in distribution (Midgley *et al.* 2001; Joubert 2011; Minter 2011). Environmental changes have occurred over millions of years and amphibians have persisted. The declines and extinctions that are now taking place are occurring because most of the threats causing them have come about in the last 100 years – a time span that has not allowed amphibians to adapt. Amphibian declines are indicative of the escalating environmental problems, including climate change, loss of biodiversity, toxification of the planet and rapid human population growth that need to be tackled urgently in order to curb the 6th mass extinction (Ehrlich 2010).

1.3.6 Emerging infectious diseases

Human mediated outbreaks of diseases threatening both wildlife and humans have increased in recent years. These outbreaks have been linked to environmental change, changes in human behaviour and changes in human demography which facilitate movement of pathogens outside of their native geographic or host range (Cunningham *et al.* 2003;

Daszak *et al.* 2007). Infectious diseases are now known to be the direct cause of many amphibian declines in relatively undisturbed areas (Laurance *et al.* 1996; Lips 1999; Carey *et al.* 2003). Diseases have always played a role in regulating populations, however, the recent amphibian die-offs attributable to emergent disease are unusual in that they are occurring on a global scale, in a wide variety of ecosystems and affecting large numbers of diverse species (Carey *et al.* 2003). Two pathogens have fulfilled Koch‟s postulates in terms of their ability to infect and kill amphibians, namely the chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), and ranaviruses. Both have been identified as being responsible for mass mortalities of all life stages of multiple amphibian species and have been recognised by the World Organisation for Animal Health (OIE) as having particular importance in the international trade of amphibians (OIE 2011).

Ranavirus is a genus of iridoviruses and that is believed to have caused die-offs of anurans and salamanders on all continents except Africa (Daszak *et al.* 1999; Daszak *et al.* 2007; spatialepidemiology.com). *Ranavirus* epidemics are associated with explosive die-offs and high mortality rates. They are able to survive for long periods in the absence of a host and appear to be more common in anthropogenically disturbed habitats. It is likely that the spread of *ranavirus* is mediated by humans, either directly (for example on fishing gear) or indirectly through the introduction of infected fish or frogs for the pet trade (Cunningham *et al.* 2003). In most incidences *Ranavirus* outbreaks occur in abundant, non-threatened host species (Daszak *et al.* 2007) and as yet there are no records of extinction of amphibian populations due to ranaviruses (Carey *et al.* 2003).

1.3.7 Chytridiomycosis

The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) causes the disease chytridiomycosis and has been identified as a major cause of amphibian mass mortality events and declines worldwide (Berger *et al.* 1998; Berger *et al.* 1999, Briggs *et al.* 2010; Skerratt *et al.* 2007). *Batrachochytrium dendrobatidis* (phylum Chytridiomycota, class Chytridiomycetes, order Chytridiales) is the only member of its phylum known to infect vertebrates while others are free-living saprobic detritivores or are pathogens of plants or insects (Kagami *et al.* 2007; Daszak *et al.* 2007). This organism was isolated from the epidermis of a blue poison dart frog (*Dendrobates azureus*) and described as a new genus and species of chytridiomycete by Longcore *et al.* in 1999. The fungus has three life stages: an aquatic, motile infectious stage known as a zoospore; a parasitic phase that grows in epidermis of the host, the thallus; and the zoosporangium that discharges new zoospores (Berger *et al*. 2005a). The discharge tubes of the sporangia generally project to the skin surface and are thus able to release zoospores to the environment. The zoospore is described as spherical with one or many cytoplasmic extensions and a posterior flagellum (Longcore *et al*. 1999). The life cycle is complete within 4 to 5 days (Berger *et al*. 2005a).

The fungus infects keratinised skin of the pelvic patches, ventral surfaces, digits and webbing of post-metamorphic amphibians (Longcore *et al*. 1999; Pessier *et al*. 1999), and the keratinizing tissue of tadpole mouthparts (Figure 1.2) (Berger *et al*. 1998; Altig 2007). Symptoms vary between species and are usually not apparent in wild animals until close to death. As infection progresses, clinical signs include sloughing and lesions of the skin, neurological abnormalities, general lethargy and sitting in a characteristic position whereby the hind-legs and drink patch are elevated above the ground (Schloegel *et al*. 2006). It is diagnosed histologically by cell loss, erosions and thickened stratum corneum containing chytrid zoosporangia (Figure 1.3) (Ouellet *et al*. 2005).

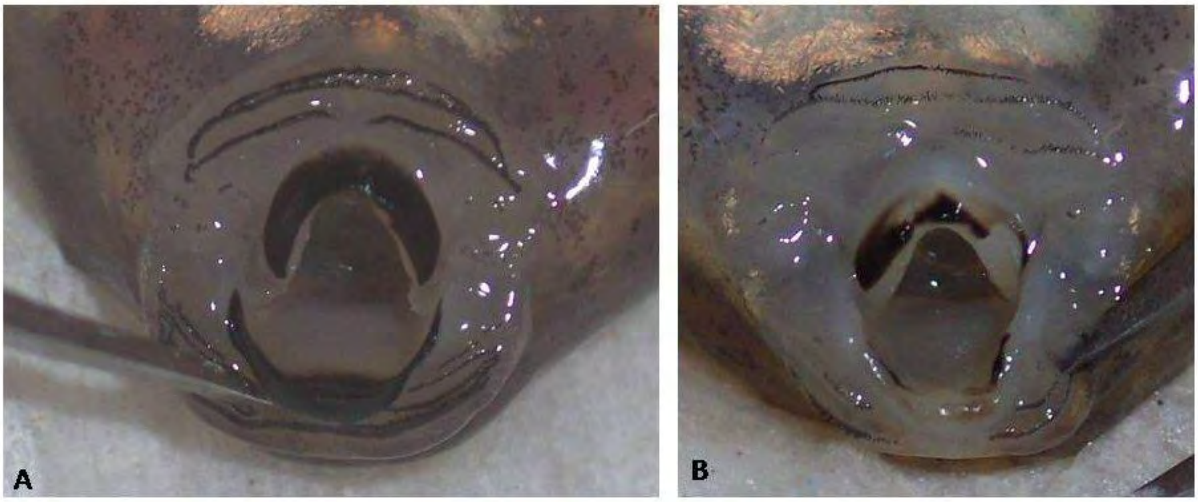

Figure 1.2: Tadpole mouthparts of *Amietia angolensis*, A) without infection with *Batrachochytrium dendrobatidis,* B) depigmentation due to *B. dendrobatidis* infection.

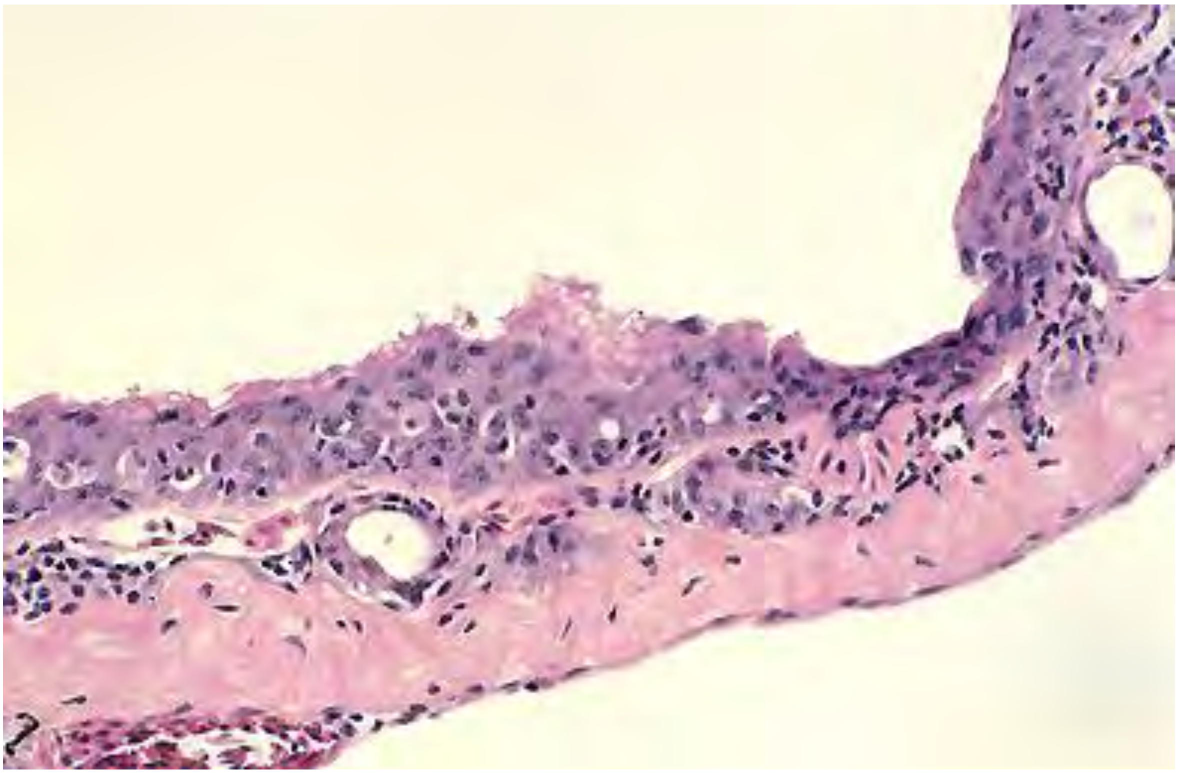

Figure 1.3: Epidermal damage in anuran skin caused by *Batrachochytrium dendrobatidis.*

It is thought that the breakdown of keratin, which plays a role in respiration and osmoregulation, may be what causes the symptoms associated with the disease. The mechanism by which *Bd* kills its host is thought to be the disruption of normal epidermal functioning by inhibiting osmosis and electrolyte transfer (Voyles *et al*. 2007). Depletion of electrolytes such as sodium, potassium and magnesium from the circulation can result in cardiac arrest and death of the host (Voyles *et al*. 2009). Since the skin is essential for maintaining homeostasis in amphibians this mechanism may explain why such a wide range of phylogenetically distinct taxa are affected by *Bd.* Experiments have shown that death of susceptible hosts occurs between 18 and 70 days following infection depending on dose, fungal strain, temperature and amphibian species (Berger *et al*. 2005a).

The usual absence of clinical symptoms makes this disease difficult to diagnose visually, especially during the early stages of infection. Histological examination of the epidermis can confirm infection but this method is painstaking and requires tissue for microscopy. Development of a *Bd* specific DNA-based assay using polymerase chain reaction has greatly enhanced detection capability and can be used for large numbers of samples (Annis *et al.*

2004). Detection by real-time Taqman PCR is even more sensitive than conventional PCR, making detection of as little as one zoospore possible, and gives both a qualitative and quantitative measure of infection (Boyle *et al.* 2004). This method allows for detection of *Bd* 1 – 2 weeks earlier than histological methods (Boyle *et al.* 2004; Hyatt *et al.* 2007). Early detection of *Bd* is vital for controlling the spread of this disease, especially in captive populations or animals intended for international trade. Acquisition of samples for PCR is also non-invasive, simply requiring a skin swab from the animal, which can then be released alive. Swabbing has also been found to be superior to other sampling methods (i.e. filtration of water and toe-clipping) in terms of ease of use in the field, efficiency in collecting *Bd* and sensitivity (Kriger *et al.* 2006b; Hyatt *et al.* 2007). Swabbing can also be used for sample collection from tadpole mouthparts, providing a non-lethal diagnostic technique for examining infection in multiple life stages (Retallick *et al.* 2006).

Ecology of *Batrachochytrium dendrobatidis*

Establishment of *Bd* at a location typically exhibits a pattern of disease emergence whereby amphibian assemblages and ecosystems are altered, followed by the pathogen becoming enzootic and maintaining low-level persistence, usually as a transient commensal in a reservoir host (Briggs *et al.* 2010; Woodhams *et al.* 2011). Acute lethal outbreaks of chytridiomycosis in amphibian communities appear to have complex causes and may be the result of underlying predisposing factors (Retallick *et al.* 2004; Ouellet *et al.* 2005). Ecological factors, particularly climate, are critical in the transition of *Bd* from an enzootic pathogen to that which causes lethal chytridiomycosis (Kilpatrick *et al.* 2009). Understanding the ecology of *Bd* is thus necessary for developing methods for controlling chytridiomycosis outbreaks and providing insight to amphibian conservation (Belden & Harris 2007). Studies have found that zoospores of this pathogen are able to survive for up to seven weeks in water in the absence of a host (Johnson & Speare 2003). A mathematical model developed by Mitchell *et al.* (2007) showed that the longer *Bd* could persist in water, the more likely it was to cause local extinction of an experimentally infected host (*Bufo bufo*). The capacity to persist for long periods may be partly responsible for the pathogen‟s ability to drive amphibian declines. The pattern of rapid die-offs observed in many infected populations may be due to saturation of the environment with zoospores as a result of frog-to-frog and frog-environment transmission and the ability of zoospores to persist outside of the host body and be transferred between host taxa.

Several studies have tested the thermal preferences of *Bd* and suggest that the fungus favours cooler temperatures: *in vitro* experiments show that the fungus can grow and reproduce at temperatures between 6 and 28 °C (Longcore *et al*. 1999), with optimal temperature between 17 and 25 °C (Piotrowski *et al*. 2004). The fungus can survive freezing, but above 29°C cultures fail to achieve growth and above 32°C the fungus is killed within 96 hours or after 4 hours at 37°C (Johnson *et al*. 2003). Pathogenicity in live frogs peaks at temperatures below 19°C and decreases steadily above 23°C (Kriger & Hero 2007). It is unable to withstand complete desiccation (Johnson *et al*. 2003). These findings support field observations that *Bd* may be more virulent at higher altitudes or during the winter months while at lower elevations and higher temperatures it behaves saprophytically (Berger *et al*. 2004; Pounds *et al*. 2006).

The link between climate change and pathogen-caused amphibian declines, known as the "climate-linked epidemic hypothesis", has been investigated by several authors (e.g. Kiesecker *et al*. 2001; Carey & Alexander 2003) and there is much controversy regarding whether global warming causes outbreaks of *Bd* (Corn 2005; Bustamante *et al*. 2010). The climate-linked epidemic hypothesis predicts that increased amphibian declines are observed in association with unusually warm years, since temperature influences disease dynamics. Chytridiomycosis, however, appears to work against this theory since it generally becomes more pathogenic at lower temperatures. Pounds *et al*. (2006) tested this hypothesis by examining declines and extinctions of Harlequin frogs (*Atelopus*) in Central and South America and suggested that climate shifts, especially cloud formation, at a local or micro-scale create favourable conditions for *Bd* despite overall warming (Herrera *et al.* 2005). Furthermore, temperatures at elevations where most *Atelopus* extinctions have occurred are shifting toward the growth optimum for *Bd*. Environmental factors thus play an important role in mediating the impact of *Bd*, indicating that seasonal sampling is necessary for determining the full extent of invasion potential of this pathogen (Carnaval *et al*. 2006; Kriger & Hero 2007). Furthermore, since the immune defences of amphibians decrease in lower temperatures, they may be more susceptible to chytridiomycosis when the fungus reaches optimal pathogenicity (Kriger & Hero 2007). Understanding the thermal restrictions of *Bd* has important implications for host-parasite ecology and the effect of potential chytridiomycosis outbreaks on wild amphibians (Kriger & Hero 2006).

Aside from international trade in animals, other anthropogenic activities such as land disturbance have been associated with the emergence of wildlife diseases, and this may also be the case with the introduction of *Bd* to previously unaffected areas (Daszak *et al*. 2004). Scientists believe that chytridiomycosis will continue to drive amphibian extinctions at an unprecedented rate and that human activity may be creating conditions that are cumulatively causing amphibians to become increasingly susceptible to this disease (Daszak *et al*. 2000; Gascon *et al*. 2007).

Infection Pathogenicity and Host Susceptibility

Not all amphibian species are equally affected by infection with *Bd*; some species, or populations within species, decline (or are even eliminated) while others persist (e.g. Hopkins & Channing 2003; Kriger & Hero 2006; Tobler & Schmidt 2010). This variability in outcome of infection can differ among species and among populations within a species (Briggs *et al*. 2010; Tobler & Schmidt 2010). Host susceptibility is due to a number of factors, including environmental conditions, host response and strain variation in *Bd*. The severity of infection is related to climatic conditions including temperature, humidity and altitude (Kriger 2007; Woodhams *et al*. 2007). Amphibians are ectotherms, and as such, their physiology is directly determined by their external environment. Their immune system, in particular, is affected by temperature and the interaction between *Bd* and the host is likely to be temperature dependent (Fisher 2007). Studies have found that changes in disease status was strongly correlated with changing climatic conditions and that infected frogs survived longer or were even able to clear infections in warmer conditions (Kriger & Hero 2006; Bustamante *et al*. 2010). As discussed above, infections have also been observed to be more severe at high altitudes. These conditions are associated with immune-suppression in frogs (Carey *et al*. 1999). Similar observations have been made in South Africa, where low-altitude populations are able to persist in the presence of infection. This implies that climatic conditions in these instances do not favour lethal outbreaks and/or that species have developed sufficient resistance to the disease.

Resistance to chytridiomycosis and disease in general, is determined by innate immune response of the host species, and this too can vary between and within species. Variation in mortality rates within populations of the same species are likely to be influenced by individual host characteristics and genetic differences between populations (Tobler & Schmidt 2010). Amphibian skin contains an array of both proteins (peptides) and natural microbiota that assist with protection from disease. Peptides are produced in granular glands of the skin and secreted into the mucous layer (Rollins-Smith & Conlon *et al*. 2005). These antimicrobial peptides vary among lineages of amphibians and thus influence survival ability among species (Apponyi *et al*. 2004; Garner 2007). The antimicrobial peptides from some amphibian families are effective at inhibiting growth of *Bd in vitro*, suggesting that some species have greater resistance than others due to their compilation of peptides (Rollins-Smith & Conlon *et al*. 2005; Conlon *et al*. 2007; Woodhams *et al*. 2007). Indeed, experimental studies have shown that species that have suffered population declines lack *Bd*-resistant peptides that are present in resistant species (Carey *et al*. 2006; Woodhams *et al*. 2006a; b). In addition, certain cutaneous bacteria have been identified as being able to inhibit *Bd* growth *in vitro* (Harris *et al*. 2006; 2009). This microbial skin community is affected by environmental changes such as climate change or chemical pollution, allowing *Bd* to more effectively colonize the host‟s skin, providing a possible explanation for why some chytridiomycosis outbreaks have been lethal.

Another factor influencing pathogen efficacy is variation in the strain and passage history of *Bd* (Berger *et al*. 2005b). Although isolates from different parts of the world have been found to be genetically very similar, some differences have been found in the physiology of different isolates. Controlled infection experiments with different isolates *in vitro* have shown that time till death and weight-loss caused by infection varies with strain (Berger *et al*. 2005b; Retallick & Miera 2007). These results suggest that variation occurs even at small geographical scales and may explain some variation in host response. Furthermore, some non-lethal effects of chytridiomycosis can be non-permanent and reversible (Retallick & Miera 2007). Other factors that can affect mortality rate include fungal dose and the age of hosts (Berger *et al*. 2005b).

Treatment and Prevention

Although growing research has revealed much about the ecology of *Bd*, more knowledge is needed on the dynamics of infection in the wild (Gascon *et al*. 2007). This information can be used in developing treatments for infected populations and hygiene protocols to limit spread. Elimination of *Bd* is not practical because of continued reintroduction risk and treatment of individuals in the wild is fraught with difficulty (Daszak *et al*. 2007; Woodhams *et al*. 2011). As such, *ex situ* treatments are more likely to be beneficial to those species facing imminent threat of extinction from *Bd* (Rödder *et al*. 2009). Given the broad host range, no single mitigation strategy is appropriate for all amphibian species (Woodhams *et al*. 2011). Antifungal bacteria and peptides isolated from species that show resistance can be used as biological control by introduction of these compounds to sensitive species (Harris *et al*. 2009; Rollins-Smith & Conlon 2005). Currently, however, the most effective form of treatment is through population management, for example, by re-introducing *Bd*-free captive-bred or drug-treated animals into areas that are deemed free of the disease (Lubrick 2010; Weldon and Fisher 2011), with the goal of ultimately encouraging survival of *Bd*-resistant individuals. Drug treatments that have so far been effective include 0.01% itraconazole baths (Nichols *et al*. 2000; Pessier & Mendelson 2010), 1% sodium hypochlorite (chlorine bleach) and quaternary ammonium compounds. Heat (60°C for 5 min) and desiccation (Johnson *et al*. 2003; Webb *et al*. 2007) is effective at killing the fungus in culture. Additional research into innate immune response will also provide knowledge necessary for conservation action by prioritising *Bd*-sensitive species for *ex-situ* breeding programs and providing information on species-specific peptide profiles that can be used in modelling disease dynamics (Woodhams *et al*. 2006b; Garner 2007).

Researchers also need to be extremely cautious of spreading chytridiomycosis whilst in the field (*in situ*) or when working with captive species (*ex situ*) (Walker *et al*. 2008). This is important to prevent the spread of the disease to areas it may not already be present, to prevent spread within and between wild populations of frogs and to control the disease in ex-situ situations. Hygiene protocols using best-practice procedures have been developed for personnel and researchers who work with frogs or in aquatic environments (e.g. NSW 2001). *In-situ* management requires that workers recognise boundaries between sites and take appropriate measures to reduce risk of spreading infection between such sites. This is particularly important in the case of threatened frog species, where protocols should be applied over very short distances (NSW 2001). Equipment must be cleaned and disinfected at the commencement of fieldwork and between sites. Even in regions where chytridiomycosis outbreaks do not appear to be problematic (such as South Africa) these practices must be implemented to prevent potential disaster. Awareness of *Bd* infection with regard to the trade of amphibians is also crucial to stem the spread of this devastating pathogen (Weldon and Fisher 2011).

1.3.8 Synergistic effects

Often, the threats discussed above work in concert to produce synergistic effects, exacerbating the problem of amphibian conservation (Gascon *et al.* 2007; Hero & Kriger 2008). For example, increased UV-B radiation can make frogs more susceptible to fungal infection (Kiesecker *et al.* 2001) or may make amphibians more susceptible to the negative effects of pesticides (Boone & Bridges 2003; Hayes *et al.* 2006); climate change may facilitate optimum temperatures for *B. dendrobatidis* in certain regions (Pounds *et al.* 2006) and different combinations of pesticides may result in amphibian deformities (Sessions 2003). Similarly, the effect of pesticide exposure is very likely to make amphibians more susceptible to pathogens, due to the inability to ellicit a proper immune response (Hayes *et al.* 2006). Habitat destruction is likely to compound the effects of climate change by limiting species‟ dispersal, while increasing temperatures are likely to facilitate the spread of disease (Pounds *et al.* 1999).

Most research however, has focussed on individual factors with little attention paid to such interactions (Sparling *et al.* 2003; Hayes *et al.* 2006). A change in this approach is necessary if problems are to be identified and solutions sought. Most of these complex interactions are context dependent and this is important to understand when attempting to mitigate such threats (Hero & Kriger 2008). In most cases, more research into life history ecology and a better understanding of habitat requirements and connectivity is required to protect amphibians (Cushman 2006). In addition, knowledge of conservation genetics, population structure and restoration of altered landscapes is lacking. A science-based landscape approach to conservation is thus needed for effective management of amphibian habitat (Dodd & Smith 2003).

1.4. Frog Conservation in South Africa

South Africa is an arid country with an average annual rainfall of 497 mm, well below that of the global average of 860 mm (Cowan 1995). Despite this, southern Africa has a relatively rich diversity of amphibians with 160 known species (du Preez & Carruthers 2009). These are represented only by the order Anura (frogs) and exhibit a vast range of morphologies and life history traits across 13 families (du Preez & Carruthers 2009). The highest species richness for frogs occurs in KwaZulu-Natal and the highest numbers of threatened species occur in the Western Cape (Measey *et al.* 2011a). Both areas are recognised as being important for both frog endemism and having high levels of human activity (Minter *et al.* 2004; Driver *et al.* 2005). Overall, 43% of South African frog species are endemic to the country and of these, 35% are in a threatened category; all Critically Endangered and Endangered species are endemics and only one species within the Vulnerable category is not endemic (Measey *et al.* 2011). Furthermore, within the Afrotropical realm (south of the Sahara) South Africa is ranked fourth in terms of number of Threatened species (Stuart *et al.* 2008).

The IUCN Red Listing procedure provides a standardised approach for focussing attention on threatened species and providing a system of setting and maintaining benchmarks for environmental impact assessments (Scott-Shaw 1999; IUCN 2011). Listings are made according to five quantitative criteria which are based on biological indicators of populations that may be threatened (IUCN Standards and Petitions Subcommittee 2011). To list a taxon in any of the threatened categories (VU, EN or CR) only one of the criteria needs to be met (IUCN Standards and Petitions Subcommittee 2011). To date, listings for South African frogs have been made primarily according to Criteria B, namely geographic range size, and fragmentation, decline or fluctuation. These assessments are primarily based on Area of Occupancy (AOO) and Extent of Occurrence (EOO). Vast improvements have been made in recent decades in terms of Red Listing of South African frogs, although more accurate knowledge on AAO and EOO for certain species is still lacking (Measey 2011). Early assessments included only select taxa, for example, McLachlan (1978) included just eight amphibians with only one considered threatened (*Breviceps gibbosus*) and Branch (1988) included 17 species, five of which were listed as Threatened. In 2004 the publication of the Atlas and Red Data book of the frogs for South Africa, Lesotho and Swaziland coincided with the GAA and contributed one of the most comprehensive datasets into the GAA (Minter *et al*. 2004).

A re-assessment was conducted in 2009 by the South African Frog Re-assessment Group (SA-FRoG 2010) and published in 2011 (Table 1.1). Of the 117 species assessed in 2004 17% were considered Threatened (significantly lower than the proportion of globally threatened species (Minter *et al*. 2004). It is these 20 species that are included in this study. Near Threatened (5 species) and Data Deficient (8) from the 2004 assessment are not included. The 2011 re-assessment included 118 species and resulted in some significant variations to the Red Listing. Table 1 shows the 2004 and 2011 listings. It was decided for the purposes of this study to retain the 2004 listing since overall most of the species remained within the threatened categories and the project was already well under way at this stage.

Only two species, *Arthroleptella rugosa** (new species, Turner & Channing 2008) and *A. subvoce* were added to the Red List. The following species were shifted from a Threatened category to a lower category: *Afrixalus spinifrons* (VU – NT), *Breviceps gibbosus* (VU – NT), *Cacosternum capense* (VU – NT), *Hyperolius horstockii* (VU – LC) and *Strongylopus springbokensis* (VU – LC). The threatened taxa are distributed through 5 regions in South Africa, namely the Western Cape (8 species), KwaZulu-Natal (6 species), Eastern Cape (3 species), the Northern Cape (2 species) and Mpumalanga (1 species). The threatened species of KwaZulu-Natal were the focus of this study and a brief description of each is given below.

Table 1.1: Red List species of South Africa listed in order of status (2004 and 2011 assessments are shown for comparative purposes). Threatened categories: CR = Critically Endangered, EN = Endangered, VU = Vulnerable, NT = Near Threatened. Bold lettering indicates changes in status between 2004 and 2011 assessments.

Frog species	Province	Red List Category 2004	Red List Category 2011
Anhydrophryne ngongoniensis	KwaZulu-Natal	**CR**	EN
Heleophryne hewitti	Eastern Cape	**CR**	EN
Heleophryne rosei	Western Cape	CR	CR
Microbatrachella capensis	Western Cape	CR	CR
Hyperolius pickersgilli	KwaZulu-Natal	EN	**CR**
Vandijkophrynus amatolicus	Eastern Cape	EN	**CR**
Leptopelis xenodactylus	KwaZulu-Natal	EN	EN
Natalobatrachus bonebergi	KwaZulu-Natal	EN	EN
Amietophrynus pantherinus	Western Cape	EN	EN
Afrixalus knysnae	Western Cape	EN	EN
Anhydrophryne rattrayi	Eastern Cape	**EN**	VU
Xenopus gilli	Western Cape	VU	**EN**
Breviceps sylvestris	Limpopo	VU	**EN**
Breviceps macrops	Northern Cape	VU	VU
Capensibufo rosei	Western Cape	VU	VU
Hemisus guttatus	KwaZulu-Natal	VU	VU
Afrixalus spinifrons	KwaZulu-Natal	**VU**	NT
Breviceps gibbosus	Western Cape	**VU**	NT
Cacosternum capense	Western Cape	**VU**	NT
Hyperolius horstockii	Western Cape	**VU**	LC
Strongylopus springbokensis	Northern Cape	**VU**	LC
Arthroleptella rugosa	Western Cape	*	**CR**
Arthroleptella subvoce	Western Cape	DD	**EN**
Breviceps bagginsi	KwaZulu-Natal	DD	**VU**

1.5 Threatened species of KwaZulu-Natal

1.5.1 *Hyperolius pickersgilli* (Raw 1982), Critically Endangered

Hyperolius pickersgilli (Pickersgill‟s Reed Frog) is a small (max size 29 mm in females) reed frog with variable colouration (Raw 1982). Males and juveniles are usually brown in colour and are characterised by having a dark-edged light dorso-lateral band running from the snout to the hind quarters (du Preez & Carruthers 2009). Females lack the dorso-lateral stripe and are usually light green in colour (Figure 1.4). The underside is smooth and pale and the concealed body surfaces (inner thighs, toes and fingers) lack pigmentation. The snout extends only just beyond the nostrils and is slightly pointed. The call is a soft insect-like chirp issued intermittently (Bishop 2004b). The behaviour and call of this species are cryptic, often making it difficult to detect even when present.

Figure 1.4: Female Pickersgill‟s Rœd Frog, *Hyperolius pickersgilli*, from Forest Lodge, Mtunzini on the North coast of KwaZulu-Natal.

Distribution

This species is endemic to a narrow strip along the coast of KwaZulu-Natal. It is currently known from only 13 isolated sites between St Lucia in the north and Kingsburgh in the south. Only two of these sites occur within protected areas (Umlalazi Game Reserve and iSimangaliso Wetlands Park) (Bishop 2004b). It occurs up to 380 m.a.s.l within 20 km of the coast. The area of occupancy (AOO) was estimated at only 9 km^2 and the extent of occurrence (EOO) is 2,303 km^2 (Measey 2011). This study provided an opportunity to thoroughly survey the distribution of this species and refine estimates of AOO and EOO (Chapter 4)

Habitat & Life history

The species prefers densely vegetated wetland areas, typically with shallow, stagnant water, within Coastal Bushveld-Grassland (Bishop 2004b). Males call from dusk until the early hours of the morning (pers. obs.). Breeding takes place in well-concealed vegetation. Out of the breeding season the species can move up to 2 km from breeding sites for foraging and over-wintering (an important observation for conservation of the species) (J. Harvey; A. Wilken pers. comm.). Additional knowledge is required to understand the breeding biology and ecological requirements of this species. It is thought not to occur within the same vicinity as the abundant *Hyperolius marmoratus* (Bishop 2004b), although this may be as a result of inability to detect the species" call when other species are chorusing loudly (pers. obs.).

Status

The species has recently been listed as Critically Endangered (IUCN 2011) from Endangered in 2004 due to its small area of occupancy (< 9 km^2), severe fragmentation of its habitat and continuing decline in the area of occupancy, extent and quality of habitat and number of locations (Measey 2011). In addition, it is a specially protected species under the KwaZulu-Natal Conservation Management Amendment Act (1999), and is considered to be a species of importance in KwaZulu-Natal (Goodman 2000). Few populations are known from formally protected areas (Armstrong 2001), and Harrison *et al.* (2001) recognised the need to identify and protect remaining breeding *H. pickersgilli* habitats. The species has been prioritised for conservation research (Measey 2011) and is also be the first Threatened frog species in South Africa to be used in a captive breeding program (Visser 2011).

Threats

It is estimated that less than 1% of this species range falls within protected areas, and this does not represent a viable population size for the long-term survival of this species in a protected area (Armstrong 2001). As such, protection of the species at the remaining unprotected sites is critical. The species is threatened primarily by habitat loss caused by urbanisation, afforestation and drainage for agricultural and urban development (Measey 2011). Many of the historically known sites have been eliminated by either sugar cane or eucalyptus plantations (Johnson & Raw 1987; Bishop 2004b). The remaining subpopulations are small and severely fragmented and are thus subject to genetic depletion, which may be reflected in lowered larval fitness, ultimately resulting in local extinction (Hitchings & Beebee 1997).

1.5.2 *Anhydrophryne ngongoniensis* (Bishop & Passmore 1993), Endangered

Anhydrophryne ngongoniensis (Mistbelt Chirping Frog) is a very small frog, with a mean SVL for males of 16.9 mm and a maximum of 22.0 mm for females (du Preez & Carruthers 2009). The pupil of the eye is horizontal. The dorsum is sandy to golden brown with four indistinct stripes composed of small dark spots (Figure 1.5). There is a dark brown band passing from the snout, through the eye, to the axilla (Bishop & Passmore 1993). The ventrum is white and the underside of the limbs and throat is pale yellow. There is no webbing on the hind feet and males do not possess a vocal sac, making the sexes indistinguishable (Bishop & Passmore 1993). The call is a very soft cricket-like “trill” and consists of eight to ten short pulses repeated about one second apart (du Preez & Carruthers 2009).

Figure 1.5: An adult Mist-belt Chirping Frog, *Anhydrophryne ngongoniensis*, from Ngele Forest. Photograph by Clifford and Suretha Dorse.

Taxonomy

Anhydrophryne ngongoniensis is a relatively recently described species (Bishop & Passmore 1993). This species was recently found to belong to the genus *Anhyrdrophryne* rather than *Arthroleptella*, which is restricted to the Western Cape (Dawood & Stam 2006).

Distribution

This species is endemic to the high-rainfall, mid-altitude grasslands of south-central KwaZulu-Natal. It is restricted to an extremely small area (total AOO is estimated to be 9 km^2) within this mistbelt region of the eastern escarpment (Measey 2011). There are currently 12 isolated subpopulations that occur within four locations: Mpur Forest, Ngele Forest, Roelton Dam and Poortjie Forest (Bishop & Passmore 1993). The most recently discovered population at Ngele Forest appears to be the largest of all known subpopulations (Harvey 2007). Sites occur between 1,020 and 1,720 m.a.s.l and EOO is estimated at 1,525 km^2 (Measey 2011).

Habitat & Life history

Anhydrophryne ngongoniensis occurs primarily in dense grassy vegetation in Midlands Mistbelt Grassland, Southern KwaZulu-Natal Moist Grassland, Drakensberg Foothill Moist Grassland and Southern Mistbelt Forest, usually above 1,000 m (Mucina & Rutherford 2006). Sites occur on slopes along riparian seepages and stream channels and the frogs are usually found in loose tunnels at the base of dense vegetation tussocks (Bishop 2004a; Harvey 2007). The breeding season extends from November to February, with calling taking place both day and night in misty conditions (Bishop 2004a). Males call from well concealed positions during the day, but at night climb to elevated positions on grass stems. Breeding takes place in decaying vegetation at the base of grass tussocks. Eggs are laid on damp soil or vegetation and develop directly without the requirement of open water at any stage in the life cycle (du Preez & Carruthers 2009). Eleven to fourteen small eggs are deposited on damp soil under vegetation. Fully formed froglets (4 mm) emerge from the eggs approximately 27 days after egg laying (Bishop & Passmore 1993).

Status

The species was listed as Critically Endangered between 2004 and 2010, but has most recently been re-assessed as Endangered (IUCN 2011) due to its extremely small area of occupancy (less than 10 km^2), small and fragmented subpopulations and ongoing decline in extent and quality of habitat (Measey 2011). The current population size is estimated to be 2,495 – 3,350 individuals (Harvey 2007). Aside from the Ngele Forest subpopulation, no known populations occur in officially protected areas (Armstrong 2001; Measey 2011).

Threats

The species is experiencing major habitat loss (50% in 50 years) due to afforestation and agriculture with the result that the already restricted population is diminishing and being further fragmented. The majority of sites occur in unprotected areas and are thus subject to alien plant infestation and inappropriate burning regimes (Measey 2011). Subpopulations at Ngele and Mpur are somewhat less threatened as they are not exposed to burning or such high levels of alien plant invasion (Harvey 2007).

1.5.3 *Leptopelis xenodactylus* (Poynton 1963), Endangered

Leptopelis xenodactylus (Long-toed Tree Frog) is a large tree frog reaching a maximum SVL of 60 mm (Figure 1.6). The dorsal skin is granular and is a uniform bright green colour. The underside is pale cream in colour. It is the least arboreal species in the genus and has much longer fingers and toes with less defined discs at their tips (Burger 2004). Males are distinguished by having a pectoral gland on the chest (du Preez & Carruthers 2009). The call consists of one or two short croaks uttered at long intervals (Passmore & Carruthers 1979).

Figure 1.6: A Long-toed Tree Frog, *Leptopelis xenodactylus*, from Lake Merthley near Greytown. Photograph by Greg Tarrant.

Distribution

The species occurs in the high-rainfall, high-altitude central and western grasslands of KwaZulu-Natal and marginally in adjacent regions of the Eastern Cape (Burger 2004). It occurs between 1,000 and 1,830 m.a.s.l and does not reach the steep slopes of the escarpment. It is known from 11 localities including Mpur Forest, Underberg and the Giant‟s Castle area (Burger 2004). The EOO is 10, 567 km^2 and AOO is 50 km^2 (Measey 2011).

Habitat & Life history

The species inhabits wetlands and marshes in Moist Upland Grassland, Short Mistbelt Grassland and North-eastern Mountain Grassland (Burger 2004). It breeds in wetlands within these grasslands, preferring thick tussocks of vegetation surrounded by a maze of channels (Burger 2004). They remain hidden during the day and at night males emerge to call from higher up on vegetation, with calls having been recorded from September to January. Additional information on breeding biology of the species is required (Measey 2011).

Status

This frog is listed as Endangered (Harrison *et al*. 2001; Minter *et al*. 2004; Measey 2011) due to its small area of occupancy, continuing decline in extent and quality of its habitat and its fragmented distribution. Over 50% of the subpopulations are isolated with the distances between them considered too great for dispersal (Measey 2011). However, of the six threatened frog species in KwaZulu-Natal, *L. xenodactylus* has the highest percentage of its predicted range (20%) fall within protected areas, namely uKhahlamba-Drakensberg Park (Armstrong 2001). Two new populations have recently been discovered at Lake Merthley near Greytown and Fort Nottingham in the midlands (J. Harvey pers. comm.).

Threats

The species is threatened by habitat loss and degradation caused by afforestation and agriculture. Other threats include the desiccation of wetlands caused by invasive plants, incorrect fire regimes and overgrazing, trampling and eutrophication caused by cattle (Measey 2011).

1.5.4 *Natalobatrachus bonebergi* (Hewitt & Methuen 1912), Endangered

Natalobatrachus bonebergi (Kloof Frog) is a slender and relatively small frog, with females up to 37 mm (Figure 1.7) and males reaching approximately 25 mm. Nuptial pads are conspicuous on males and they do not have a vocal sac (du Preez & Carruthers 2009). Dorsal colouration is variable, ranging from brown to grey, usually with a broad vertebral band. A distinct dark stripe runs from the snout over the eye to the top of the arm. The snout is pointed, with the upper jaw projecting markedly over the lower (du Preez 2004). The digits are long, ending in conspicuous T-shaped terminal discs to facilitate their semi-arboreal lifestyle. The webbing of the toes is not extensive (3 phalanges free of webbing) (du Preez 2004).

Figure 1.7: A Kloof Frog female, *Natalobatrachus bonebergi*, keeping an egg clump moist, in Vernon Crookes Nature Reserve. Photograph by Adrian Armstrong.

Taxonomy

Natalobatrachus bonebergi is a species of the Pyxicephalidae. The genus is monotypic and genetic analysis studies have shown it to be a sister to *Arthroleptella* (Bossuyt *et al*. 2006; van der Meijden *et al*. 2011).

Distribution

Natalobatrachus bonebergi is restricted to lowland riparian forests of southern KwaZulu-Natal and the northern Eastern Cape with Dwesa Nature Reserve as its southernmost locality (du Preez 2004). The species is known from only nine locations, between 50 and 900 m.a.s.l. Its EOO is approximately 15, 000 km^2 and AOO is estimated as 1 % of this, at 150 km^2 (Measey 2011).

Habitat & Life history

Natalobatrachus bonebergi inhabits rocky streams and adjacent vegetation in densely forested ravines (Passmore & Carruthers 1979). The species is a semi-arboreal specialist, requiring clear shallow streams with overhanging vegetation. They are also good swimmers and are well camouflaged in their environment of leaf-litter and rocks. They have an extended breeding season from October – May. Males have a very quiet call which they issue from the river bank or elevated positions on rocks or vegetation above the water. Gelatinous masses consisting of 75-95 eggs are deposited on rock surfaces or vegetation overhanging pools. Females have been observed to keep the egg clutches moist with liquid from their cloacas (Figure 1.7). Tadpoles hatch after about 6 days and drop into the water to complete development, which takes approximately two months (Kok & Seaman 1989; du Preez 2004).

Status

The species is listed as Endangered due to its relatively small AOO (150 km^2) and the continuing loss and decline of its habitat resulting in severe fragmentation of its distribution. It occurs in Vernon Crookes, Oribi Gorge, Krantzkloof, Umtamvuna, Dwesa and Cwebe nature reserves (du Preez 2004).

Threats

The habitat of *Natalobatrachus bonebergi* is being heavily impacted by clearing for agriculture (especially sugar cane) as well as urbanisation, particularly in KwaZulu-Natal (Measey 2011). As a result populations of this species are becoming severely fragmented. In addition, its stream-dependant lifestyle may make this species vulnerable to chytrid infection (C. Weldon pers. comm.). The species has been Red Listed as Endangered since 2001 (IUCN 2011).

1.5.5 *Hemisus guttatus* (Rapp 1842), Vulnerable

Hemisus guttatus (Spotted Shovel-Nosed Frog) is a large and distinctive species, reaching 80 mm in length (du Preez & Carruthers 2009). It has a small head, small eyes and shovel-shaped snout adapted to its fossorial lifestyle (Figure 1.8). The limbs are muscular and the toes lack webbing (Alexander 2004). The dorsal colouring is uniform dark brown to olive and covered in small yellow spots. The ventrum is white. The call is a cricket-like trill (Alexander 2004).

Figure 1.8: The Spotted Shovel-nosed Frog, *Hemisus guttatus*, from Durban. Photograph by Marius Burger.

Distribution

This species is distributed in KwaZulu-Natal between Durban in the south to Hluhluwe in the north and occurs at a few inland points in southern Mpumalanga (Alexander 2004). It ranges from sea level to 1000 m.a.s.l in the Lebombo Mountains. Its EOO is 51 000 km^2 (Measey 2011).

Habitat & Life history

This species is fossorial and spends large amounts of time burrowed below ground. It inhabits Coastal Bushveld and Grassland along the coast and North-eastern Mountain Grassland and Natal Central Bushveld in the interior (Alexander 2004). It breeds on wetland edges and along rivers with slight gradients. Breeding occurs between October and December and males usually call from within burrows, making them very difficult to find. Amplexus is initiated on the surface and the pair retreat underground where the female lays up to 2000 eggs in a brood chamber. She remains with the eggs during their development. Once hatched, tadpoles move to water to complete their development (Alexander 2004).

Status

Hemisus guttatus is listed as Vulnerable due to an estimated AOO of 510 km^2, severely fragmented distribution and continuing decline in the extent and quality of its habitat (Measey 2011). The species is protected within iSimangaliso Wetland Park (Greater St Lucia), Umlalazi and Bluff Nature Reserves, Hluhluwe-Umfolozi Park and Bonamanzi Private Nature Reserve (Alexander 2004).

Threats

The species‟ habitat is being subjected to high levels of urbanisation and agricultural activities, especially sugar cane cultivation in the coastal region of its distribution, sylviculture and the effect of alien vegetation on the water table (Measey 2011).

1.5.6 Afrixalus spinifrons (Cope 1862), Near Threatened

Afrixalus spinifrons is comprised of two subspecies, *A. s. spinifrons*, which occurs at low altitudes of the KwaZulu-Natal and Eastern Cape coast lines, and *A. s. intermedius*, which is endemic to the midlands of KwaZulu-Natal and occurs at altitudes above 1000m in KwaZulu-Natal (Figure 1.9) (Pickersgill *et al*. 2004). Within this range populations are fragmented and numbers appear to be in decline (Measey 2011). Populations of *A. spinifrons* have been reduced as a result of habitat loss, habitat fragmentation and the introduction of alien plants (Pickersgill *et al*. 2004; 2007). The *Afrixalus spinifrons* complex, including *A. knysnae*, has been identified as requiring a taxonomic review to determine whether they constitute full species, the results of which will be important for directing conservation efforts (Measey 2011). This study aims to address these requirements and this species is described fully in Chapter 5. The pertinence of systematic reviews to conservation is discussed below.

Figure 1.9: Male *Afrixalus spinifrons* from Hilton Wetland, KwaZulu-Natal Midlands. The yellow throat that distinguishes males is displayed. Photo by Adrian Armstrong.

1.6 The importance of systematics for effective conservation

Knowledge of systematics is crucial to understanding and documenting species diversity and, ultimately, for protecting species adequately (Hunter 2006; Kremen *et al*. 2008; Padial *et al*. 2010). In general, systematic methods use phylogenetics and taxonomy to infer the biogeographical patterns and evolutionary processes behind biological diversity. Phylogenetics involves generating hypotheses regarding the evolutionary relationships between biological entities, while taxonomy uses these phylogenies to provide classifications of the taxa concerned (Hillis *et al*. 1996). The necessity for comprehensive systematic records is illuminated by the fact that despite continuous new discoveries and significant advances in the technologies used to assess biological diversity, it is estimated that as many as 90% of living species remain undocumented (Hanken 1999; Wheeler 2004). Ironically, there are major concerns that many of these species will be eliminated due to human activity before they are described, underscoring the need to document new species and highlighting the necessity of systematic studies for conservation biology (Hanken 1999; Hopkins & Freckleton 2002).

This is especially pertinent for amphibians, which despite having one of the highest rates of description of new species of all vertebrate groups, are experiencing massive population declines, and species extinctions, globally (Glaw & Köhler 1998; Hanken 1999; Köhler *et al*. 2005; Vieites *et al*. 2009). Aside from exploration of poorly known areas of the world (Sangster 2009), this increase is largely attributable to new technologies such as virtual access to museum collections, DNA sequencing and geographical information systems (Metzker 2009; Padial *et al*. 2010). Advances in molecular techniques have been particularly relevant in revealing previously unrecognised relationships between taxa that are otherwise morphologically and ecologically diverse (Knowles 2004; Glaw and Vences 2006; van der Meijden *et al*. 2011). In addition to genetic analysis, the traditional methods of morphological and call analysis for anuran species delimitation are important for producing robust systematic studies. Comparison of morphological characteristics has been the primary method used to describe and classify organisms throughout history (Linnæas 1758; Mayr & Ashlock 1991; Adams *et al*. 2004) and it remains the most general criterion used to define amphibian species (Vences & Wake 2007).

Bioacoustic analysis of male advertisement calls has also been recognised as an extremely practical tool for delimiting anuran species (Ryan 1990; Backwell 1991; Minter 1998; Cannatella 2007). The majority of frogs use vocalisation primarily for breeding functions (Arak 1983a; Duellman & Trueb 1994). The advertisement call of males primarily functions to attract gravid females to a breeding site (Wells 2007), and secondarily to advertise the caller"s position to conspecific males, thereby facilitating inter-male spacing in the chorus (Littlejohn 1977; Telford 1985; Backwell 1988; Backwell & Passmore 1991). Most species have a unique call, allowing females to locate males in an otherwise noisy and complex environment. This individuality provides an exceptionally useful approach for identifying species by means of distinguishing specific mate recognition systems (Paterson 1985; Backwell 1991; Minter 1998).

Using a combination of molecular, morphometric and call analyses provides the most comprehensive means of resolving taxonomic and phylogenetic uncertainties for anurans (Kluge & Farris 1969; Vences *et al.* 2002; Vieites *et al.* 2009) and adopts the approach of integrative taxonomy that is becoming increasingly popular (Padial *et al.* 2010). While the systematics of southern African amphibians has received increased attention in recent years, especially in terms of molecular analysis (e.g. Cunningham & Cherry 2004; Scott 2005; van der Meijden *et al.* 2005; 2011; Tarrant *et al.* 2008; Tolley *et al.* 2010), there remain gaps in the knowledge of the phylogeny of many genera (Channing *et al.* 2011).

Defining species

Species is the taxonomic level most frequently dealt with regarding issues concerning biodiversity (Mallet 2007; Moritz 2002), and the definition of species is important when trying to resolve systematic uncertainties (de Queiroz 2005). The species is the basic unit for taxonomy and is therefore also the easiest unit by which biodiversity can be quantified (Mayr 1982; Baum 1998; Wheeler 2004; Hunter 2006). Defining what constitutes a species, however, has been one of the most important and longest-standing debates amongst biologists and is known as "the species problem" (Mayr 1957; 2001; Dobzhansky 1976; Coyne *et al.* 1988; de Queiroz 1998; Barton 2001; Hey 2006), resulting in over twenty recognised concepts (Mayden 1997; de Queiroz 2005).

A species concept refers to the biological definition of the word "species" and the criteria upon which this definition is based (Mayr 2001). Various species concepts have dominated different eras and biologists and taxonomists are yet to agree on a single definition (Mallet 2007). Darwinian evolutionary theory (1859) replaced Aristotelian essentialist (or typological) thinking, which defined each species by its unvarying essence, with that of the "morphological species concept", which included an appreciation for variation. Darwin argued that species were fundamentally varieties that acquired their well-defined state only when intermediates died out, leaving a morphological gap. This morphological gap criterion was accepted by most early evolutionists (e.g. Wallace 1865) and remained the leading concept until the "interbreeding species concept" put forward by Poulton (1904) and further popularised by Dobzhansky (1937) and Mayr (1942) as the "biological species concept". Under the biological species concept (BSC), species are defined as "groups of interbreeding natural populations that are reproductively isolated from other such groups" (Mayr 1970) and is commonly applied to sexually reproducing organisms (Dobzhansky 1937; 1976; Mayr 1942; 1982; Mayr & Ashlock 1991).

The BSC appeared to solve the species problem and was widely accepted (and still is) by many biologists. However, its definition based on interbreeding and gene flow could not account for "difficulties" such as asexual organisms (Hutchinson 1968), hybridisation or intermediates, and re-opened the species concept debate amongst biologists with even more vigour (Mallet 2007). The BSC"s reliance on "isolating mechanisms" is also problematic in that it implies that reproductive isolation is adaptive. The lack of a clear universal species concept has led to different groups of biologists adopting concepts most suited to their particular study interest. The ecological species concept, for example, is based on niche occupancy, and can be applied to organisms that reproduce asexually or by hybridisation (van Valen 1976).

The recognition concept, defined as "the most inclusive population of individual biparental organisms which share a common fertilization system" (Paterson 1985) is based on the prezygotic compatibility of species, including mating signals and fertilisation signals and relies on "specific mate recognition systems" (SMRS) rather than the isolating mechanisms necessary for application of the BSC. Under the recognition concept, the characters which delineate the gene pool are restricted to the adaptations of the SMRS, which serve the purpose of facilitating successful fertilisation rather than ensuring reproductive isolation

(Minter 1998). The recognition concept has been recognised as being most appropriate for anurans since mating behaviour, in particular acoustic communication and female choice, is integral to sexual selection with the advertisement call the primary SMRS in most frog species (Ryan *et al*. 1990; Backwell & Passmore 1991; Minter 1998).

Conflict arises since phylogenetic concepts that emphasise historical divergence of populations will define different species than those defined by concepts which emphasise ecological replaceability (Cunningham 2001; de Queiroz 2005). Application of different definitions or concepts can thus alter species counts by an order of magnitude or more. The implications of this problem are far-reaching, and are pertinent both to systematics, and conservation (Frost & Kluge 1994; Mallet 2007). Whether a group of animals is protected often depends on its red-list status as determined per species (Hunter 2006).

Speciation

Species are continually changing as a result of evolutionary processes such as genetic drift, sexual selection and environmental adaptation (Mayr 1963). These changes are reflected in the phenotype and different aspects of the phenotype (such as reproductive and ecological compatibility) tend to diverge concurrently (Hillis *et al*. 1996; Cunningham 2001). Speciation is the diversification of all aspects of the phenotype to form discrete entities (Barton 2001; Cunningham 2001). In particular, speciation is the acquisition of reproductive incompatibility (Carson 1986; Littlejohn 1993; Buckley *et al*. 1997). Typically speciation occurs via allopatric speciation (vicariant species formation), where geographically isolated populations evolve separately to become incompatible with one another (Via 2001). Recently, the plausibility of parapatric and sympatric modes of speciation (adaptive species formation), whereby populations within range of each other can diverge through disruptive selection, is becoming more widely accepted (Turelli *et al*. 2001; Via 2001; Vences & Wake 2007). Species recognition studies have been integral to furthering our understanding of anuran evolution and the role of the advertisement call as an isolating mechanism is of paramount importance in speciation (Blair 1958; Ryan 1990).

Subspecies represent the transition of populations of a species to taxonomically distinguishable entities, in that members of subspecies differ to some extent morphologically or genetically, yet are able to interbreed and produce fertile offspring (Mallet 2007).

Interbreeding however is usually inhibited by geographic isolation or other factors (Mayr & Ashlock 1991). Because of the practical issues regarding species concepts, the role of subspecies in zoology has compounded the difficulties in taxonomic delimitation (Mallet 2007). Application of polytypic species concepts (such as the BSC) results in a reduction of recognised species as taxa previously recognized as full species become subspecies. On the other hand, application of phylogenetic species concepts more recently have resulted in taxonomic inflation as former subspecies are re-elevated to species level, despite intergradation at their boundaries (Mallet 2007).

While the use of mitochondrial DNA is relevant to the study of speciation in reconstructing lineages, inclusion of morphological and bioacoustic data is important for delimiting species, since in terms of population biology speciation is the acquisition of phenotypic differences, primarily reproductive incompatibility (Templeton 1981; Coyne & Orr 1989; Littlejohn 1993; Schluter 1996). For anurans, these phenotypic data are mostly morphological measurements (usually size) and call analyses. An applicable definition of species is thus “populations which are both genetically distinct and phenotypically discrete, relative to other populations” (Cunningham 2001), and this is the approach I apply in this study. In terms of conservation, the term “Evolutionarily Significant Units” (ESU) has been proposed as being more appropriate for prioritising taxa and habitats for protection given that current taxonomy may not accurately reflect genetic diversity (Ryder 1986; Moritz 1994; 2002). The goal of conservation must be to “maintain evolutionary processes and the viability of species and functional landscapes necessary to achieve this” (Frankel 1974).

Molecular techniques

Analysis of genetic data has become an indispensible tool for species discrimination (Vences & Wake 2007). The expansion of molecular phylogenetics, and the production of phylogenetic trees, has been a useful tool for providing an indirect record of speciation events that have led to extant species (Barraclough & Nee 2001). Molecular techniques have become particularly useful in anuran taxonomy since they have one of the most conserved body plans of all vertebrate groups with the result that morphological homoplasy among different adaptive clades often obscures phylogenetic relationships (Emerson 1986; Bossuyt & Milinkovitch 2000; Hoegg *et al.* 2004; van der Meijden *et al.* 2011).

Knowledge from DNA sequences is used increasingly over that of other biological functions for studies of amphibian phylogeny, especially in cases where taxa recognised on morphological and bioacoustic evidence have proven to be invalid (Shaffer & McKnight 1996; Hall 2004; Vences & Wake 2007; Vences *et al*. 2010). Such knowledge has been valuable in revealing homologies not apparent from morphological examination (Williams 1993) and has been especially useful for identifying cryptic species (Frost *et al*. 2006; Vences *et al*. 2004; Vences & Wake 2007; van der Meijden *et al*. 2005; 2011). The use of mitochondrial genes that encode ribosomal RNA are especially useful for inferring phylogenies because they are easily accessible, have a wide range of evolutionary rates and as such can provide resolution across a large time scale (Hillis *et al*. 1996). However, data from non-molecular sources is by no means obsolete and synthesis of various datasets is preferable (Cannatella 2007). The combination of both mitochondrial and nuclear markers strengthens the power of phylogenetic analyses by providing more potentially informative nucleotide sites for and by accessing variation at different levels since the evolutionary rate of different genes often varies (Cummings et al. 1995; Makokha 2007).

Bioacoustic analysis

Anurans use vocalisation extensively, and especially, for mating functions (Bogart 1960; Gerhardt & Bee 2007), and the advertisement call is a function of mate recognition (Littlejohn 1965; Backwell 1988; Ryan *et al*. 1990; Minter 1998; Gerhardt & Bee 2007). Evolution of acoustic communication is driven by sexual selection, including the morphology and physiology associated with call production and call features such as intensity, pitch and rate (Ryan 1990; Rand 2001; Wells and Schwartz 2007). For most anurans, the male advertisement call is the most frequently used call type, but some species employ a diverse range of calls depending on the social situation, including, aggression calls (between completing males), courtship calls (upon female approach), release calls (by both males and females following mating) and distress calls (for example, if seized by a predator) (Wells & Schwartz 2007).

Analysis of advertisement calls provides a powerful tool for distinguishing species since each species has a unique advertisement call issued by males to attract females (Gerhardt & Bee 2007; Wells 2007). Knowledge of calls is useful in systematics as it allows evaluation of the species own recognition strategy with the idea that if two distinctive call types are observed in a single population (and size of caller and environmental factors such as temperature have been accounted for) then it is assumed that two separate species are present (Vences & Wake 2007). Advertisement call structure is theorised to be adapted to the species‟ environment, for example grassland species usually have pulsed calls (Passmore 1981). The call “note” is usually issued repeatedly during a call bout and may be evenly spaced or grouped. Call repetition can vary or remain constant within a call bout, and can depend on temperature, proximity of competing males or on female approach (Minter 1998). Calls may be pulsed or unpulsed, monophasic or biphasic. Important factors for analysis include call duration, call repetition rate, pulse rate and dominant frequency. Correction for body size and temperature are usually necessary for call comparisons of large datasets (Littlejohn 1965).

1.7 Project Aims

The broad aim of this study is to provide information on threats facing South Africa"s Critically Endangered, Endangered and Vulnerable frog species at two levels. On a national level, the objective is to assess the potential risk of *Batrachochytrium dendrobatidis* infection in these species. On a regional scale, information on the distribution, systematics and biology of KwaZulu-Natal"s threatened species is provided, as well as recommendations regarding conservation actions for these species.

Objectives:

1.7.1 Provide national baseline data on the presence and prevalence of *Batrachochytrium dendrobatidis* infection in South Africa"s threatened species

To date very little data has been accumulated on the occurrence, distribution and impact of *Batrachochytrium dendrobatidis* (*Bd*) in South Africa"s threatened species. Despite the absence of major mortality events caused by *Bd* in South Africa, such information is essential for prevention of additional spread and for contributing to management strategies concerning response and control of outbreaks of virulent strains. Samples were collected by means of skin swabs and analysis by quantitative real-time PCR. From these results prevalence data were obtained, and together with all records of infection in South Africa used to model the predicted distribution of the pathogen in South Africa.

1.7.2 Compile a review of threats to KwaZulu-Natal species and assist with development of conservation plans

KwaZulu-Natal"s threatened species require improved knowledge and resources in order to be adequately protected. This study aims to identify and contribute to areas of research that are lacking and thus inhibiting conservation strategies. Specific conservation recommendations are given, including monitoring programmes to assess population trends, threat assessments and ground-truthing based on predictive distribution modelling. This information will be used to contribute to Biodiversity Management Plans (BMP-S) for certain species so that conservation plans for these species are gazetted, and therefore, legally binding.

1.7.3 Model the predicted distribution of *Hyperolius pickersgilli*

Knowledge about the distribution of a species is essential for assessing the threat status of a species. For South African species, Red Listing criteria have primarily been determined by the Extent of Occurrence (EOO) and Area of Occupancy (AOO) of each species (Measey 2011). In many cases such information is incomplete and obtaining this data is a priority for conservation research. This study makes use of the ecological niche modelling to predict the distribution KwaZulu-Natal"s Critically Endangered *Hyperolius pickersgilli*. This species has a restricted distribution and its cryptic behaviour makes detection difficult. The predicted distribution model was used to guide surveys of potential additional habitat for this species. In addition all historically known sites were surveyed to determine species existence. Results of surveying were used to recalculate EOO an AOO and to prioritise sites for conservation action.

1.7.4 Contribute to the systematics of the *Afrixalus spinifrons* complex

The *Afrixalus spinifrons* complex requires taxonomic clarification. The complex is comprised of two sub-species, *A. s. spinifrons* and *A. s. intermedius* and the species *A. knysnae*. *A. s. spinifrons* is restricted to coastal lowlands of KwaZulu-Natal and the coastal region of the Eastern Cape, while *A. s. intermedius* occurs at higher elevations in the KwaZulu-Natal midlands. *Afrixalus knysnae* occurs marginally on the eastern coast of the Western Cape. The members of this group are very similar in morphology and have similar calls. In addition to examining morphology and calls, this study makes use of molecular techniques for the first time to resolve the phylogeny within the group. Gene sequencing was conducted for specimens from throughout the distribution using the mitochondrial gene fragment 16S. Determining whether the subspecies are separate species will contribute to conservation decisions.

1.7.5 Improve public awareness

Public awareness and education is a vital part of all conservation strategies. In South Africa, the public is largely ignorant of the importance of frog biodiversity and the fact that this group of animals is under severe threat. Compounding the problem in some sectors of South African society are the many superstitions and fears surrounding frogs (Tolley *et al.*

2011). During the course of this study awareness was raised and misguided beliefs dispelled through talks to the public (including scholars and students), media and specialised workshops. Establishing and strengthening networks with provincial conservation authorities, zoos and other stakeholders involved in amphibian conservation as well as engaging with landowners (particularly in KwaZulu-Natal) was an additional key objective in order to pave the way for long-term conservation plans for South Africa‟s threatened species.

CHAPTER 2

PREVALENCE AND PREDICTED DISTRIBUTION OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN THE THREATENED FROGS OF SOUTH AFRICA

2.1 Abstract

Chytridiomycosis has been identified as a major cause of global amphibian declines. Despite widespread evidence of *Batrachochytrium dendrobatidis* (*Bd*) infection in South African frogs, sampling for this disease has to date not focused on threatened species or whether this pathogen poses a disease risk to these species. This study provides baseline data on the occurrence and prevalence of *Bd*-infection in South African Red List species. In addition, all known records of infection from throughout the country were used to model the ecological niche of *Bd* in South Africa to give a better understanding of potential distribution and associated disease risk. Presence and prevalence of *Bd* was determined through collection of 360 skin swab samples from 17 threatened species from 38 sites across the country. Swabs were tested for *Bd* using quantitative real-time PCR. Average prevalence was 14.8% for threatened species, compared with 33.9% for all occurrence records in South Africa. Pathogen load varied considerably between species, with *Afrixalus spinifrons* (Hyperoliidae) exhibiting the highest infection intensity. The prevalence in South African species is consistent with other regions where the pathogen is considered endemic, and the lack of major mortality events supports the "out of Africa" hypothesis. Distribution of *Bd* was modelled using maximum entropy (Maxent) based on all known occurrence records for South Africa. The resultant probability threshold map indicated that *Bd* is largely restricted to the wet eastern and coastal regions of South Africa. All South African threatened species occur within the limits of the predicted area for *Bd* and are thus potentially exposed to *Bd*-associated risk factors. Although no clinical symptoms or mortality events as a result of *Bd* were observed in any of the populations studied, the influence of other stressors on the incidence of disease requires additional research, and the possibility of introduction of a novel virulent strain of *Bd* remains a potential threat to South African anurans. Predicting pathogen distribution patterns and potential impact is therefore necessary for prioritising research and guiding management decisions.

2.2 Introduction

Large-scale, enigmatic amphibian declines in the 1990s in undisturbed regions of the tropics and protected areas prompted research into possible causes, resulting in the identification and description of a novel chytrid fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*) (Longcore *et al.* 1999). This non-hyphal zoosporic fungus causes the skin disease, chytridiomycosis, in amphibians and is now recognised as a significant contributor to global declines (Berger *et al.*1999, Daszak *et al.* 1999; Skerratt *et al.* 2007; Fisher *et al.* 2009; Briggs *et al.* 2010). Chytridiomycosis has been identified as one of the most important drivers of the amphibian crisis in terms of the number of species impacted and its“ potential to cause extinction (Gascon *et al.* 2007). The pathogen is widespread, occurring on all amphibian-inhabited continents, and is capable of infecting an extremely wide range of host species (Berger *et al.* 1998; Hyatt *et al.* 2007). The pathogen has to date been detected in 516 species worldwide (Aanensen 2012), approximately half of which are species experiencing declines (Daszak *et al.* 2007; Hyatt *et al.* 2007; Skerratt *et al.* 2007). It is one of the most virulent pathogens of amphibians and is responsible for amphibian population declines in Australia (Berger *et al.* 1999; Schloegel *et al.* 2006), Europe (Bosch *et al.* 2001), New Zealand (Waldman *et al.* 2001; Bell *et al.* 2004), the Americas (Bradley *et al.* 2002; Garner *et al.* 2005; Lips *et al.* 2006; Rachowicz *et al.* 2006; Crawford *et al.* 2010), Canada (Schock *et al.* 2009; Forzán *et al.* 2010) and Africa (Weldon & Du Preez 2004a; b; Hopkins & Channing 2003). It has also now been linked to several species extinctions in some of these regions (Daszak *et al.* 2003; Burrowes *et al.* 2004; Hyatt *et al.* 2007). The disease has also been reported in captive amphibians in zoos, research collections and commercial collections (Ouellet *et al.* 2005). Strategies to protect amphibians from this disease are therefore essential in the overall campaign to conserve amphibians.

Early records of infection roughly coincide with the first observations of significant amphibian declines (Carey 2000; Carnaval *et al.* 2006), with retrospective studies showing that the disease had likely already become a global threat by the 1970s (Skerratt *et al.* 2007; Soto-Azat *et al.* 2009). Initial disease outbreaks on various continents have all been characterised by an epidemic-type pattern of rapid spread and high mortality in a wide range of species (Laurance *et al.* 1997; Lips *et al.* 2006; Fisher & Farrer 2011). The earliest records of infection are from Africa in the 1930s (Weldon *et al.* 2004; Soto-Azat *et al.* 2009), followed by infection records associated with population declines in the United States and Canada in the 1960s (Koonz 1992; Carey 2000; Oullet *et al.* 2005), the tropics in the 1990s (Berger *et al.* 1998; Lips 1998; 1999), New Zealand (Waldman *et al.* 2000) and Europe in the late 1990s (Mutschmann 2000; Bosch *et al.* 2001). Multi-locus sequencing of isolates of *Bd* from disparate regions of the world exhibit no or very low genetic diversity, supporting the hypothesis that the pathogen has only recently become a widespread pandemic (Morehouse *et al.* 2003; Daszak *et al.* 2007; Farrer *et al.* 2011). These patterns of spread, and the highly conserved genome, provide support for the hypothesis that the disease has recently emerged and has not been naturally distributed around the world (Oullet *et al.* 2005; Kriger 2007; Fisher & Farrer 2011).

These findings imply that the introduction of this pathogen to regions where it has had the most damaging consequences (North America, South America and Australia) has been facilitated by humans through the global movement of either infected animals or vector material for the laboratory, pet and food trades (Cunningham *et al.* 2003; Mazzoni *et al.* 2003; Daszak *et al.* 2004; Pounds *et al.* 2006; Kriger & Hero 2009). A review by Fisher & Garner (2007) concluded that trade in amphibians is driving the emergence of chytridiomycosis through the spread of infected animals, introduction of infected animals into naive populations, and amplification of infection caused by release of untreated discharge into water supplies. In particular, the international trade of the African Clawed Frog (*Xenopus laevis*) from South Africa has been identified as one of the major sources of dissemination of amphibian chytrid (Weldon *et al.* 2004, Soto-Azat *et al.* 2009). This species is known to carry *Bd* without being adversely affected by it and thus acts as a reservoir for the disease (Johnson & Speare 2005; Weldon *et al.* 2007).

The origin of amphibian chytrid as being African was first hypothesised by Weldon *et al.* (2004). This hypothesis was based on a histological study of the presence of *Bd* in archived *Xenopus* specimens. The earliest record of infection in South Africa was from 1938 in a *X. laevis* specimen from the Western Cape (Weldon *et al.* 2004). Since the 1940s the prevalence of chytrid in the South Africa has remained stable and from about 1973 the pathogen has occurred in all regions of southern Africa (Weldon *et al.* 2004; Soto-Azat *et al.* 2009). The "out of Africa" hypothesis is further supported by additional studies of museum specimens showing that a consistently low prevalence of infection over time has existed in Africa (Soto-Azat *et al*. 2009; Walker *et al*. 2008) and by additional historical reports of occurrences from Africa (Goldberg *et al.* 2007; Greenbaum *et al.* 2008; Kielgast *et al.* 2010; Bell *et al.* 2011). The widespread trade of *X. laevis*, beginning with its use in human pregnancy testing in the mid-twentieth century (Shapiro & Zwarenstein 1934), and its resistance to chytridiomycosis, makes this species an ideal candidate for spreading *Bd* from Africa to the rest of the world (Weldon *et al*. 2004; 2007; Soto-Azat *et al*. 2009; Weldon & Fisher 2011). Subsequent surveying throughout Africa has restricted the origin of *Bd* as being from southern and eastern Africa, with much of western Africa remaining free of the pathogen (Penner *et al*. In press).

Xenopus laevis is notorious for establishing feral populations (Weldon *et al*. 2007), some of which have been documented in the United Kingdom, United States (Tinsley & McCoid 1996), Chile (Lobos & Measey 2002) and Europe (Fouquet 2001; Fouquet & Measey 2006). Feral *X. laevis* carrying *Bd* could undoubtedly come into direct contact with local frog species and pass on infection (Garner *et al*. 2006). Ultimately, the negligence of unabated trade in live amphibians by national and international authorities warrants new approaches to amphibian conservation due to consequent disease spread implications (Weldon & Fisher 2011). Despite the long-term presence of *Bd* in South Africa, and considerable risk associated with outbreaks of the disease, little has been done by way of investigating the threat it may pose to indigenous species, in particular those that are Red Listed. Die-offs in a number of taxa have been associated with *Bd* infection (Hopkins & Channing 2003; Weldon & du Preez 2004) and the possibility of outbreaks of lethal novel strains may have serious impacts on local amphibian fauna. Little or no surveying for *Bd* in threatened South African frogs has been conducted and this study aims to fill this gap. In addition, by including the most recent records from South Africa, an objective of this study is to validate the "out of Africa" hypothesis.

The impact of *Bd* varies widely between species and even among populations within a species (Briggs *et al*. 2005; Tobler & Schmidt 2010). Some are particularly susceptible, with declines, and in some cases, extinctions, due to severe outbreaks taking place very rapidly (several weeks) while conversely, others appear to suffer no negative effects upon infection (Crawford *et al*. 2010; Vredenburg *et al*. 2010). The disease has been predicted to occur in regions of the world with the highest amphibian diversity, likely due to its association with cool and wet habitats (Ron 2005; Smith *et al*. 2009; Murray *et al*. 2011; Bielby *et al*. 2008).

Understanding the distribution of *Bd* within a region is paramount for implementing protocols for disease management and for determining disease or extinction risk (Bielby *et al*. 2008). Occupancy and ecological niche modelling for determining species‟ distribution has become an increasingly used tool for conservation planning (Stillman & Brown 1994; Anderson *et al*. 2003; Araújo & Guisan 2006; Elith *et al*. 2011). Models have been used for predicting *Bd* distribution and associated disease risk on both a global (Rödder *et al*. 2009) and regional scale for the New World (Ron 2005), United States (Adams *et al*. 2010) and Australia (Murray *et al*. 2011). Correlative approaches to niche modelling extrapolate from associations between point occurrences and environmental data to identify areas of predicted presence (Soberón & Peterson 2005). Here, the maximum entropy (Maxent) method is employed (Phillips *et al*. 2006) to correlate South African *Bd* occurrence records (presence-only) with environmentally suitable parameters. Risk assessments of potential *Bd* introduction and anthropogenic spread based on distribution information are becoming a widely used method in efforts to minimise impact of this pathogen (St-Hilaire *et al*. 2007; Bielby *et al*. 2008). On a national scale, conservation management plans for controlling the spread of *Bd* and directing identification of candidate species for monitoring or *ex-situ* breeding require improved knowledge of the current distribution of *Bd* as well as identification of areas/populations not yet affected (Rödders *et al*. 2009; Murray *et al*. 2011; Woodhams 2011).

Because *Bd's* ecological preferences are well understood (Berger *et al*. 2004; 2005a; Johnson & Speare 2003; 2005), the use of ecological niche modelling provides an ideal tool for predicting potential distribution of this pathogen (Bielby *et al*. 2008; Murray *et al*. 2011). Furthermore, unlike many other pathogens which rely on specific internal host conditions, *Bd* is an ideal candidate for modelling since infections occur on ecothermic amphibian hosts and the pathogen is directly influenced by external environmental conditions, especially temperature and moisture (Mitchell *et al*. 2007). Risk assessments based on ecological models are becoming an increasingly valuable method of identifying both previously naive areas and species that are most likely to be afflicted with chytridiomycosis, as well as optimising detection probability and data collection (Lubrick 2010; Murray *et al*. 2011).

Although a global model for *Bd* predicted distribution exists (Ron 2005), its application is limited because it was based on very few samples from South Africa (with all but one from the Western Cape), is on too broad a spatial scale to be useful on a regional scale, and appears to exaggerate suitable areas in South Africa. For these reasons, predictions on a finer scale, using up-to-date data, is necessary to assess *Bd* distribution in South Africa. This study makes use of a comprehensive database of records of *Bd* infection in the country to estimate where *Bd* is likely to occur in addition to where it has already been located. This information can be useful for predicting disease risk.

The broad aims of this chapter are to:

1. Provide baseline data on the occurrence and prevalence of *Bd*-infection in South Africa‟s threatened frogs.
2. Model the predicted distribution of *Bd* using Maxent to improve the understanding of which species and regions may be at higher infection risk.

2.3 Methods

2.3.1 Prevalence assessment

Sample collection using skin swabs is an effective non-lethal and repeatable technique for testing for presence of *Bd* (Retallick *et al*. 2006). Threatened species (IUCN Vulnerable, Endangered and Critically Endangered) were targeted for surveys based on SAFAP data (Minter *et al*. 2004) as well as sampling of additional populations found on an ad hoc basis during the course of the field work. Individuals (adults and, where appropriate, tadpoles) of each species were detected via visual, acoustic or opportunistic searches and caught by hand or net. Sample size per site depended on detection likelihood of the target species, but where possible, 20 samples were taken per site per species (Table 2.1).

Table 2.1: Sites sampled for *B. dendrobatidis* testing in threatened South African species. *n* = sample number.

Site Name	Province	Latitude	Longitude	Target species	*n*
Cape Agulhas	WC	-34.74106	19.67883	*X. gilli*	25
Cape Point	WC	-34.30603	18.44133	*X. gilli*	25
Silvermine Nature Reserve	WC	-34.10095	18.44809	*C. rosei*	35
Kirstenhof	WC	-34.08555	18.4525	*A. pantherinus*	7
Bergvliet	WC	-34.04864	18.44789	*A. pantherinus*	22
Youngsfield Military Base	WC	-34.00419	18.49025	*A. pantherinus*	2
Kennilworth	WC	-33.99637	18.48486	*M. capensis*	20
Disa Stream	WC	-33.98586	18.39072	*H. rosei*	26
Skeleton Gorge	WC	-33.98586	18.39072	*H. rosei*	8
University of Cape Town	WC	-33.95818	18.45746	*B. gibbosus*	2
Geelhoutboom River	EC	-33.79434	25.06377	*H. hewitti*	32
Martins River	EC	-33.79326	25.03819	*H. hewitti*	2
Hogsback 2	EC	-32.59892	26.94552	*A. rattrayi*	3

Hogsback 1	EC	-32.54774	26.91443	*V. amatolicus*	1
Dwesa NR	EC	-32.25348	28.87046	*N. bonebergi*	1
Coffee Bay	EC	-31.93496	29.08826	*A. spinifrons*	1
Vernon Crookes	KZN	-30.2786	30.59596	*N. bonebergi*	17
Umkomaas	KZN	-30.21718	30.795353	*H. pickersgilli*	1
Isipingo	KZN	-29.99185	30.9056	*H. pickersgilli*	46
Adam's Mission	KZN	-29.99183	30.78328	*H. pickersgilli*	1
Prospecton	KZN	-29.98328	30.938	*H. pickersgilli*	14
Tala Nature Reserve	KZN	-29.82954	30.53535	*A. spinifrons*	2
Cowies Hill	KZN	-29.82436	30.59567	*N. bonebergi*	1
Mt. Moreland	KZN	-29.6382	31.09754	*H. pickersgilli*	28
Cedara	KZN	-29.55784	30.255406	*A. spinifrons*	1
Hilton	KZN	-29.53916	30.28625	*A. spinifrons*	2
Fort Nottingham	KZN	-29.44493	29.90642	*A. spinifrons*	9
Kamberg Nature Reserve	KZN	-29.37361	29.725	*A. spinifrons*	3
Rosetta	KZN	-29.30417	29.9625	*A. spinifrons*	2
Lake Merthley	KZN	-29.02242	30.58106	*L. xenodactylus*	10
Forest Lodge, Mtunzini	KZN	-28.96782	31.753219	*H. pickersgilli*	4
Umlalazi Nature Reserve	KZN	-28.95805	31.76472	*H. pickersgilli*	1
Port Durnford	KZN	-28.90521	31.85801	*H. pickersgilli*	6
Haernertsburg	LP	-23.93619	29.93916	*B. sylvestris*	2
Woodbush	LP	-23.8111	29.96365	*B. sylvestris*	16
Hanglip	LP	-22.99959	29.88353	*B. sylvestris*	1
Soutspanberg	LP	-22.99590	29.88353	*B. sylvestris*	2
MacDougal's Bay	NC	-29.26172	16.87107	*B. macrops*	4
38 Sites	**5**			**17**	**360**

Sampling was conducted in the rainy months (August – March) between 2008 and 2012. Field sampling protocol followed Brem *et al.* 2007 (although for this study swabs were air-dried instead of being placed into alcohol). The swabbing procedure is non-invasive compared to tests that rely on cytology: using a fresh pair of latex gloves for each animal, the caught frog was held in one hand and swabbed using the other. The cotton tip of the swab was gently stroked five times each over the ventral surfaces of the thighs, tibia, ventrum and webbing of the live frog (Fig 2.1), which was then released at the point of capture.

Figure 2.1: Ventral surfaces of frog that were targeted for swabbing for *Batrachochytrium dendrobatidis* detection (Adapted from Berger *et al.* 2005c).

For the *Heleophryne* genus, tadpole mouthparts, owing to their large size, could be swabbed (61 samples were obtained this way). Re-sampling of the same individual was avoided by keeping individuals in separate bags and postponing swabbing until all frogs had been captured. Hygiene protocol to prevent the spread of *Bd* between sites according to St-Hilaire *et al.* (2007) was adhered to. Measures to reduce the risk of spreading infection between such sites are particularly important in the case of threatened frog species, where protocols should be applied over very short distances. Equipment and footwear was cleaned and disinfected at the commencement of fieldwork and between sites. This was done by removing excess mud and rinsing or spraying boots with disinfectant (5% bleach solution) or allowing them to air dry before visiting the next site. Swabs were kept refrigerated at approximately 4°C until testing.

Detection of *Batrachochytrium dendrobatidis* DNA with Real-Time PCR

Recommended protocols were applied for the detection of *Bd* in order to obtain results that meet with international standards (Hyatt *et al*. 2007). Detection with real-time TaqMan PCR has been found to be superior to diagnosis by histology (Kriger *et al*. 2006b; Hyatt *et al*. 2007) and was conducted at the NRF Research Laboratory, National Zoological Gardens of South Africa. DNA from skin swabs was extracted using PrepMan Ultra (Applied Biosystems™, Foster City, CA, protocol described in Hyatt *et al*. 2007). Extracted DNA was analysed for *Bd* using quantitative real-time TaqMan PCR assays (Boyle *et al*. 2004) with *Bd*-specific primers *Bd*1a (5‟-CAGTGTGCCATATGTCACG-3‟) and *Bd*2a (5‟-CATGGTTCATATCTGTCCAG-3‟) (Annis *et al*. 2004). The StepOnePlus™ real-time PCR system from Applied Biosystems™ was used for the TaqMan assay. The assay is extremely sensitive and capable of detecting as little as 1 zoospore or 10 pg of DNA from *Bd*. Samples were processed in duplicate and standards for quantification of *Bd* followed Boyle *et al*. (2004). Amplification in both samples was considered positive. No amplification was considered negative. Quantity mean provides a genomic equivalent of *Bd* density and results were interpreted as follows:

CT mean	Quantity mean	Result
40 and above	Less than 0.05	Negative
Less than 39	Less than 0.05	Low – Positive
Less than 39	0.05-0.1	Medium – Positive
Less than 39	More than 0.1	High-positive

2.3.2 Statistical analysis

STATISTICA (Ver. 10; Statsoft 2011) was used to conduct Fisher‟s exact test to calculate significance between infection prevalence in the threatened species tested for *Bd*.

2.3.3 Predictive Distribution Modelling

Predictive modelling software Maxent (ver. 3.3.3k; Phillips *et al*. 2004, 2006; Phillips & Dudík 2008) was used to model the predicted distribution of *Bd* in South Africa. Maxent output correlates environmental suitability for the target organism, where higher values correspond to a better prediction of better conditions (Phillips *et al*. 2006; Elith *et al*. 2011) and has been shown to outperform other correlative approaches (Elith *et al*. 2006; Austin 2007; Heikkinen *et al*. 2011). One hundred and twenty six (excluding duplicates) *Bd*-positive presence records with high resolution geo-referencing data (GPS co-ordinates) were compiled from the Africa *Bd* dataset (Weldon *et al.* unpublished data), including all samples from this study.

For the Maxent model, environmental variable data was obtained from BIOCLIM (Hijmans *et al*. 2005). For the initial modelling, 24 variables were used, of which 19 were bioclimatic variables (continuous), four geo-physical variables and one a biome-type variable (categorical). Of these, 15 variables with the highest contribution to the model were selected for the final run (Table 2.2). The variables were re-sampled to 250m grids using ArcGIS v10 (ESRI 2011). The following model parameters were used: to allow adequate time for convergence, the number of iterations were set to 5000; number of replicates was set to 100 using bootstrapping with 30% of the data used for testing and 70% for training. In an effort to reduce sampling bias, a bias file was used to guide background point selection (see Phillips *et al.* 2009). Model performance was evaluated by the area under the curve (AUC) statistic of the receiver operating characteristic (ROC) plots (Fielding & Bell 1997; Phillips *et al*. 2006). Jack-knife tests of variable importance, using regularised training gain, test gain and AUC for test data, determined which variables made the greatest contribution to the model. From the results of the first model the top ranking variables contributing 90% of the information for the model were identified and used in subsequent models. Four further models were run using the above parameters, but with different combinations of environmental variables. Environmental variables were excluded based on the Jack-knife analysis results of previous models.

Table 2.2: Environmental variables, and their percentage contribution, included in the final MAXENT niche model for predicted distribution of *Batrachochytrium dendrobatidis*.

Key	**Variable**	**Contribution to final model (%)**
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp)	3.7
BIO5	Max Temperature of Warmest Month (°C)	2.6
BIO6	Min Temperature of Coldest Month (°C)	3.5
BIO9	Mean Temperature of Driest Quarter (°C)	0.6
BIO10	Mean Temperature of Warmest Quarter (°C)	2.7
BIO11	Mean Temperature of Coldest Quarter (°C)	13.9
BIO13	Precipitation of Wettest Month (mm)	4.8
BIO15	Precipitation Seasonality (Coefficient of Variation)	5.7
BIO16	Precipitation of Wettest Quarter (mm)	4.5
BIO19	Precipitation of Coldest Quarter (mm)	8.1
Topo	Topography	13.2
dist to water	Distance to water	20.3
Biomes	Biomes	6.0
Slope	Slope	3.4
Aspect	Aspect	6.8

2.4 Results

2.4.1 Prevalence of *Batrachochytrium dendrobatidis* infection in South Africa"s Threatened Frogs

In total, 360 swab samples were obtained from 17 threatened species and an additional 130 swabs were obtained from 26 non-threatened (Least Concern) species that occurred in sympatry with threatened species in order to ascertain *Bd* presence at sites in which threatened species occurred (Appendix A). Of the 38 sites sampled, *Bd* was present at 20, with an overall prevalence of 26.6%. Of the 17 threatened species tested, eight were found to be positive for *Bd*, with an average prevalence of 14.8%. Prevalence in non-threatened species from sympatric sites was 19.8% for a total of 23 species (n = 91) (Appendix A). Sampling was targeted at sites of threatened species and as such was not evenly distributed throughout the country. The majority of samples were therefore obtained in KwaZulu-Natal and the Western Cape lowlands. Figure 2.2 shows sampling effort at the 38 sites and gives an indication of *Bd*-infection prevalence per threatened species at each sampling locality.

Prevalence varied considerably among taxa (Figure 2.3), from 79.4% in *Heleophryne hewitti* (n = 34) to 2.4% in *Xenopus gilli* (n = 41), with an average prevalence among threatened species of 14.5% (Table 2.3). Quantity mean for positive samples varied widely and for statistical purposes values less than 0.05 were excluded, leaving 66 samples for analysis. Average quantity mean ranged between 1375.6 for *Afrixalus spinifrons* (n = 12; from 7 sites) and 0.16 for *Xenopus laevis* (n = 1) (Table 2.3).

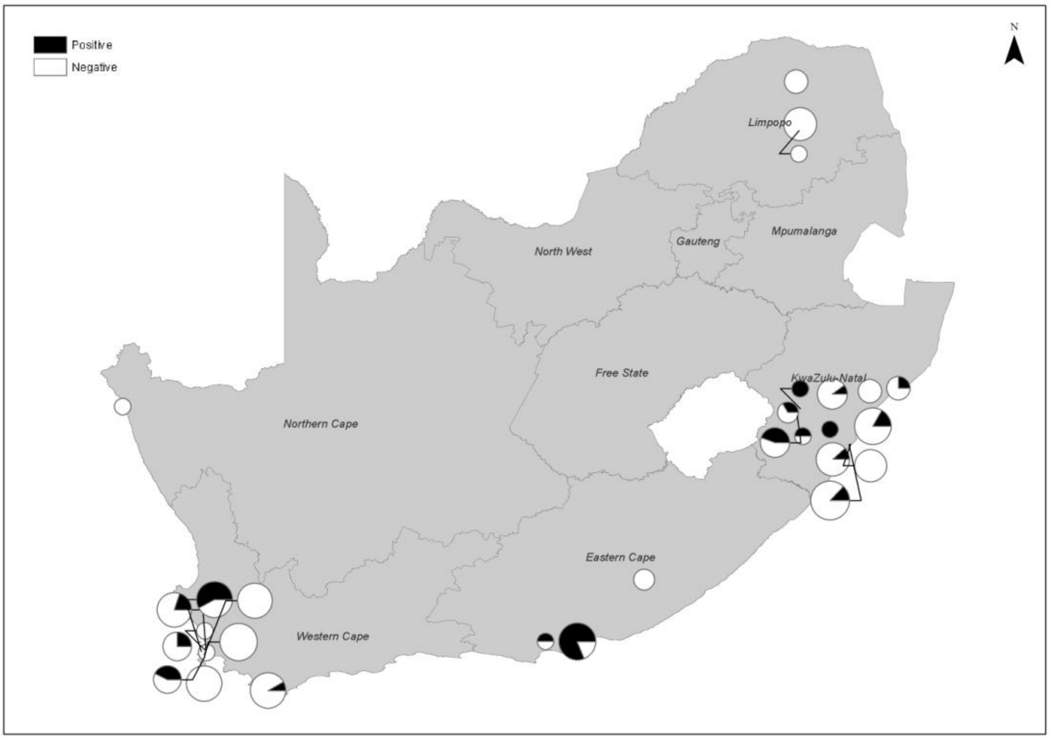

Figure 2.2: Map showing swab sample collection points for threatened species in South Africa. Pie-charts represent prevalence (Black = positive samples, White = negative); size gives an indication of sample size.

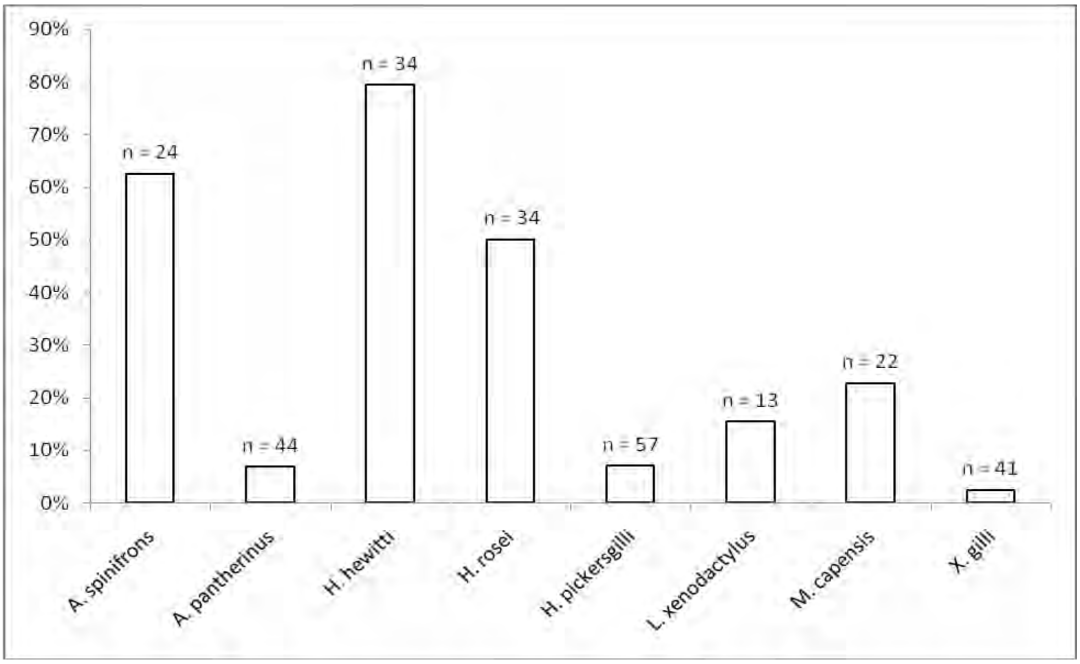

Figure 2.3: Prevalence (%) of infection in South African threatened species that tested positive for *Batrachochytrium dendrobatidis*.

Table 2.3: Swab samples from threatened (Red List 2011) species and prevalence of *Batrachochytrium dendrobatidis* infection (percentage of samples positive for the pathogen) and average quantity mean per infected species. *n* = sample size.

Species	**Status**	***N***	**Province**	***Bd* Prevalence (%)**	**Average Quantity mean**
Afrixalus spinifrons	NT	24	KZN, EC	62.5	1375.6
Amietophrynus pantherinus	EN	44	WC	6.8	7.792
Anhydrophryne rattrayi	VU	3	EC	0	-
Breviceps macrops	VU	2	NC	0	-
Breviceps gibbosus	NT	2	WC	0	-
Breviceps sylvestris	EN	28	LP	0	-
Capensibufo rosei	VU	31	WC	0	-
Heleophryne hewitti (tadpoles)	EN	34	EC	79.4	49.78
Heleophryne rosei (tadpoles)	CR	34	WC	50	18.97
Hemisus guttatus	VU	2	KZN	0	-
Hyperolius horstockii	VU	5	WC	0	-
Hyperolius pickersgilli	CR	57	KZN	7	2.92
Leptopelis xenodactylus	EN	13	KZN	15.4	61.12
Microbatrachella capensis	CR	22	WC	22.7	6.81
Natalobatrachus bonebergi	EN	16	KZN	0	-
Vandijkophrynus amatolicus	CR	1	EC	0	-
Xenopus gilli	EN	41	WC	2.4	0.16
Total: 17		**359**		**Average: 14.8%**	**190.4**

2.4.2 Statistical analysis

Due to distinct differences in sample size (13 to 57 for infected species) of the various threatened species the Fisher‟s exact test was run to determine whether some species were significantly more infected ($p<0.05$) than others when taking sample size into account (Table 2.4). *Heleophryne hewitti* was significantly more infected than all other species except *Afrixalus spinifrons*, both species with exceptionally high prevalence in spite of sample sizes in the mid range. Similarly, *A. spinifrons* was significantly more infected than all other species except *H. hewitti* and *H. rosei*. Together these species represent the three species with the highest prevalence (≥50%). *Amietophrynus pantherinus*, *Hyperolius pickersgilli* and *Xenopus gilli* had the three lowest prevalences that resulted from larger samples sizes ($n > 40$) and did not differ significantly from each other, only from above mentioned species and in the case of *A. pantherinus* and *X. gilli* from *Microbatrachella capensis* as well.

Table 2.4: Fisher‟s exact test for infection prevalence for threatened South African species. P<0.05 = significantly different.

	A. spinifrons	*A. pantherinus*	*H. hewitti*	*H. rosei*	*H. pickersgilli*	*L. xenodactylus*	*M. capensis*	*X. gilli*
A. spinifrons								
A. pantherinus	0.0001							
H. hewitti	0.1615	0.0001						
H. rosei	0.5993	0.0001	0.0432					
H. pickersgilli	0.0001	0.3837	0.0001	0.0001				
L. xenodactylus	0.0165	0.1271	0.0002	0.0463	0.3081			
M. capensis	0.0072	0.039	0.0001	0.0237	0.2086	1		
X. gilli	0.0001	1	0.0001	0.0001	0.3962	0.1405	0.0464	

2.4.3 Predictive Modelling

The *Bd* occurrence data was compiled from the Africa *Bd* database (Weldon *et al.* Unpublished data), which for South Africa, included a total of 1, 858 samples that were tested for *Bd* both by both PCR (n = 1163) and histological assessment (n = 695). Of these, 630 samples were positive for *Bd* infection, with an overall average prevalence of 33.9% (Table 2.5). The samples were obtained from both wild-caught and archived specimens, from across all nine provinces, and included approximately 62 species (25 genera), from 20 contributors spanning occurrence dates between 1938 and 2012 (Table 2.6).

Table 2.5: Summary of Africa *Batrachochytrium dendrobatidis* database and occurrence points used for predictive distribution modelling (*Bd* + = infected). *each species was counted in every province that it occurred.

Province	Tested individuals	Number of species (of which threatened)	*Bd*+ Samples	Geo-referenced *Bd*+ localities
EC	81	15 (5)	45	9
FS	133	7 (0)	26	14
GP	10	1 (0)	0	0
KZN	348	20 (5)	79	48
LP	219	8 (1)	12	7
MP	89	6 (0)	5	12
NC	137	10 (1)	108	3
NW	225	8 (0)	155	0
WC	616	15 (7)	200	28
South Africa	**1858**	**62***	**630**	**121**

Table 2.6: *Batrachochytrium dendrobatidis* (*Bd*) occurrence records from frog genera in South Africa used in the Maxent model, as obtained from the Africa *Bd* dataset (1938 – 2012, Weldon *et al.* unpublished data). N = samples tested for *Bd*; Prevalence = % of total positive for *Bd*. *Geo-referenced localities (GPS co-ordinates) include duplicates.

Genus	N	Prevalence (%)	Geo-referenced
Amietia	451	36.8	39
Afrixalus	38	55.2	27
Amietophrynus	106	12.3	62
Anhydrophryne	4	0	2
Arthroleptis	2	0	0
Breviceps	32	0	32
Cacosternum	164	56.1	9
Capensibufo	32	0	32
Chiromantis	5	20	5
Hadromophryne	8	50	8
Heleophryne	70	62.8	70
Hemisus	7	42.8	3
Hyperolius	346	31.8	65
Kassina	28	21.4	4
Leptopelis	25	20	13
Microbatrachella	22	22.7	22
Natalobatrachus	13	0	13
Phrynobatrachus	17	52.9	5
Ptychadena	12	33.3	4
Schismaderma	22	63.6	9
Semnodactylus	2	0	0
Strongylopus	71	46.5	35
Tomopterna	122	59.8	10
Vandijkophrynus	2	50	2
Xenopus	257	10.1	212
Total	**1858**	**Avg: 33.9 %**	**683***

Model accuracy was assessed by area under the curve (AUC) of the receiver operator characteristic (ROC), where 1 = perfect prediction and 0.5 = no better than random. For this model, mean test AUC for the *Bd* model was 0.824 indicating that the model provided a good fit to the data (Wisz *et al*. 2008). Analysis of variable contributions (Jack-knife tests) showed that “Precipitation of wettest month” (Bio 13), “Precipitation of Wettest Quarter” (Bio 16) and “Distance to water” had the highest predictive power when used in isolation (Figure 2.4).

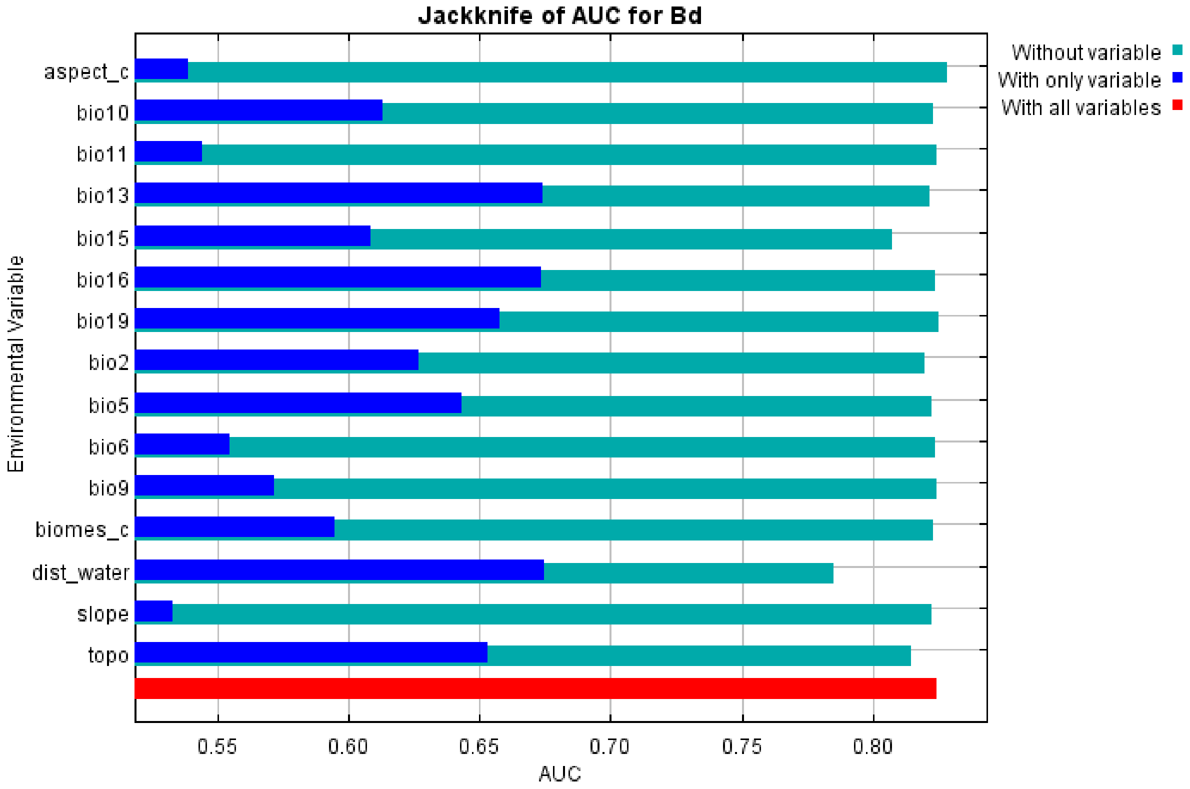

Figure 2.4: Jack-knife test showing the contributions of the various bioclimatic variables to the area under curve (AUC) of the final predictive model for *Batrachochytrium dendrobatidis* distribution in South Africa. „Only variable‟ indicates the results of the model when a single variable is run in isolation; „without variable‟ indicates the effect of removing a single variable from the full model (Jack-knife). Values are means from 100 replicates.

The resultant threshold map of the predictive model (Figure 2.5) indicates that occurrence of *Bd* infection is likely to be highest in the eastern and coastal regions of South Africa, with much of the drier central and northern inland regions unsuitable due to low precipitation and few water bodies that limit occupancy by amphibians and thus *Bd*. An exception is the Orange River basin in central South Africa that provides climatic refugia for *Bd* from the surrounding semi-arid environment. Infection is especially concentrated along the KwaZulu-Natal escarpment and highlands surrounding Lesotho as well as the lowlands of the Western Cape.

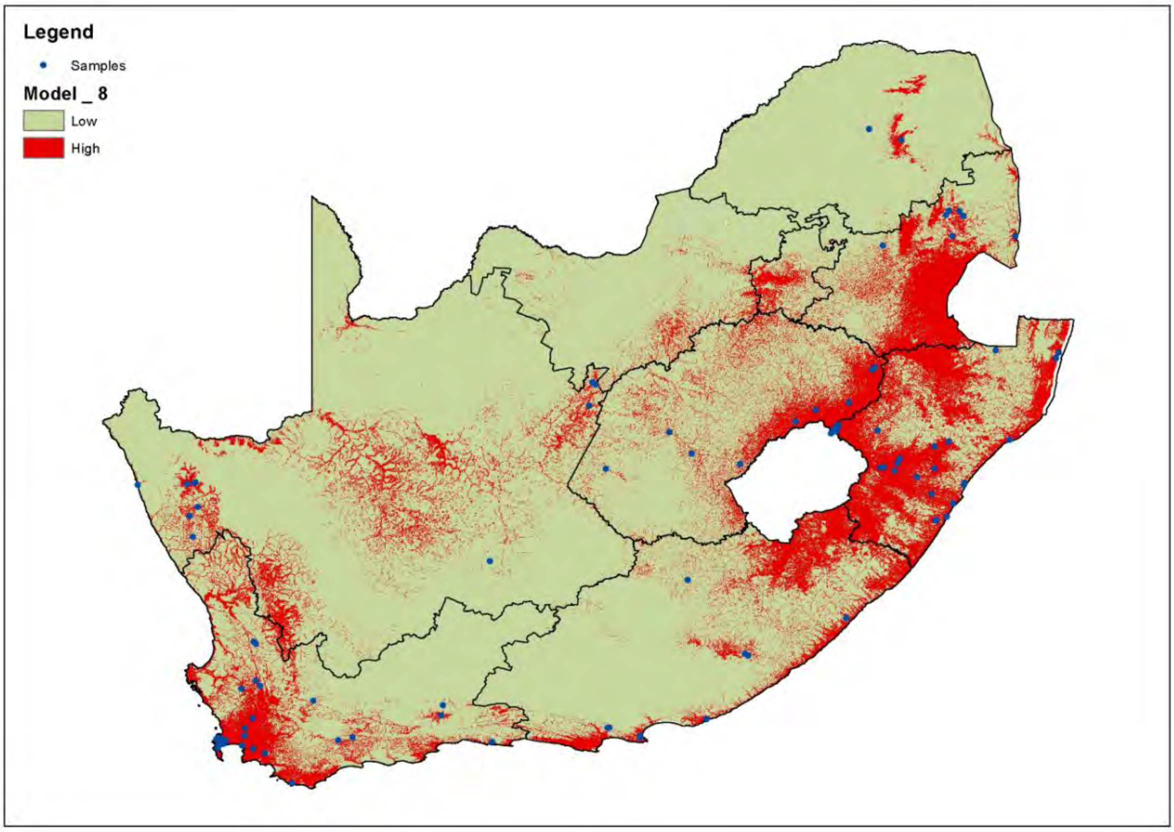

Figure 2.5: Probability threshold map for predicted occurrence of *Batrachochytrium dendrobatidis* in South Africa. Red indicates areas of higher probability.

2.5 Discussion

2.5.1 Infection prevalence

The significant role that *Bd* plays in amphibian declines has meant that understanding its prevalence and distribution is important for managing its spread and preventing introduction to suitable areas that remain disease-free (Adams *et al*. 2010; Woodhams 2011). Knowledge of prevalence and distribution of *Bd* across South Africa has, until now, not been thoroughly assessed, in particular in terms of infection in Red List species. This study provides the first quantitative data on *Bd* infection prevalence in South Africa"s threatened species and makes use of *Bd* occurrence data from all known records in the country to model the potential geographic distribution of infection. A proactive approach to conservation strategies for preventing introduction of *Bd* to novel areas and awareness regarding its effect on indigenous species through exposure experiments provides vital knowledge for managing a potential crisis (Andreone *et al*. 2008; Kremen *et al*. 2008). For example, a comprehensive national monitoring plan has been developed for the island of Madagascar that will serve the purpose of detecting *Bd* early should it be introduced, and will lead to targeted rapid response (Weldon *et al.* submitted). Although a similar strategy does not yet exist for South Africa, surveillance takes place in the form of intermittent conservation research by various research groups.

Based on examination of archived specimens of *Xenopus laevis* only, Weldon *et al*. (2004) found prevalence to be very low (1.5 – 3.8%) across the country. These low infection levels can be ascribed to the nature of archived collections that often consist of small sample sizes from a range of sites and dates, as well as to the lower detection probability of *Bd* by the histological methods used on specimens as compared with PCR techniques (Kriger *et al.* 2006b). Such samples violate the minimum sample size rule proposed by DiGiacomo & Koepsell (1986) for assumed prevalence-based detection probability, resulting in underestimated detection. Subsequent studies, including the present, augment previous research on infection data in South Africa significantly by including samples from multiple species from many additional sites (Africa *Bd* database, Weldon *et al*. unpublished data).

Based on these data, overall prevalence for *Bd* infection in South Africa is 33.9% across all species and 14.8% for threatened species. This surveying effort indicates that approximately 39% of South African species have been screened for *Bd* infection to date. The majority of sampling has been conducted in the Western Cape and KwaZulu-Natal provinces, and minimal sampling has been done in Gauteng, the Eastern Cape and Mpumalanga. This information can be used to direct future surveying for *Bd.*

Within South African species, prevalence varied significantly among genera, for both threatened and non-threatened species. With regard to the threatened species that were tested for *Bd* infection, of the nine species for which no infection was detected, six were represented by samples of up to five individuals only. This number is below the minimum objective of 20 individuals and therefore *Bd* status in these species could be a consequence of small sample size (DiGiacomo & Koepsell 1986). Furthermore, *Bd* was not detected in any threatened species for which fewer than 13 individuals were collected. Interestingly, none of the three *Breviceps* species, which employ direct development and are strictly terrestrial, were infected, including *B. sylvestris* (n = 28). Compared with prevalence in non-threatened species (19.8%) at the same sites for which samples from Red List species were obtained, prevalence among threatened species was lower (14.8%), possibly as a result of smaller sample sizes for non-threatened species (range = 0 – 13 samples per site).

With regard to infection in threatened species, the two *Heleophryne* species (*hewitti* and *rosei*) and *Afrixalus spinifrons* exhibit the highest prevalence of *Bd* infection (79.4, 50 and 62.5% respectively). These species have life histories highly dependent on close associations with the aquatic environment. *Heleophryne* are a riverine species that spend the majority of their lives in the vicinity of water (du Preez & Carruthers 2009) and as a consequence may be exposed to *Bd* spores more frequently. Another factor influencing the high prevalence in *Heleophryne* may be that the swab samples for these species were all taken from tadpole mouthparts for which high concentrations of zoospores have been detected in the keratin of infected tadpoles (Altig 2007). Susceptibility of tadpoles to infection varies widely and does not necessarily result in mortality (Blaustein *et al*. 2005). The closely related *Hadromophryne* has been found to possess skin peptides which offer resistance to *Bd* despite high prevalence and infection intensity (Gericke and Weldon, unpublished data).

Afrixalus spinifrons breeds in open water bodies (ponds, farm dams and wetlands) and males call from emergent vegetation at, or close to, the water‟s surface (pers. obs.; Pickersgill *et al.* 2004). This frequent contact with water may makes this species more susceptible to infection (Johnson & Speare 2003; Berger *et al.* 2005c). By contrast, the more terrestrial species showed either no (e.g. *Breviceps* and *Capensibufo*) or low infection intensity and prevalence (*Amietophrynus pantherinus*). The low prevalence and infection intensity in *X. gilli* may be an indication of the resistance of this genus to the pathogen (Gibble *et al.* 2007; Weldon *et al.* 2007). Neither clinical symptoms nor mortality was observed in any of the populations sampled. It should be noted however that three of the four Critically Endangered species tested positive for *Bd*, with the only exception being *V. amatolicus* for which only one specimen was screened, and which, again is largely a terrestrial species (Boycott 2004a). This places the Critically Endangered taxa in a particularly high risk category should pathogen virulence be exacerbated through sudden changes in environmental conditions. Discounting *Bd* as a threat to non-terrestrial and direct breeding threatened species should also not be taken for granted considering that some species could be susceptible and that there is an overlap in distribution with predicted *Bd* range (see below).

Briggs *et al.* 2005 showed that long-term persistence with *Bd* may be dependent on survival of a proportion of infected post-metamorphic individuals within a population. Whether the pathogen becomes lethal is subject to a complex array of factors, including life-history, immune defence system of host species, infection intensity and environmental context that influence the host-pathogen response (Longo & Burrowes 2010). Furthermore, host mortality is dependent on host age and size, duration of exposure to *Bd*, length of hibernation period and pathogen virulence (Garner 2007). It is important to recognise the distinction between infection with *Bd* (carrying the fungus but with no ill-effects) and disease that may cause mortality (chytridiomycosis) (Forzán *et al.* 2010). Data from this study indicates that the presence of *Bd* does not necessarily cause disease or declines (see also Woodhams *et al.* 2011). The ease with which *Bd* infection can be detected with modern techniques has meant that investigations into disease-caused declines may have become somewhat neglected (Duffus 2009). However in South Africa it does not appear that population declines have gone unnoticed due to the comprehensive database that comprises more than a decade of survey data.

2.5.2 Predicted distribution

Predictive distribution models provide a practical solution for minimising the rate of false negatives at the population scale and for optimising sample collection (Adams *et al.* 2010). Distribution of *Bd* is often not homogenous even in regions in which the pathogen is widespread and *Bd* is able to tolerate a broad range of climatic variables across varying altitudes (Adams *et al.* 2010; Murray *et al.* 2011). Until now, a fine scale model of predicted distribution for *Bd* in South Africa has not been available. The Maxent model generated here with up-to-date data shows patterns consistent with models from other regions of the world, (e.g. Murray *et al.* 2011) in that *Bd* distribution is correlated with generally cooler and wetter areas and excluded from arid areas. The areas with the highest predicted distribution of *Bd* coincide with the areas that host the highest frog diversity and endemicity in South Africa (KwaZulu-Natal and the Western Cape) (Measey 2011). However, these regions are also those with the highest number of samples and this may have influenced the model. The model also indicated that *Bd* can occur in a wide range of locations with conditions ranging from the warm and wet lowlands of KwaZulu-Natal to the more extreme conditions of the Drakensberg escarpment. The central and north-western regions of South Africa are least suitable for *Bd*. More records are needed from the Orange River basin to test the extent to which this relatively wet area is able to expand the distribution of *Bd* from the more suitable east of the country to the less suitable western extreme.

The model indicated that the distribution of *Bd* is most strongly influenced by precipitation of the wettest season and distance from water. This is expected given *Bd*‟s low tolerance for desiccation (Berger *et al.* 2005; Johnson *et al.* 2003) and dependence on the presence of permanent water for the transmission of aquatic zoospores (Berger *et al.* 1998). The variables "Mean temperature of coldest quarter (°C)" (13.9%) and "Topography" (13.2%) also contributed significantly to the model, indicating *Bd*‟s tolerance for cool temperatures between 10 and 23 °C (Longcore *et al.* 1999; Piotrowski *et al.* 2004), which fall into the range of mean winter temperatures for South Africa (-3 to 15 °C). Kriger & Hero (2006) showed that prevalence levels for *Bd* peaked between 12.3 and 19.7 in Australia, for which regions of highest *Bd* occurrence match those in South Africa, i.e. cool and wet areas (Murray *et al.* 2011). The influence of topography can be explained by *Bd*"s habitat requirements of low-lying water-bodies and generally cooler temperatures that often persist in valleys, especially when vegetation cover is high.

Without exception, all of South Africa"s threatened species have distribution ranges that overlap with the predicted distribution for *Bd* and as such are thus potentially exposed to *Bd* infection. As has been shown by the results of the prevalence of *Bd* infection in threatened species, those with more aquatic life-histories and that are associated with permanent water bodies are most susceptible to infection and risk of disease (Berger *et al.* 2004). Disease risk is further influenced by species-specific susceptibility and the additional risk factors discussed above. Incorporating life-history factors and knowledge of pathogen distribution will thus be useful for determining disease risk (Bielby *et al.* 2008; Murray et al. 2011).

2.5.3 Conservation Recommendations

The results of this model provide a clearer indication of where *Bd* infection occurs within South Africa, and can be used for identifying species that are at risk of *Bd*-related declines. Bielby *et al.* (2008) identify high-altitude, range-restricted, aquatic species with low fecundity as being those most susceptible to rapid declines. Cognisance of this, together with knowledge of pathogen distribution can help identify species that may be at risk of experiencing *Bd*-related declines. Of the threatened South African species that fall under these criteria, and which exhibit high infection intensity, *Heleophryne rosei* (CR) and *Afrixalus spinifrons* (particularly the subspecies from the KZN Midlands) (NT) may be susceptible to outbreaks of chytridiomycosis. Another species that warrants attention is *Amietia vertebralis* (NT) which is endemic to the north-western Drakensberg of KwaZulu-Natal and Lesotho. Although declines have not yet been detected, populations of *A. vertebralis* are the only southern African species for which intermittent mortality has been observed (Meyer *et al.* In prep.). The effects of external stressors, such as climate change, may exacerbate the impact of *Bd* on this species. Presently, the only consistent monitoring of *Bd* infection in South Africa is that being carried out by North-West University for *A. vertebralis* (Measey 2011) and it is imperative that this should be continued.

Infection with *Bd* does not always imply that lethal chytridiomycosis will result and presence of the pathogen ranges between endemic and lethal. As such, management of this disease should be context specific. Woodhams *et al*. (2011) recommend following a three-step process for mitigating disease at population level: 1) identifying mechanisms of disease suppression; 2) parameterise epizootiological models of disease and population dynamics for testing under semi-natural conditions and; 3) adaptive management in field trials with natural populations. Kriger & Hero (2009) also discuss treatment options and preventative measures to remove the threat of disease, namely the complete ban on all unnecessary long-distance trade and transport of amphibians. Despite this recommendation, and the fact that chytridiomycosis being recognised by the Office International des Epizooties (OIE), standards pertaining to the trade of amphibians remain to be developed (Hyatt *et al*. 2007). The export of wild-caught *Xenopus laevis* from South Africa is ongoing, without screening for *Bd* being conducted, despite this being identified as a major means of initial dissemination of the pathogen (Weldon *et al*. 2007; Weldon & Fisher 2011). This undoubtedly poses a risk to native amphibians for importing countries. Developing stringent regulations for the international trade of amphibians should be a priority as should implementing policy for the ban on export of *X. laevis* from South Africa (Vogel 2008). Trade of other species identified as vectors of the pathogen, for example, *Rana catesbeiana* should also be monitored and controlled (Hanselmann *et al*. 2004).

A vigilant approach should also be employed to prevent the introduction of a novel strain that could be potentially lethal to certain South African species. Although *Bd* appears to now be endemic to many regions of the world, with populations able to persist with low levels of infection (e.g. Briggs *et al*. 2005; Tobler & Schmidt 2010), experimentation has shown that infection with novel strains of *Bd* can cause death even when local strains do not (Gahl *et al*. 2011). Introduction of novel *Bd* strains into South Africa could prove devastating to local frog populations. Coupled with the potential unknown effect of additional stressors such as climate change and anthropomorphic habitat transformation, South Africa could still experience population declines as a result of chytridiomycosis. As such, identification of potentially susceptible species and likely areas of infection are essential first steps for any form of mitigation from *ex-situ* population management to the development of a surveillance program.

CHAPTER 3

STATUS OF AND CONSERVATION RECOMMENDATIONS FOR KWAZULU-NATAL'S THREATENED FROGS

3.1 Abstract

There are six threatened frog species in KwaZulu-Natal; one Critically Endangered (*Hyperolius pickersgilli*), three Endangered (*Anhydrophryne ngongoniensis, Leptopelis xenodactylus, Natalobatrachus bonebergi*) and one Vulnerable (*Hemisus guttatus*). *Afrixalus spinifrons* (Near Threatened) is also included for the purposes of this study. Half of these species are endemic to the province and the remainder occur only marginally in bordering provinces. All are endemic to South Africa. Much of KwaZulu-Natal forms part of the Maputaland-Pondoland-Albany hotspot, which hosts both high levels of biodiversity and is greatly threatened by various anthropogenic activities, and has been identified as a region in need of collaborative conservation efforts. Conservation plans are primarily reliant on knowledge of a species distribution and identification of threats based on research. This knowledge can then be used to guide specific conservation actions. Here, a synopsis of threats to frog biodiversity in the province is provided and threat assessments conducted for selected sites for three of the threatened species. The status of each species is discussed and recommendations on specific conservation actions going forward are outlined. *Hyperolius pickersgilli*, in particular, has been prioritised for such research, much of which has been provided by this study, and as such is examined in the most detail.

3.2 Introduction

3.2.1 KwaZulu-Natal as a biodiversity hotspot

South Africa is the third most biologically diverse country in the world, after Brazil and Indonesia (Endangered Wildlife Trust 2002; Driver *et al*. 2005). Despite occupying only 2% of the world‟s land surface, South Africa hosts a disproportionately high share of global diversity with 10% of the Earth‟s plant species and 7% of the mammal, bird and reptile species represented in the country (Dahlgren & Van Wyk 1988; King *et al*. 2005). There are three globally recognised biodiversity hotspots within South Africa, namely the Cape Floristic Region, the Succulent Karoo and the Maputaland-Pondoland-Albany hotspot, which is shared with Mozambique and Swaziland (Figure 3.1).

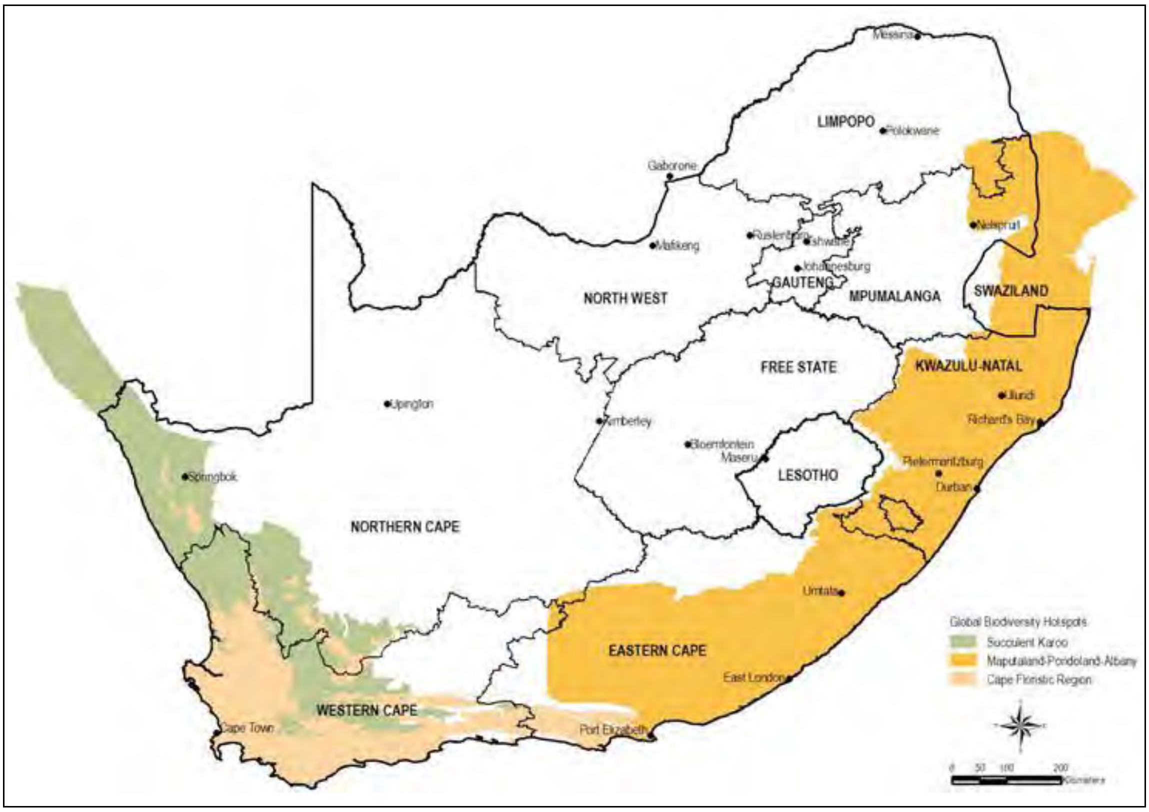

Figure 3.1: The biodiversity hotspots of South Africa. The Maputaland-Pondoland-Albany hotspot covers much of the KwaZulu-Natal province (Source: Mittermeier *et al.* 2005).

Nearly all of KwaZulu-Natal Province falls within the Maputaland-Pondoland-Albany hotspot, which has considerable levels of diversity and endemism (Bruton & Cooper 1980; Mittermeier *et al*. 2005). This hotspot is the second richest floristic region in southern Africa after the Cape Floristic hotspot (Goodman 2000; Driver *et al*. 2005; Conservation International 2008). Over 6, 000 plant species occur in the province, of which 16% are endemic (Acocks 1953; Low & Rebelo 1998). The floral species richness represented by the plant communities (ranging from sub-tropical swamp forest at sea level to alpine tundra at 3300 m.a.s.l) is reflected by the diversity of the fauna (Le Roux 2002). Overall, 23% of species that occur in the province are endemic. Disturbingly, much of this extraordinary natural heritage is at risk from a variety of human activities (as discussed below) (Hilton-Taylor 1996). The climate of KwaZulu-Natal ranges from subtropical in the northern coastal areas to temperate at the high elevations of the Drakensberg Mountains (Mucina & Rutherford 2006). Summers are typically warm (21°C to 28°C on the coast) and humid while winters are colder (ranging between 0°C and 23°C across the province) and drier (Van Riet *et al*. 1997). Overall, the province is the wettest in South Africa, receiving a mean annual rainfall of 845 mm, with a maximum of 1355 mm in coastal regions (Van Riet *et al*. 1997). This varied climate is due to a complex topography, and has important implications for the biodiversity within the province, which is a hotspot for many taxonomic groups (Low & Rebelo 1998; Tolley *et al*. 2008; Hilton-Taylor 1996; Conservation International 2008).

This region has been listed as one of nine priority areas for conservation in South Africa due to its high levels of diversity and endemism, but also because it faces ongoing and increasing pressure from human population growth and subsequent development (Raw 1995; Mittermeier 1999; Le Roux 2002; Goodman 2000; Armstrong 2009). The extent and rate of land transformation in the province is greater than that of any other country or province in southern Africa (Scott-Shaw 1999; Jewitt 2012). KwaZulu-Natal has the second largest human population (21.3 %) in South Africa at approximately 10.65 million (SSA 2010). This increasing population places ever increasing demands on land and natural resources. Mining, timber and sugar-cane production are the main historical economic drivers that have led to significant fragmentation of the landscape, particularly along the coast (Johnson and Raw 1987).

An estimated 90% of the Coastal Forest (important habitat for *Natalobatrachus bonebergi*) has been destroyed for sugar cane cultivation (Cooper 1988). More recently, there has been a substantial increase in tourism leading to residential and resort development, again especially along the coast (Le Roux 2002). Conflict between human needs and preservation of natural resources arises because human populations generally coincide with areas that are most valuable in terms of biodiversity and ecosystem services as they are both correlated with higher rainfall areas (Turpie *et al*. 2009).

In terms of amphibian diversity, KwaZulu-Natal hosts the greatest species richness in the country (Channing 2001; Measey 2011). There are a total of 70 frog species in KwaZulu-Natal of which 10% are endemic (Armstrong 2001). The rich species diversity together with ongoing high levels of human activity makes the province an important hotspot requiring a long-term amphibian conservation plan. After the Western Cape, KwaZulu-Natal has the second highest concentration of threatened frog species in South Africa (Armstrong 2001; Measey 2011).

3.2.2 Threatened Frogs of KwaZulu-Natal

Five of the 18 threatened South African frog species occur within the KwaZulu-Natal province. For the sake of this study the Natal Leaf Folding Frog, *Afrixalus spinifrons* (Near Threatened) is also included, due to research priorities accorded to it by Measey (2011) and because it was previously listed as Vulnerable, to total six threatened species for KwaZulu-Natal (Table 3.1).

Half of KwaZulu-Natal"s threatened frog species are endemic to the province, with those that occur outside the province only marginally so (Table 3.1). Most of these endemics are enigmatic due to their preference for inhabiting dense vegetation and/or because of their weak calls (Armstrong 2001). Furthermore, of the six species only one, *Leptopelis xenodactylus*, is considered to be adequately protected in terms of formally protected areas in which it occurs (Armstrong 2001). Species descriptions of each of the six threatened KwaZulu-Natal species are outlined in Chapter 1.

Table 3.1: The six threatened frog species found in KwaZulu-Natal, South Africa (IUCN 2011). Their general distribution and endemic status is shown (CR = Critically Endangered; EN = Endangered; VU = Vulnerable; and NT = Near Threatened).

Species	**Common Name**	**Region**	**Endemic**	**Red List Category 2011**
Hyperolius pickersgilli	Pickersgill‟s Reed Frog	Coastal	Yes	CR
Anhydrophryne ngongoniensis	Mistbelt Chirping Frog	Southern KZN	Yes	EN
Leptopelis xenodactylus	Long-toed Tree Frog	Southern KZN and Midlands	Yes	EN
Natalobatrachus bonebergi	Kloof Frog	Coastal Forest of southern KZN	Extends to coastal Eastern Cape	EN
Hemisus guttatus	Spotted Shovel-nosed Frog	Northern Coastal	Occurs marginally in Mpumalanga	VU
Afrixalus spinifrons spinifrons *A. s. intermedius*	Natal Leaf-folding Frog (comprised of two subspecies)	Coastal Midlands	Extend to Eastern Cape Yes	NT

3.2.3 Threats to KwaZulu-Natal‟s Anuran Fauna

Over the past century more than one third of KwaZulu-Natal has been transformed (where soil is turned and natural plant cover removed) as a result of anthropogenic land use (Armstrong *et al*. 1998; Goodman 2000; Jewitt 2012). It is in the most transformed regions (the central midlands and central coast) that the highest concentrations of threatened species occur (Mittermeier *et al*. 1999; 2005; Goodman 2000; Ashton *et al*. 2005). Based on land cover categories, as of 2008, 45.6% of the province had been completely and permanently transformed (Figure 3.2) (Jewitt 2012). This estimate does not include degraded vegetation types or include alien-infested areas, and as such, is likely to be conservative (Jewitt 2012). Should the rate of transformation continue on the current trajectory, it is predicted that only 22% of the province will remain in a natural state by 2050 (Jewitt 2012).

Once levels of transformation exceed certain thresholds, the consequences for biodiversity become increasingly severe: a 30 – 50% loss of natural habitat impairs landscape connectivity, which significantly impacts on population dynamics and species interactions (Dobson *et al.* 2006); transformation above 50% results in a rapid decline in the ability of the landscape to support viable populations (Flather & Bevers 2002); and a 70 – 80% loss of habitat exacerbates the effects of fragmentation, such as spatial configuration and habitat linkages, and affects species survival (Fahrig 2003). In KwaZulu-Natal, the first threshold has already been reached and the second level is predicted to be reached this year (2012).

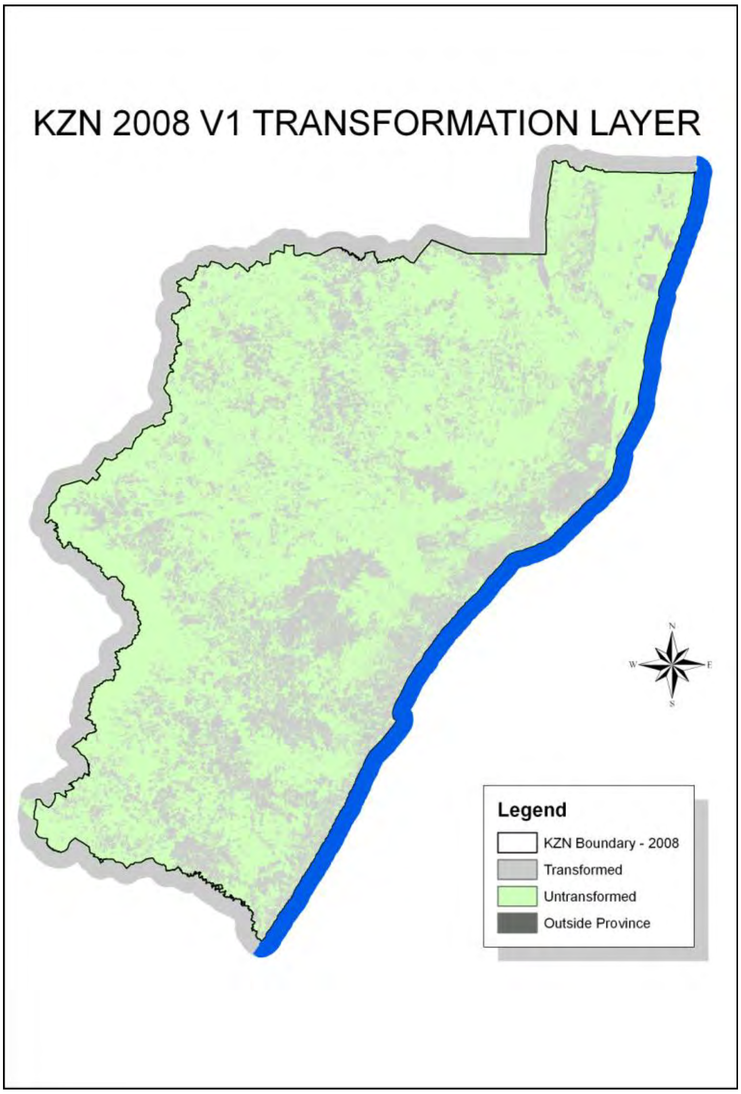

Figure 3.2: Percentage land transformation in KwaZulu-Natal as of 2008. Transformed areas are shown in grey and natural areas are shown in green (Source: Ezemvelo KZN Wildlife 2011).

The main factors driving land transformation and habitat modification in KwaZulu-Natal are crop farming and sylviculture (forestry) (Armstrong *et al.* 1998). Much of KwaZulu-Natal is arable and historically transformation of large areas for commercial farming has been a principal threat to the province"s biodiversity (Hilton-Taylor 1996; Driver 2005; Mittermeier *et al.* 2005). Much of this has been for irrigated or dryland crops, especially in the Midlands and Highland Grassland corridors. Sugar cane accounts for 12 % of the Natal Coast corridor being transformed (Johnson & Raw 1987; SANBI 2010). Additional threats caused by large-scale agricultural practices include over-abstraction of water, the impacts of fertilizers and pesticides, soil erosion and invasive weeds. Countrywide, 84 % of increased water abstraction since 1995 is attributed to irrigated agriculture (SANBI 2010). Livestock farming has led to the degradation of 66 % of ecosystems in the region. Poor management practices including overstocking and too-frequent burning have resulted in overgrazing and a loss of floristic biodiversity. Water systems for stock negatively impact stream-flow into wetlands by impounding excess water (SANBI 2010).

Sylviculture (commercial timber production) has resulted in irreversible transformation and associated loss of biodiversity in much of KwaZulu-Natal (Armstrong *et al.* 1998). Afforestation threatens many of South Africa"s Red List frog species and in KwaZulu-Natal *Anhydrophryne ngongoniensis* and *Leptopelis xenodactylus* are particularly impacted by habitat loss caused by afforestation (Minter *et al.* 2004; Measey 2011). Significant portions of the Midlands, Highland Grassland and Zululand have been converted for timber, resulting in high levels of fragmentation (SANBI 2010). Associated with the direct habitat loss caused by exotic plantations are the indirect effects of disrupted hydrodynamics and increased fire frequency. Plantations are estimated to use up to 1500 million litres per annum more water than the vegetation they replace, significantly reducing stream flow. Furthermore, plantations encourage encroachment of alien vegetation which increases the occurrence and intensity of fires (Henderson 2010). Alien invasive plants also severely disrupt the normal functioning of ecosystems by out-competing and replacing indigenous vegetation (Henderson 2001).

Encroachment by alien vegetation is a major threat to biodiversity in South Africa with over 10 million hectares (8%) of the country having been assessed as invaded by invasive alien plants. Within KwaZulu-Natal, 9.75% of the province was assessed as being invaded

(WESSA 2008). The majority of KwaZulu-Natal‟s threatened species occur in areas that are impacted by invasive alien vegetation. For example, *Anhydrophryne ngongoniensis* is a species reliant on grassland vegetation, much of which is being replaced by not only exotic plantations of *Pinus*, but also the associated encroachment of bugweed (*Solanum mauritianum*) and black wattle (*Acacia mearnsii*). *Hemisus guttatus* and *Hyperolius pickersgilli* are also threatened due to the lowering of the water table caused by invasive alien plants, and plantations of *Eucalyptus* (Minter *et al.* 2004; Measey 2011).

The loss of wetlands is possibly the single biggest threat to frog species in KwaZulu-Natal (Channing & Van Dijk 1995; Measey 2011). Anecdotal accounts abound about how species, now rare, used to be found in abundance in semi-urban areas (Pickersgill 2007; N. Passmore, pers. comm.; A. Lambiris, pers. comm.). Despite the importance of wetlands, more than half of the wetlands in South Africa have been destroyed, mostly due to agricultural development (Kotze *et al.* 1995). Wetland loss can be deliberate (such as drainage for pasture production) or inadvertent (usually due to poor management which results in erosional degradation) (Higgins 2003; Amis *et al.* 2007). The majority of South African frogs are associated with wetlands for at least part of their life cycle (Channing & van Dijk, 1995; du Preez & Carruthers 2009). Many species make use of temporary wetlands, which are often lost unintentionally due to their seasonality. Small wetlands, which are also more readily destroyed, are also important to frogs, especially in terms of meta-population dynamics (Channing & van Dijk, 1995). Aside from *Natalobatrachus bonebergi* (which is a riverine species), all of KwaZulu-Natal‟s threatened frog species are threatened by wetland loss. Protection of wetlands is thus essential for inclusion in systematic conservation plans for amphibians (Cowan 1995; Kotze *et al.* 1995).

3.2.4 Threat Identification and Assessment

In addition to knowledge about the distribution and behaviour of a species, an assessment of direct threats is essential for producing holistic conservation plans (Ashton *et al.* 2005; Salafsky *et al.* 2008). For most frog species in South Africa, threats have not been formally documented and their identification is largely anecdotal (Minter *et al.* 2004; Measey 2011). Direct threats are defined as human activities that cause the destruction, degradation and/or impairment of biodiversity (Salafsky *et al.* 2008). By removing or reversing direct threats a species may be conserved for as long as that conservation action is implemented. Indirect threats (stressors and contributing factors) may predispose or make certain species more vulnerable (Measey 2011). Distinguishing between direct and indirect threats can be difficult, since the line between them is often unclear (Salafsky *et al*. 2008). Assessment of direct threats has been identified as the most important priority for South Africa"s threatened frogs (Measey 2011).

Threat identification should be ongoing during research into threatened anurans (Salafsky *et al*. 2008). Ideally determination of threats should be conducted using a standard approach and be quantified so that appropriate conservation actions can be taken (Salafsky *et al*. 2008). Conservation actions can be targeted at reversing direct threats, contributing factors or biodiversity targets (Figure 3.3). Targeting direct threats is usually the most successful approach as in most cases these are caused by human activity.

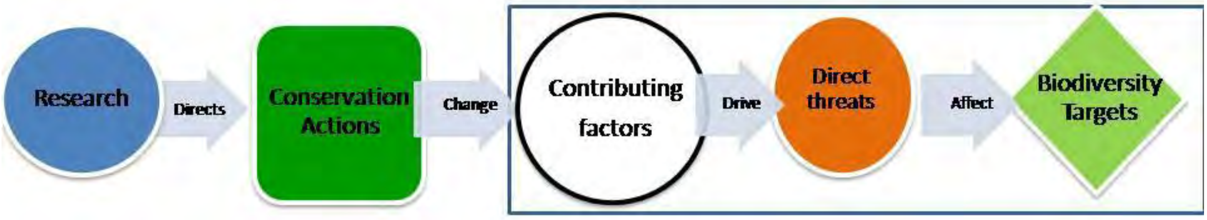

Figure 3.3: A general model for a conservation project (Adapted from Salafsky *et al.* 2008).

The aims of this chapter were to:

1) Identify threats to threatened frog species in KwaZulu-Natal;

2) Provide recommendations on additional research requirements; and

3) Recommend conservation actions for these species, particularly *Hyperolius pickersgilli,* which is the province"s only Critically Endangered frog species.

3.3 Methods

3.3.1 Study sites

During the course of this study, multiple localities (Appendix B) for each of KwaZulu-Natal"s threatened frogs were visited and threats assessed. Habitat and threat assessments were conducted for *Afrixalus spinifrons, Hyperolius pickersgilli* and *Natalobatrachus bonebergi* at select sites which were revisited throughout the study period (between 2008 and 2012). The sites assessed occurred in the following areas:

Mount Moreland "Froggy Pond"

Mount Moreland is situated north of Durban and immediately south of King Shaka International Airport (Figure 3.4). The large (7.19 Ha) reed-bed wetland known as "Froggy Pond" hosts one of the largest known populations of *Hyperolius pickersgilli*, estimated at 1928 adults, as of February 2011 (Bowman 2011). Other notable species at the site include *Hemisus guttatus* (VU) and a multitude of Least Concern species including *Afrixalus delicatus*, *Hyperolius tuberilinguis*, *Leptopelis natalensis*, *Strongylopus fasciatus*, *Ptychadena oxyrhynchus* and *Phrynobatrachus natalensis*.

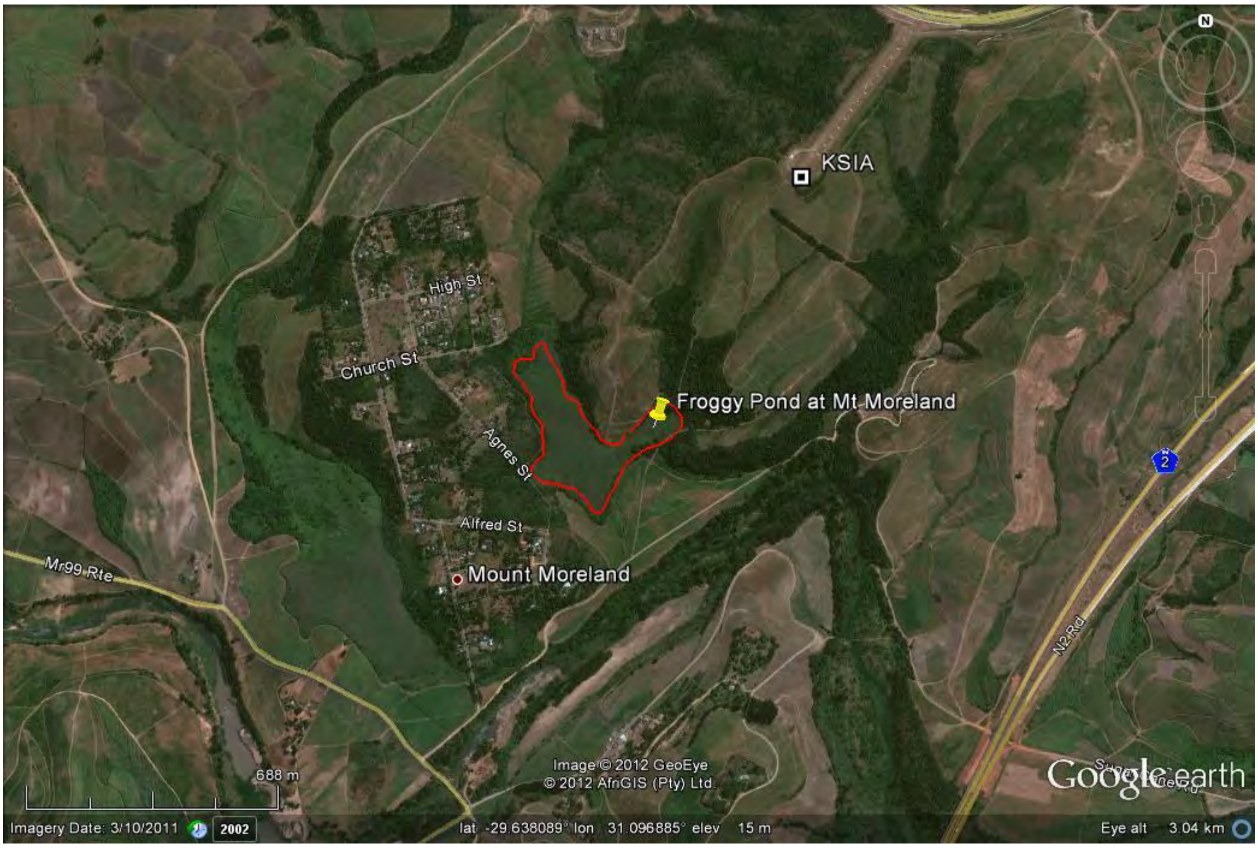

Figure 3.4: Mount Moreland and surrounding wetlands, with "Froggy Pond" indicated by the red outline. The runway for King Shaka International Airport (KSIA) is visible to the North.

Vernon Crookes Nature Reserve

Vernon Crookes is a 2189 hectare Reserve located on the South Coast of KwaZulu-Natal, approximately 15 km inland from Scottburgh (Figure 3.5). Although small, the Reserve is a combination of natural riparian forest (along the Mnyengelezi River) and grassland and as such hosts a vast array of species. It provides an island haven for biodiversity in an otherwise transformed landscape. In terms of frog diversity it is home to two threatened species *Natalobatrachus bonebergi* (EN) and *Afrixalus spinifrons* (NT). In addition, other least concern species that were noted here during the study include *Hyperolius marmoratus*, *Leptopelis natalensis*, *P. natalensis*, *Arthroleptis wahlbergi*, *Amietia angolensis* and *Breviceps verrucosus*. The reserve is surrounded by sugarcane plantations and a portion of the reserve has been reallocated under a land-claim and is now potentially threatened by overgrazing by domestic cattle, poaching and increased use of natural resources (R. Uys, pers. comm.).

Figure 3.5: Vernon Crookes Nature Reserve showing two sites for *Afrixalus spinifrons* (a pond to the north-east of the reserve) and *Natalobatrachus bonebergi* (in riparian habitat along the Mnyengelezi River).

Durban South (Prospecton and Isipingo wetlands)

The Prospecton and Isipingo wetlands occur in the highly industrialised zone of Durban South. Both are surrounded, and effectively isolated, by a sea of heavy development (Figure 3.6). The Isipingo site hosts an apparently healthy population of *H. pickersgilli* and is situated between a busy road, water treatment works, electrical substation and low-income housing, which is accessed directly through the wetland area. As such, this population is very isolated and its long-term survival is uncertain. The site at Prospecton is situated immediately south of the old Durban airport and also appears to host a relatively large population of *H. pickersgilli* as well as *A. spinifrons*. A large area surrounding and including the site is proposed to be developed for the new Durban South Port. This is a massive development, which in all likelihood will extirpate the population of *H. pickersgilli* here (as well as all other biodiversity at the site). These sites were visited on numerous occasions at night to search for *H. pickersgilli* and *A. spinifrons*.

Figure 3.6: Durban South area showing the wetland areas at Isipingo to the west and Prospecton to the east (outlined in red).

Krantzkloof Nature Reserve

Proclaimed in 1950, Krantzkloof Nature Reserve protects 584 hectares of land within an otherwise suburban area on the Kloof plateau (Figure 3.7). It is situated on the coastal escarpment inland of Durban and is the meeting place of the Molweni and Nkutu Rivers, which are tributaries of the Umgeni River. The plateau is comprised of Natal Group sandstone. In terms of biodiversity, the reserve contrasting aspects and disjunct moisture regimes make Krantkloof an area of high diversity and it is home to many species that are threatened or rare (Ezemvelo KZN Wildlife). In terms of amphibians, Krantzkloof is the type locality of *Natalobatrachus bonebergi* as well as the Natal Cascade Frog *Hadromophryne natalensis*. The reserve was visited during daylight hours as well as at night on several occasions.

Figure 3.7: Image of Krantzkloof Nature Reserve showing riverine valleys surrounded by steep krantzes. This reserve is the type locality for both *Natalobatrachus bonebergi* and *Hadromophryne natalensis.*

3.3.2 Site assessments

Habitat assessments were conducted at two sites per species (Table 3.2) using a standardised form to give an overall score for habitat integrity. The aim of these paired assessments was to compare a relatively intact, or where appropriate, Protected Area (Site A) with a more disturbed site (Site B). Sites were selected based on knowledge of occurrence of target species as determined through Atlas data (Minter *et al*. 2004) and through knowledge gained during the study period. For each site assessment time, weather conditions, species recorded and observed threats were recorded. The assessment form template can be found in Appendix C.

Table 3.2: Details of sites assessed for the six threatened frog species in KwaZulu-Natal. Two sites per species were assesses, with Site A representing a more intact locality and Site B, one that was more degraded. Species status and locality co-ordinates are given.

Target Species	Status	Site A	Site B
Hyperolius pickersgilli	CR	Mount Moreland S -29.63816° E 31.09746°	Isipingo S -29.991050° E 30.904940°
Natalobatrachus bonebergi	EN	Vernon Crookes Nature Reserve S -30.270427° E 30.600048°	Krantzkloof Nature Reserve S -29.777492° E 30.83088°
Afrixalus spinifrons spinifrons	NT	Vernon Crookes S -30.270427° E 30.600048°	Prospecton S -29.982446° E 30.937997°
Afrixalus spinifrons intermedius	NT	Kamberg NR S -29.373280° E 29.722850°	Kingussie Farm S -29.432000° E 29.905870°

The standard habitat and threat assessment form was compiled using various sources including the Atlas and Red Data book of the frogs of South Africa, Lesotho and Swaziland (Minter *et al*. 2004) and online sources (http://www.vasos.org/habitatguidelines.htm; U.S. EPA 1999). Assessments were conducted once at each site for all sites, but for those sites visited frequently assessments were updated yearly to determine any changes over time. Assessments took place throughout the study period (2008 to 2012) during the breeding season (between August and April) and involved at least two researchers/assistants including myself.

Habitat variables assessed included air and water temperature; water depth; substrate composition; vegetation composition (including alien vegetation); an estimate of percentage of suitable breeding area and surrounding terrestrial habitat and land-use in the catchment area. All anuran species detected during the site visit (either by audio or visual detection) were recorded. Threat criteria assessed included physical threats (proximity to roads or other transportation corridors); human influence (proximity of human habitation, habitat modification, agricultural/industrial activities and natural resource use) and evidence of pollution. Each was rated according to high, medium or low impact. An overall habitat score for each site was given as follows: 1 = Optimal; 2 = Suboptimal; 3 = Marginal; 4 = Poor; and 5 = Severely degraded. Optimal habitats are those in which habitat integrity is stable (over 70% is intact) and minimum threats were perceived, while severely degraded systems are those in which less than 20% of the habitat was deemed stable and the threats were perceived to be limiting to the species.

3.4 Results & Discussion

3.4.1 Site Threat Assessments

Table 3.3 shows the overall habitat integrity rating for each of the 8 assessed sites. As could be expected, Category A sites (those in Protected and relatively undisturbed areas) achieved higher habitat integrity ratings (with an average of 1.5) than Category B sites (those in disturbed areas), with an average of 3.5. The condition of each site is described below.

Table 3.3: Habitat integrity scores of assessed sites for KwaZulu-Natal"s threatened frog species. 1 = Optimal, 2 = Suboptimal, 3 = Marginal, 4 = Poor.

Site A	**Date Assessed**	**Habitat Integrity Score**	**Site B**	**Date Assessed**	**Habitat Integrity Score**
Mount Moreland	1 September 2011	2	Isipingo	4 October 2011	4
Vernon Crookes (Mnyengelezi River)	7 April 2009	1	Krantzkloof Nature Reserve	24 March 2009	3
Vernon Crookes (Plateau Dam)	7 April 2009	2	Prospecton	28 January 2011	4
Kamberg Nature Reserve	21 December 2011	1	Kingussie Farm Dam	16 December 2011	3
Average Score (A)		**1.5**	**Average Score (B)**		**3.5**

Site comparisons for *Hyperolius pickersgilli*

Site A

Mount Moreland (Froggy Pond), which was assessed for *Hyperolius pickersgilli,* received an overall habitat integrity score of 2 (sub-optimal). This score gives an indication of the relatively high quality of habitat at this site. The vast reed-beds and immediate surroundings provide important frog habitat, which hosts a wide variety of species (at least 21), and is almost certainly a refuge for many species that were historically widespread in the Durban area, for example, *H. pickersgilli, Afrixalus delicatus*, *Hemisus guttatus*. Vegetation is comprised predominantly of the indigenous Common Reed (*Phragmites australis*), with an understory of the Bristly Snakeroot (*Persicaria attenuata*), and Buffalo grass (*Stenotaphrum secundatum*). Approximately 80 % of the wetland provides suitable breeding habitat for *H. pickersgilli*, which appears to require both long, standing reeds (such as *P. australis*) and a moribund understory from which males call from low positions. The wetland is surrounded by extensive sugar cane (*Saccharum sp.*) plantations and bordered to the West by Mount. Moreland village. There is relatively high infestation of alien vegetation including exotic *Canna* encroaching the wetland itself. The wetland is surrounded by large gum trees (*Eucalyptus sp.*), bugweed (*Solanum mauritianum*) and lantana (*Lantana camara*). The proportion of alien vegetation appears to be increasing (A. Wilken; pers. obs.).

The construction of the King Shaka International airport in 2009/2010 and associated infrastructure potentially poses a significant threat to Mount Moreland‟s biodiversity, particularly at "Froggy Pond" which is used as the run-off site for effluent from the airport. Although the effluent is treated prior to being released into the wetland it may still introduce harmful contaminants from sewage, fuel and other pollution into the wetland. Recent water testing however has shown that potassium and nitrate levels in the water from the wetland are low (I. Visser pers. comm., January 2012). Other threats from the airport include direct deaths of frogs caused by increased traffic in the area, loss of habitat as a result of both the airport itself and any concomitant construction. The wetland is also in the direct flight path of aeroplanes, the noise from which may affect calling and breeding behaviour (Bee & Swanson 2007; Parris *et al*. 2009; Cunnington & Fahrig 2010). Currently only an estimated 20% of the surrounding habitat is suitable for terrestrial activities (foraging and over-wintering). The species has been found in gardens bordering the wetland (A. Wilken pers. comm.) and also 1.2 km away at the airport site (J. Harvey pers. comm.). The inevitable expansion of infrastructure related to the new airport will undoubtedly impact on this species‟ terrestrial habitat needs. The nearest known occupied site is 38 km away to the south at Isipingo (see below). The wetlands at Mount Moreland are currently offered minimal protection under the Mount. Moreland Conservancy and are being rezoned as conservation areas.

Site B

The wetland at Isipingo is small (2 ha) and is isolated within a highly industrialised area. It received an overall habitat rating of 4 (Poor). It is bordered by a sewage works and an electrical sub-station to the South and low-cost housing in the North and is close (100 m) to a very busy road (M35). A footpath running directly through the wetland to the East end is used to access this housing. Some informal draining of the wetland has already taken place to allow for subsistence farming on the periphery. This proximity to human habitation and activity poses a potentially high level of threat to the biodiversity of the wetland. Despite this, the wetland itself appears to be relatively intact and has the highest density of *H. pickersgilli* of any of the other sites (Figure 3.8).

Figure 3.8: Habitat at the Isipingo wetland. Despite highly industrial surroundings and threats from proximity to human activities including housing and subsistence agriculture, this wetland is a haven for biodiversity.

The wetland is dominated by Bulrushes (*Typha capensis*), Sedges (*Cyperus textilis*) and Bristly Snakeroot (*P. attenuata*). The periphery of the wetland is becoming increasingly encroached by *Solanum mauritianum* and *Lantana camara* as well as thistle, which gives an indication of disturbance (A. Armstrong pers. comm.). The nearest site hosting a population of *H. pickersgilli* is at the Prospecton wetland (3.4 km to the North-East), although there may be limited connectivity between the sites. Ironically, of all 16 known localities for *H. pickersgilli* the two that are in closest proximity to each other are those in the most disturbed landscape. The Prospecton site will in all likelihood be completely eliminated as a result of the proposed new Durban South Port development (see below).

Site comparisons for *Natalobatrachus bonebergi*

Site A

This stretch of the Mnyengelezi River in Vernon Crookes Nature Reserve was visited frequently (8 times) during the study period (2008 to 2012) between October and May of the frog breeding season (Figure 3.9a). This site received an overall habitat integrity rating of 1 (optimal) for *N. bonebergi*. The species was detected at the site at each visit, with adults, egg clutches and/or tadpoles in evidence at each visit. The stream is slow-flowing has variable stretches of different water depths as well as numerous positions for egg-clutch attachment including rocks and overhanging vegetation (Figure 3.9b). These conditions are ideal for *N. bonebergi*.

Figure 3.9: (a) Ideal habitat for *Natalobatrachus bonebergi* at the Mnyengelezi River in Vernon Crookes Nature Reserve, (b) egg clutch of *N. bonebergi* attached to a twig.

Site B

Krantzkloof Nature Reserve, although reported to host *N. bonebergi* was not found to be highly suitable to the species during the course of this study. Stretches of the Molweni and Nkutu Rivers were searched on various occasions, but the species was not detected. The stretch that was assessed on 24 March 2009 received an overall habitat integrity score of 3 (marginal) for this species. When compared to Vernon Crookes, the river here is fast-flowing, silted and there are little or no suitable surfaces for egg-attachment.

Notes on new sites

During this study three new localities were found for *N. bonebergi*, namely at Cowies Hill (-29.82436, 30.89609); Armadale Farm near Melville and Umbumbazi Nature Reserve (Figure 3.10). The presence of the species was reconfirmed at Oribi Nature Reserve and Dwesa-Cwebe Nature Reserve in the Eastern Cape. It was not detected in Palmiet Nature Reserve, from which there are also historical records.

Figure 3.10: An amplecting pair of the Endangered Kloof Frog *Natalobatrachus bonebergi* at Umbumbazi Nature Reserve near Paddock. Photo courtesy Leon Meyer.

Site comparisons for *Afrixalus spinifrons spinifrons*

Site A

The plateau dam site at Vernon Crookes Nature Reserve was given an overall habitat integrity score of 2 (sub-optimal) for *A. s. spinifrons*. The water body is surrounded by emergent vegetation comprised primarily of sedges and provides ideal habitat for *A. s. spinifrons*. A possible threat may be posed by trampling from cattle from the neighbouring rural area, which now have access to the reserve. The dam is also in close proximity to a reserve road, although traffic is minimal.

Site B

The wetland at Prospecton received an overall habitat score of 4 (Poor). This medium-sized wetland (4 ha including adjacent reed beds) is situated at the south end of the old Durban airport runway, adjacent to the very busy Prospecton Road and is surrounded by a highly developed industrial zone (oil storage and car manufacturing primarily). The wetland is dominated by the Common Reed (*Phragmites australis*), the Bristly Snakeroot (*Persicaria attenuata*), and Buffalo grass (*Stenotaphrum secundatum*). Considering its location and overall rating as poor, this substantial wetland remains relatively intact, and provides a refuge for biodiversity and hosts two threatened frog species (*H. pickersgilli* and *A. spinifrons*) and at least 7 Least Concern species including *H. tuberilinguis* and *L. natalensis*. There is a drainage ditch splitting the wetland into two halves, which is where frogs are concentrated towards the start of the breeding season as this is where water accumulates. After rain the reed-bed area becomes wetter in patches. *A. s. spinifrons* is most easily located along the drainage ditch. This site has been ear-marked for development for the new Durban South Port and is likely to be completely eliminated (see below for conservation actions and Chapter 4 for more detail).

Site comparisons for *Afrixalus spinifrons intermedius*

Site A

The assessment site at Kamberg Nature Reserve is a wetland area in the Stillerus section of the reserve. The site received an overall habitat integrity rating of 1 (optimal). The area had been visited three weeks later (January 2011) a year before without success. On 21 December 2011 numerous individuals of *A. s. intermedius* were detected. This frog was last recorded at Stillerus in 1987. In addition, the Rhythmic Caco, *Cacosternum spp. A* of du Preez and Carruthers (2009) was recorded at the same locality, the first record of this narrow-range endemic frog from the uKhahlamba-Drakensberg Park World Heritage Site. The site is comprised of low, inundated vegetation along a stream. No immediate threats were perceived.

Site B

The site at Kingussie Farm is a large dam with extensive areas of emergent vegetation including *Phragmites* and *Carex sp*., which provides ideal habitat for *A. s. intermedius*. The site was assessed on 16 December 2011 and received an overall habitat integrity rating of 3 (marginal). The species was first detected at this site in November 2008 at another section of the dam Potential threats include trampling from cattle, eutrophication and agricultural activities, such as ploughing of the surrounding terrestrial habitat which may impact the foraging and over-wintering activities of the frogs.

Additional surveys for *A. s. intermedius*

Numerous other localities were searched for *A. s. intermedius* during the course of the study (see Chapter 5). Notable rediscoveries include that at a dam on Cedara College property near Hilton, the first record in 35 years and at the Type locality on the Mooi River floodplain at Rosetta on 21 December 2011.

3.4.2 Conservation actions for threatened anurans

Measey (2011) outlined conservation research requirements for all of South Africa‟s threatened frogs. Research provides the necessary scientific basis from which conservation actions can be developed (Salafsky *et al.* 2008). This study provides threat identification for certain of KwaZulu-Natal‟s threatened frog species. Based on habitat and threat assessments, conservation actions for *H. pickersgilli*, *N. bonebergi* and *A. spinifrons* are outlined below. Additional aspects of research requirements for *H. pickersgilli* and *A. spinifrons,* including knowledge of distribution and taxonomy respectively, are provided in subsequent chapters. Research requirements for *A. ngongoniensis, L. xenodactylus* and *H. guttatus* were given in Measey (2011) and these still need to be implemented. As such, these species are not dealt with in extensive detail here.

Conservation actions must be implemented and managed by provincial and regional conservation authorities and non-government organisations. Involvement of these, and other relevant stakeholders, such as landowners and other interested and affected parties, will be essential for the success of long-term conservation strategies. Fundraising is necessary to carry out the majority of actions described. Priority is given to the Critically Endangered species in the Province, namely Pickersgill‟s Reed Frog *Hyperolius pickersgilli.* An overall objective for all threatened species is to raise their public profile through media, awareness events and developing general information material for schools. Conservation actions for threatened frog species in South Africa include:

Habitat protection

Habitat loss is the biggest threat to amphibians so it follows that protection of habitat remains the key strategy for conserving amphibians (Cushman 2006). The effect of habitat loss and fragmentation on populations requires knowledge about population viability and connectivity, as determined by juvenile dispersal, which is lower in fragmented landscapes (Cushman 2006). Protection of non-breeding terrestrial habitat and corridors is thus as important as that of breeding sites. Landscape-level conservation strategies are therefore likely to be the most successful for ensuring long-term species survival (Gibbs 1998;

Löfvenhaft *et al*. 2004; Cushman 2006, Ernst *et al*. 2006). Methods for securing land for protection of biodiversity include land acquisition, stewardship programmes and Protected Area expansion programmes. Rehabilitation of sites may be necessary in some cases (e.g. clearing of alien vegetation, wetland restoration and buffer-zone management). Sound management practices must be put in place to ensure ongoing security of the site.

Biodiversity Management Plans for Species (BMP-S)

Legal conservation actions are required to develop, change or influence policy at all levels. On a national level, in cases that require formal agreement between multiple stakeholders a Biodiversity Management Plan (BMP-S) provides a useful tool for outlining co-ordinated, strategic conservation measures for threatened species. This is a relatively new tool provided by the National Environmental Management Biodiversity Act (NEMBA) and ensures that conservation plans are legally ratified and therefore enforceable. A BMP-S can be developed for one or more species, or for one or more populations or a meta-population. All relevant stakeholders (for example, land owners, conservation authorities and other interested parties) must be invited to take part in the development of the BMP-S (DEAT 2009). To date, only one BMP-S has been developed for a South African threatened frog, namely the Western Leopard Toad (*Amietophrynus pantherinus*) in the Western Cape. Of KwaZulu-Natal‟s threatened species, *H. pickersgilli* is an ideal candidate for a BMP-S. Development of a BMP-S can help guide all other conservation actions.

Monitoring Programmes

Global amphibian population declines, some of which have occurred in protected areas, dictate that a vigilant approach is needed for implementing monitoring regimes and to identify stressors if declines are discovered (Dodd 2003). Long-term monitoring programmes are an essential means of assessing population trends for a species or assemblage of species and are particularly relevant and applicable to threatened frog species, both for detecting fluctuations in population size and for measuring success of conservation actions (Dodd 2004; Measey *et al.* 2011b). There are typically multiple factors that must be considered when designing monitoring protocols including method of sampling technique; site selection; effect of climatic variables, species characteristics and life-stage on detectability; natural population fluctuations; meta-population processes (particularly juvenile dispersal); and human-based constraints (available funds, consistency of training personnel and time) (Gibbs 1998; Dodd 2004).

Detection ability is seldom perfect and varies widely between species, sites and observers (Mackenzie *et al*. 2002; Field *et al*. 2005; Smith *et al*. 2006) and methods must be adapted to suit target species/assemblages, for example, multi-species call surveys have been found to be inefficient for rare species (Pellet & Schmidt 2005). As a general observation; the majority of threatened species in South Africa tend to have quiet, inconspicuous calls and cryptic behaviour, for example, *H. pickersgilli* has a call that could easily be mistaken for an insect, and is issued only intermittently so is easily overlooked, while *N. bonebergi* also has an extremely soft call akin to water dripping slowly. Species with highly specialised habitat requirements or localised distribution may also be difficult to detect. However, it is often these species for which declines have been noted, so it is necessary to include them in monitoring programmes (Dodd 2004).

Amphibians have complex life cycles and as such may require multiple sampling techniques. There are numerous monitoring techniques including active sampling (e.g. visual encounter, call surveying, egg mass counts) and passive sampling (traps, covers and data loggers). For community assemblages it is usually best to employ more than one method (Dodd 2004). Monitoring protocols should be standardised where possible and repeatable in the long-term (at least 10 years) in order to detect true population changes as a result of human disturbance as opposed to natural fluctuations (Dodd 2004; Rödel & Ernst 2004; Veith *et al*. 2004). Monitoring protocols must also always employ biosecurity measures to prevent introduction and/or spread of disease, particularly chytrid (Chapter 2). The goal of long-term monitoring is to provide reliable data to demonstrate trends, for example by using population viability analysis (Akçakaya & Sjögren-Gulve 2000; Field *et al*. 2005; Measey 2011). Optimising protocol design under given resource constraints is thus key to successful monitoring (Field *et al*. 2005). Currently, the only long-term monitoring programme in South Africa is that carried out by Cape Nature at three sites in the Western Cape (Measey 2011).

Hyperolius pickersgilli received the highest score in terms of priority for implementation of a monitoring programme for South African threatened species (Measey 2011).

Education and awareness

There exists a great need in South Africa to raise the public profile of amphibians, as well as significantly build on capacity to increase research output (Measey 2011). Improving public awareness can be carried out at all levels, from school education to citizen science. Awareness can also be driven through print and broadcast media and specific awareness events. Building capacity requires attracting new young researchers in the field of amphibian research and improved training within provincial and national conservation authorities, which in turn can help promote awareness. South Africa is entering an exciting period where collaboration between local and global conservation authorities and non-government organisations can really begin to drive such initiatives. During the course of this study, I gave numerous talks to school and community groups, as well as wrote a number of articles for popular publications regarding the amphibian crisis and threatened frogs in South Africa. In addition, the chytrid project was featured on South Africa television and I gave an interview on the plight of frogs in a further interview (See Appendix C for details of presentations and articles).

Ex-situ breeding programmes

Captive breeding (*ex situ*) programmes are becoming widely used as a conservation tool for threatened species, with the ultimate aim of future reintroduction into the wild (Tenhumberg *et al*. 2004). As with all conservation measures such programmes need to be carefully planned to optimise long-term species persistence, and translocation strategies, in particular, should be based on empirical research (Rout *et al*. 2009).

In terms of *ex-situ* conservation for amphibians, 2008 saw the globally coordinated “Year of the Frog” campaign successfully bring public awareness to the plight of amphibians through Zoos, Aquaria and Botanical Gardens (Amphibian Ark). An Amphibian Species Prioritisation Workshop was held in Johannesburg in April 2008. The workshop brought attention to the role that *ex-situ* programmes for threatened species can play in conservation. The process of evaluating an *ex-situ* role for species is also integral for distinguishing *in-situ* (in the field) conservation needs and the two disciplines should benefit from one another (Amphibian Ark 2008). For the majority of South African species assessed additional knowledge with regard to species range and identification of the threats within that range was identified as a requirement. Depending on the severity of these threats, conservation roles can be assigned to each species. In some cases the species may provide an educational ex-situ role, while in the most extreme cases an Ark (when the species is effectively extinct in the wild) or Rescue program is required if threats are not reversible within a time frame. Furthermore, captive survival assurance programmes will play a vital role in disease management through the treatment of infected animals, breeding of disease-free offspring and eventually release into pathogen free environments. Seven categories (for each of which there are a number of options) are assessed in order to determine a prioritisation score for each species. These categories are:

1. Extinction Risk (Status)
2. Phylogenetic Significance
3. Threat Mitigation (level of management)
4. Conservation Role in terms of Ex-Situ Value
5. Biological Significance (uniqueness)
6. Cultural/Social/Economic Significance
7. Scientific Importance

From this assessment, a Taxon Management Plan can be compiled for each species. Working in conjunction with zoos will be an important aspect of the conservation for threatened South African species. Amongst those species prioritised for an *ex-situ* programme in KwaZulu-Natal, *Hyperolius pickersgilli* was highlighted as qualifying for a rescue/supplementation *ex situ* role, and this has now been initiated by the Johannesburg Zoo. The remainder of KwaZulu-Natal‟s threatened species were allocated the role of Conservation Research for *ex-situ* programmes.

3.4.3 Conservation recommendations for *Hyperolius pickersgilli*

This study has provided improved knowledge of the distribution of, and threats to *H. pickersgilli* (also see Chapter 4). Of the assessed sites, Mount Moreland is a large and relatively undisturbed reed-bed wetland that would be well suited to a stewardship programme. Isipingo is small and isolated within a highly transformed landscape; conditions which do not bode well for its long-term survival (Cushman 2006). Ideally this area can be acquired through a purchase agreement and managed by local conservation authorities. Maintenance of terrestrial sites is crucial to the survival of amphibians since individuals spend most of their lives as terrestrial predators (Dodd 2003). The discussion on dispersal and meta-populations of *H. pickersgilli* at Mount Moreland is expanded in Chapter 4.

The following actions are recommended for *H. pickersgilli*, to be implemented over the next three years, with the aim of protecting at least 30% of the population (Figure 3.11):

1) Research

Additional research requirements for *H. pickersgilli* include refining population estimates for all localities, determining the impact of habitat fragmentation, particularly on gene flow, and improved understanding of breeding biology. Based on this knowledge, sites with the highest conservation value can be identified.

2) Development of a Biodiversity Management Plan for Species

Developing a BMP-S for *H. pickersgilli* has been identified as a priority for this species and will provide guidelines for all other conservation actions as well as facilitating relationships between all relevant stakeholders. *Hyperolius pickersgilli* will be only the second frog species for which this is done in South Africa (the first being *Amietophrynus pantherinus*). An initial workshop including the majority of relevant stakeholders (provincial and municipal conservation authorities, land owners and interested parties) was held on 4 August 2011 to introduce the concept of a BMP-S for this species.

The following steps are now necessary to implement developing a BMP-S for *H. pickersgilli*:

- Drafting a framework for the BMP-S (process initiated).
- Identify all stakeholders.
- Circulating the draft to all relevant stakeholders and inviting them to a second workshop.
- Hold stakeholder workshop to expand draft and ensure buy-in from all stakeholders.
- Finalise BMP-S for submission to Minister of Environment and Water Affairs.
- Facilitate ratification by Minister of Environment and Water Affairs.
- Facilitate implementation of BMP-S by stakeholders (monitor progress).

3) Initiate stewardship programme for selected sites

Mount Moreland, and possibly a number of other sites (see Chapter 4), have been identified as a possible location for a stewardship programme, which will entail:

- Facilitation of meetings with landowners and the province, as needed, for stewardship negotiations.
- Developing conservation management plan for *H. pickersgilli* for each site.
- Implementing long-term monitoring programme.
- Participate as required on the development of the stewardship contract.

4) Initiate partnerships for land acquisition/offsets for sites ear-marked for development

The Prospecton wetland and surrounds (including reed bed adjacent to N2) are likely to be lost to development of the new Durban South Port Development. A rescue and translocation of this population to a new/rehabilitated site is likely to provide the only solution to ensuring survival of this population:

- Engaging with key stakeholders (Transnet) to open dialogue about impact, solutions and biodiversity offsets.
- Identify sites for purchase that are suitable for re-introduction/rehabilitation.
- Compile budget for above and for long-term costs.
- Devise translocation strategy to optimise survival of the majority of this population.
- Implement rescue and translocation process (to take place over 3 seasons) and test success through monitoring.
- Manage new site under a stewardship programme and have it proclaimed as a protected area.

A number of additional sites, including Isipingo and Umkomaas, may provide an opportunity for land acquisition:

- Identify land-owners.
- Identify partners for land acquisition.
- Approach partners with proposal for land acquisition.
- Develop conservation management plan for *H. pickersgilli* for each site based on relevant threats (e.g. alien clearing, identification of upstream development impact on wetland hydrology).

5) Develop and implement standardized monitoring programme

Setting up a long-term monitoring programmes and population estimates for all populations, and particularly within the eThekwini Municipal Area.

- Develop standard monitoring protocol for *H. pickersgilli.*
- Implement standard monitoring protocol for *H. pickersgilli* across all sites for 2012/2013 season.
- Provision of training for KZN Wildlife and eThekwini staff to facilitate long-term monitoring.

6) Ensure high profile of *H. pickersgilli* in the public domain

- Use *H. pickersgilli* as Durban "Frog Mascot" for annual awareness day.
- Relevant signage at sites (Protected sites and Mount Moreland) to include *H. pickersgilli*.
- Media (print, broadcast, awareness day).
- Presentations at schools and to general public.
- Ongoing fundraising.
- Raise awareness at zoos regarding *ex-situ* programme for *H. pickersgilli*.

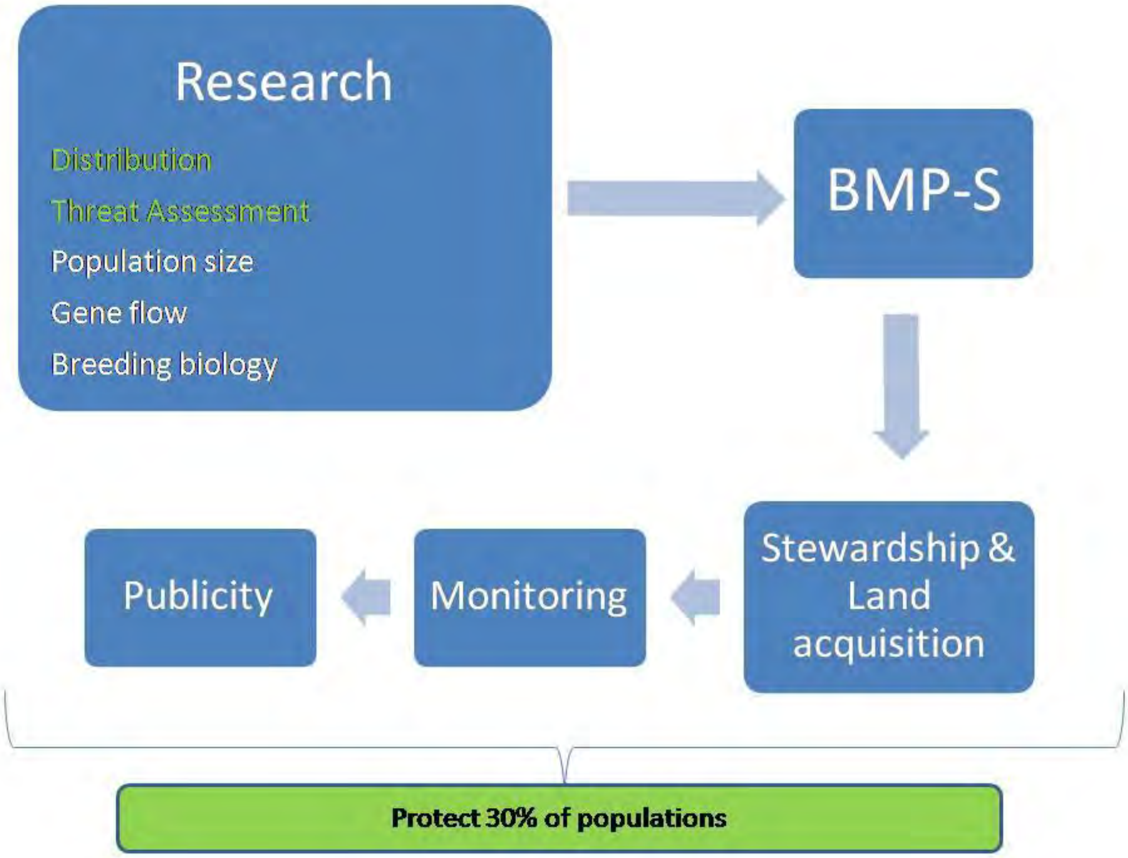

Figure 3.11: A flow-chart for the conservation management plan for *Hyperolius pickersgilli* with the aim of conserving at least 30% of the total population. Research priorities that have been completed during this study are indicated in green text.

3.4.4 Conservation recommendations for *Natalobatrachus bonebergi*

Although both of the sites (Vernon Crookes and Krantzkloof Nature Reserves) assessed for *N. bonebergi* occur in Protected Areas, habitat in Vernon Crookes is far more suitable than that in Krantzkloof. The latter has been degraded as a result of urbanisation upstream of the river catchments, resulting in heavy siltation and increased water flow. Removal of vegetation as a result of flooding has also destroyed crucial habitat. The situation is very similar at Palmiet Nature Reserve. Additional surveys within these reserves are recommended. On a positive note, the species was detected at a number of new localities during the study period and its presence was reconfirmed in various protected areas. It is now known to occur in at least five protected areas. It will be essential that staff in these areas are trained to identify and monitor *N. bonebergi* and to report any immediate threats. Engagement with regional managers and district conservation officers of KZN Wildlife was initiated during the study period. Additional research requirements and conservation recommendations are given below:

1) Research

- Gain a better understanding of distribution through additional surveying and predictive modelling.
- Estimation of population size.

2) Initiate stewardship programme for selected sites

- Identification of stakeholders.
- Open dialogue with land-owners.
- Prioritisation of sites with conservation value.

3) Develop and implement standardized monitoring programme

- Maintenance of habitat
- Staff training (Protected areas) on identification and monitoring.
- Implementation of long-term monitoring programmes.

3.4.5 Conservation recommendations for *Afrixalus spinifrons*

Afrixalus spinifrons is comprised of two subspecies, the taxonomy of which is covered extensively in Chapter 5. Determining the species boundaries between these taxa has important consequences for conservation plans (see Chapter 5). *Afrixalus spinifrons spinifrons* occurs along the KwaZulu-Natal and Eastern Cape coastal regions. Much of the coastal region is under heavy development pressure (Johnson & Raw 1987; Armstrong *et al.* 1998; Goodman 2000), as such, this subspecies appears to be experiencing population declines (Pickersgill 2007; Measey 2011). Based on the site comparisons done in the KwaZulu-Natal midlands and on general observations during the course of this study *A. s. intermedius* is inadequately protected. Armstrong (2001) predicted that just 2.8% of this subspecies‟ range occurs within protected areas and it is in need of further protection strategies to ensure long-term survival. On the other hand, the subspecies appears to have benefited from the construction of farm dams throughout the midlands region. Indeed, aside from the localities in the uKhahlamba-Drakensberg Park and the Type locality at Rosetta, all records obtained for this subspecies during this study were from farm dams. Furthermore, molecular results (see Chapter 5) indicate that populations from the Eastern Cape Province in fact belong to *intermedius*, thereby expanding the known distribution considerably. Conservation actions for *A. spinifrons* are not considered urgent at this time (Measey 2011). Specific research conservation recommendations include:

1) Research

- Taxonomic investigation of the two subspecies using morphology, call analysis and genetics was give high priority by Measey (2011). Chapter 5 provides a review of the *A. spinifrons* complex based on these methods.
- Gain a better understanding of distribution through predictive modelling and additional surveying.
- Ongoing investigation of threats.
- Recalculate AOO and EOO based on current species delimitation ranges (see Chapter 5).

2) Habitat protection

- Prioritisation of sites with conservation value.
- Engage with farm-owners on whose properties the species is known to occur.

3) Develop and implement standardized monitoring programme for selected sites

- Implementation of long-term monitoring programmes at the extremes of the distribution.

3.6.6 Notes on *Leptopelis xenodactylus* and *Anhydrophryne ngongoniensis*

Although habitat and threat assessments were not specifically conducted for *L. xenodactylus* and *A. ngongoniensis* during this study, various localities for these species were visited and threats noted. *Anhydrophryne ngongoniensis* was not detected during the study period. Harvey (2007) conducted a population estimate for this species, surveyed historical localities and detected a number of new localities. The species does not occur in any officially protected areas and, as such requires improved conservation strategies. Management recommendations were outlined by Harvey (2007) and made available to the private forestry companies with *A. ngongoniensis* on their properties.

These recommendations included guidelines of fire practices, control of alien vegetation, grazing practices and riparian zone management. Although a detailed assessment of these recommendations was not conducted as part this study, it appeared that these management practices were being put into place within the forestry areas. Research requirements for the species are outlined in Measey (2011). *Leptopelis xenodactylus* is considered adequately protected within the province, with 19.7% of its predicted range occurring in Protected Areas (Armstrong 2001). For both species, many of their localities occur within forestry-owned land and therefore provide a good opportunity for stewardship programmes. Further investigations into distribution, population size, breeding biology and threats are required for both species.

CHAPTER 4

USING ECOLOGICAL NICHE MODELLING TO PREDICT THE DISTRIBUTION OF *HYPEROLIUS PICKERSGILLI* (RAW 1982)

4.1 Abstract

Hyperolius pickersgilli has recently (2011) been listed as Critically Endangered, based on an estimated area of occupancy (AOO) of less than 9 km^2. It is endemic to the KwaZulu-Natal coastal area and is known only from limited localities, the majority of which face increasing pressure from anthropogenic activities. Given its status, cryptic behaviour and restricted range, this species is an ideal candidate for predictive modelling. Here, maximum entropy (Maxent), based on limited occurrence data, was used to model a predicted distribution for this species and to identify potential dispersal linkages between meta-populations. Predicted sites with an occurrence-probability greater than sixty percent were surveyed during the species‟ breeding season of 2010-2011 and 2011-2012. Despite extensive surveys, few new localities were revealed: in total just 5 new subpopulations were discovered during the study period. In addition, 15 known historical locations (pre-2007) were re-visited to verify the species‟ presence there. Of these sites, *H. pickersgilli* was deemed absent at eight. Taking this into account, and including additional subpopulations discovered between 2007 and 2010, the total number of localities now known is 17. Based on these results, the AOO and extent of occurrence (EOO) for this species were recalculated to be 14.17 km^2 and 2081.5 km^2 respectively. These increases are expected given a range extension to the south and an overall addition of five new subpopulations. Based on this knowledge, it is recommended that the species be reclassified as Endangered under the IUCN Red List criteria B2ab(iii). However, the degree of fragmentation between localities, ongoing decline in quality of habitat and threats from development pressure facing the majority of localities, make the long-term survival of these subpopulations uncertain and conservation action for this species remains urgent.

4.2 Introduction

Ecological niche modelling is becoming increasingly useful as a tool for application in conservation biology and wildlife management (Anderson *et al*. 2003; Araújo & Guisan 2006; Elith *et al*. 2011). In terms of conservation planning for threatened species, models are valuable for identifying suitable habitat and revealing potential additional populations (Stillman & Brown 1994; Guisan *et al*. 2006); predicting potential impacts of future climate change (Araújo *et al*. 2004; Beale *et al*. 2008) and pathogen infection (Rödder *et al.* 2009; Murray *et al.* 2011); guiding conservation prioritisation and protected-area selection (Leathwick *et al*. 2005); guiding re-introduction of threatened species (Pearce & Lindenmayer 1998); and directing IUCN listings or risk assessments (Rondinini *et al*. 2005; Tinoco *et al*. 2009; Pfab *et al*. 2011). Rare species, in particular, have been identified as being most in need of predictive distribution modelling (Lomba *et al.* 2010). Given the general dearth in resources for conservation planning, modelling techniques also provide an economical method for determining distributions and directing surveying expeditions (Fielding & Bell 1997; Funk *et al*. 2005).

Predictive species distribution modelling utilises the relationship between species‟ occurrence data and environmental variables to estimate suitable distribution based on the species‟ fundamental niche requirements (Pearson 2007). Occurrence data are typically presence-only records since systematic biological survey data is usually limited (Elith *et al*. 2011) and absence data is rarely inferred with certainty (Stockwell & Peters 1999; Elith *et al*. 2006). As such, use of absence data can bias analyses (Pearson 2007; Elith *et al*. 2011). An important source of occurrence records is systematic data in the form of collections data from museums, atlasing projects and other historical records (Funk & Richardson 2002; Pearson 2007). Environmental variable layers of high resolution are also becoming more readily available from a variety of sources, making ecological niche modelling increasingly accurate (Elith *et al*. 2006).

The maximum entropy method of generating a model is a correlative approach that uses presence-only data with environmental variables that can be expected to influence the species‟ distribution through their effect on physiology and probability of persistence

(Pearson 2007). Maxent software (Phillips *et al*. 2006) has been shown to have performance capabilities that are competitive with other correlative methods in terms of its ability to predict habitat, work well with low numbers of occurrence records and distinguish between grades of habitat suitability (Elith *et al*. 2006; Wisz *et al*. 2008). Furthermore, Maxent is able to make use of both continuous and categorical environmental variables and thereby generate finer scale predictions than other methods (Phillips *et al*. 2006).

Pickersgill‟s Reed Frog, *Hyperolius pickersgilli*, is a cryptic and obscure species, discovered in 1977 from the coastal lowlands of KwaZulu-Natal (Raw 1982). At the time of its description, it was known from just seven localities and since then has been recorded at a further eleven sites (Minter 2004; Biodiversity Database of Ezemvelo KZN Wildlife). *Hyperolius pickersgilli* is a habitat specialist favouring very dense reed beds in Coastal Bushveld-Grassveld (Mucina & Rutherford 2006) at altitudes below 380 m.a.s.l. (Raw 1982; Armstrong 2001; Bishop 2004b). It requires an understory of thick vegetation such as Snakeroot (*Persicaria attenuata*), from which males call, and taller reed vegetation, including the Common Reed (*Phragmites australis*), Bulrushes (*Typha capensis*), and sedges (including *Cyperus dives* and *C. papyrus*) (Bowman 2011; pers. obs.). The species requires perennial standing water of between 20 and 60 cm in depth. Much of this species‟ habitat has been degraded and is continuing to decline in quality as a result of urban and agricultural development in the coastal region (Johnson & Raw 1987; Armstrong *et al*. 1998). As such, the remaining populations of *H. pickersgilli* are under significant pressure and may be experiencing declines and even possible extirpation. The high degree of fragmentation between the majority of localities is of particular concern (Scott-Shaw 1999; Armstrong 2009). The cryptic behaviour, small size, quiet call and favoured habitat of *H. pickersgilli* make the species difficult to locate in the field (Bishop 2004b). As such, the use of predicted distribution modelling for this species may provide a valuable tool for directing field surveys and revealing the presence of new populations.

During the 2011 IUCN re-assessment for South African frogs, *H. pickersgilli* was uplisted from Endangered in 2004 to Critically Endangered B2ab(ii, iii) based on its small area of occupancy (AOO) of 9km^2, fragmented distribution and continuing decline in habitat (SA-FRoG & IUCN 2011; Measey 2011). This species is endemic to a narrow strip within 16 km of the coast of KwaZulu-Natal between St Lucia in the north to near Kingsburgh in the south (Raw 1982; Bishop 2004b). Of the twelve localities known to have extant populations as of 2010 (Measey 2011), only two occurred within formally protected areas, namely at Umlalazi Game Reserve and iSimangaliso Wetland Park World Heritage Site. Two sites in Mtunzini are offered a minimal level of conservation by the Twinstreams-Mtunzini Natural Heritage Site (Bishop 2004b). However, the remaining 8 sites are largely isolated within heavily industrialised, agricultural or urbanised areas (Measey 2011). The purpose of this study was to verify the species‟ existence at known historical sites (pre-2007) and to use predictive modelling to assist in finding new populations.

The aims of this chapter were to:

1) Predict the distribution of *H. pickersgilli* using Maxent and to ground-truth the model;

2) Revisit historically known sites of *H. pickersgilli* to determine site status and confirm presence of the species;

3) Delimit potential meta-populations of the species to guide conservation measures such as sourcing individuals for *ex situ* conservation breeding and re-introductions, and to;

4) Revisit the IUCN red-list status of the species in the light of new information gathered during the study.

4.3 Methods

4.3.1 Predictive modelling

Species occurrence records were obtained from the South African Frog Atlas Project (Minter *et al*. 2004) and the Biodiversity Database of Ezemvelo KZN Wildlife. Records with a spatial resolution of between 0 and 250 m were used. In total, 39 presence records were available for use in the modelling. Environmental predictors likely to influence the distribution of the species were collated from the literature (Poynton 1964; Duellman & Trueb 1994; Bishop 2004b; du Preez & Carruthers 2009; Franklin *et al*. 2009) and from additional knowledge of the species habitat requirements gathered during the study period (pers. obs.). For the purposes of this model only continuous variables were used, with categorical variables over-layed at a later stage (see below). The continuous variables used were mean minimum and maximum daily temperatures and relative humidity January and July, and mean annual temperature and precipitation for KwaZulu-Natal (Table 4.1). The coverage of these predictors were developed at a scale of 1′ x 1′ using the decimal degree Cape (1880) datum, and were re-projected to the WGS84 datum, transverse mercator lo31 central meridian, and then re-sampled to a 20 m x 20 m (400 m^2) grid, based on the Ezemvelo KZN Wildlife 2008 version 2 landcover coverage. No increase in the resolution accuracy of the climatic variables was assumed. A resolution of 400 m^2 was found to be appropriate for wetlands (B. Escott, pers. comm.). Re-sampling was done to allow the incorporation of finer scale data in the form of wetland and hard transformation (100 % loss of native habitat) coverages. Otherwise, many of the wetlands and associated land transformation would have been lost from the analysis. Minor shifts, of up to 10 m, at mismatched interfaces between the 1′ x 1′ grid and the 20 x 20 m grid in climatic variables will occur. However, due to the initial scale of the mapping of climatic variables and the scale of the research domain, these errors were considered negligible (Elith *et al*. 2011).

Maxent version 3.3.3e (Philips *et al*. 2004, 2006; Philips & Dudík 2008; Elith *et al*. 2011) was used to develop the environmental niche model for *H. pickersgilli*. Maxent requires predictor variables to be in ASCII format and occurrence points in CSV file format. Five replicates were then run in Maxent using the cross-validate setting. The maximum number of iterations was set at 1000 to ensure algorithm convergence and the default settings were used for all other relevant parameters: feature selection = automatic; regularisation multiplier = 1; convergence threshold = 10^{-5} (Jackson & Robertson 2010). Twenty five percent of the occurrence points were randomly selected by the model as testing data for comparison against the model output (Phillips & Dudík 2008). A mask was used to ensure that the background samples were selected from the general region in which the species occurs. This general region was taken to be the Indian Ocean Coastal Belt, which spans altitudes between 0 and 450 m.a.s.l. (Mucina & Rutherford 2006). *Hyperolius pickersgilli* is a coastal species and has not been recorded above 340 m (Bishop 2004b). Areas of the Province of KwaZulu-Natal above 450 m altitude were therefore masked out of the background selection and also out of the predicted geographical range of the species.

Model accuracy was assessed by the area under the curve (AUC) of the receiver operator characteristic (ROC), which is a measure of discrimination ability (presence from background), where an AUC of 1 = perfect prediction, 0.5 indicates prediction no better than random and AUC values > 0.75 are considered useful predictors of distribution (Fielding & Bell 1997; Elith *et al*. 2006). An AUC > 0.9 is considered outstanding (Hosmer & Lemeshow 2000; Van Gils & Kayijamahe 2010). Jack-knife tests performed by the model are used to evaluate the importance of each predictor variable in explaining the observed species distribution (Philips *et al*. 2006; Monterosso *et al.* 2009). The response of *H. pickersgilli* to each variable was evaluated from the response curves generated by the model (Philips *et al.* 2006; Monterosso *et al.* 2009).

The resultant Maxent probability map (Figure 4.1) was imported into ArcMap 10.0 (ESRI Inc. 2011), multiplied by 1000 and exported to the Idrisi Geographic Information System (GIS; The Andes Edition, Version 15, 2006; Eastman 1999), in which further overlays were made with the land transformation and wetland coverages for KwaZulu-Natal (Ezemvelo KZN Wildlife) and the ground-truthing data. Wetland types suitable for *H. pickersgilli* were determined from an overlay of the distribution record locations on the wetland coverage in the Idrisi GIS, and the probability of occurrence of *H. pickersgilli* in the suitable wetlands obtained from the Maxent probability map through overlay with the suitable wetlands map.

Hard-transformed land was subtracted from the Maxent probability map to eliminate as many transformed wetlands from the resultant map as possible.

From a previous version of the probability occurrence map, created without the use of a mask and the cross-validate parameter, wetlands with probability of 60 % or greater occurrence for *H. pickersgilli* were selected and over-layed with 1:50 000 topographical maps (Chief Directorate: National Geo-spatial Information, Mowbray, Cape Town) for the purposes of directing the ground-truthing exercise.

Potential meta-populations of *H. pickersgilli* were delimited using RAMAS GIS (Akçakaya 2005). The scale and size of the occurrence probability wetland map was adjusted through pixel thinning to a pixel size of 40 m, to enable the GIS program to carry out the required analysis. The resized map was then re-classed to boolean, with wetlands having a probability of occurrence for *H. pickersgilli* of more than zero being assigned a value of one. Although this may be an overestimate of the extent of occurrence of *H. pickersgilli*, any other cut-off probability was considered to be arbitrary. The maximum dispersal distance was estimated to be 2 km, as based on the distances to the nearest probable breeding wetland at known sites, and on observations of *H. pickersgilli* outside of wetlands up to 1.6 km (A. Wilken; J. Harvey pers. comm.).

A friction map for the movement of *H. pickersgilli* was developed from the KwaZulu-Natal 2008 landcover coverage, according to an order of magnitude scale, and the values for the landcover classes estimated were as follows: 1 – wetlands, grassland / bush clumps mix, grassland; 10 – natural water, irrigated permanent orchards (banana, citrus), commercial sugarcane, emerging farmers‟ sugarcane, forest, dense bush (70-100% canopy cover), bushland (< 70% canopy cover), woodland, forest glade; 100 – golf courses, low density settlement, subsistence (rural), annual commercial crops irrigated, degraded forest, degraded bushland (all types), degraded grassland, old cultivated fields (secondary grassland), old cultivated fields (secondary bushland), smallholdings (grassland), airfields, old plantation (high vegetation), old plantation (low vegetation), rehabilitated mines (high vegetation), rehabilitated mines (low vegetation); 1000 – plantation, mangrove wetlands, dryland permanent orchards (cashew nuts), built-up dense settlement, annual dryland commercial crops, KZN main & district roads, KZN railways, 10 000 – clearfelled plantation, permanent dryland pineapples, mines and quarries, bare sand, erosion, bare rock, alpine grass-heath, KZN national roads, dams, estuarine water, sea water, bare coastal sand, outside KZN boundary. The friction map was used to illustrate potential linkages between wetlands in terms of maintaining meta-population dynamics.

4.3.2 Ground-truthing and historical site verification

Seventy one predicted sites with occurrence probability for *H. pickersgilli* ≥ 60 % were visited during the breeding seasons of 2010 (October 2010 – February 2011) and 2011 (August 2011 – January 2012) (see Appendix D for details). Records of historical locations (1977 – 2007) were obtained from the Ezemvelo KZN Wildlife Biodiversity Database. These sites were also visited during the same period to verify species presence (Table 4.2).

Sites were visited from dusk to a few hours after sunset on the same nights (mainly between 19:00 and 01:00). *Hyperolius pickersgilli* is usually one of the first species to start calling, commencing at dusk and continuing to the early hours of the morning (pers. obs.). Presence/absence was determined by listening for *H. pickersgilli* male advertisement calls and where possible, visual confirmation was conducted by actively searching for individuals. If present, their position was recorded using a handheld Garmin Geko^{TM} 201 GPS. The behaviour and ecology of the frogs and records of other frog species were also noted. The specific conditions such as plant species, water depth and prevailing weather conditions were recorded at each wetland. Air and water temperatures were recorded using an Extech Instruments waterproof thermometer. Any immediate possible threats, such as alien vegetation, were also noted. Absence of *H. pickersgilli* was presumed in cases in which no suitable habitat was present or where habitat had been obviously destroyed. In cases where suitable habitat was present but absence could not be ascertained, the site was revisited where possible. Also included in the ground-truthing dataset were recent records collected by James Harvey (pers. comm.) and Morgan Trimble (pers. comm.) during the course of their fieldwork.

4.3.3 Area of occupancy (AOO) and extent of occurrence (EOO)

Based on the results from ground-truthing, area of occupancy (AOO) and extent of occurrence (EOO) were recalculated using IUCN Red List guidelines (Version 9.0, September 2011). The EOO of *H. pickersgilli* was calculated in Cartalinx (Hagan *et al*. 1998) by joining the appropriate distribution locality points (of ≤ 250 m spatial resolution) to form a minimum convex polygon. The area of this polygon was considered the EOO. However, much of the polygon fell over the adjacent Indian Ocean, so the area of ocean enclosed in the polygon was subtracted from the EOO to form the second estimate of the EOO.

Area of occupancy (AOO) is a measurement that represents the area of suitable habitat currently occupied by the taxon (IUCN Standards and Petitions Subcommittee 2011). Area of occupancy is an important parameter for inclusion in Red List criteria as it gives an indication of species with restricted distribution and hence, of restricted habitat, as is the case for *H. pickersgilli*. Habitat specialists are considered to have an increased risk of extinction. It can also be useful as a proxy for population size. The AOO for *H. pickersgilli* was calculated in two ways. Firstly, the number of 4 km² (2 km x 2 km) cells into which the distribution locality points used to determine the EOO fell was calculated, and the total area of these cells calculated, as recommended by the IUCN Standards and Petitions Subcommittee (2011). Secondly, the areas of the extant wetlands in which one or more of the distribution locality points fell were summed to provide another estimate of the AOO (IUCN Standards and Petitions Subcommittee 2011). However, the second approach did not consider dispersal routes or potential non-breeding habitat, and so may result in conservative estimates of AOO.

4.4 Results

4.4.1 Maxent Model

The average test AUC for the replicate runs was 0.970 ±0.009 S.D, indicating that the data was an extremely good fit to the model (Hosmer & Lemeshow 2000). The average Maxent model predicted that the variable "July means of daily average relative humidity (%)" was important in describing the realized climatic niche of *H. pickersgilli* (Table 4.1). The jackknife tests of variable importance on the training data and on the test data indicated that the variables "July means of daily average relative humidity (%)" and "July means of daily minimum temperature (ºC)" were the variables that decreased the contribution the most when omitted from the model. Therefore, these variables appear to have the most information that is not present in the other variables. The predicted distribution of *H. pickersgilli* indicates a greater probability of occurrence of the species towards the coast (Figure 4.1). The probability of occurrence of *H. pickersgilli* in suitable coastal wetlands is indicated in Figure 4.2, indicating a preference for low altitude, perennial wetlands with dense reed vegetation.

Table 4.1: Respective contributions of variables included in the final MAXENT climatic niche model for *Hyperolius pickersgilli*.

Variable	Percent contribution	Permutation importance
July means of daily average relative humidity (%)	76.1	85.9
July means of daily minimum temperature (ºC)	13.4	1.9
January means of daily average relative humidity (%)	5.8	0.4
January means of daily maximum temperature (ºC)	2.4	3.2
Mean annual precipitation (mm)	1.3	0.2
January means of daily minimum temperature (ºC)	0.7	2
July means of daily maximum temperature (ºC)	0.4	0.3
Mean annual temperature (ºC)	0	0
Mask	0	0

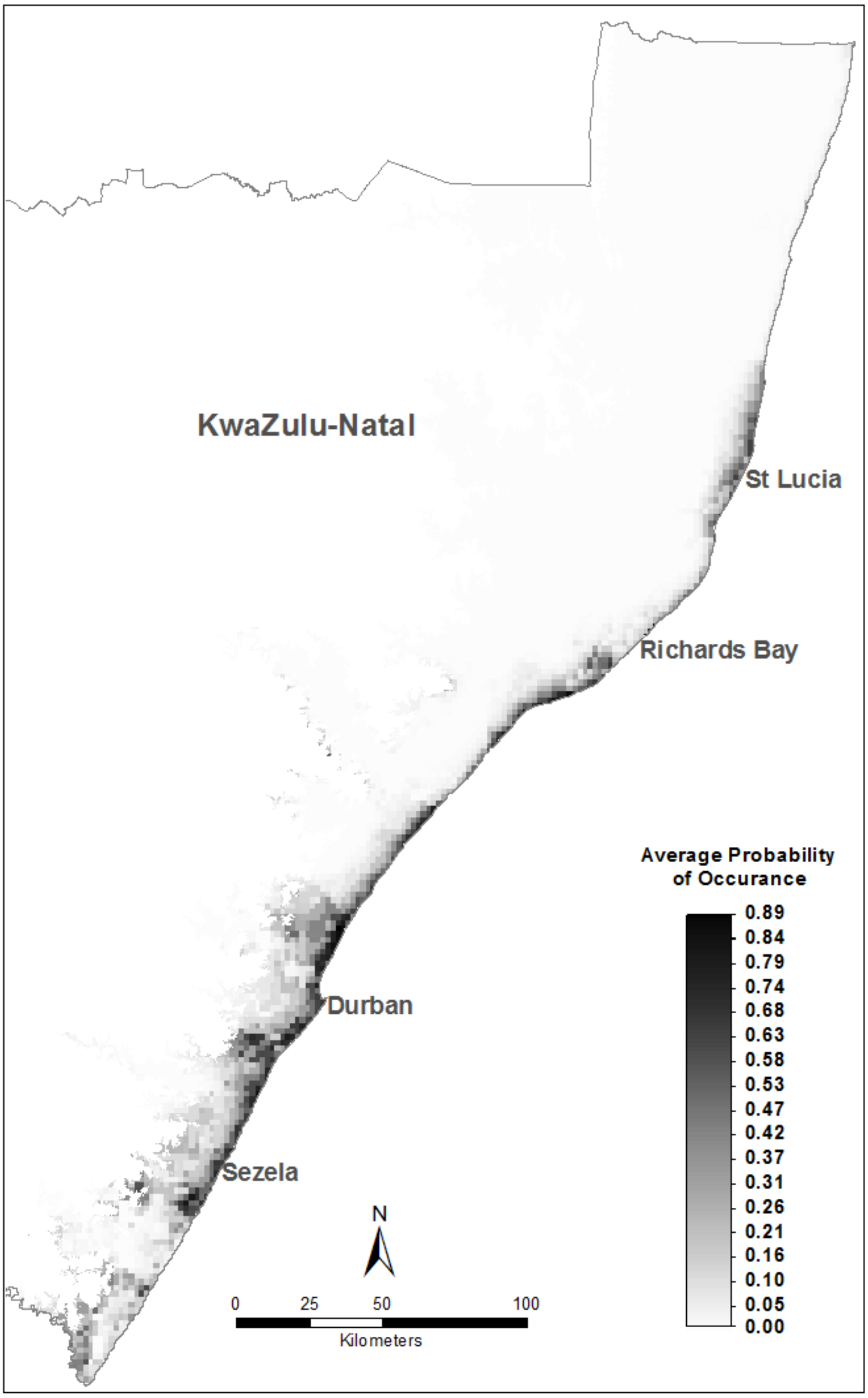

Figure 4.1: Map of the average probability of occurrence of *Hyperolius pickersgilli* according to the Maxent model of its climatic niche in KwaZulu-Natal. The map indicates that *H. pickersgilli* is restricted to the central coastal region of the province.

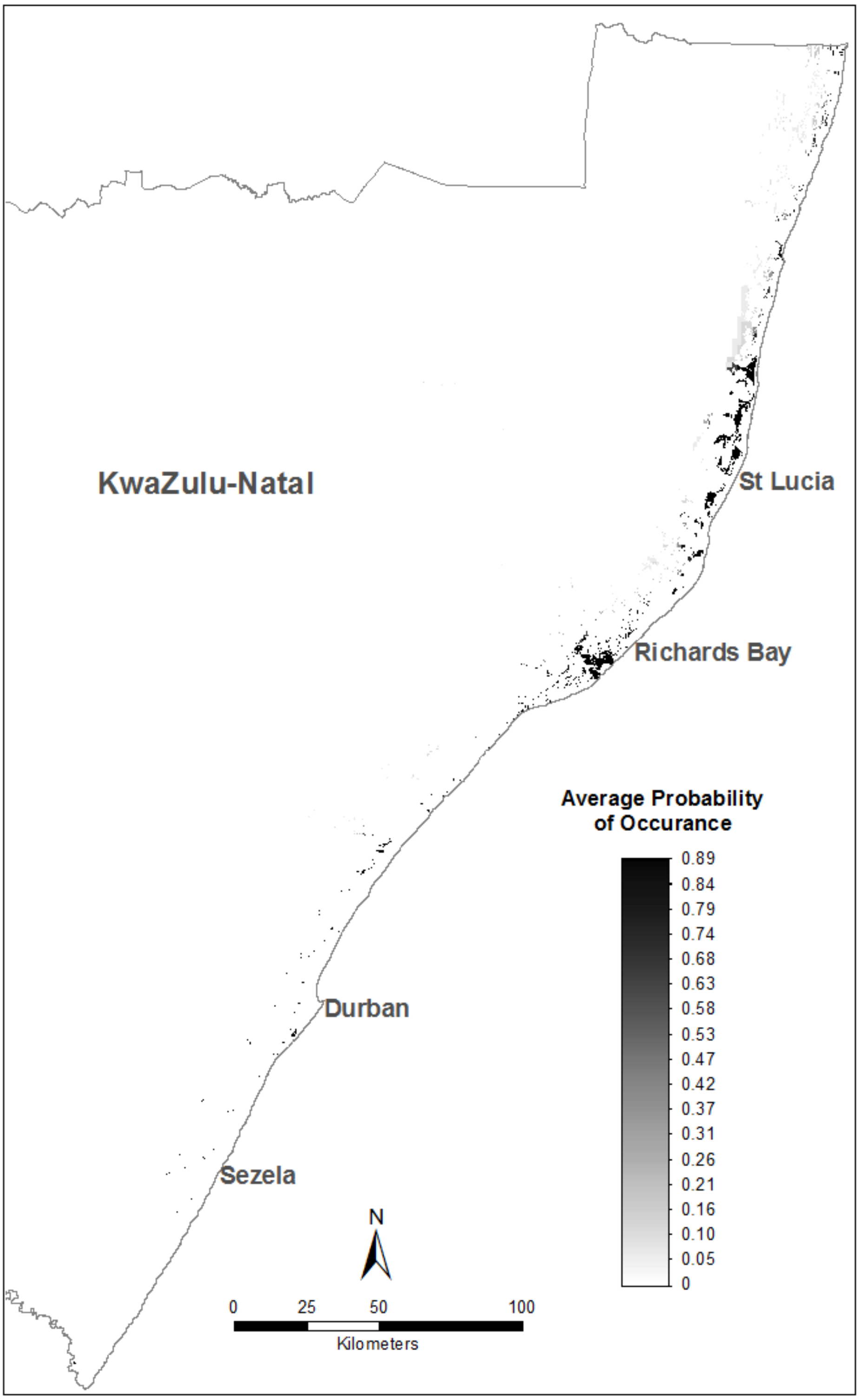

Figure 4.2: Map of the probability of occurrence of *Hyperolius pickersgilli* in coastal wetlands of suitable type in KwaZulu-Natal.

4.4.2 Potential meta-populations

One hundred and forty-nine potential meta-populations were distinguished based on the Maxent model, which can be divided into eight regions from North to South, with 1 the most northerly and 8 the most southerly (Figure 4.3). Although no occurrence records exist for *H. pickersgilli* in the most northern regions (1 – 3), there are potentially 26 meta-populations at suitable wetlands here. The bulk of the suitable wetlands, and therefore meta-populations, (108) occur in the central regions 4 – 7, with the remainder (15) occurring to the south of the distribution (region 8).

An example of potential linkages at Mt. Moreland based on the friction map that *H. pickersgilli* could use while dispersing between wetlands within the meta-population area is presented in Figure 4.4. No complete linkages of least friction value between the wetlands within that meta-population range remains. Any *H. pickersgilli* inhabiting the wetland in the South-eastern corner of the Figure 4.4 is probably effectively cut off from the other wetlands by the national freeway (N2), indicated as a curved white stripe.

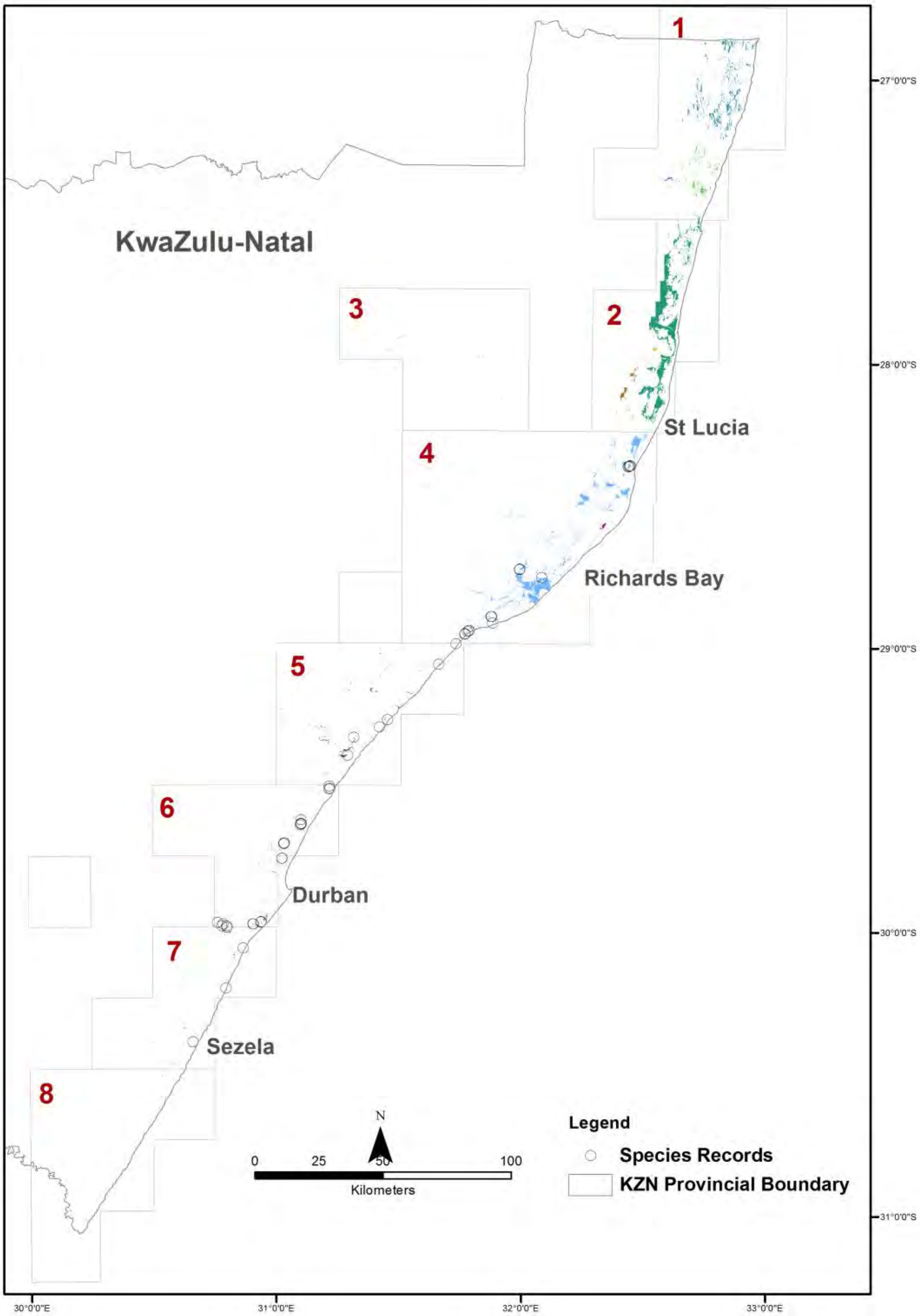

Figure 4.3: (A) Distribution of potential *Hyperolius pickersgilli* meta-populations (1-8) in KwaZulu-Natal. Blocks 1 – 8 represent regional meta-populations. Circles represent species occurrence records.

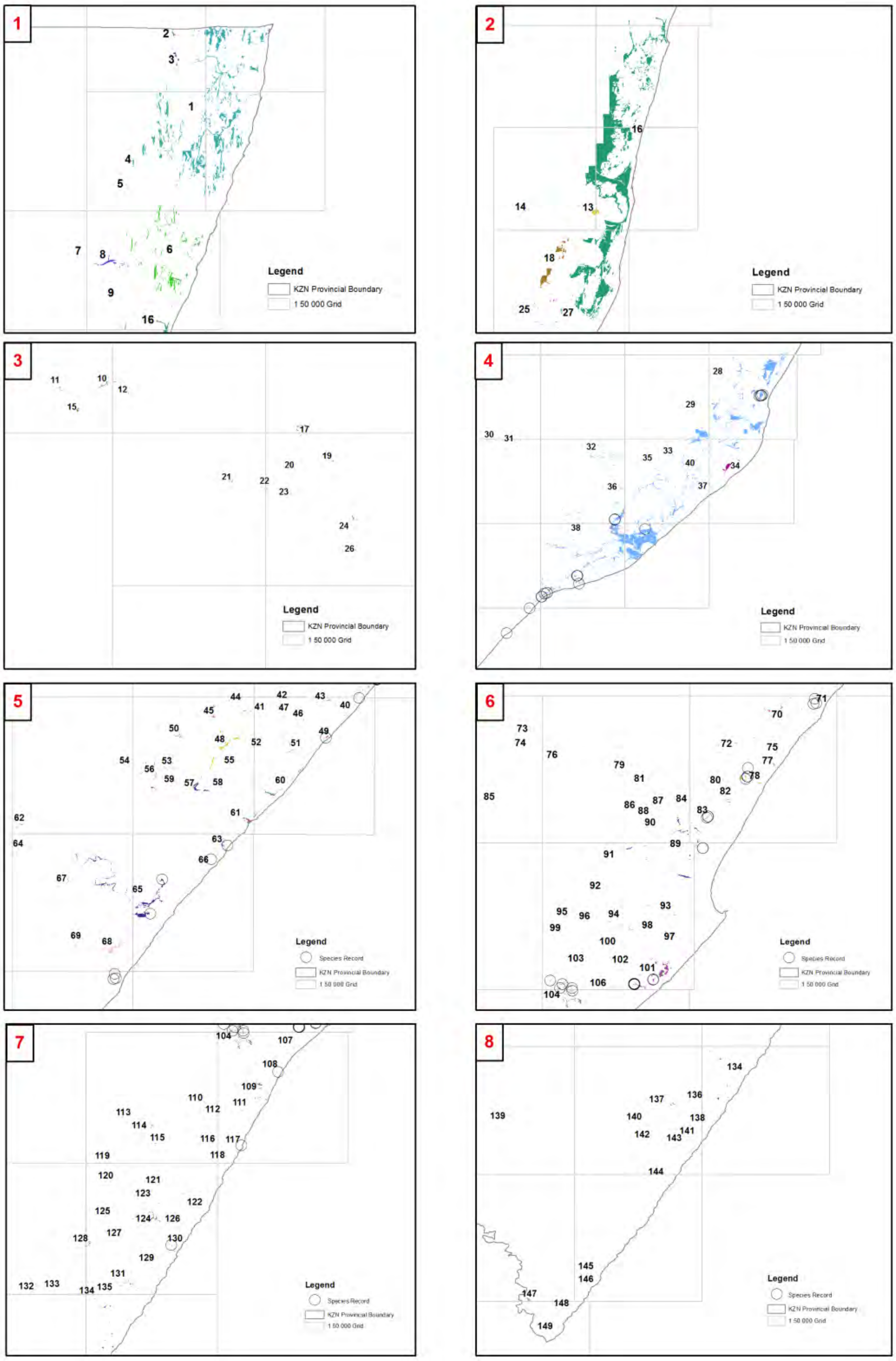

Figure 4.3: (B) close-up views of the potential meta-populations of *Hyperolius pickersgilli* throughout its distribution. Each meta-population is represented by a different colour.

Figure 4.4: Wetland habitat at Mt. Moreland suitable for a potential meta-population of *Hyperolius pickersgilli* indicating linkages (areas delimited by dotted lines) between three of the wetlands via low friction pathways. Wetlands are in black, least-friction landcover class areas are in dark grey; landcover classes assigned a friction value of an order of magnitude higher are indicated by light grey; transformed areas and landcover classes with higher friction values are white.

4.4.3 New localities for *Hyperolius pickersgilli*

Overall, only a relatively small number of localities visited during the ground-truthing (Appendix D) appeared suitable for *H. pickersgilli*, and of these, the species was present at only six (one of which was a rediscovery): the St Lucia Estuary (iSimangaliso World Heritage Site; -28.37183º, 32.40335, altitude 4 m.a.s.l.), Port Durnford (-28.90521°, 31.85801°, altitude 21 m.a.s.l.), Nonoti (-29.29718º, 31.41226º; altitude 29 m.a.s.l.), Umkomaas (-30.217183°, 30.795353°, altitude 13 m.a.s.l.) and Sezela (-30.40670°, 30.66145°, altitude 21 m.a.s.l.). Although the wetland at Prospecton (-29.97837°, 30.93617°;

altitude 6 m.a.s.l.), was not predicted by the model it is included as a new site which was discovered during the period of ground-truthing (December 2010). This wetland occurs in the industrial zone of Durban south and is described in Chapter 3.

The St Lucia Estuary location (rediscovered on 3 February 2011) is a tall, thick *Phragmites australis* reed-bed, straddling both sides of the main tar road into St Lucia, and is protected as part of the iSimangaliso World Heritage Site (Bishop 2004b) (Figure 4.5). Although the wetland is bisected by the road, the ability of *H. pickergilli* to disperse to the north and south, still remains.

Figure 4.5: Habitat at the St Lucia Estuary area at which a population of *Hyperolius pickersgilli* was found on 3 February 2011. Photograph by Adrian Armstrong.

The population near Port Durnford was discovered on 12 October 2011. The large perennial wetland comprised of *P. australis* and *Cyperus latifolius* with deep (50 cm in parts), stagnant water (Fig. 4.6). Numerous males were heard calling and visual confirmation was made though observation of both males and females (Fig. 4.7). Preliminary call records indicate that this site may host one of the largest known populations of *H. pickersgilli*. This land belongs to a timber company, Mondi-SiyaQhubeka, and is bordered by pine plantations and rural habitation. A 40 ha compartment of trees has already been removed from the area opposite the wetland and the timber company is planning to rehabilitate this area back to wetland, which could possibly provide a re-introduction site for rescued or captive-bred individuals of *H. pickersgilli*.

Figure 4.6: Habitat at the Port Durnford wetland at which a new population of *Hyperolius pickersgilli* was found on 12 October 2011.

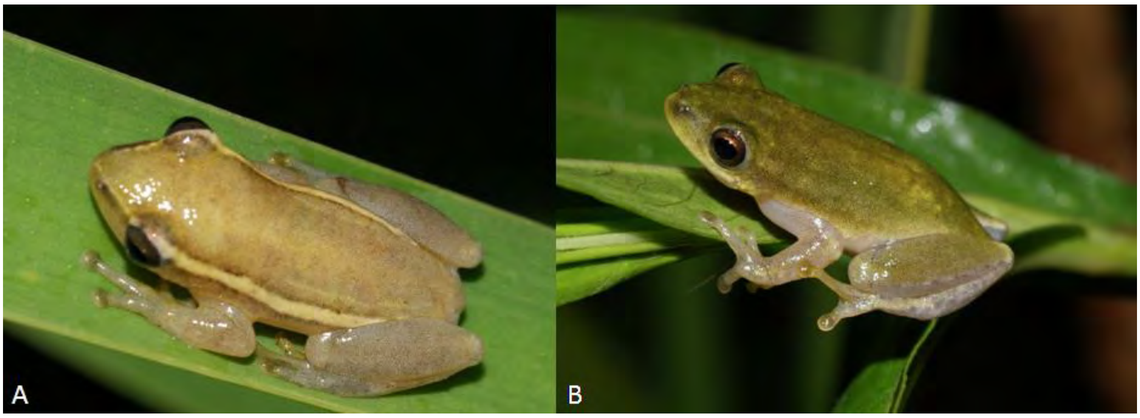

Figure 4.7: (A) Male and (B) female *Hyperolius pickersgilli* at Port Durnford, 12 October 2011.

A population on the south coast near Umkomaas was discovered on 6 January 2012. A few males were heard calling (and one visually detected) on either side of the coastal R102 road in a densely vegetated wetland comprised largely of *P. australis*, *Persicaria attenuata* and *C. dives*. This small wetland is surrounded on all sides by transformed landscapes (urbanisation and roads) (Figure 4.8). The site is currently facing pressure from a housing development plan. Furthermore, the area upstream of the catchment has been ear-marked for further urban development (R. Boon, pers. comm.). The wetland is also heavily infested with alien vegetation including Brazilian pepper (*Schinus terebinthifolius*), Syringa (*Melia azadarach*), Wild canna (*Canna indica*) and Lantana (*Lantana camara*) (J. Vermuelen pers. comm.). Calling was very intermittent and ceased altogether within 15 minutes, emphasising how easy it is to overlook this species. Although other species, including *Hyperolius tuberilinguis,* were calling strongly, the environmental temperature of 24.2 °C at 22:00 and extremely high humidity may have inhibited calling of *H. pickersgilli*. After searching the wetland, it became apparent that only a small portion of the area seems to host *H. pickersgilli*.

Figure 4.8: Site for the new population of *Hyperolius pickersgilli* discovered on 6 January 2012 near Umkomaas, on the KwaZulu-Natal south coast. The wetland area is indicated by the red line.

Another new population was located near Zinkwazi Beach at Nonoti Sugar Estate on 10 January 2012. This is a medium-sized, densely vegetated perennial wetland that appears highly suited to *H. pickersgilli*. Although no calling males were detected at the time of the survey, many juveniles resembling *H. pickersgilli* (Fig. 4.9) were present on the wetland periphery. Three of these were collected for rearing at the Johannesburg Zoological Gardens to verify their identity and for inclusion in the *ex situ* captive breeding program (Visser 2011). All three have survived in captivity to date and appear to be *H. pickersgilli*. The wetland at Nonoti appears to be highly suitable for the species and is in good condition, but is surrounded by sugarcane.

Figure 4.9: Juvenile *Hyperolius pickersgilli* from the locality discovered on 10 January 2012 at Nonoti Farm, Zinkwazi Beach.

On 19 January 2012, a further population on the south coast was discovered in the Sezela area. A number of males were heard calling from a very dense reed-bed of *C. dives*, *P. australis* and *P. attenuata* with ~ 60 cm deep stagnant water. Other species present included *H. tuberilinguis* and *Afrixalus fornasinii*. This medium-sized wetland is surrounded by extensive sugar-cane fields. This population at Sezela is now the southernmost known site of this species, approximately 40 km south of the now extinct Warner Beach site, and is also completely surrounded by sugar cane and in the vicinity of industrial operations including an electrical substation and large sugar mill. On 20 January 2012 numerous males were heard calling in a relatively extensive *P. australis* reed-bed immediately adjacent to the busy N2 highway in the Prospecton area (-29.97837°, 30.93617°). This system is an extension of the Prospecton population discovered in December 2010.

Both the probabilities of occurrence at the ground-truthing locations where *H. pickersgilli* was present and the probabilities of occurrence of the locations at which *H. pickersgilli* was not detected followed a Normal distribution (Shapiro-Wilks $W=0.93$, $p>0.05$, $W=0.95$, $p>0.05$, respectively). The mean probability of occurrence for *H. pickersgilli* at the ground-truthed sites where it was present (0.5373) was greater than the mean probability of occurrence for *H. pickersgilli* at the ground-truthed sites where it was not detected (0.4177; $t = 1.506185$, df = 31, $p = 0.071$; one-tailed test).

4.4.4 Historical sites

Fifteen of a total of 17 historical sites were visited, including the Type locality at Avoca. The presence of *H. pickersgilli* was ascertained at only seven of these sites (Table 4.2). For the remaining eight sites habitat had either been completely destroyed or was transformed to such an extent that it was no longer suitable as breeding habitat for *H. pickersgilli*. The only historical sites not revisited were those at Monzi, from which a specimen was collected by D.G. Broadley in 1978, of which verification was uncertain (M. Pickersgill, pers. comm.) and at the Tongaat-Hulett sugar estate near Zinkwazi Beach due to difficulties with gaining access permission at the time.

Habitat at the original site of discovery of *H. pickersgilli* in 1977 by Martin Pickersgill at Mt. Edgecombe appears to have been lost entirely. What must have been the original wetland is now dry (possibly as a result of intentional drainage) and is used for cultivation of subsistence crops. There is very little remaining suitable reed-bed vegetation and no remaining standing water. The area is heavily littered and dominated by alien vegetation.

The Type locality at Avoca (Raw 1982) has been completely eliminated. The site is situated next to the Seekoei Spruit River and has been completely lost to urbanisation, including a petrol station and the Avoca railway station. Figure 4.10 provides a comparison of the area in 1978, (the year H. pickersgilli was discovered there) when no development existed directly on the site, to how it currently appears. Warehousing and a petrol station have been constructed on the floodplain wetland adjacent to the river.

The southernmost historical locality at Warner Beach, discovered by Martin Pickersgill in 1982, has been destroyed by a housing development (M. Pickersgill, pers. comm.). Potential habitat in the vicinity is also not suitable for the species. The area was visited three times during this study. The site is in close proximity to a busy road intersection and is also adjacent to the N2 highway. The original wetland area appears to have been drained and has been heavily invaded by alien vegetation, in particular Spanish reed (*Arundo donax*) (Henderson 2001). There is a deep pond below what must have been the original wetland area and although other anuran species including *A. fornasinii*, *H. tuberilinguis* and *Amietophrynus gutturalis* are present, this is not suitable for *H. pickersgilli.* A drainage line in the vicinity also hosts these species, but again, no *H. pickersgilli* were detected. Road construction nearby has resulted in tar being pushed into this system. Based on these observations it seems reasonable to suggest that this historical population is no longer extant.

Figure 4.10 (A): Type locality of *Hyperolius pickersgilli* at Avoca, Durban, as at 2012. The wetland area of the floodplain has been destroyed by heavy development in the area; (B) The site in 1978, free of the development at the type locality.

Historical sites north of Durban were also surveyed. The locality given for the original Twinstreams, Mtunzini site may be the same that matches the "Matatiele Pan" site discovered by Neville Passmore sometime later (exact date unknown) in the vicinity of the current Twinstreams Indigenous Nursery. This area was visited several times during this study, and the only remaining reeded areas are completely dried, most likely as a result of the extensive surrounding *Eucalyptus sp.* plantations and long-term drought in recent years, and therefore no longer suitable for *H. pickersgilli*. There are three other extant populations in the Mtunzini area, namely at Forest Lodge, at the Raphia Palms Monument and in Umlalazi Nature Reserve, where the presence of *H. pickersgilli* was confirmed during the 2010 and 2011 breeding periods. The latter is a large, dense reed-bed wetland which is formally protected, although it may be impacted somewhat through reed-harvesting (*P. australis* and *Juncus spp.*) and sewage disposal from the nearby staff compound (R. Uys, pers. comm.).

The site near Tugela mouth (Mandini area, Lambiris 1989) is situated on a sugarcane farm and the wetland has been completely drained. There is a small man-built pond in the vicinity, but any suitable reed-bed habitat has been lost. The site at Groutville (Charlottedale) is a ditch with sedges, and a single male was calling there when visited on 4 February 2011 (A. Armstrong & R. Bowman, pers. comm.). On the same day, the population at Stanger was visited and found to be extant in a small reed-bed wetland adjacent to a dam. There are three small populations within the Adam"s Mission (Golokodo) area. One population persists along a drainage ditch adjacent to a tar road, while another appears to be a relatively intact population in a sedge wetland. The area is becoming increasingly urbanised and there was evidence of illegal refuse dumping on the periphery of the wetland.

A number of historical sites were not used in the model due to their relatively poor spatial resolution (less than 200 m^2). In some cases, resolution was only to quarter degree grid resolution scale. A record for Fairbreeze can be discounted as the purported collector had never been to the site nor collected *H. pickersgilli* at any time (W. Branch, pers. comm.). A further record from 1997 at Port Durnford also had very low spatial resolution, and no area within a 2 km^2 radius of the location appears suitable. Whether this record matches the discovery in October 2011 is uncertain. The Richard"s Bay historical site is comprised of dense swamp forest in which a single specimen was located in 1982 (M. Pickersgill pers.

comm.). The surrounding area was an extensive reed-bed, much of which appears to have been lost to urbanisation and roads. There is a suitable wetland in the vicinity (-28.765620°, 32.055178°), although *H. pickersgilli* was not detected at the time of the survey undertaken for this study. A photographic record dating to 2001 (G. Upland, pers. comm.) of *H. pickersgilli* from the Amatikulu/Dokodweni area (-29.074720°, 31.648890°) appears instead to be of juvenile *H. marmoratus* (L.H. du Preez, pers. comm.). A visit to the site on 10 January 2012 revealed that the wetland areas on the property were badly disturbed as water is routinely extracted for use in the pet-food production and tropical fish-rearing operations that are conducted on the premises. Contaminated water is subsequently returned to the reed beds for filtration. The required understory of moribund/low-lying vegetation is also lacking and thus this site does not appear suitable for *H. pickersgilli*. No calls of *H. pickersgilli* were heard during the survey. Another historical site at "Senla Sugar Estates" (now Tongaat-Hulett) near Zinkwazi Beach appears suitable, but we were unable to gain access at night at the time of surveying due to indemnity requirements.

4.4.5 Additional locations

Five additional locations for *H. pickersgilli* were located on an ad hoc basis between 2007 and 2010. A large population of *H. pickersgilli* was discovered at "Froggy Pond", Mt. Moreland (-29.639758°, 31.095865°) in January 2007, during the EIA process for the King Shaka International airport at La Mercy (J. Harvey, pers. comm.). The population is present in a large perennial, reed-bed wetland located immediately south of the new airport and which is surrounded by sugarcane plantations. This site is described in Chapter 3. A further population was discovered in the highly industrialised Isipingo area in November 2007 (-29.991050°, 30.904940°). This site is described in Chapter 3.Three additional populations of *H. pickersgilli* were discovered as part of other surveys and are included in the overall count for populations now known: in early January 2011 the species was found at Simbithi Eco Estate (-29.513222°, 31.215000°) and on 22 December 2011 it was recorded where the Enseleni River enters the western edge of Lake Nsezi near Richards Bay (-28.739167°, 31.969028°) (J. Harvey, pers. comm.). On 13 March 2012 the species was reported from near Mahunu (-28.92845º, 31.8641º) (M. Trimble & A. Prins, pers. comm.).

Table 4.2: Details of historical localities (1977 – 2007) which were re-visited between October 2010 and January 2012 (South – North).

Site name, date of discovery and source	**Date re-visited**	**Coordinates**	**Threats**	**Size**	**Apparent Status**	***Hyperolius pickersgilli* detected**
Warner Beach , 1982 (Raw 1982)	Nov 2011 Jan 2012	-30.07644° 30.86504°	Habitat loss (possible wetland drainage); proximity to road; urbanisation	Small	Extinct	No
Adam‟s Mission, 2001 (Minter 2004)	Jan 2011	-30.00276° 30.80039°	Rural urbanisation; proximity to road	5 small populations within 4 km of each other	Poor	Yes
Avoca, 1981 (Type locality) (Raw 1982)	Oct 2011	-29.76010° 31.02199°	Complete habitat loss; urban development	n/a	Extinct	No
Mt. Edgecombe, 1977 (Raw 1982)	Oct 2011	-29.70835° 31.02867°	Habitat loss; urbanisation; cultivation in historical river bed	n/a	Extinct	No
Stanger, 2002 (Minter 2004)	Dec 2010	-29.33239° 31.31027°	Rural urbanisation; soil dredging	Small	Poor	Yes
Charlottedale, 2002 (Minter 2004)	Dec 2010 Feb 2011	-29.39611° 31.28556°	Rural urbanisation	Small	Poor	Yes
Tugela River Mouth, 1983	Dec 2010	-29.20833°	Sugarcane	n/a	Extinct	No

(Lambiris 1989)		31.46667°				
Amatikulu Prawn farm, 2007 (Ezemvelo KZN Wildlife Biodiversity Database)	Jan 2012	-29.074720° 31.648890°	Habitat alteration; water drainage; contamination from industrial activities (pet food manufacture) and tropical fish breeding) on premises	Medium	Poor	No
"Matatiele Pan" Twinstreams (late 1980s N Passmore)	Multiple visits	-28.98562° 31.72981°	Eucalyptus plantations; drought	n/a	Extinct	No
Forest Lodge, Mtunzini, 1994 (Minter 2004)	Oct 2011	-28.96782° 31.75322°	None perceived	Medium	Intact (protected within a conservancy)	Yes
Raphia Palms, Mtunzini, 2001 (Minter 2004)	Multiple visits	-28.95853° 31.76228°	Dry conditions in the region over the past decade.	Very Small	Intact (protected within a conservancy)	Yes
Umlalazi Nature Reserve, 1997 (Minter 2004)	Dec 2010	-28.95805° 31.76472°	Sewage run-off; reed harvesting	Large	Protected	Yes
Richards Bay, 1982 (Raw 1982)	Jan 2012	-28.767620° 32.058410°	Habitat loss; dumping of refuse	Medium	Intact	No

St Lucia Estuary (Raw 1982)	Various	-27.63614° 32.58520°	Main road bisects the wetland	Large	Protected	Yes
"Senla" Tongaat-Hulett Sugar Estate (Raw, 1982)	Jan 2012	-29.27120° 31.44533°	Surrounded by sugarcane; pesticide runoff	Medium	Intact	No To be re-visited at night

4.4.6 Extent of occurrence and area of occupancy

EOO for *H. pickersgilli* was recalculated as 2081.5 km² (when the portion of the EOO that falls over the sea is excluded), and the AOO as 120 km². The area of wetlands with extant populations or known to have been inhabited by *H. pickersgilli* is 14.17 km².

4.5 Discussion

4.5.1 Predictive modelling and surveying

Hyperolius pickersgilli has been highlighted as species requiring improved knowledge on its distribution and population size (Measey 2011). Even in the original description, Raw (1982) notes that within the distributional range of *H. pickersgilli* "only an infinitesimal amount would actually be suitable habitat for this species". This lowland area has been fairly well surveyed in terms of amphibian fauna, but even experts in the field have overlooked populations of this species due to its inconspicuous nature (N. Passmore, pers. comm.). In addition, very little of this species‟ original habitat remains along the KwaZulu-Natal coastline as a result of urbanisation and agriculture (Johnson & Raw 1987; Armstrong 2001). As such, additional surveys of breeding habitat have been recommended (Bishop 2004b; Measey 2011). The predictive model generated here enabled surveys to be targeted at wetlands that are most likely to be suitable for *H. pickersgilli*. Surveys were undertaken over two breeding seasons to detect the species at predicted wetlands, as well as at historical sites. Despite intensive surveying during this study, only six new localities for *H. pickersgilli* were discovered, namely at Prospecton, St Lucia (a rediscovery), Port Durnford, Zinkwazi Beach Umkomaas, and Sezela. The populations at Lake Nsezi, Mahunu, Simbithi and Prospecton were not discovered as a direct result of the model, but were found during the study period, and are included in the total of subpopulations below. This low result relative to survey effort gives an indication both of the rarity of the species as a result of its habitat specificity, as well as its low detectability.

Detection of any species is typically not perfect, and this is particularly true for species that are difficult to observe such as most anurans (MacKenzie *et al*. 2002; Smith *et al.* 2006). Furthermore, calling is strongly influenced by temporal variation and environmental factors, such as rainfall, temperature and humidity, which can all affect detectability (Bridges & Dorcas 2000; Oseen & Wassersug 2002; Bishop 2004b). Imperfect detection is highly likely for a species such as *H. pickersgilli* due to its small size, inconspicuous call and cryptic behaviour (Bishop 2004b). *Hyperolius pickersgilli* has a soft, cricket-like call which does not carry far, and is usually issued from hidden positions low down in dense vegetation (Raw

1982; du Preez & Carruthers 2009). Raw (1982) attributes “the voice of this species [as] probably one of the main reasons for the species not having been found early”. As such, it is often difficult to detect, particularly if sympatric species are chorusing loudly (Bishop 2004b). The fact that only few subsequent discoveries of new populations have been made since the species was described in 1982 gives an indication of both its rarity and obscurity. There appears to be much variation in the probability of occurrence of *H. pickersgilli* at sites where it was detected and at sites where it was not detected. This may be a result of stochasticity in the presence or absence of *H. pickersgilli* at sites caused by anthropogenic factors such as drainage of water or lowering of the water table, pollution and the introduction of alien plants (Griffiths *et al.* 2010). Alternatively, the predictive model may not be refined enough to account for variations in microclimate that may or may not be conducive to the existence of the species at certain sites (Araújo & Guisan 2006; Kearney & Porter 2009). In some instances, sites were visited where *H. pickersgilli* is known to occur, but no calls were heard.

Although the Maxent model was developed from relatively few distribution records, support was given to the model by the ground-truthing exercise. The mean probability of occurrence for *H. pickersgilli* at sites where the species was recorded was higher than the mean probability of occurrence for the species where it was not detected. High relative humidity was an important variable influencing the distribution of *H. pickersgilli* according to the Maxent model. *H. pickersgilli* is a small frog that occurs in warm areas (Raw 1982; du Preez & Carruthers 2009), and as a result, high relative humidity is likely to assist with reducing water vapour loss from the frog. Small frogs like *H. pickersgilli* are liable to higher rates of water loss than larger frogs because boundary layer resistance decreases with decreasing body size, surface-to-volume ratios increase with decreasing body size, and more elongate frogs have a larger surface area in comparison with species with a rounder body shape (Wells 2007).

The main strength of the distribution model in guiding survey effort, was not only that it could pinpoint wetlands with high probability of occurrence for *H. pickersgilli*, but also that it could guide decisions of what regions should be surveyed. The model indicated that the probability of occurrence for the species was higher towards the coast, and that potentially suitable habitat for the frog could be found both North and South of its known distribution. Indeed, two new populations of the species were discovered to the South of its known range. However, no new populations have yet been discovered to the North of its known range. Whether this is because the St Lucia Estuary acts as a barrier to the species due to its large size and salinity remains unknown. *Hyperolius pickersgilli* has also not been recorded far inland (maximum 16 km from the coast) and therefore the species may not have been able to skirt the Estuary and lake by moving westwards inland far enough for it to reach suitable wetland habitat North of the Estuary. The wetland coverage for KwaZulu-Natal missed some smaller and some larger wetlands in the coastal region, and even though the wetlands coverage has been in development over many years, the coverage of the coastal wetlands is not yet complete. On the other hand, surveying for *H. pickersgilli* has assisted in ground-truthing the wetlands coverage and has resulted in more wetlands being added to the wetland coverage.

4.5.2 Fragmentation and dispersal corridors

Of the potential wetlands surveyed, 53% have been destroyed or transformed, in the main, as a result of drainage for sugarcane (Johnson & Raw 1987; Armstrong *et al.* 1998). Most of these sites are isolated within a landscape of otherwise largely unsuitable habitat. Habitat loss and fragmentation are some of the primary threats to amphibian populations (Fahrig 2003; Cushman 2006). Fragmentation has serious consequences for the overall survival of amphibian species through its effect on population connectivity by hindering juvenile dispersal (Griffiths *et al.* 2010). Consequently, dispersal is likely to be severely limited for *H. pickersgilli*. The friction maps (Figures 4.3 and 4.4) give an indication of remaining dispersal routes for *H. pickersgilli*. Much of the landscape surrounding breeding habitat has been transformed and as such is impassable to *H. pickersgilli*. For example, increased development of roads, such as in the vicinity of the wetlands in Durban south, can act as a barrier to dispersal by increasing mortality (Gibbs & Shriver 2005).

At the known localities, potential meta-population linkages exist at Mt. Moreland, Adam"s Mission, Simbithi Eco-estate, Richard"s Bay and Mtunzini, where the three sites form a meta-population between which dispersal may take place along the Siyaya River. Of concern is the proposed mineral mining that will occur south of Mtunzini, which may result in significant changes to the hydrology of the area as well as contamination of water resources (A. Armstrong pers. comm.). The remainder of localities with extant populations of *H. pickersgilli* are small and isolated, criteria which do not bode well for long-term survival (Harrison *et al*. 2001; Griffiths *et al.* 2010). Not only is there considerable distance between most sites, but the landscape between subpopulations is generally highly unsuitable to allow for dispersal between them.

4.5.3 Status of historical sites

Raw (1982) lists seven localities in the original description of *H. pickersgilli*. Of these, only the one which occurs within a formally protected area (iSimangaliso World Heritage Site) at St Lucia Estuary has been ascertained to still be extant (although the exact location details of the original discovery are unknown). Both the Type locality and site of original discovery have been destroyed. Subsequent to the species description, a further 8 localities were reported up until 2007. During this study most of these sites were revisited. *Hyperolius pickersgilli* was not detected at 8 of the 15 known historical localities, with the species deemed to be extinct at no less than 5 of these sites (Table 4.2).

Prolonged drought may have caused the decline or extinction of populations of aquatic-breeding frogs in various places (Wells 2007). It appears that *H. pickersgilli* is confined to permanent wetlands that survive prolonged periods of drought that occur from time to time in parts of the distribution range of the species (Bruton & Cooper 1980). *H. pickergilli* lacks granular areas on the belly that assist with water uptake from damp surfaces, indicating that the species likely relies on water bodies and wet surfaces rather than damp surfaces for water uptake through the skin (Wells 2007). Movement of metamorphs or adults between wetlands may occur mainly during wet weather as a result, and drought conditions may curtail such movement. However, the resistance of the skin of *H. pickersgilli* to water loss is unknown, and some species of *Hyperolius* are known to have relatively high resistance to water loss (Wells 2007).

4.5.4 Conservation Implications

Hyperolius pickersgilli occurs within an area that is being heavily impacted by human activity (Johnson & Raw 1987; Armstrong *et al*. 1998; Goodman 2000). Based on the results generated from the surveying efforts of this study EOO was recalculated for *H. pickersgilli* as 2081.5 km² and the AOO as 14.17 km² (or 120 km² when non-breeding habitat is taken into account). As could be expected from the range expansion to the south and an overall increase from 12 to 17 localities, the AOO has increased somewhat. The recalculated EOO is approximately 220 km² less than the figure given in the 2011 assessment, possibly as a result of the large amount of ocean area that was clipped from the total polygon. The discrepancy in results indicates that more refined methods are probably necessary before making any decisions regarding a status update for *H. pickersgilli*. Increases in range size are expected with increased survey intensity and, if relevant, updates to species status (Botts *et al*. 2012). Although the AOO now exceeds 10 km^2, there is still severe fragmentation between localities, and a continuing decline in habitat quality (IUCN Red List Guidelines Committee 2011). Based on the results of surveying, and on assessment of the level of threat at each locality for the species, it is recommended that the Red List status of *H. pickersgilli* be reassessed as Endangered (EN B1ab(iii)+2ab(iii)). The AOO now exceeds 10 km^2, but the population remains severely fragmented, and habitat area, extent and quality are continuing to decline (cf. IUCN Red List Guidelines Committee 2011).

In addition to producing positive results in terms of finding new populations, species distribution models are useful for examining potential dispersal routes between sites within meta-populations. Ideally, such corridors can be translated into long-term conservation plans for *H. pickersgilli*. Meta-population dynamics, especially of low-vagility organisms such as frogs is adversely affected by fragmentation (Carr & Fahrig 2001; Cushman 2006), and should be taken into account when designing conservation plans. The meta-population maps produced here can be used to support conservation plans.

Overall, the species is currently present at seventeen localities. Only two populations, those at Umlalazi Nature Reserve and iSimangaliso World Heritage Site, are offered formal provincial protection. The Mtunzini populations at Forest Lodge and the Raphia Palms monument are afforded some protection under the Twinstreams-Mtunzini Natural Heritage

Site and conservancy area. All remaining populations, namely Isipingo, Mt Moreland, Zimbithi, and Port Durnford, Adam"s Mission, Stanger and Charlottedale face varying levels of threat. It is important to recognise that each of these sites has conservation value and to work with relevant authorities, stakeholders and land-owners to secure long-term protection of these populations. Table 4.3 provides suggestions for land protection methods for each site.

The Prospecton site occurs within the proposed area that is to be developed for the Durban South Port Development, which will essentially result in complete habitat removal and extirpation of this subpopulation of *H. pickersgilli*. Taking into account that each locality comprises approximately five percent of the total range of *H. pickersgilli,* loss of any site has serious implications for the overall population. The recommendation for Critically Endangered species is no further loss of habitat (Driver *et al*. 2009; Pfab *et al*. 2011), however, in this case preservation of the site seems untenable. The only solution will be a rescue and translocation programme funded by offsets by the developer. This will be a major undertaking requiring careful planning throughout, including identification of a number of possible re-introduction sites in the vicinity; rehabilitation thereof if necessary; and long-term management and monitoring (Soorae 2011).

An *ex-situ* programme, which commenced in January 2012, is being implemented and headed by Johannesburg Zoo for *H. pickersgilli*. This is the first such programme for a threatened amphibian in southern Africa with the long-term goal of creating an assurance population of this species for eventual re-introduction into the wild (Visser 2011). Additional conservation actions are discussed in Chapter 3.

Table 4.3: Recommendations for land protection of the currently known *Hyperolius pickersgilli* populations (North to South).

Locality Name	**Approximate Size**	**Status and Ownership**	**Management Recommendation**
St Lucia	Large (~ 20 ha)	Protected (iSimangaliso Wetland Heritage Site)	Long-term monitoring (KZN Wildlife)
Richard‟s Bay	Medium (~ 10 ha)	Not protected	Stewardship
Port Durnford (2 sites)	Medium (~5 ha)	Not protected (Mondi)	Rehabilitation and Stewardship
Umlalazi Nature Reserve	Medium (~ 5 ha)	Protected (KZN Wildlife)	Long-term monitoring (KZN Wildlife)
Mtunzini (2 sites)	Small (~ 2 ha)	Not officially protected (Forest Lodge and Mtunzini conservancy)	Stewardship
Zinkwazi Beach	Medium (~ 5 ha)	Not protected (Private)	Stewardship
Stanger and Ethafeni	Small (~ 2 ha)	Not protected (Private)	Land acquisition
Simbithi Eco Estate	Small (~ 2 ha)	Not protected (Private)	Stewardship
Mt Moreland	Large (17 ha)	Not protected (ACSA)	Stewardship
Prospecton	Medium (~5 ha)	Not protected (ACSA)	Translocation (new site necessary)
Isipingo	Small (2 ha)	Not protected (Private)	Land acquisition
Adam‟s Mission	Small (2 ha)	Not protected (Private)	Land acquisition
Umkomaas	Small (2 ha)	Not protected (Private)	Land acquisition
Sezela	Small (1 ha)	Not protected (Private)	Land acquisition
Total	80 ha		

Currently approximately 31% of *H. pickersgilli*"s range is officially protected. Successful stewardship programs for Mt. Moreland, Port Durnford and the Mtunzini sites would secure a further 30% for long-term survival. Stewardship agreements require long-term commitment from land-owners and are an onerous process to implement, but are becoming increasingly popular for protection of habitat with high biodiversity value on privately owned land. This method of securing habitat will be well suited to Mt. Moreland, which in addition to hosting *H. pickersgilli* is also an important bird area, and its protection will involve multiple stakeholders. Smaller, privately owned sites would be better suited to land acquisition options, whereby the land is purchased and managed by a local conservation authority or non-government organisation. The Isipingo and Umkomaas sites are most suitable for this option currently. Development plans for housing units, including a sewage processing plant and parking areas are currently being considered for the land at Umkomaas. Presence of *H. pickersgilli* here will ideally prevent this from going ahead. Land acquisition similarly is only viable if long-term management procedures are put in place, including monitoring (Field *et al*. 2005).

4.5.5 Conclusions

Although these recent finds indicate that ongoing surveys will likely continue to reveal unknown populations, for each new discovery made, loss of an historical site is verified. *Hyperolius pickersgilli* is both habitat-specific and very easily overlooked, with the consequence that sites hosting the species may be ear-marked for development. Despite an overall increase in the number of localities at which the species is present, its Critically Endangered status remains warranted due to the severe level of fragmentation between populations, ongoing decline in habitat quality and very few populations in Protected Areas (IUCN 2011). As a consequence urgent conservation actions, including long-term monitoring, are needed (Chapter 3). In addition to producing positive results in terms of finding new populations, species distribution models are useful for examining potential dispersal routes between sites within meta-populations. Ideally, such corridors can be translated into long-term conservation plans for *H. pickersgilli*. Further research on the life history and dispersal abilities of *H. pickersgilli* will be useful for contributing further to conservation strategies for this species.

CHAPTER 5

THE IMPORTANCE OF SYSTEMATICS FOR CONSERVATION ISSUES: THE CASE OF THE *AFRIXALUS SPINIFRONS* (ANURA: HYPEROLIIDAE) COMPLEX IN THE LIGHT OF MOLECULAR, BIOACOUSTIC AND MORPHOLOGICAL ASSESSMENT

5.1 Abstract

The *Afrixalus spinifrons* complex is comprised of three taxa which have an intricate taxonomic history: *A. spinifrons spinifrons*, *A. s. intermedius* and *A. knysnae*. The complex represents the southernmost range of the African genus *Afrixalus*. The taxonomy of this group has been controversial because of disagreements over taxon range delimitations as well as a high degree of morphological homogeneity between members of the complex. Resolution of these issues has been highlighted as a conservation research requirement. Here, for the first time, molecular phylogenetic analysis is used in conjunction with morphological and bioacoustic data, to elucidate relationships within the complex. Specimens were obtained from various institutions for morphological assessment and new collections were made from 15 localities throughout the range for molecular analysis. Sequences of the mitochondrial 16S gene were used to investigate phylogenetic relationships at the species level. In terms of morphology, the results largely agree with previous studies, with *A. s. intermedius* intermediate in terms of size relative to *A. knysnae* and *A. s. spinifrons.* However, specimens of *A. s. spinifrons* from the Eastern Cape resemble *A. s. intermedius* more closely in terms of morphology than they do specimens from the KZN coast. This finding was supported by the molecular results, which indicated that the Eastern Cape form is more closely related to *A. s. intermedius* than to *A. s. spinifrons* from the KZN coast. *Afrixalus spinifrons spinifrons* and *A. s. intermedius* form two distinct, but closely related clusters. *Afrixalus knysnae* is confirmed as a member of the clade, but divergent at the species level.

5.2 Introduction

5.2.1 General

Advances in techniques for assessing the patterns of amphibian diversity have been effective in revealing cryptic species and previously unknown relationships between taxa that are otherwise phenotypically similar (Nevo & Beiles 1991; Glaw and Vences 2006; Vences & Wake 2007). Often, this has lead to a substantial increase in species descriptions, and this has important applications for conservation, since species are the fundamental unit that is considered for protection purposes (Wheeler 2004; Elmer et al. 2007; Kremen et al. 2008; Padial et al. 2010). The members of the southern African "dwarf" *Afrixalus* are morphologically very similar, and in some cases virtually identical. Resolution of the phylogeny of the *Afrixalus spinifrons* complex, which is endemic to South Africa, has been highlighted as a research conservation priority (Measey 2011). This study aims to elucidate the systematics within this group through the use of molecular, morphological and call assessment.

The systematics of *Afrixalus* groups throughout Africa has been reviewed a number of times in recent decades, based primarily on morphology, and in some instances, on bioacoustics (Schiøtz 1974; Pickersgill 1984; 1996; 2005; Poynton & Broadley 1987). Since anurans, and particularly hyperoliids, show high levels of morphological similarity (Emerson 1986; Glaw and Vences 2006), the advent of molecular analysis has been invaluable in detecting divergences between closely related taxa (Funk & Omland 2003; Van der Meijden *et al.* 2005; Wollenberg *et al.* 2007). Despite an interest in understanding the relationships within the *spinifrons* group, a phylogeny based on molecular markers is so far lacking. Mitochondrial markers are useful in systematic and population studies, especially among closely related taxa (Avise 2000; Boore 1999; Moritz *et al.* 1987). This is especially relevant where population data is limited due to small samples sizes (Vences & Wake 2007). Advertisement calls are the primary mechanism of mate recognition in anurans (Paterson 1985; Minter 1998). As such, bioacoustic characters are of excellent use in systematic studies, although the rate at which different call parameters evolve can vary among groups, as is the case with molecular divergence (Ryan 1990; Ryan *et al.* 1990; Wollenberg et al. 2007).

The term “species complex” refers to a group of closely related species, where the exact demarcation between species is often unclear or cryptic owing to their recent and usually still incomplete reproductive isolation (Knowlton 1993). Superspecies and cryptic species complexes are examples of species complexes. “Superspecies” is applied to a group of at least two more or less distinctive species with approximately parapatric distributions (Haffer 1986). Such groupings may occur in a lineage undergoing rapid speciation or where speciation recently occurred, so that species separation mechanisms have yet to be fully developed (Haffer 1986). These groupings recognise the process of speciation and that not all species are equal in terms of their delimiting parameters (Pickersgill 1996). Within *Afrixalus*, there are a number of distinct species clusters that are recognised as superspecies including the *brachycnemis*, *sylvaticus* and *septentrionalis* complexes (Pickersgill 1996). The spinifrons complex represents the southernmost extension of the genus (Pickersgill 1996). The *Afrixalus spinifrons* complex is comprised of *Afrixalus knysnae* and two subspecies, *Afrixalus spinifrons spinifrons* and *Afrixalus spinifrons intermedius* (*sensu* Pickersgill 1996). The complex has been identified as requiring taxonomic investigation with a priority on calls, phylogeography and systematics (Measey 2011).

With regard to *Afrixalus*, Pickersgill (2005) defines subspecies as “two or more morphologically different taxa sharing the same vocal recognition system and if there is no permanent zoogeographical barrier separating them”. Pickersgill‟s definition contrasts with other definitions of subspecies in that it requires no geographical barrier. Usually, the subspecies taxon refers to morphologically divergent allopatric populations (Mayr & Ashlock 1991). For this study, I adopt the traditional views of Mayr & Ashlock (1991) and the necessity of reproductive incompatibility for speciation (Littlejohn 1993).

In zoological nomenclature when a species is split into subspecies the originally described population is referred to as the nominate subspecies; in this case *Afrixalus spinifrons spinifrons* is the nominate subspecies and *A. spinifrons intermedius* the subspecies (ICZN 1999). Uncertainty regarding this complex arises in that the two forms intergrade geographically within KwaZulu-Natal (Pickersgill 1996) and that species boundaries between the Eastern Cape form of *A. s. intermedius* and *A. knysnae* are unclear. Establishing whether these taxa constitute separate species has been deemed a high conservation priority and will have implications for the conservation actions required to protect them (Pickersgill *et al.* 2004; Measey 2011). Since the taxa contained within this complex have different geographical ranges, and possibly differing ecological requirements, protection plans should be precisely defined in order to increase their impact on target populations.

5.2.2 Taxonomic history of the *Afrixalus spinifrons* complex

Afrixalus (Laurent 1944) is a sub-Saharan genus of the African family *Hyperoliidae* (Laurent 1943) and is comprised of about 35 species and subspecies. These are small (16 – 40 mm) tree and sedge dwelling frogs, distinguishable from *Hyperolius* spp. by having vertical pupils (Schiøtz 1974; Pickersgill 2007). They are commonly referred to as Leaf-folding Frogs owing to their unique method of oviposition, whereby the eggs are enclosed in a sealed leaf nest (Figure 5.1) (du Preez & Carruthers 2009). The taxonomy of *Afrixalus* has been the subject of numerous reviews, especially with regard to the eastern African „dwarf" forms (Schiøtz 1974; Pickersgill 1984; 1996; 2005; Poynton & Broadley 1987). Dwarf refers to the trend in which modifications associated with the miniaturising process are clearly evident; in this case adults seldom exceed 25 mm in length (Pickersgill 2005). Species of this group are remarkably similar, and are indeed difficult to distinguish from one another (du Preez & Carruthers 2009). Aside from the Greater leaf-folding frog *A. fornasinii*, all southern African *Afrixalus* species are usually less than 26 mm in length (Pickersgill 1996). The component taxa of the *Afrixalus spinifrons* complex are isolated from the more northerly dwarf species to form a discrete complex or superspecies (Pickersgill 1996).

Figure 5.1: Newly hatched *Afrixalus spinifrons* tadpoles in a leaf-nest. Photo Louis du Preez.

Afrixalus spinifrons was originally described as *Hyperolius spinifrons* (Cope 1862) from Umvoti, KwaZulu-Natal. The Holotype (ANSP 11320) is housed at the Academy of Natural Sciences, Philadelphia. Cope (1862) describes the species as having a narrow head and rounded muzzle. The tympanum is concealed. The skin of the upper surfaces is smooth other than at the front and snout, which are studded with "acute tubercles". Over the years, various synonyms have been applied including *Megalixalus spinifrons* (Ahl 1930); *Megalixalus fornasinii spinifrons* (Power 1935) and *Afrixalus spinifrons* (Rose 1962; Poynton 1964; Pickersgill 1984). General external characteristics for members of the complex include a smooth gular disc; dorsal and ventral asperities, a swollen and tapering snout; subarticular tubercles of the outer fingers entire; a broad lateral band and no occipital patch (Pickersgill 1996).

Afrixalus spinifrons spinifrons is confined to the central KwaZulu-Natal coast, at low to intermediate altitudes (up to approximately 700 m above sea level). It is characterised by having a swollen snout densely covered by black asperities (Pickersgill 1996; du Preez & Carruthers 2009). The bulbous snout and concentrations of asperities on the snout have been suggested to represent an adaptation to living in reedbeds (Pickersgill 1996). It is distinguished from *A. knysnae* by its swollen snout, smaller size, and in having more extensive dorsal markings (Pickersgill 1996).

Specimens from inland and southern KwaZulu-Natal and the Eastern Cape were previously included as a variation of *A. knysnae* (at the time considered to be a southern form of what was then known as *A. brachycnemis*) and were considered distinct from *A. spinifrons* (Poynton 1964). Pickersgill (1984) was able to distinguish between the southernmost members of the genus, i.e. *A. spinifrons* and *A.* (*brachycnemis*) *knysnae*, but still had difficulties in identifying material from altitudes above 700 m in southern KwaZulu-Natal and the Eastern Cape since they shared characteristics exclusive to *spinifrons* (i.e. the swollen snout and extensive dorsal markings). Compounding this was that *A. knysnae* in the Western Cape was geographically isolated from eastern populations of "*A. knysnae*" and no obvious link could be established (Pickersgill 1996). As a result, Pickersgill (1996) erected the subspecies *Afrixalus spinifrons intermedius* based on type specimens from the Mooi River floodplain in Rosetta (altitude 1400 m) in the KwaZulu-Natal midlands. The description is based on an adult male holotype (MP 531, Senckenberg Museum, Frankfurt, Germany) and 49 paratypes from the type locality. Pickersgill"s (1996) study found that in certain characters (body length, build and pattern), *intermedius* was midway between *spinifrons* and *knysnae*, but in others characters that were diagnostic for *knysnae*, was more similar to *spinifrons*. The subspecific designation was supported by specimens from the Umvoti Vlei area, where *spinifrons* and *intermedius* seemed to intergrade (Pickersgill 1996). The subspecies does not have the swollen snout typical of *spinifrons* and the dorsal asperities are homogenous, both in size and distribution (Pickersgill 1996). Apart from the broad, dark lateral band, markings are confined to the posterior half of the dorsum. *A. knysnae* has similar dorsal patterns but can be distinguished by its larger size and more robust build.

Afrixalus knysnae was described by Loveridge (1954) as *Hyperolius knysnae* from Knysna, Western Cape. The description is based on a gravid female (SVL 24 mm, holotype: MCZ 10884, Museum of Comparative Zoology, Harvard University) and three adult female paratypes (24 – 25 mm, Natal Museum, NM 217-218 from the original series of the type). The holotype specimen had originally been described by Cope (1862) as *Hyperolius cinciventris* but was recognised by Loveridge as a separate species, which he was able to describe once obtaining the paratypes from Dr R.F. Lawrence of the Natal Museum. Loveridge notes that the entire dorsum is covered with “minute, black punctuations, some of which are slightly raised like the spines of *Afrixalus*.” The snout is obtuse and the skin of the throat and chest is smooth, while that of the belly is granular. The dorsal colour is uniform other than a dusky vertebral line that terminates on the parietal region in a tulip-shaped blotch and is also covered in black punctuations (Loveridge 1954).

5.2.3 Species Descriptions

Species descriptions of this complex vary among sources and this has certainly contributed to the taxonomic confusion surrounding the group (Poynton 1964; Schiøtz 1974; Pickersgill 1984; 1996; 2005; Poynton & Broadley 1987; Pickersgill *et al.* 2004; du Preez & Carruthers 2009). The descriptions that follow are based on the most recent literature (Pickersgill 1996; 2007; du Preez & Carruthers 2009; Measey 2011). Table 5.1 summarizes the main differences between the three members of the *Afrixalus spinifrons* complex based on both females and males (*sensu* Pickersgill 1996).

Table 5.1: Comparison of the three members of the *Afrixalus spinifrons* complex (*sensu* Pickersgill 1996).

Characteristic	***A. knysnae***	***A. s. spinifrons***	***A. s. intermedius***
Length	Large (22 – 25 mm)	Small (16 – 23 mm)	Medium (17 – 24 mm)
Build	Stocky	Slender	Sturdy
Snout shape	Not swollen	Bulbous	Not swollen
Asperities	Homogenous	Concentrated on snout	Homogenous
Dorsal Markings	Sometimes forward of mid-back	Forward of mid-back	Not forward of mid-back
Range	Eastern Western Cape Coast	KwaZulu-Natal and Eastern Cape coast	KwaZulu-Natal midlands above 700 m.a.s.l

Afrixalus spinifrons spinifrons

The nominate subspecies reaches a maximum body size of 25 mm in females (du Preez & Carruthers 2009). Pickersgill (2007) describes the species as having a relatively stocky body shape. The snout is swollen and bulbous. The dorsal skin is covered with very large, dense black asperities. These are denser anteriorly, but not necessarily more concentrated on the snout (Figure 5.2). Asperities are present on the ventral side, usually confined to the gular area (Pickersgill 1996; 2007). Colouring above can vary from ivory to light brown with a darker brown vertebral band running from the tip of the snout broadening at the rear and split by a light-coloured marking above the cloaca. The underside is creamy and thighs yellow. Males have a yellow gular disc. The advertisement call is comprised of two components, the short, sharp „zick!“ and a soft, prolonged rattle lasting two or more seconds (Pickersgill 1996; 2007). Figure 5.1 shows a male from Mtunzini on the KwaZulu-Natal coast.

Figure 5.2: Male *Afrixalus spinifrons spinifrons* from Forest Lodge, Mtunzini (-28.96782° S, 31.75322° E) on the KwaZulu-Natal coast. Asperities are concentrated anteriorly and on the bulbous snout.

Afrixalus spinifrons intermedius

The body shape of *A. s. intermedius* is stockier than that of *A. s. spinifrons* (Pickersgill 1996). Pickersgill (2007) describes the snout as never swollen with more abundant asperities while du Preez & Carruthers (2009) claim the asperities are evenly distributed over the dorsum (Figure 5.3). Dorsal colouring is ivory to pale gold and markings are either lacking or usually confined to the posterior of the back. The ventrum is similar to that of *A. spinifrons*. The tibia has a dark band on its lower surface and a narrow transverse streak may also be present (Pickersgill 2007). The call is similar to *A. s. spinifrons*, but has a longer duration. Subsequent examination of additional specimens by Pickersgill (2007) revealed similarities to *A. s. spinifrons* (namely extensive dorsal markings) which he suggests are due to movement of *spinifrons* into the *intermedius* range due to warming temperatures. Figure 5.2 shows a male from Fort Nottingham in the KwaZulu-Natal Midlands (-29.4320° S, 29.90587° E).

Figure 5.3: Male *Afrixalus spinifrons intermedius* from Kingussie Farm, Fort Nottingham (-29.4320° S, 29.90587° E). Spines are not concentrated on the snout but are homogenous over the dorsum and colouring is uniform.

Afrixalus knysnae

The body of *A. knysnae* is larger than the other two complex members (up to 25 mm in females) and more robust with a relatively broad head (Figure 5.4). The canthus rostralis is angular and the snout is tapering (not swollen as in *spinifrons*). The dorsum has prominent asperities evenly distributed on the back and head. They may be more abundant on the snout, but unlike *spinifrons* they do not form tubercles (Pickersgill 2007). Ventrally, the gular disk is smooth with weakly scattered asperities which become more concentrated and better developed posteriorly. The gular disk is relatively small compared to that of the other members of the complex (Pickersgill 1996). Colouring is ivory to yellow-brown above, with a darker lateral band running from the nostril through the eye to the groin. The ventral survaces are usually creamy white. Darker paravertebral stripes on the back are present on most specimens (du Preez & Carruthers 2009). The call is a soft prolonged *trill* lasting for one or two seconds, followed by a short *zip* similar to *spinifrons* and *intermedius* (Burger *et al*. 2004; du Preez & Carruthers 2009).

Figure 5.4: *Afrixalus knysnae* from Covie, Western Cape (Photograph by Vincent Carruthers).

5.2.4 Distribution

Afrixalus spinifrons inhabits Coastal Bushveld-Grassland and Moist Upland Grassland in KwaZulu-Natal and the Eastern Cape Provinces. *Afrixalus spinifrons spinifrons* occurs at low to intermediate altitudes (below 1000 m) from Cintsa Bay in the Eastern Cape to St Lucia in northern KwaZulu-Natal (Pickersgill *et al*. 2004). *Afrixalus spinifrons intermedius* occurs at altitudes above 1000 m in KwaZulu-Natal between Underberg and Pietermaritzburg (Pickersgill *et al*. 2004). New populations were discovered in Franklin (KwaZulu-Natal) and Umtata (Eastern Cape) during the Atlas survey (SAFAP 2004; Pickersgill *et al.* 2004). Those populations from the Eastern Cape and KwaZulu-Natal previously assigned to *A. knysnae* were re-assigned to *A. s. spinifrons* by Pickersgill (1996). According to Pickersgill (2007) *A. s. spinifrons* and *A. s. intermedius* appear to intergrade on the escarpment at about 700 m.

Afrixalus knysnae occurs at low altitudes (below 750 m.a.s.l) on the south coast on either side of the border between the Eastern and Western Cape Provinces (Measey 2011). According to this distribution, *A. knysnae* does not occur in sympatry with any other *Afrixalus* species. The closest known *A. spinifrons* site is at Kei Road, 400 km to the east. As with *A. spinifrons*, there is some contention regarding the distribution of this species, with some authors (e.g. Passmore & Carruthers 1979; Channing 2001) including the central to northern Eastern Cape coastal belt as part of this species range, while others restrict the distribution to the Western Cape (Burger *et al*. 2004). The species occurs in Mountain Fynbos and Afromontane Forest and has been recorded from glades, clearings and roadside pools (Burger *et al*. 2004).

5.2.5 Ecology and Life-history

The genus is characterised by a unique method of oviposition; following coupling, the amplectant pair move from the call site and position themselves along the axis of a leaf or grass close to the surface of the water (Pickersgill 2007; du Preez & Carruthers 2009). As they move along the axis of the blade the female deposits between 20 and 50 white eggs which are fertilised by the male. The male then folds the leaf with his hindlegs to encase the eggs within a tube which is sealed with an adhesive substance deposited with the eggs (du Preez & Carruthers 2009) (Figure 5.1). The eggs remain in the folded leaf until tadpoles hatch and drop into the water, usually after 4-6 days (Pickersgill *et al*. 2004; du Preez &

Carruthers 2009). Metamorphosis is completed in approximately 6 weeks (Pickersgill *et al*. 2004). Breeding occurs between August and February for *A. s. spinifrons* and September and January for *A. s. intermedius* (Pickersgill *et al*. 2004). Males call from emergent vegetation, usually close to the water"s surface, in choruses of between 4 and 20 individuals between sunset and 03:00 (Backwell & Passmore 1991).

Afrixalus spinifrons spinifrons breeds in standing water (including dams and ponds), sedge beds and grassy wetlands (Figure 5.5). In winter it can be found in the leaf axils of *Strelitzia* spp. *Afrixalus spinifrons intermedius* occurs in marshes, dams, floodplains and riverbanks and females and juveniles of the subspecies can be found sunbathing in arum lilies during the day (Pickersgill 1996; 2007).

Figure 5.5: Habitat of *Afrixalus spinifrons spinifrons* at a small natural pond at Tala Nature Reserve, KwaZulu-Natal with emergent sedge and bulrush vegetation.

5.2.6 Advertisement call characteristics

Afrixalus spinifrons is a prolonged breeding species with a complex, functionally partitioned, two-part advertisement call (Backwell 1988; 1991). Phonotaxis experimentation with the closely related *A. delicatus* has shown that the "*trill*" component (a long buzz) serves to attract females and that the "*zip*" component (a series of short ticks) is used for establishing male distribution during calling (Backwell 1988). In *A. spinifrons*, the two call components are not linked in a single call nor are they issued in a set sequence. Consequently, they may be more easily modified depending on distribution of males and the availability of females (Backwell 1988). *Afrixalus knynae* also exhibits the two-part call, comprised of a short *zip* and longer *trill* (Burger *et al*. 2004). Most *Afrixalus* species are particularly sensitive to pulse rate since their calls are comprised of successive pulses (Backwell 1991; Pickersgill 2007).

5.2.7 Conservation Status

Afrixalus spinifrons is listed as Near Threatened (IUCN 2011) due to its area of occupancy being less than 1900 km^2, the continuing decline in the quality of its habitat and its severely fragmented distribution. Many of the historical sites of this once common species have disappeared under development (Pickersgill *et al*. 2004; Pickersgill 2007). Remaining sites face various threats which may seriously impact on population viability in future (Pickersgill *et al*. 2004; discussed in Chapter 3). Loss of certain sites could result in less than 10 remaining locations triggering the criteria for Vulnerable status (Measey 2011). Coastal populations of *A. s. spinifrons* are at high risk of habitat loss due to intensive development pressure in the region, in particular transformation from sugar cane agriculture (Johnson and Raw 1987; Armstrong 2001) (see Chapter 3). *Afrixalus spinifrons intermedius* has been highlighted as having particular conservation significance for KwaZulu-Natal since it is endemic to the province (Armstrong 2001). Separation of these taxa will have understandable implications for their protection since it will inevitably raise their conservation status.

Afrixalus knysnae is listed as Endangered (IUCN 2011) due to its limited distribution and ongoing development of the coastal area in which it occurs. Habitat at most of the known localities has been altered to some extent and alien vegetation is common at the majority of sites. Only a few populations occur in pristine habitats. It is known from only seven localities and has an extent of occurrence (EOO) of just 1756 km^2 (Measey 2011). Area of occupancy (AOO) has not been calculated, but is believed to be in decline (Measey 2011). The distribution is considered to be highly fragmented and there is concern that some subpopulations have recently become extinct since recent surveys have failed to detect the species (Measey 2011; M. Pickersgill pers. comm.). It is threatened by habitat loss as a result of coastal development, agriculture and chemical pollution. According to Measey (2011), this species is ranked amongst the highest in need of conservation-oriented research, in particular with regard to taxonomy, breeding phenology and threat identification.

Thus there is certain contention with regard to species delimitations in the *Afrixalus spinifrons* complex regarding their physical descriptions, distributions and calls. This study aims to provide additional knowledge on these aspects in order to clarify taxonomic boundaries by introducing molecular analysis for phylogenetic analysis. Because the ecological divergence and differences in geographical distribution between species of this complex could influence the relative conservation efforts, a clear description of their taxonomic status is necessary by way of various independent criteria.

The aims of this chapter were to:

1) Develop the existing morphological and bioacoustic data sets available to describe various lineages within the *Afrixalus spinifrons* complex;

2) Investigate the molecular phylogeny of the different identified population within the complex (particularly with regard to *A. s. spinifrons* and *A. s. intermedius*) using mitochondrial DNA sequences;

3) Contribute to future taxonomic revisions of this complex.

5.3 Methods

5.3.1 Study Area

Specimen collections were made from both known (SAFAP 2004) sites and new sites (Appendix E) discovered as a result of field work during this study throughout the distributional range of *A. spinifrons* (Figure 5.6). Field work was conducted during the breeding season (August to February) of *A. spinifrons* between 2009 and 2012. Since *A. spinifrons* is nocturnally active, fieldwork was typically conducted after nightfall between 19:00 and 00:00. One to 6 specimens were collected per site for morphological assessment and tissue samples. Some additional samples were generously supplied by other researchers (Appendix E).

5.3.2 Morphometric Assessment

Specimens

A total of 96 specimens of *A. s. spinifrons* (n = 56), *A. s. intermedius* (n = 37) and *A. knysnae* (n = 3) from the following collections were examined for morphological assessment (See Appendix E for details). This includes 36 specimens that were collected during the course of this study for call and molecular analysis (accessioned to the AACRG collection):

AACRG - African Amphibian Conservation Research Group Collection, North West University, South Africa (37 specimens).
DM – Durban Natural Science Museum, Durban, South Africa (2 specimens, damaged)
NM – KwaZulu-Natal Museum, Pietermaritzburg, South Africa (39 specimens).
PEM – Port Elizabeth Museum at Bayworld, Port Elizabeth, South Africa (20 specimens).

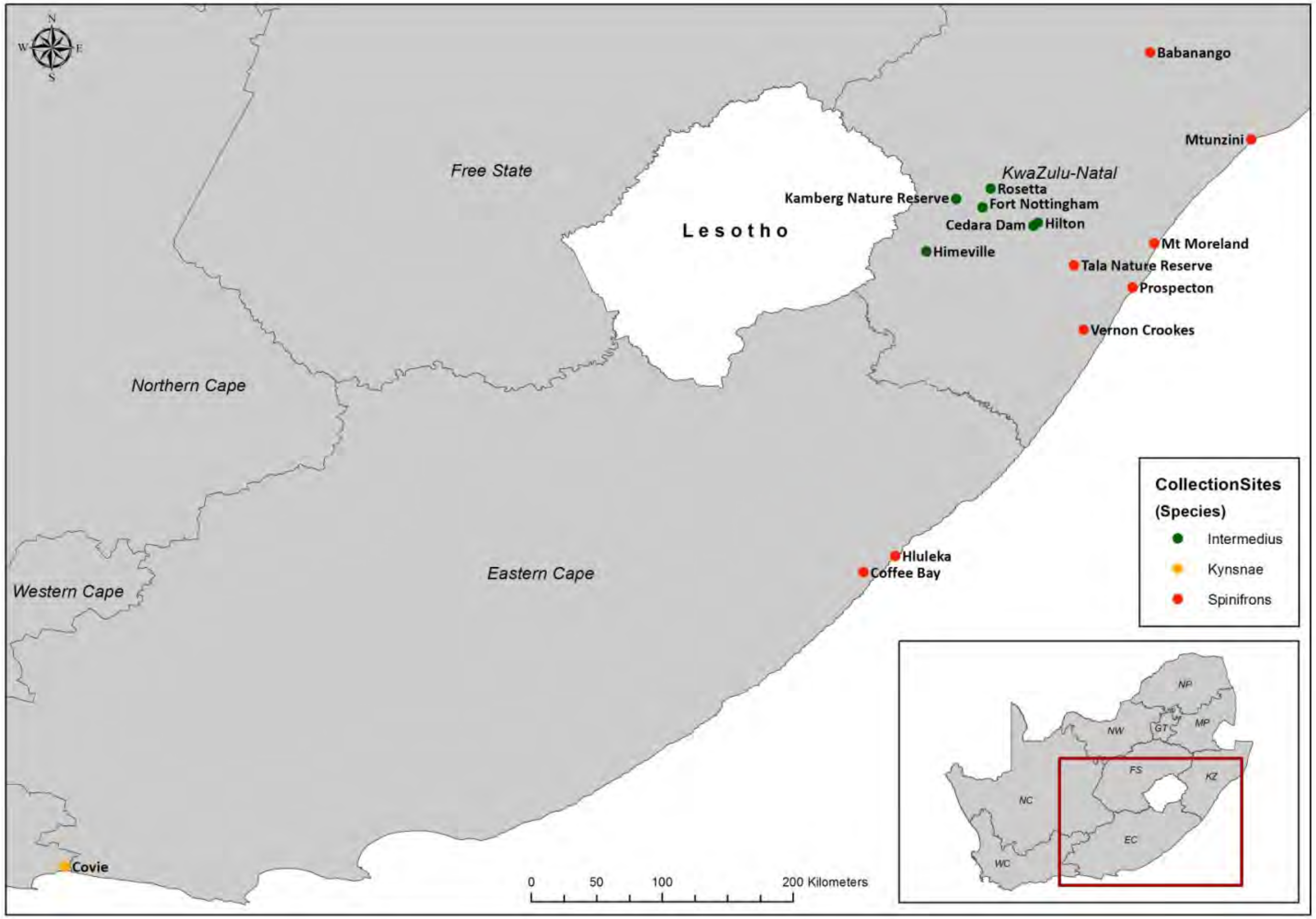

Figure 5.6: Map of sample localities in southern and eastern South Africa for *Afrixalus spinifrons spinifrons* (red dots), *A. s. intermedius* (green dots) and *A. knysnae* (yellow dot). Tissue samples for molecular analysis were obtained from each site.

The following characters deemed to be useful for diagnosis of *Afrixalus* (particularly in adult males) (*sensu* Pickersgill 1984) were examined for the purposes of this study:

1. **Snout-vent length (SVL):** The distance from the tip of the snout to the vent, measured with the specimen pressed lightly on a flat surface.
2. **Head width (HW):** Distance across the head taken at the widest point of the mouth.
3. **Snout shape:** Can vary between being swollen to tapering.
4. **Tibia length (TL):** The maximum length, measured on the flexed hind limb. Tibia length as a percentage of snout-vent length can be used to differentiate between some *Afrixalus* species.
5. **Spinosity**: Pickersgill (1984) regards asperities as the most important diagnostic character for *Afrixalus*, particularly in adult male specimens. Spines or asperities are defined as small, usually darkly pigmented spinules which differ in composition from the surrounding skin.
6. **Gular disc texture:** Can be either smooth or granular. For members of the *A. spinifrons* complex it is smooth, with or without sparse asperities (Pickersgill 1996).
7. **Shape of gular disc:** Used by both Schiøz (1967) and Pickersgill (1984; 1996) as a useful character.
8. **Ventral granulation:** the shape and size of ventral granulation can be useful in distinguishing certain species.
9. **Colouration (in life) and dorsal pattern:** This can be highly variable and differences between species are subtle, however can be useful as a character.

The locality, date of collection, sex (where determinable from external characteristics) and any other label information were also recorded. Specimens collected for this study were photographed in life prior to euthanizing. Collected animals were euthanized using a solution of either MS222 (®Sigma Aldrich) or chlorobutanol (Sigma) (Ethics clearance: NWU 02D02). Thigh tissue was removed and stored in ethanol and the specimen fixed on a fixing tray with 10% neutral buffered formalin. Once fixed, specimens were kept in 10% neutral buffered formalin.

External characters were measured to the nearest 0.01 mm using electronic digital callipers for all specimens. Each measurement was repeated 3 times to minimise personal error. The average of these three measurements was used for statistical analyses (Appendix E). All measurements were taken on the left side of the specimen unless otherwise stated.

Statistical analysis was performed using STATISTICA version 10 (StatSoft, Inc. 2011). Analysis of the ratios of Tibia Length as a percentage of Snout-Vent Length (TL/SV), Head Width as a percentage of Tibia Length (HW/TL) and Head Width as a percentage of Snout-Vent Length (HW/SV) provided meaningful correlations between individuals and these data were therefore used for comparative analysis using one-way Anova tests. Three One-Way Anovas were run (with species as the independent variable) to see if there were any differences among the species for tibia length as a proportion of SVL. Percentage data were arcsine square-rooted prior to analysis to satisfy the assumption of normality.

5.3.3 Molecular analysis

DNA Sequencing

Tissue samples were taken from thigh muscle or toe clippings of 30 adults of the *Afrixalus spinifrons* complex from 15 localities in KwaZulu-Natal, the Eastern Cape and Western Cape and stored in 95-100% ethanol. Whole genomic DNA was extracted from a small amount of tissue using the Qiagen Animal Tissue spin-column protocol. The following gene fragments were selected for sequencing: a partial sequence of 545 base pairs (bp) of the mitochondrial gene fragment 16S ribosomal RNA and two partial nuclear protein-coding genes; 300 bp recombination activating gene (RAG1) and 450 bp proopiomelanocortin (POMC). These markers were selected based on their suitability to deep and shallow phylogenetic inference (Vieites *et al*. 2007). Double-stranded PCR was performed in 25µl reactions with 0.110µM of primer to amplify the target gene fragments using the primers and PCR conditions shown in Table 5.2. PCR product was sent to Inqaba Biotech, Pretoria for sequencing.

Table 5.2 Primer sequences and PCR conditions used for molecular analysis in this study. Sources: 1. Palumbi *et al.* 1991; 2. Chiari *et al.* 2004; 3. Vieites *et al.* 2007.

Gene	Primer name	Sequence (5‟→ 3‟)	Source	PCR conditions
16S	16S AL	CGCCTGTTTATCAAAAACAT	1	94(90), [94(45), 55(45), 72(90) x 33], 72(300)
16S	16S BH	CCGGTCTGAACTCAGATCACGT	1	
Rag1	AmpF1	ACAGGATATGATGARAAGCTTGT	2	94(300), [94(30), ↑52-57 (40), 68(180) x 10], [94(10), 57(40), 68(180) x 20], 68(300)
Rag1	AmpR1	AACTCCGCTGCATTKCCAATRTCACA	2	
POMC	DRV F1	ATATGTCATGASCCAYTTYCGCTGCAA	3	95(120),[95(60),58(60),72(90) x 35], 72(600)
POMC	DRV R1	GGCRTTYTTGAAWAGAGTCATTAGWGG	3	

Data Analysis

Ultimately, only partial sequences of the 16S gene were analysed due to low PCR yields of the RAG1 and POMC fragments. 16S sequences were aligned using the MUSCLE algorithm implemented in MEGA 5 (Tamura *et al*. 2011). The Maximum Parsimony (MP) analysis was performed with MEGA 5 following a branch-and-bound search on 43 equally-weighted informative characters, with gaps considered as missing data. Bootstrap percentage support values were calculated following heuristic search and stepwise addition with 1,000 replicates using the close-neighbour-interchange method. A T92 + I + G model was selected by modeltest 3.06 (Posada & Crandall 1998) and used for Maximum Likelihood (ML) and Bayesian analysis. Bayesian analysis was conducted using the software MrBayes 3.04b (Huelsenbeck & Ronquist 2001; 2004), with four chains running for a million generations, sampling each 100 cycles. Bayesian posterior probabilities were computed after removing the first 1,000 trees as the burn-in phase. Bayesian Inferences (BI) was run three times independently to assess for convergence, using the Tracer software available at http://tree.bio.ed.ac.uk/software.

5.3.5 Bioacoustic analysis

Recordings of male *A. s. spinifrons* and *A. s. intermedius* advertisement calls were made in the field from October 2011 to January 2012 at various localities (Table 5.3) using a NAGRA ARES-ML recorder (Software Version 3.24 2009). Air temperature was recorded using an ExTech Instruments waterproof thermometer since temporal call features such as call duration and pulse rate and duration can be affected by ambient temperature (Gerhardt & Bee 2007). Calls were edited using WaveSurfer version 1.8.8p4 (Sjölander & Beskow 2000). Call components were separated, and only the *trill* component was used for analysis as this has been shown by phonotaxis experimentation on *A. delicatus* to be the component that functions in female attraction (Backwell 1988). Sound analysis was performed with Sound Ruler Acoustic Analysis (Version 0.9.6.0) using default settings (Gridi-Papp 2007). The *trills* of each call bout were analysed separately and the mean values of key temporal factors, namely pulse rate (number of pulses per second), pulse duration and the spectral factors, dominant and fundamental frequency were measured for each call. The average of each factor was used for statistical analysis.

Table 5.3: Localities at which call recordings were made for *Afrixalus spinifrons intermedius* (*n* = 19) and *Afrixalus spinifrons spinifrons* (*n*= 11).

Species	**Location**	**Date**	**Air Temp (°C)**	**# of males recorded**
A. s. intermedius	Fort Nottingham (Kingussie Dam)	16/12/2011	16.2	5
	Hilton Wetland	18/10/2011	19.2	4
	Himeville	16/01/2012	17.5	1
	Kamberg Nature Reserve	21/12/2011	19.3	5
	Rosetta, Moor River floodplain	21/12/2011	19.6	4
A. s. spinifrons	Coffee Bay	18/11/2011	16.5	4
	Mtunzini (Forest Lodge)	05/11/2011	20.5	2
	Mt Moreland	07/12/2011	21.3	1
	Prospecton	30/11/2011	22.8	2
	Tala Nature Reserve	16/11/2011	17.6	2

For comparison of trill components of the two subspecies, statistical analysis with STATISTICA (Ver. 10; Statsoft, Inc. 2011) was performed to determine whether there was any significant difference between the call parameters, pulse rate, fundamental frequency, dominant frequency and call duration. Tests for normality were performed for each variable to determine whether parametric or non-parametric testing was necessary for the data. T-tests to compare the means of each parameter were performed for each parameter. Only „call duration" was not normally distributed, so the non-parametric equivalent to the t-test for that variable (the Mann-Whitney U-test) was run for this variable. Standard deviation and coefficient of variation was calculated for each variable.

5.4 Results

5.4.1 Morphology

Since the morphology of the *Afrixalus spinifrons* complex has already been assessed comprehensively (Pickersgill 1984, 1996, 2007), it was not deemed necessary to re-examine all type material and other museum specimens for the purposes of this study. A subset of specimens were selected from the most important collections in South Africa, and along with specimens collected during the study period for examination. In particular, attention was paid to specimens from the Eastern Cape, which were referred to *A. s. spinifrons* based on morphological examination (Pickersgill 1996). Paratypes were loaned from the Durban Natural Science Museum and Natal Museum. Unfortunately, those from the former were in an unexaminable state (completely dried out). Collection of 30 new specimens (KZN Wildlife permit 5080/2011) also provided a good opportunity to examine colouration in life, since colouration in preserved specimens can differ markedly from live specimens (McDiarmid 1994).

Size, Colouration and Sexual Dimorphism

Snout-Vent measurement data shows that *A. knysnae* specimens were significantly larger than *A. s. spinifrons* and *A. s. intermedius* (15.7% and 14.4% respectively). Sexual dimorphism traits appear to be similar between *spinifrons* and *intermedius*. Female SVL was on average (21.42 mm, n = 15) 12.7% larger than males (19.01 mm, n = 35). For the average measurements for each taxon, both males and females were included (*A. s. intermedius* females = 10, males = 27; *A. s. spinifrons* females = 13, males = 43; *A. knysnae* males = 3). In general, females were paler in colour than males and had fewer, less dense asperities. Where asperities were apparent, they were usually uniform in distribution. Females frequently exhibited pale (bluish) dots on the lower surfaces of the limbs and lateral surface. This characteristic was observed for females of both *A. s. spinifrons* and *A. s. intermedius* from throughout the range (Fig 5.7). Males are characterised by having a brightly coloured yellow gular sac (du Preez & Carruthers 2009). Asperities on males were distributed over the entire dorsum, including the limbs and were more highly concentrated, particularly on the snout (although there is significant variation within these patterns).

Figure 5.7: (A) a male *Afrixalus spinifrons spinifrons* from Tala Nature Reserve showing defined dorsal colouration and pronounced asperities) and (B) a female *Afrixalus spinifrons intermedius* from the Type locality at Rosetta, showing pale colouration and few asperities.

Males were generally darker in colour and had more clearly defined dorsal and lateral banding, however colour is also highly variable both between individuals of the different taxa and for the same individual depending on exposure to light (Figure 5.8).

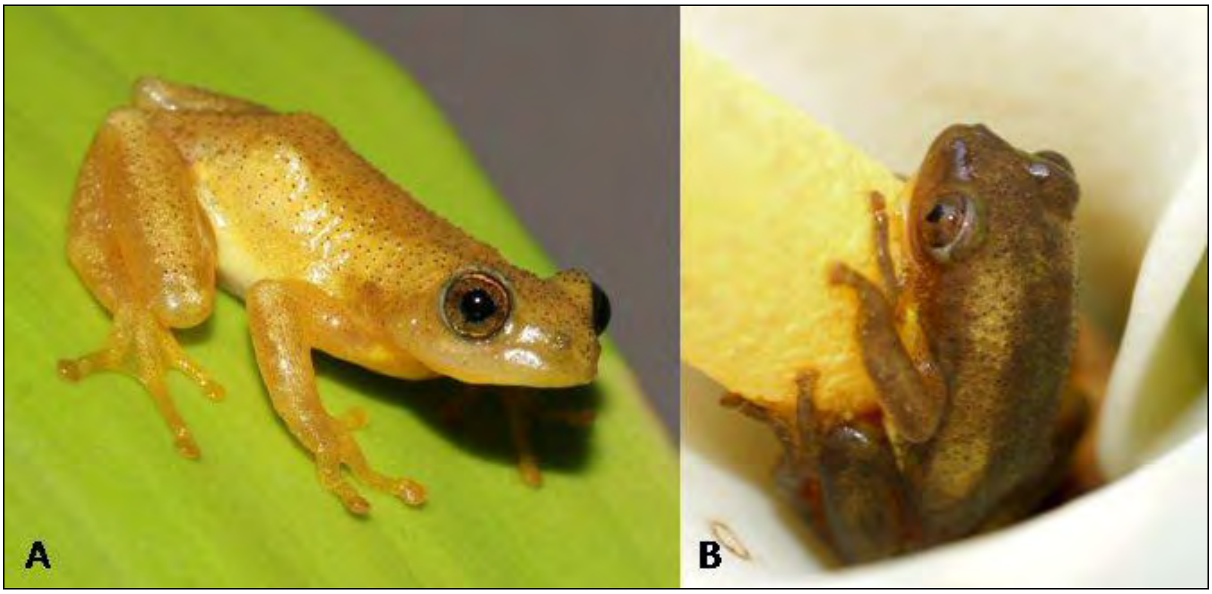

Figure 5.8: Colour variation in a single *A. s. intermedius* male from Himeville following (A) exposure to dark conditions, (B) exposure to light.

Overall, colouration in *A. s. spinifrons* was darker with more defined patterning than in *A. s. intermedius*. Snout characteristics and degree of spinosity were found to be highly variable between and within taxa, with blunt, tapering and rounded snout shapes being represented in both *A. s. spinifrons* and *A. s. intermedius*. On average, snout shape was found to be tapering in specimens from the Eastern Cape (Figure 5.9) and midlands and more rounded and swollen (bulbous) in coastal specimens (Fig 5.10). Colouration was usually paler and more uniform in *A. s. intermedius*, while asperities were less pronounced and more evenly distributed across the dorsum. The pattern of spinosity was also inconsistent, ranging from being concentrated on the snout to evenly distributed over the entire dorsum, with no clear pattern discernable for specimens from the different regions. Similarly, gular shape was also found to be highly variable. These results are summarised in Table 5.4.

Figure 5.9: Male *Afrixalus spinifrons spinifrons* from Coffee Bay area, Transkei, Eastern Cape showing pale lateral bands, evenly distributed asperities and tapering snout. Photograph, Donnavan Kruger.

Figure 5.10: Male *Afrixalus spinifrons spinifrons* from Prospecton, Durban showing bulbous snout and dense asperities.

Table 5.4: Comparison of characteristics of *A. s. spinifrons* from the KwaZulu-Natal coast (*n* = 33) and Eastern Cape coast (n = 21); *A. s. intermedius* from the KwaZulu-Natal midlands (*n* = 37); and *A. knysnae* from the Western Cape (*n* = 3). x̄ = Mean.

Characteristic	***A. s. spinifrons* (KZN coast)**	***A. s. spinifrons* (EC coast)**	***A. s. intermedius* (KZN midlands)**	***A. knysnae* (Covie)**
Length	Medium (range: 15 – 23.5)	Medium (range: 16.5 – 23 mm)	Medium (range: 13 – 23 mm)	Large (range: 20.7 – 22 mm)
TL/SV	36 – 50% (x̄ = 42 %)	36 – 50% (x̄ = 43%)	39 – 49 % (x̄ = 44%)	44%
Snout shape	Short and bulbous	Tapering, not swollen	Relatively long, not swollen	Tapering, not swollen
Asperity concentration	Concentrated on snout and between eyes.	Evenly distributed	Evenly distributed	Evenly distributed
Colouration	Dark yellow to brown. Defined dorsal and lateral bands	Dorsum uniform yellow - brown. Dark lateral bands	Pale yellow, uniform	Dark yellow. Dorsal markings incomplete.

Statistical analysis of proportions

The one-way analysis of variance (Anova) tests on the ratios for snout-vent length (SVL), head-width (HW) and tibial-lenth (TL) showed that for TL/SVL *A. s. intermedius* (n = 37) was significantly larger than *A. s. spinifrons* (n = 56): (f(2, 93) = 6.44; p = 0.02). There was no significant difference among *A. s. spinifrons, A. s. intermedius* and *A. knysnae* (n = 3) for HW/SV ratio: (f(2, 93) = 0.73; p > 0.05) and there was no significant difference among the three species for HW/TL ratio: (f(2, 93) = 2.56; p = 0.08).

5.4.2 Molecular Phylogeny

The tree based on the 16S sequences generated using maximum likelihood (ML) is shown in (Figure 5.11). This provided an initial preview based on only one gene fragment, but represents intermedius as paraphyletic with respect to spinifrons. The 16S tree shows that the three taxa of the *A. spinifrons* complex form a clade, with *A. s. spinifrons* from coastal KwaZulu-Natal and *A. s. intermedius* from the midlands grouping as closely related, but separate lineages. *Afrixalus knysnae* (samples from Covie, Western Cape) is confirmed as a member of the clade, but clearly distinct, with a more deeply divergent lineage.

The branch support values for these relationships are relatively high with 100% Bayesian posterior probability (BP) for the coastal *A. s. spinifrons* cluster and >98% for Bayesian posterior probability and MP and ML bootstrap values for the *A. knysnae* branch. The *A. s. intermedius* cluster has 71% ML bootstrap support (the MP and BP values were below 50% and therefore not highly supported). Interestingly, the Eastern Cape populations are placed with *intermedius* from the KwaZulu-Natal midlands. A further unexpected result was the placement of the Vernon Crookes samples within the *intermedius* cluster, as it has been presumed that since their distribution is coastal, they belonged to *spinifrons*. A sample from Babanango, which is a significant range extension to the interior of northern KwaZulu-Natal, also groups with the coastal *spinifrons* cluster.

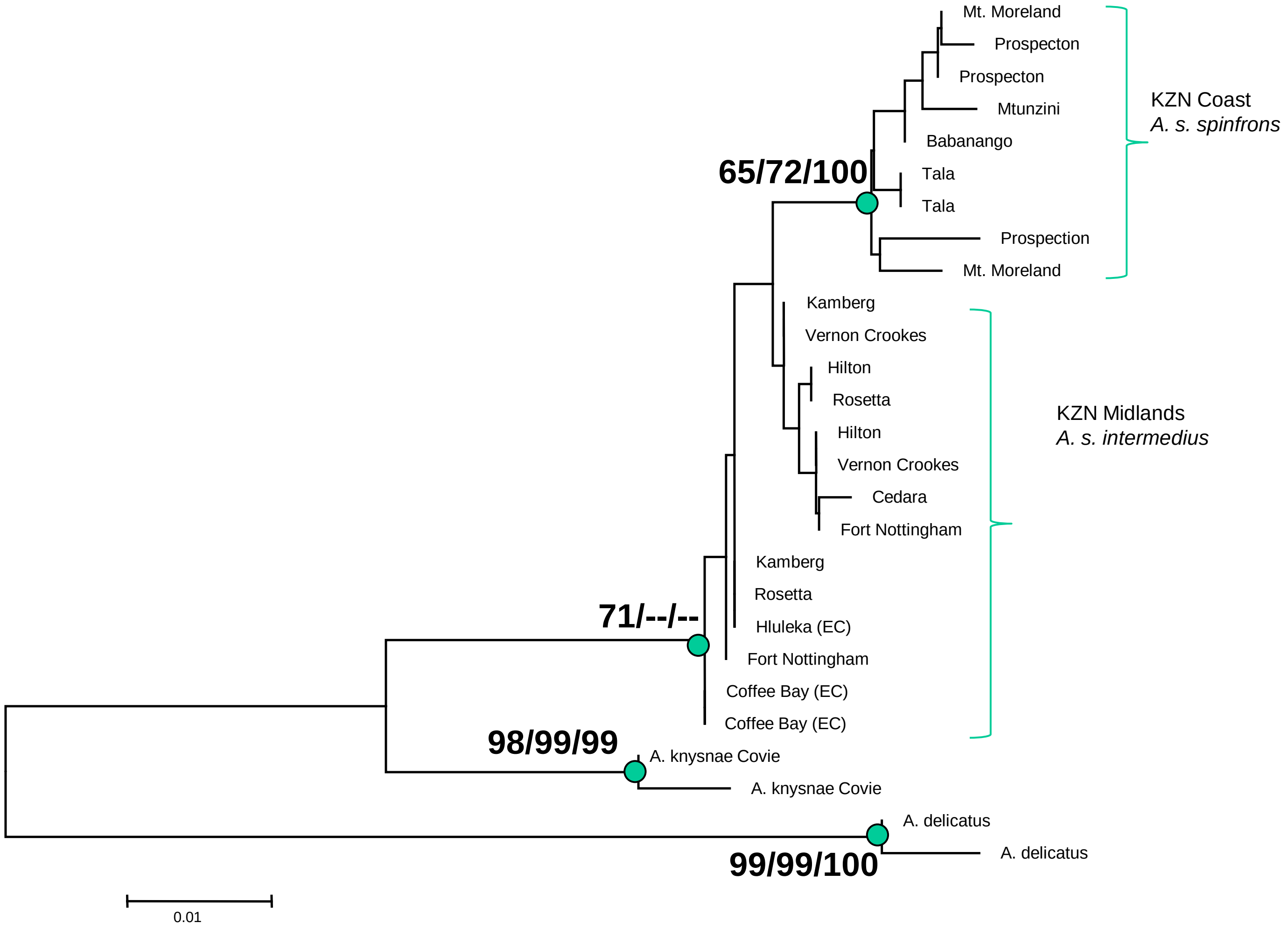

Figure 5.11: Maximum likelihood tree of *Afrixalus spinifrons* based on 545 base pairs of the 16S mitochondrial gene fragment. Node values indicate bootstrap proportions and Bayesian posterior probabilities in this order: ML/MP/PB. *Afrixalus delicatus* represents the outgroup.

5.4.3 Bioacoustic analysis

Call structure varied considerably between localities for both *A. s. spinifrons* and *A. s. intermedius*. The typical call of both subspecies consists of a regularly pulsed *zip* and a sustained *trill* of variable duration: 2.3 – 30 s for *A. s. intermedius* (SD = 5.8) and 3.6 - 46.8 s for *A. s. spinifrons* (SD = 14.5). There was not a fixed pattern of alternation of the two components and they were not linked in a single unit or produced in a set sequence. Males could also produce only one of the components for a long period. *Afrixalus spinifrons intermedius* was observed to produce the *zip* component less frequently than *A. s. spinifrons*. The call of *Afrixalus knysnae* is comprised of short (1.3 – 1.8 s) *trills*. Figure 5.12 shows typical advertisement calls of *A. s. spinifrons*, *A. s. intermedius* and *A. knysnae*.

Because of the small sample sizes (*A. s. intermedius* n = 19; *A. s. spinifrons* n = 11) and the relatively narrow difference in average temperature for the two regions (18.7°C in the midlands and 19.5°C on the coast), the original uncorrected data were used for analysis. Table 5.5 shows the descriptive statistics including means, standard deviation and coefficient of variation for pulse rate, fundamental frequency, dominant frequency and call duration for both subspecies. Pulse rate was significantly different (p = 0.000031), being more rapid in *A. s. spinifrons* than in *intermedius*, as indicated by the box plot in figure 5.13.

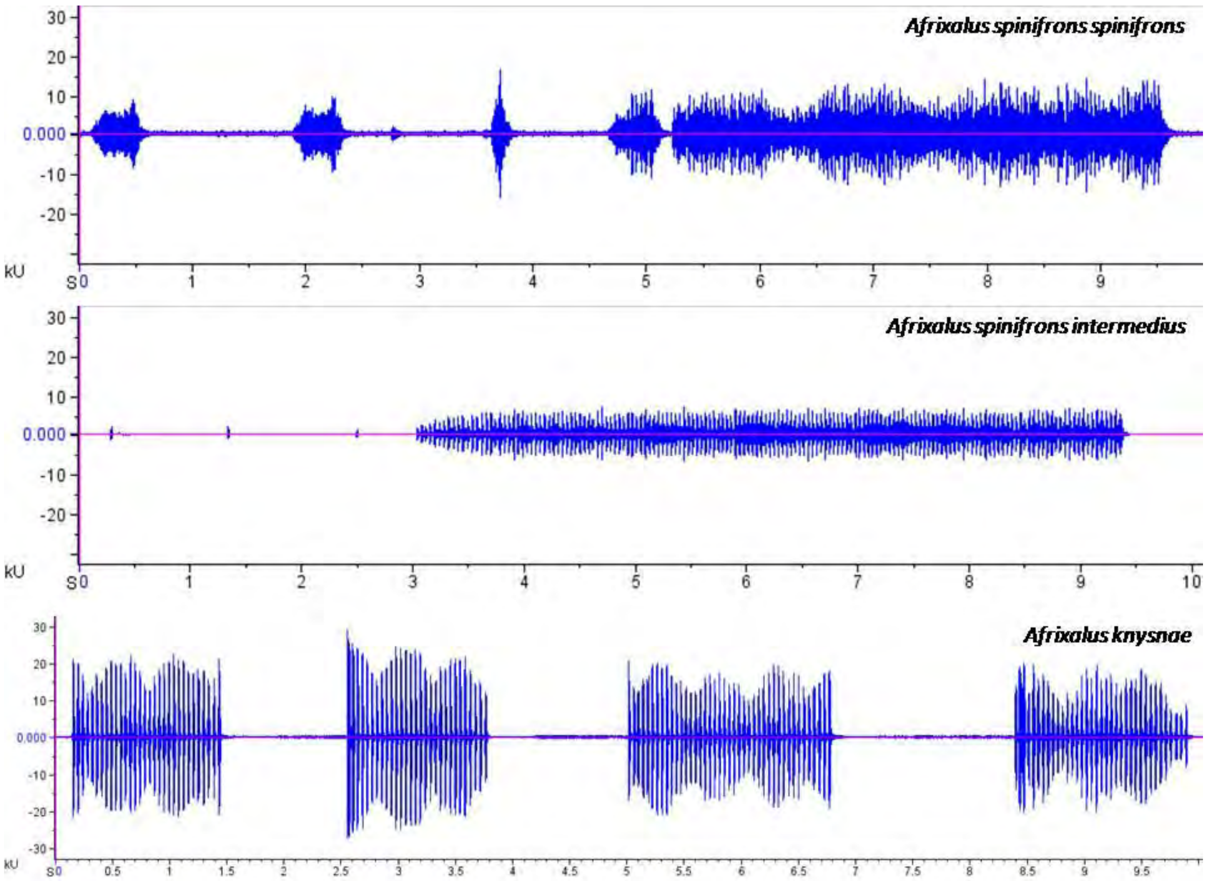

Figure 5.12: Comparison of waveform of the advertisement calls of male: *Afrixalus spinifrons spinifrons* (top) from the KwaZulu-Natal coast showing the short introductory *zip* pulses followed by the longer rapidly pulsed *trill*; a long *trill* of *A. s. intermedius* (middle) and the short *trills* typical of *A. knysnae* (bottom). Time (seconds) is shown on the x axis, and kilounits (kU) on the y axis. Produced using RavenLite version 1.0 (Charif *et al*. 2006).

Table 5.5: Descriptive statistics of advertisement call parameters of *Afrixalus spinifrons spinifrons* (AS; *n* = 11; mean temperature = 19.5°C, range 16.5 – 22.8°C) from the KwaZulu-Natal coast and *A. s. intermedius* (AI; *n* = 19; mean temperature = 18.7 °C, range 16.2 – 19.6°C) from the Natal midlands. Pulse rate = pulses per second; Pulse duration = pulse duration between 50% amplitude marks; SD = Standard Deviation. CV = Coefficient of variation.

	Mean	Minimum	Maximum	SD	CV	Standard Error
AS_Pulse_Rate (p/s)	31.67	21.00	46.10	7.98	25.21	2.41
AI_Pulse_Rate (p/s)	20.83	15.00	29.20	4.05	19.42	0.93
AS_Pulse_dur (s)	1.45	1.130	2.06	0.27	19.21	0.08
AI_Pulse_dur (s)	1.93	1.04	3.63	0.75	38.88	0.17
AS_Fund_Freq (Hz)	2035.41	1895.90	2203.60	96.12	4.72	28.98
AI_Fund_Freq (Hz)	2125.27	1641.70	2721.30	327.22	15.40	75.07
AS_Dom_Freq (Hz)	4070.82	3791.70	4407.20	192.27	4.72	57.97
AI_Dom_Freq (Hz)	4250.56	3283.30	5442.60	654.43	15.40	150.14

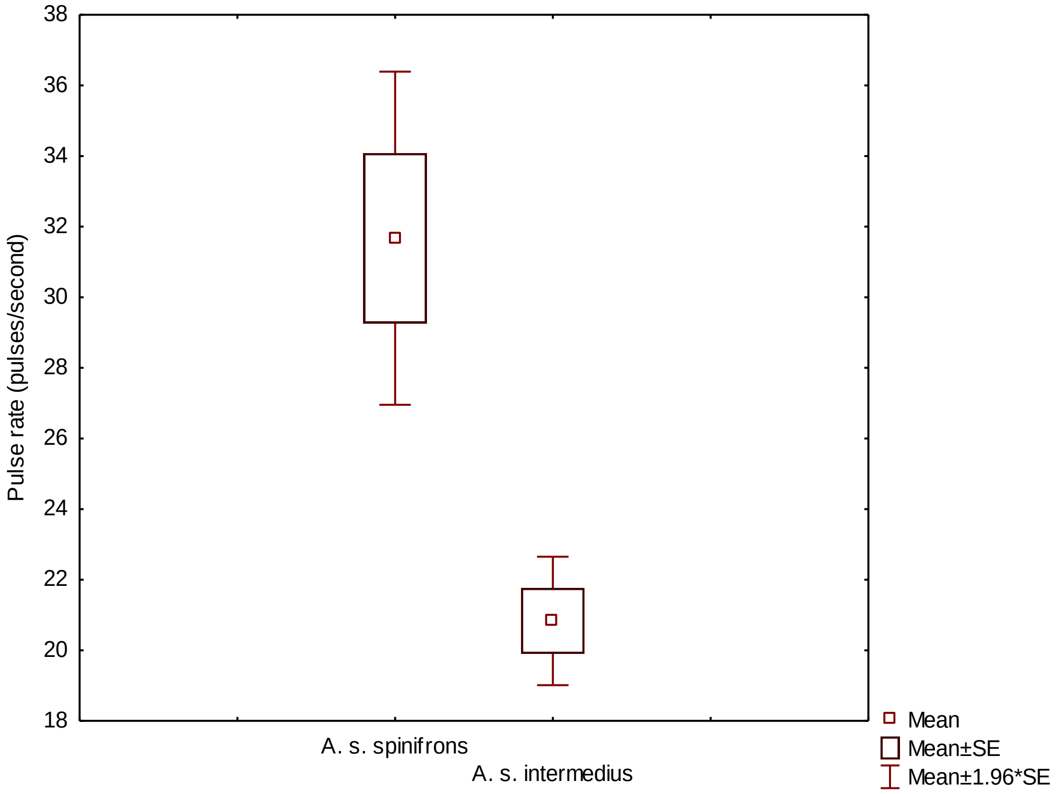

Figure 5.13: Box and whisker plot of pulse rates of trill notes of advertisement calls in *Afrixalus spinifrons spinifrons* (above) vs. *A. s. intermedius* (below).

5.5 Discussion

5.5.1 Morphology

In his description of three new *Afrixalus* taxa, Pickersgill (1984) remarked that colour and other morphological variation had been overlooked by previous authors, and that this variation was significant enough on which to distinguish cryptic species and subspecies. Later, Pickersgill (1996) used this argument to differentiate the three taxa within the *A. spinifrons* superspecies. It is apparent from Pickergill‟s (1996) comparison (Table 5.1) that species delimitations based on morphological characters show significant overlap, and the characters used are thus possibly not reliable in their diagnostic value. The inclusion of both males and females may also obscure the results. The terminology used, for example "stocky and sturdy" is also not quantified.

Morphological assessment from this study largely agrees with Pickersgill‟s (1996, 2007) diagnosis of the *A. spinifrons* complex based on morphological evidence in that *A. knysnae* is significantly larger than the other taxa, and *A. s. intermedius* is larger than *A. s. spinifrons*. In terms of colouration and spinosity, specimens from populations in the Eastern Cape (Hluleka and Coffee Bay) resembled *A. s. intermedius* from the KwaZulu-Natal midlands more closely than they did *A. s. spinifrons* from the KwaZulu-Natal coast. This result differs from Pickersgill‟s (1996) placement of these populations with *A. s. spinifrons*.

5.5.2 Molecular Phylogeny

The phylogenetic analysis based on sequences of the 16S mitochondrial gene confirms the view that *A. knysnae* is a separate species within the *A. spinifrons* complex, thereby agreeing with a-priori species definitions (Pickersgill 1996). The *spinifrons* vs. *intermedius* clusters are geographically separate, and on the limited sampling they do not show much phylogeographic structure within each. Divergence between them is quite small so it must be a relatively recent split, especially given that genetic divergence precedes the population divergence (Funk & Omland 2003; Felsenstein 2004). The results provide new insight into the placement of Eastern Cape populations with *intermedius*. This placement may indicate that the uMkomaas River is a possible boundary to *spinifrons*, which would also explain the placement of the Vernon Crookes specimens with the *intermedius* cluster. In terms of the morphology of the Vernon Crookes specimens, this also matches *intermedius* more closely than *spinifrons* in terms of colouration, snout shape and dorsal asperity distribution (Fig 5.14).

Figure 5.14: Specimen from Vernon Crookes showing colouration and asperity distribution similar to *Afrixalus spinifrons intermedius* from the midlands and Eastern Cape.

The molecular results also indicate a surprisingly high degree of variation within each lineage. Even samples from the same locality (e.g. Mt. Moreland and Prospecton) display high divergences. Divergence between populations within the same clusters may be a result of patchy suitable habitat and populations could have been historically isolated and fragmented. More recently, fragmentation would be caused by human disturbance and resulting in loss of habitat connectivity, which can ultimately have species- and population-level implications (Cushman 2006). As to the species status question, mitochondrial DNA alone is not enough to diagnose species (Moore 1995; Hall 2004). The additional sequences from POMC and RAG 1 will be necessary to further elucidate these relationships. These results are, however, enough to confirm that groups that are distinct morphologically (phenotypically) are genetically separate lineages.

5.5.3 Call analysis

The waveforms of the advertisement calls of the three taxa within the *A. spinifrons* complex (Figure 5.12) show that the structure of the calls of each taxon varies considerably. The components of biphasic calls reflect sexual differences in the auditory sensitivity of males and females (Narins & Capranica 1976). For example, females are not influenced or attracted to the *zip* component and males do not alter behaviour in response to the *trill* as has been demonstrated in *A. delicatus* by Backwell (1988) and presumed in *A. spinifrons.* The advantage of the functionally partitioned advertisement call appears to lie in the ability of males to selectively modify the proportions of the call components depending on the social context (Arak 1983b) and may enhance the efficiency of vocal communication and hence increase the individual"s probability of mating (Backwell 1988). Should an approaching male get too close to a calling male within the chorus, the resident male will escalate to encounter calling, which is a more rapidly pulsed and slightly longer version of the zip component (Backwell 1988). The results of the call analysis for *A. s. spinifrons* and *A. s. intermedius* showed that overall, the two subspecies have very similar calls. There was, however significant difference in pulse rate between the two, which has been established as a sufficient key factor for distinguishing advertisement calls of sympatric populations (Loftus-Hills & Littlejohn 1971). Temperature can influence pulse rate, although in this comparison of the populations, the difference in average temperature was minimal (1.2 °C). Additional sampling of calls from a wider distribution, including the Eastern Cape populations, with corrections for temperature, will be necessary to fully understand the divergence of advertisement calls within this species complex.

5.5.4 Conservation Implications

Moritz (1999) suggests that the goal of conservation should be to conserve evolutionary and ecological processes, rather than specific phenotypic variants. To this end, what is important in terms of conservation, are evolutionary significant units (ESU), which may define species, subspecies or smaller population groups that are considered distinct for purposes of protection (Moritz 1994; 1995; Conner & Hartl 2004). Such a view point may be appropriate for *A. s. intermedius* and *A. s. spinifrons*, which have distinct parapatric distributions for which reproductive isolation is incomplete. Treatment of these taxa in this way also supports the mate-recognition system, emphasising that species limits can be relevant at different microtaxonomic levels (Haffer 1986).

Conservation strategies that incorporate evolutionary processes such as gene flow and population connectivity, at multiple levels across broad landscapes are likely to be more beneficial than species- and site-specific actions (Cushman 2006). The effect of habitat fragmentation on juvenile dispersal has been identified as a key issue, particularly regarding pond-breeding amphibians (Sjögren-Gulve 1994; 1998), as in the case of *A. s. intermedius* especially. Despite the species boundaries between *A. s. intermedius* and *A. s. spinifrons* being somewhat blurred, separate conservation plans are recommended for the populations in coastal and inland areas, with *A. s. spinifrons* from the KwaZulu-Natal coastal region requiring the most immediate attention. The Eastern Cape populations should also be treated under a separate broad conservation plan.

The results of this study confirm certain previous assumptions regarding species delimitations within *Afrixalus spinifrons* that were based primarily on morphological data (Pickersgill 1996), but through the inclusion of molecular analysis, help to further elucidate relationships within this group of morphologically very similar taxa.

CHAPTER 6

GENERAL CONCLUSIONS & MANAGEMENT RECOMMENDATIONS

6.1 Contribution to conservation plans

This chapter is aimed at integrating the information gained from this study and to discuss the important general conclusions thereof. Management recommendations based on these conclusions are also included. The broad aim of this study was to provide information which was previously lacking with regard to aspects of South Africa‟s threatened species and the threats facing them. The topics were broad, ranging from potential disease threat, to improving knowledge of species distribution, to revising systematics. Each of these objectives had been highlighted as research priorities in the conservation research strategy for South African frogs compiled by Measey (2011). The findings of this study can be used to contribute to conservation strategies for South Africa‟s threatened frog species, with additional emphasis on those in KwaZulu-Natal.

6.1.1 Assessment of *Bd* infection as a threat

Prior to this study, no research had focussed on *Batrachochytrium dendrobatidis* (*Bd*) infection in South Africa‟s threatened frogs. Although evidence of widespread distribution of the pathogen was beginning to accumulate, including reports of die-offs in some species (Hopkins & Channing 2003; Weldon & du Preez 2004a), studies had not specifically included any Red List species, apart from *Xenopus gilli*. To this end, I surveyed 17 of the 20 Red List species (2004) for *Bd* infection. Of these, *Bd* was detected in 8 species, with an overall prevalence of 14.8%. The highest infection prevalence and pathogen load was, in most cases, associated with species with strongly aquatic life-histories, namely *Heleophryne* *rosei*, *H. hewitti* and *A. spinifrons*. The *Heleophryne* species inhabit montane habitats and are closely associated with perennial mountain streams (Boycott 2004b), which are also known to be highly suitable for transmission of *Bd* (Lips 1998; Johnson & Speare 2005). The majority of infection records for *A. spinifrons* came from the midland regions of KwaZulu-Natal, indicating *Bd's* preference for cooler climates (Berger *et al.* 2004; Kriger & Hero 2007). Surprisingly, no infection was found in *Natalobatrachus bonebergi*. This species has a riverine life-history, which is often associated with the presence of *Bd* (Johnson & Speare 2003; Drew & Allen 2006). Most of the records were from Vernon Crookes Nature reserve where the presence of *Bd* has been confirmed in non-threatened species and *A. spinifrons* (Meyer *et al.* unpublished data; this study). It is possible that *N. bonebergi* exhibits resistance to infection and/or that the coastal climate in which it occurs does not facilitate advanced infection of *Bd* in this species. Additional sampling may be necessary to provide a more accurate representation. Similarly, no infection was found in *Capensibufo rosei* which is a temporary winter breeder restricted to montane regions of the Western Cape Peninsula (de Villiers 2004c). Concern over possible population declines linked to impaired resistance to disease in high-altitude species have been raised for this species (de Villiers 2004c). During this study, swab samples from *C. rosei* were collected over two breeding seasons at Silvermine Nature Reserve and no *Bd* was detected, in spite of the fact that *Amietia fuscigula* showed heavy infections in the Reserve. Although additional sampling would be advantageous, this study likely indicates that chytridiomycosis is not a threat to *C. rosei*.

Infection was also found in *Leptopelis xenodactylus, Amietophrynus pantherinus, Microbatrachella capensis*, and at very low levels*, in Xenopus gilli. Leptopelis xenodactylus* had a 15.4% prevalence of infection. It is a wetland-associated species of the KwaZulu-Natal midlands and highlands (Burger 2004). Toads such as *A. pantherinus* have predominantly terrestrial life-histories, but are usually found in the vicinity of wetlands throughout the year (de Villiers 2004a). During the breeding season, they spend intensive periods near or in permanent water bodies such as vleis, dams, ponds and slow-moving rivers (de Villiers 2004a). This association with water at times that is optimal for *Bd* growth conditions (early spring) undoubtedly bring the species into direct contact with the pathogen (ref). *Microbatrachella capensis* which exhibited relatively high prevalence of infection (22.7%, n = 22) is also a Cape low-lands species closely associated with wetlands in the Fynbos biome (de Villiers 2004d). It is a winter breeder, which potentially avails it to *Bd* exposure under optimum conditions for the pathogen. *Xenopus gilli* inhabits similar habitat to *M. capensis* (de Villiers 2004e) and this study showed it to exhibit very low levels of prevalence (2.4%), which are consistent with other infection studies for the genus (Weldon *et al*. 2004; Soto-Azat *et al*. 2009; Ramsey *et al*. 2010). Samples were not obtained from *Anhydrophryne ngongoniensis, Hemisus guttatus* (although two swabs from tadpole mouth parts were both negative) and *Cacosternum capense*. *Anhydrophryne ngongoniensis* and *Hemisus guttatus* are primarily terrestrial and as such unlikely to be exposed to *Bd* infection. *Cacosternum capense* is a Western Cape endemic that breeds in winter in low-lying temporary water bodies (de Villiers 2004b). Surveying of the sympatric *C. platyus* found a 50% *Bd* infection prevalence in samples from Klipheuwel (Appendix A), indicating that *C. capense* may also harbour infection.

The epidemiological data, and the results of the predictive modelling, suggest that *Bd* is indeed widespread in South Africa in frogs from all taxonomic groupings with no apparent adverse effects. Moreover, a considerable proportion of the threatened species were infected with *Bd* and some with very high infection intensities. Despite pressures from phylogenetic, environmental or anthropogenic origins that threaten these species, the additional burden of infectious disease by chytridiomycosis does not seem to cause any direct threat to any of the populations surveyed during this study. No clinical symptoms of *Bd* infection were apparent in any of the populations surveyed and no population crashes were detected or reported during the study period. However, recent phylogenetic analysis of multiple *Bd* isolates indicate that strains exist with varying degree of pathogenicity (Farrer *et al*. 2011) of which both hypovirulent and hypervirulent strains are present in South Africa. Data from this study suggest an endemic infection, which is further supported by the "out of Africa" hypothesis (Weldon *et al*. 2004), and which is reiterated by the lack of population declines in spite of the presence of a hypervirulent strain of *Bd*. Vigilance should however be maintained as introduction of a foreign hypervirulent strain into South Africa may result in indigenous amphibian species reacting in a way that naïve hosts react to novel pathogens leading to population declines. Proven susceptibility to *Bd*, suitable environmental conditions and the risk of introduced strains provide sufficient ground for regarding *Bd* as a threat to South Africa"s threatened frogs. Since *Bd* was not listed as a threat to any of the species that were reviewed during the most recent Red List Assessment of South African frogs, we recommend that *Bd* be added to the list of threats for all relevant threatened frog species.

The predicted distribution map produced by MaxEnt in this study gives a good indication of where *Bd* is likely to occur within South Africa. This is a useful tool for guiding future surveys, including areas that have not previously been well sampled. Additional sampling for species with high infection prevalence is recommended and population monitoring of these species, particularly *H. rosei*, should be ongoing. Compilation of a management plan for *Bd* in South Africa, incorporating these findings, is planned for this year (C. Weldon pers. comm.).

6.1.2 Conservation plans for KwaZulu-Natal‟s threatened anurans

KwaZulu-Natal hosts the highest anuran biodiversity of all of South Africa‟s provinces (Measey 2011) and has the second highest number of threatened species (5) in the country after the Western Cape (11) (IUCN 2011). It is crucial that threatened species and the habitat that supports them, is managed appropriately and management plans should take into consideration the species‟ life history, habitat requirements, threats, assessment of literature and engagement with relevant stakeholders. Understanding of the effect of management practices on fauna in South Africa is, however, still in its infancy (Ashton *et al*. 2005). A primary objective of this study was to provide recommendations on conservation plans for threatened species in KwaZulu-Natal. Surveys during the course of this study confirmed the presence/absence of Red List species at many known sites across the province, as well as identified new populations for some of these.

As is the situation globally, habitat loss is the primary threat to Red List frog species in KZN. In, particular, wetlands in the coastal region are under heavy pressure from development. *Hyperolius pickersgilli* (CR) is especially vulnerable to this threat. Much of this study was dedicated to this species and, as a result, comprehensive conservation plans have been recommended and are currently being implemented (see section 6.1.3 below). Loss of coastal riverine forest as a result of agricultural activity is the major contributing threat to populations of *N. bonebergi* and this species remains primarily in fragmented patches of this habitat in protected areas. Despite discovery of additional populations, the Endangered status of this species remains warranted. Several new populations of *L. xenodactylus* were discovered between 2008 and 2011, many of them in forestry areas (J. Harvey pers. comm.) These areas are generally well managed and long-term survival of this species should be secure. Additional surveying of predicted habitat may reveal further populations, possibly warranting a status change in the future. Conservation recommendations for *Afrixalus spinifrons* are discussed below (Section 6.1.4).

Close collaboration during the course of this study with Adrian Armstrong at Ezemvelo KZN Wildlife has meant that the province"s threatened species have been prioritised within the conservation authority"s management plans, with the objective of conducting additional species distribution modelling for *Afrixalus spinifrons*, *Leptopelis xenodactylus* and *Natalobatrachus bonebergi*. Additional recommendations include training of KZN Wildlife staff at protected areas that host populations of Red List species, and that monitoring of these species is put in place.

6.1.3 Distribution of *Hyperolius pickersgilli*

Hyperolius pickersgilli is KwaZulu-Natal"s only Critically Endangered frog species (IUCN 2011). Its obscurity has meant that, since its discovery, very little subsequent work has been conducted that has been specifically focused on this species (Bishop 2004b). Measey (2011) prioritised *H. pickersgilli* for conservation research including 1) an evaluation of extent of occurrence (EOO); 2) improved understanding of threats; 3) population estimates; 4) improved understanding of basic biology and ecology; 5) improved understanding of phylogeography and tissue collection. Of all the Red List species assessed in the document, *H. pickersgilli* received the highest priority (21 of a possible 25) for conservation actions in terms of monitoring, accurate threat identification and compilation of a stakeholder agreement (BMP-S) (Measey 2011).

This study has provided much of this information, and laid the groundwork for conservation actions that need to follow. Based on extensive surveying and assessments of historically known sites, the EOO of *H. pickersgilli* has been recalculated as 2081.5 km², which is a decrease of approximately 222 km² from the 2011 assessment, despite a range extension to the south of 40km. This may be the result of a different methods used here to calculate EOO and the fact that a large area of the coast was excluded from the polygon to exclude the portion which fell into the ocean. Area of occurrence (AOO) was recalculated as being slightly larger than that in the 2011 assessment (14.17 km² as opposed to 9 km²), which is expected given the increase in localities from 12 to 17. Refinement of the methods used to calculate AOO and EOO may be needed to improve these estimates. Despite the increase in AOO, the Critically Endangered status of *H. pickersgilli* remains valid, given the severe level of fragmentation between the majority of localities, the ongoing threats at many sites and the concomitant decline in habitat quality. At least one site, Prospecton, will be completely destroyed in the near future as a result of development, with the outcome that the population of *H. pickersgilli* here will require a major translocation plan. The effects of genetic variation and the maintenance of evolutionary processes should be considered for such programmes to prevent reducing population viability (Moritz 1999).

As a result of the surveying carried out during this study, the threats facing each locality are now well understood (Table 4.2). Bowman (2011) conducted a population estimate at Mt. Moreland which now needs to be repeated for the duration of the breeding season, and extrapolated for all other localities. *Hyperolius pickersgilli* has been prioritised for work under a newly formed amphibian programme (see below), for which drawing up a biodiversity management plan (BMP-S) is the first consideration. This important step will facilitate the implementation of all subsequent actions. Protection measures for each site have been identified and grant applications are currently under way with the goal of securing an additional 30% of the species habitat for protection. The species has also been prioritised for the first *ex-situ* programme of a Red List species in South Africa, and I assisted the Johannesburg Zoo in identifying sites for collection and obtaining the first animals for the captive programme (January 2012). As part of this study, tissue samples were collected for application in phylogeograpy and for determining the impact of habitat fragmentation on gene flow.

6.1.4 Taxonomy of *Afrixalus spinifrons*

This study makes use of molecular analysis for the first time in order to better understand the relationships within the *Afrixalus spinifrons* complex, particularly between the subspecies *intermedius* and *spinifrons*, which are primarily distributed within KwaZulu-Natal. Both *Afrixalus spinifrons* and *A. knysnae* have been identified as requiring some level of taxonomic investigation pertaining to systematics, bioacoustics and phylogeography (Measey 2011). The results of the molecular analysis based on mitochondrial and nuclear gene segments largely support Pickersgill"s (1996) species delimitations, with the interesting difference being that Eastern Cape populations group with *A. s. intermedius* as opposed to with *A. s. spinifrons* as proposed by Pickersgill (1996). This was also supported by the overall morphological characteristics, i.e. distribution of dorsal spines and colouring. The molecular results confirm that *Afrixalus knysnae* is a member of the *Afrixalus spinifrons* clade, but as a distinct species, which supports existing diagnosis (Pickersgill 1996). The phylogenetic tree based on 16S gene fragment sequences shows *A. s. spinifrons* and *A. s. intermedius* to be two separate clusters. Divergence between them however, is small, indicating a relatively recent split. Unfortunately time constraints did not allow for completion of sequencing with the additional gene fragments (the nuclear gene RAG1 and mitochondrial gene POMC), which will allow for better resolution of these relationships. This work is planned for the near future. Additional work using bayesian coalescent estimates of divergence times, migration rates and population sizes along with estimates of the variance would be useful for better understanding the phylogeography of these taxa. This can be done using Isolation with Migration models (Hey & Nielsen 2004; 2007) and could provide a useful tool in combination with species distribution modelling to understand more about historical habitat change in the midlands and coastal areas of KwaZulu-Natal. These methods can also be used for estimating population sizes, which has been recommended for both *A. spinifrons* and *A. knysnae* (Measey 2011). Bioacoustic analysis of *A. s. intermedius* and *A. s. spinifrons* calls showed significant difference in pulse rate, which can be used for delimiting species (Littlejohn2001).

In conclusion, at this stage, splitting of the two taxa into separate species based on the combination of morphology, bioacoustics and molecular phylogeny would be premature. As such, their current status of Near Threatened appears warranted. However, the two clusters are geographically separate and on the limited sampling they don't show much phylogeographic structure within each. As such, they could be treated as evolutionary significant units (ESU)‟s for the purposes of conservation (Moritz 1994; 1999; 2002; Conner & Hartl 2004). In terms of conservation planning, the coastal populations of *A. s. spinifrons* are under greater threat of habitat loss from development than those of *intermedius,* which has possibly benefited from construction of farm dams in the midlands (Armstrong 2001; Pickersgill *et al.* 2004). If the taxa are split into separate species their EOO and AOO will require recalculation and their status reassessed accordingly. *Afrixalus knysnae* was ranked by Measey (2011) as amongst the highest in need of conservation research and this remains the case, with additional research required for better understanding the species‟ biology and threats.

6.2 Amphibian Conservation in South Africa: Moving Forward

Amphibian conservation biology in South Africa appears to be mirroring the trends now taking place worldwide in conservation thinking, that is, following a period of “crisis fatigue”, we are entering a period of action in response to catastrophe (Woodhams *et al.* 2011). The zeitgeist of “doom and gloom” scenarios is slowly being replaced by one of hope and positivity. The publication of the strategy document for conservation research in South Africa (Measey 2011) has laid the foundation for research requirements for each of South Africa‟s threatened species, from which conservation objectives and actions can be based.

During the course of this study I have formed working relationships with provincial conservation authorities (particularly Ezemvelo KZN Wildlife), local municipalities (eThekwini) and a number of NGO‟s as well as private companies. These relationships, and the knowledge I have gained over the past four years, has positioned me to participate in devising and implementing conservation actions within a new programme for the Endangered Wildlife Trust focussed on amphibians. Initially, Pickersgill‟s Reed Frog *Hyperolius pickersgilli* and the Critically Endangered Amatola Toad *Vandijkophrynus amatolicus* have been prioritised. The latter was recently “re-discovered” following a period of 13 years since it was last recorded (Conradie & Tarrant 2011; Tarrant & Cunningham 2011). The programme will also continue to raise the public profile of frogs in South Africa. The overarching objectives of the programme will be to:

1) Raise funding for amphibian conservation projects in South Africa;
2) Work with relevant stakeholders, research institutions and individuals to prioritise conservation action for African amphibians through the development of a strategy and action plan and strengthen the network of conservation partners and authorities involved in amphibian conservation;
3) Identify, develop and implement amphibian conservation projects as per the strategy and action plan;
4) Support certain existing projects involving threatened frog species by working with all relevant partners/stakeholders and research institutions;
5) Conduct, co-ordinate and assist with specific research objectives necessary for identifying threats and providing ecological knowledge to enable implementation of conservation actions. This includes supporting young researchers, through post-graduate support, where possible;
6) Provide logistical services for dialogue between the research, conservation, management and media sectors for matters pertaining to amphibian conservation;
7) Promote awareness and appreciation among the general South African public about the importance of amphibians and the need for conservation thereof;
8) Provide relevant educational material on amphibians to be built into school programmes/curricula or extra-mural programmes including printed material, posters and teaching packs;
9) Provide training on frog identification, monitoring and conservation to relevant stakeholders such as provincial conservation authorities;
10) Engage on a community and landowner level in areas important for amphibian conservation and encouraging “citizen science” pertaining to amphibian conservation.
11) Work with zoos and aquaria to promote awareness of amphibian conservation and to develop *ex-situ* breeding programmes, where relevant, for threatened frog species.

6.3 Concluding remarks

This study provides important information that had been prioritised for conservation research. It also provides baseline information on the distribution and prevalence of *Bd* in South Africa, which can be used in management decisions. The knowledge and contacts gained as a result of this study has provided me with an opportunity to expand on these findings and implement conservation actions for the South African frog species most in need of them. An important and exciting aspect of this work will be continuing to raise the public profile about frogs in South Africa, especially at an educational level. Whilst writing this conclusion, I was notified as to yet another development threatening destruction of a wetland in which the Critically Endangered *H. pickersgilli* is present (near Umkomaas). Hopefully, the information generated in this study can, in part, be used to prevent this development from going ahead. It is time frogs started getting the recognition they deserve!

CHAPTER 7

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APPENDICES

Appendix A: Least Concern species sampled for *Batrachochytrium dendrobatidis* infection at sympatric sites to Threatened species. * Data deficient.

Genus	Species	Location	Province	Lat	Long	*Bd* positive	*Bd* negative	Total samples
Amietia	*fuscigula*	Hogsback	EC	-32.56013	26.91240	1	0	1
Amietophrynus	*rangeri*	Hogsback	EC	-32.56013	26.91240	0	2	2
Strongylopus	*grayii*	Hogsback	EC	-32.54774	26.91443	2	4	6
Afrixalus	*delicatus*	Mtunzini	KZN	-28.96782	31.75322	0	1	1
Afrixalus	*fornasinii*	Isipingo	KZN	-29.99167	30.90545	0	1	0
Afrixalus	*fornasinii*	Mt Moreland	KZN	-29.63845	31.09790	0	2	2
Amieta	*angolensis*	Krantzkloof	KZN	-29.77492	30.83088	1	1	2
Amietia	*angolensis*	Vernon Crookes	KZN	-30.26325	30.59357	0	3	3
Amietia	*angolensis*	Lake Merthley	KZN	-29.02242	30.58106	0	1	1
Amietia	*angolensis*	Fort Nottingham	KZN	-29.44493	29.90642	0	1	1
Amietia	*angolensis*	Cobham	KZN	-30.26325	30.59357	0	1	1
Amietophyrnus	*rangeri*	Vernon Crooks	KZN	-30.27035	30.60529	0	1	1
Arthroleptis	*wahlbergi*	Vernon Crooks	KZN	-30.27035	30.60529	0	2	2
Cacosternum	*striatum**	Lake Merthley	KZN	-29.02242	30.58106	2	0	2
Hadromophryne	*natalensis*	Sani Pass	KZN	–	–	0	1	1
Hadromophryne	*natalensis*	Krantzkloof	KZN	-29.77492	30.83088	0	1	1
Hemisus	*marmoratus*	Malala – Zululand	KZN	–	–	0	1	1
Hyperolius	*marmoratus*	Vernon Crookes	KZN	-30.27306	30.62485	0	1	1
Hyperolius	*marmoratus*	Vernon Crookes	KZN	-30.16375	30.37.522	0	4	4
Hyperolius	*pusillus*	Vernon Crookes	KZN	-30.27035	30.60529	0	2	2
Hyperolius	*tuberilinguis*	Mt Moreland	KZN	-29.63845	31.09790	0	1	1

Hyperolius	*tuberilinguis*	Vernon Crookes	KZN	-30.27306	30.62485	0	2	2
Hyperolius	*tuberilinguis*	Mt Moreland	KZN	-29.63820	31.09754	0	1	1
Hyperolius	*tuberilinguis*	Isipingo	KZN	-29.99105	30.90494	0	1	1
Leptopelis	*mossambicus*	North Coast	KZN	–	–	0	2	2
Leptopelis	*natalensis*	Mt Moreland	KZN	-29.63845	31.09790	0	4	4
Leptopelis	*natalensis*	Isipingo	KZN	-29.99099	30.90494	0	2	2
Leptopelis	*natalensis*	Vernon Crookes	KZN	-30.27860	30.59596	0	1	1
Phrynobatrachus	*natalensis*	Vernon Crookes	KZN	-30.27946	30.59066	0	2	2
Phrynobatrachus	*natalensis*	Mt Moreland	KZN	-29.63820	31.09754	0	6	6
Ptychadena	*oxyrhynchus*	Mt Moreland	KZN	-29.63845	31.09790	0	1	1
Strongylopus	*fasciatus*	Vernon Crooks	KZN	-30.27306	30.62485	0	1	1
Strongylopus	*grayii*	Lake Merthley	KZN	-29.02242	30.58106	0	2	2
Xenopus	*laevis*	Krantzkloof	KZN	–	–	0	1	1
Amietia	*dracomontana*	Sani Top	Lesotho	-29.58136	29.28859	0	2	2
Amietia	*umbraculata*	Sani Top	Lesotho	-29.58136	29.28859	5	8	13
Amietia	*fuscigula*	Kamieskroon	NC	–	–	1	0	1
Vandijkophrynus	*robinsoni*	Kamieskroon	NC	–	–	0	1	1
Xenopus	*laevis*	Kamieskroon	NC	–	–	1	0	1
Amietia	*fuscigula*	Skeleton Gorge	WC	-33.98586	18.43483	0	1	1
Cacosternum	*platys*	Klipheuwel	WC	–	–	3	3	6
Heleophryne	*orientalis*	Tradouw's Pass	WC	–	–	2	0	2
Vandjikophrynus	*angusticeps*	Cape Point	WC	-34.30603	18.44133	0	1	1
Xenopus	*laevis*	Pearly Beach	WC	–	–	0	1	1
Total:	**23 species**	**44 locations**				**18**	**74**	**91**

Appendix B: KwaZulu Natal's Threatened Species

Table 1: Sites visited for threatened species in KZN. Additional sites visited for *H. pickersgilli* are shown in Appendix D.NR = Nature Reserve

Locality	**Co–ordinates**	**Date visited**	**Target species**	**Red List Status 2011**
Adam's Mission/Folweni	S 29.99183, E 030.78328	20/01/2011	*H. pickersgilli*	CR
Armadale Farm	S 30.64985, E 030.50914	14/11/2011	*N. bonebergi*	EN
Cato Ridge	S 29.73623, E 030.58161	02/03/2009	*H. guttatus*	VU
Cedara	S 29.55785, E 030.25541	13/12/2011	*A. s. intermedius*	NT
Chase Valley	Various sites	01/04/2009	*N. bonebergi*	EN
Cowies Hill	S 29.82436, E 030.89609	28/01/2011	*N. bonebergi*	EN
Craigee Lea	S 30.07778, E 30.03250	03/03/2009	*A. ngongoniensis*	EN
Fort Nottingham	S 29.43200, E 29.90587	Multiple	*L. xenodactylus/ A. s. intermedius*	EN/NT
Hilton	S 29.53916, E 030.28625	18/10/2011	*A. s. intermedius*	NT
Isipingo	S 29.99099, E 30.90494	Multiple	*H. pickersgilli/A. s. spinifrons*	CR/NT
Kamberg NR	S 29.37328, E 029.72285	21/12/2011	*A. s. intermedius*	NT
Krantzkloof NR	Various sites	Multiple	*N. bonebergi*	EN
Lake Merthley, Grey Town	S 29.02250, E 030.57389	08/10/2009	*L. xenodactylus*	EN
Lynford Valley	S 30.11615, E 030.05052	04/12/2009	*A. ngongoniensis*	EN
Midmar Dam	S 29.54391, E030.18397	13/12/2011	*A. s. intermedius*	NT
Mpenjati NR	Various sites	19/01/2012	*H. pickersgilli/A. s. spinifrons*	CR/NT
Mt Moreland	Various sites	Multiple	*H. pickersgilli/A. s. spinifrons/H. guttatus*	CR/NT/VU

Mtunzini	Various sites	Multiple	*H. pickersgilli/A. s. spinifrons*	CR/NT
Nottingham Road	Various sites	22/01/2011	*A. s. intermedius*	NT
Palmiet NR	Various sites	Multiple	*N. bonebergi*	EN
Poortji Wetland	S 30.33222, E 029.51750	05/03/2009	*A. ngongoniensis/ L. xenodactylus*	EN
Port Edward	S 31.05163, E 030.21734	08/11/2011	*A. s. spinifrons*	NT
Prospecton	S 29.98245, E 030.93799	Multiple	*H. pickersgilli/A. s. spinifrons*	CR/NT
Queen Elizabeth Park	S 29.56620,E 030.32121	18/10/2011	*A. s. intermedius*	NT
Roelton Dam	S 30.13138, E 029.98778	03/03/2009	*A. ngongoniensis*	EN
Rosetta	S 29.30593, E 029.96224	21/12/2011	*A. s. intermedius*	NT
Tala NR	S 29.82954, E 030.53535	16/12/2011	*A. s. spinifrons*	NT
Umlalazi NR	S 28.95805, E 031.76472	18/12/2010	*Hyperolius pickersgilli*	CR
Vernon Crookes NR	Various sites	Multiple	*N. bonebergi/A. s. spinifrons*	EN/VU
Umbumbazi NR	S 30.70663, E 030.26944	15/11/2011	*N. bonebergi*	EN
Underberg	Various sites	Multiple	*A. s. intermedius*	NT
St Lucia	Various sites	Multiple	*H. pickersgilli*	CR

Threat Assessment Form Template

1. GENERAL

Site Name: ……………………………………………………………………………

Co–ordinates: …………………………………………………………………………

Date: ……

Time: ……………………….to…………………………..

Present: ………

Target species: ……

2. SITE OBSERVATIONS & ENVIRONMENTAL VARIABLES

2.1 Site description: ……………………………………………………………………………………………

2.2 River name if appropriate: ………………………………………………………………………………

2.3 Protected area if appropriate: …………………………………………………………………………

2.4 Weather conditions: ……………………………………………………………………………………

2.5 Most recent rainfall at site: ………………………………………………………………………………

2.6 Air temp: ……………………………………………………………………………………………………

2.7 Water appearance/odour: Clear / muddy stagnant / flowing

………

2.8 Water depth: ………………………………………………………………………………………………

2.9 Water temp: ………………………………………………………………………………………………

2.10 Substrate composition: ……………………………………………………………………………………

2.11 Land use in catchment area, High (H), Moderate (M) or Slight (S): ……………………………

………

2.12 Estimate of percentage of suitable breeding habitat: ....

2.16 Estimate of percentage of suitable terrestrial habitat surrounding breeding site:

....

2.17 Closest other suitable sites: ....

....

2.18 Closest inhabited site: ....

....

3. ANURAN SPECIES DETECTED:

Species	
Call intensity /5	
Estimated n	
n observed	**_______Juveniles ________ Males ________Females**
Amplexus observed	
Egg observed	
Bd/other symptoms	
Comments	

4. VEGETATION DESCRIPTION

4.1 Aquatic: ....

....

....

4.2 Terrestrial: ....

....

5. THREAT ASSESSMENT

5.1 PHYSICAL THREATS

5.1.1 Proximity of closest roads (m): ....

5.1.2 Type of road: ....

5.1.3 Other transportation corridors (e.g. flight path): ………………………………………………………

5.2 HUMAN INFLUENCE

5.2.1 Nearest human habitation (m): …………………………………………………...........................……

5.2.2 Human presence without habitat modification: …………………………………………………….....

5.2.3 Comment on the type and scale of intensity L,M,H) of each of the following:

5.2.4 Recreational activities: ……………………………………………………………………………………………

5.2.5 Property development (housing/commercial/industrial):
……
……

5.2.6 Agricultural activities (crops/livestock/aquaculture/siviculture):
……
…………………………………………..

5.2.7 Industrial activities:
……

5.2.8 Human natural resource–use activities:
……

5.2.9 Natural system modifications:

5.2.10 Fire ………..........

5.2.11 Dam/water use system ……………………………………………………………………………………………

5.3 POLLUTION (type and source)

5.3.1 Domestic: ……

5.3.2 Agricultural: ……

5.3.3 Industrial: ………

5.3.4 Litter: ………

5.3.5 Noise: ………

5.3.6 Other observable threats e.g. invasive species & erosion: ………………………………………

……

5.3.7 Other comments: ……………………………………………………………………………………………………

……

OVERALL HABITAT RATING

1 **Optimal**	**2** **Suboptimal**	**3** **Marginal**	**4** **Poor**	**5** **Severely degraded**

Additional Comments (Future visits) ……………………………………………………………………………

……

……

Appendix C: Public awareness

Table 1: List of talks given to scholars, students and general public

Date	**Title**	**Venue**
19 February 2009	The Amphibian Crisis and South Africa's Threatened Frogs	Underberg Wildlife Society
7 May 2009	The Amphibian Crisis and South Africa's Threatened Frogs	Ethekwini Municipality forum – Paradise Valley
18 June 2009	The Amphibian Crisis and South Africa's Threatened Frogs	Pinetown Rotary. General public.
08 July 2009	The Amphibian Crisis and South Africa's Threatened Frogs	WWF Staff – Stellenbosch
10 July 2009	The Amphibian Crisis and South Africa's Threatened Frogs	Westville Wildlife Society. General public
18 July 2009	eThekwini Conservancies meeting	Marianhill Landfill Conservancy
23 July 2009	South Africa's Threatened Frogs. Could chytrid be a threat?	ZSSA 50th Anniversary Conference. Illovo, KZN
21 September 2009	The Fascinating World of Frogs	Underberg Junior School – Grade 6 & 7
22 September 2009	South Africa's Threatened Frogs. Could chytrid be a threat?	KZN Wildlife IRDF Meeting. Midmar Dam
30 October 2009	The Amphibian Crisis and South Africa's Threatened Frogs	Rhodes University, 3rd year Zoology Students. Grahamstown
27 February 2010	South Africa's threatened frogs & Mt Moreland	Mt Moreland – general public
27 March 2010	The Fascinating World of Frogs	High school girls from KwaMashu. Botha's Hill
09 April 2010	The Fascinating World of Frogs	High school girls from Highflats. Botha's Hill

31 July 2010	South Africa's Threatened Frogs. Could chytrid be a threat?	KZN Wildlife – Freshwater Fishing Liaison Committee Meeting. Pietermartizburg
2 September 20 10	The Fascinating World of Frogs	Crawford Preparatory School, La Lucia. Grade 1 – 7
15 September 2010	South Africa's Threatened Frogs. Could chytrid be a threat?	Hogsback. General Public.
01 June 2011	The Fascinating World of Frogs	Ashley Primary School, Pinetown
24 August 2011	The Amphibian Crisis and South Africa's Threatened Frogs	Ezemvelo KZN Wildlife conservation day, Paddock
27 October 2011	Frogs!	Speckled Frogs Daycare, Gillitts
02 November 2011	Amphibians in Crisis: Frogs and Climate Change	What's up with the Weather? Climate Change seminars, Durban Natural History Museum
16 February 2012	Amphibians in Crisis	Danville Girls High School, Durban
14 March 2012	The Fascinating World of Frogs	Ashley Primary School, Pinetown

Each talk was modified according to the group and relevancy of subject to location of talk.

Table 2: List of popular articles

Date	Title	Publication
December 2008	Assessment of threatened South African frogs. Is chytridiomycosis a threat?	African Herp News Number 46
January 2009	Amphibian Declines and South Africa’s Threatened Frogs	Enviropaedia
06 April 2009	“Frogs play a key role in ecosytems”	WWF online news http://www.panda.org.za/index.php?section=News_AboutUs&id=157
June 09	Amphibian Declines and South Africa’s Threatened Frogs	Science in Africa – online journal http://www.scienceinafrica.co.za/2009/june/frogs.htm
15 June 2009	Amphibian Declines	Mountain Echo – Underberg local newspaper
18 June 2009	Frog Research	Pinetown Rotary Newsletter
November 2009	South Africa’s Threatened Frogs and chytrid	KZN Wildlife – Lion’s River newsletter
November 2009	Hogsback’s Threatened Frogs	Hogsback Newsletter
December 2009	Frog Project Update	WWF Green Trust Newsletter
January 2010	Frog declines & Global Warming	Palmnut Newsletter – Durban Natural History Museum
March 2010	Mt Moreland : A haven for Frog Biodiversity	http://www.barnswallow.co.za/
April 2010	Giba Gorge as a Sanctuary for Frogs	Giba Newsletter April 2010
November 2010	Mount Moreland : Ahaven for frog Biodiversity	Mount Moreland Website http://www.mountmoreland.com/Mount–Moreland–Frogs.html

July 2011	Amatola Toad AWOL: Thirteen years of futile searches	FrogLog 97 pp. 24 (Conradie & Tarrant)
December 2011	Rediscovery of the elusive Amatola Toad *Vandijkophrynus amatolicus*: twice seen in twenty-six years	African Herp News 55 (Tarrant & Cunnigham)
December 2011	A rare sighting of Amatola Toad *Vandijkophrynus amatolicus* near Hogsback	SA Forestry Magazine
In Press	Amphibians in Crisis: Is climate change yet another threat?	Thola Vol 14: Durban Natural Science Museum

Media Documentaries/Interviews

29 June2009	SABC2 “Wild Ltd” Documentary: Episode 18 – “Swimming Upstream”.
15 May 2011	SABC3 “Greenline” interview: Episode 11

Appendix D: *Hyperolius pickersgilli* ground–truthing.

Table 1: Wetlands with > 60% occurrence probability for *Hyperolius pickersgilli* as predicted by the Maxent model (Chapter 4). These sites were ground–truthed between October 2010 and January 2012. The species was detected at only 5 sites out of a total of 71 surveyed. The species was deemed absent at 24 sites where wetland habitat had been completely eliminated. A further 14 sites appeared unsuitable for the species (Unlikely) and the species was not detected at 24 sites which may have been suitable for the species.

Date	Area	lat	long	Site description	Likelihood of presence of *H. pickersgilli*
28/10/2010	Umlazi	-29.94502	30.92430	The general area is heavily built up with industrial, commercial and residential land–use.	Unlikely
28/10/2010	Umlazi	-29.96492	30.92770	Not suitable (wetland no longer present, housing built in the vicinity).	Absent
28/10/2010	Umlazi	-29.94790	30.93428	Wetland adjacent to Mlazi river. Disturbed by roadworks for new road. Other species heard calling (*Afrixalus fornasinii*, *H. marmoratus* and *L. natalensis*) but does not appear suitable to *H. pickersgilli*	Not detected
28/10/2010	Umlazi	-29.96765	30.93925	Not suitable. Wetland no longer present.	Absent
28/10/2010	Umlazi	-29.95243	30.94182	Large wetland area below newly constructed pedestrian bridge. Wetland disturbed and partially transformed by footbridge construction, dykes and subsistence gardening.	Unlikely
28/10/2010	Umlazi	-29.95179	30.94577	Possible a historical wetland – no longer suitable. Housing built in the area.	Absent
28/10/2010	Umlazi	-29.94049	30.94918	Wetland partially disturbed by rubbish dumping and other human	Not detected

				activities.	
28/10/2010	Umlazi	-29.95450	30.95357	Not suitable. Wetland no longer present.	Absent
11/11/2010	Roosfontein	-29.85733	30.92598	Conservation area with wetland surrounded by grassland, but no standing water or reedbeds as required by *H. pickersgilli*.	Not detected
11/11/2010	Cato Manor	-29.86012	30.93706	All Cato Manor sites in low–income housing development areas. Wetlands either no longer in existence or badly littered.	Absent
11/11/2010	Cato Manor	-29.84708	30.93947	Site completely dry.	Absent
11/11/2010	Cato Manor	-29.85707	30.94135	Not suitable.	Absent
11/11/2010	Cato Manor	-29.85969	30.95382	Not suitable.	Absent
11/11/2010	Cato Manor	29.85642	30.95999	Not suitable.	Absent
11/11/2010	Cato Manor	-29.85908	30.96174	Not suitable.	Absent
18/11/2010	Mobeni/Clairewood	-29.90414	30.95785	Dry river bed – not suitable	Absent
18/11/2010	Mobeni/Clairewood	-29.90785	30.97150	Not suitable. Wetland no longer present.	Absent

09/12/2010	Prospecton	-29.98412	30.93696	Extensive reed bed comprised of suitable vegetation adjacent to Prospecton Rd. Drainage line running through centre. Call & sighting.	**Present**
17/12/2010	Nyoni River, Amatikulu NR	-29.13370	30.59369	River system – visited by boat. Numerous other hyperolid species calling, but not suitable for *H. pickersgilli*	Not detected
18/12/2010	Amatikulu NR	-29.13461	31.59398	Not suitable. Wetland area completely dry.	Unlikely
18/12/2010	Amatikulu NR	-28.95706	31.76307	Coastal dune thicket, mostly undisturbed. Very dry – only a small amount of reed vegetation present (*Papyrus* and *Phragmites*).	Unlikely
18/12/2010	Amatikulu NR	-29.01413	31.69252	Swamp forest along small river, no proper wetland or veg. Very dry. Surrounded by pine and gum plantation.	Unlikely
18/12/2010	Amatikulu NR	-29.00727	31.65930	Forested stream within sugar cane fields. No reed vegetation.	Unlikely
18/12/2010	Amatikulu NR	-29.03838	31.66700	Swamp forest ravine within gum plantation	Unlikely
12/10/2011	Twinstreams	-28.99461	31.72471	Dry reed bed below *Eucaluptus* plantation.	Unlikely
12/10/2011	Twinstreams	-29.00095	31.71548	Possible historical wetland. Very dry. Area burnt in 2006.	Unlikely
12/10/2011	Twinstreams (Gwalagwala campsite)	-29.00679	31.7152	Destroyed wetland; grass now.	Absent
12/10/2011	Port Durnford	-28.90522	31.85848	New site. Many calling in large reed *Phragmites australis* & sedge wetland with deep stagnant water. Call and sighting.	**Present**

12/10/2011	Port Durnford	-28.90942	31.81816	Reed and sedge wetland	Not detected
02/02/2011	iSimangaliso	-28.20162	32.48896	Short bulrush wetland with deep standing water	Not detected
02/02/2011	iSimangaliso	-28.20802	32.49292	Short–grass & sedge wetland	Not detected
02/02/2011	iSimangaliso	-28.19836	32.5027	Reed wetland	Not detected
02/02/2011	iSimangaliso	-28.17175	32.49435	Dry short–grass & sedge wetland	Unlikely
13/10/2011	Umlalazi Nature Reserve	-28.96965	31.755	Reed wetland in the Siyaya River at the bridge	Not detected
13/10/2011	iSimangaliso	-28.349848	32.411071	Part of estuary within walking distance from Croc centre. Along river system.	Unlikely
13/10/2011	iSimangaliso	-28.29722	32.43881	"Space" site. Dense reedbed of tall sedges and ferns.	Not detected
13/10/2011	iSimangaliso	-28.31048	32.44502	Vlei Loop	Not detected
13/10/2011	Lake Teza, Umfolozi	-28.48532	32.16041	Large lake surrounded by emergent vegetation (*Phragmites* and *Papyrus*)	Not detected

22/10/2011	Amanzimtoti, San Gabriel Ave.	-30.07431	30.86199	Coastal forest. Close to “Warner Beach” (1982) historical site	Not detected
22/10/2011	Clansthal	-30.239145	30.768241	Drained wetland within sugar cane plantation.	Absent
22/10/2011	Clansthal	-30.240076	30.768241	Drained wetland within sugar cane plantation.	Absent
22/10/2011	Clansthal	-30.247855	30.767896	Drained wetland within sugar cane planation.	Absent
22/10/2011	Scottburgh/ Park Rynie	-30.305011	30.728704	Dry and surrounded by sugar cane.	Absent
22/10/2011	Scottburgh/ Park Rynie	-30.314265	30.724542	On sugar cane farm. Limited access.	Unlikely
22/10/2011	Park Rynie	-30.320475	30.764368	Very small sedge wetland surrounded by sugar cane.	Unlikely
22/10/2011	Park Rynie	-30.335084	30.731506	Small drainage line in sugar cane.	Unlikely
08/11/2011	Amanzimtoti, Fynn Road	-30.039641	30.879553	Steep slope dominated by Spanish reed. Housing built in area of historical wetland.	Absent
08/11/2011	Amanzimtoti, Fynn Road	-30.03154	30.879149	Dominated by Spanish reed. Not suitable	Absent
08/11/2011 06/12/2011	Croc world – South of Scottburgh	-30.25825	30.76772	*Phragmites* reed bed below Crocworld grounds. Appeared suitable at initial visit, but none on second night visit, possibly due to insufficient ground–cover.	Not detected

08/11/2011	Hibberdene	-30.57091	30.57537	Reedbed wetland	Not detected
08/11/2011	Hibberdene, near turn–off	-30.558473	30.582478	Sedge and dwarf papyrus reedbed wetland	Not detected
08/11/2011	Karridene	-30.12364	30.82865	Dry and surrounded by sugar cane	Not detected
08/11/2011	Karridene	-30.12706	30.82451	Reeds adjacent to road	Not detected
06/01/2012	Umkomaas	-30.21717	30.79542	Dense reedbed of *Typha*	**Present**
06/01/2012	Umkomaas	-30.22707	30.78991	Wetland	Not detected
09/01/2012	Port Durnford	-28.90403	31.8680	Vlei with dwarf sedges. Not suitable for *H. pickersgilli*	Not detected
10/01/2012	Amatikulu	-29.07870	31.63973	*Phragmites* and *Typha* wetland. Suitable	Not detected
10/01/2012	Zinkwazi Beach (Nonoti)	-29.29659	31.41242	Perennial wetland densely vegetated with *Phragmites*. Highly suitable	**Present**
19/01/2012	Elysium	-30.44585	30.61935	Drained wetland within sugarcane plantation (furrowed)	Absent
19/01/2012	Elysium	-30.46386	30.63477	Drained wetland within sugarcane plantation (furrowed)	Absent
19/01/2012	Sezela	-30.40612	30.63641	Spartan reedbed	Not detected
19/01/2012	Sezela	-30.40827	30.64943	Drained wetland within sugarcane plantation (furrowed)	Absent

19/01/2012	Sezela	-30.40679	30.64038	Scraggly reedbed	Not detected
19/01/2012	Mpenjati	-30.97502	30.28037	Wetland with bulrushes *Typha capensis*, ferns and reeds *Phragmites australis* but little standing water	Not detected
20/01/2012	Sezela	-30.40670	30.66145	Medium wetland. Very dense: *Cyprus*, *Persicaria* and *Phragmites*	**Present**
20/01/2012	Sezela	-30.39988	30.67799	Drained wetland within sugarcane plantation (furrowed)	Absent
20/01/2012	Sezela	-30.40208	30.67404	Drained wetland within sugarcane plantation (furrowed)	Absent
20/01/2012	Prospecton	-29.97837	30.93617	Dense reedbed running parallel to N2	**Present**
20/01/2012	Prospecton	-29.98182	30.93495	Dense *Phragmites australis* reedbed running parallel to N2	**Present**
20/01/2012	Prospecton	-29.98396	30.93425	Dense *Phragmites australis* reedbed running parallel to N2	**Present**
20/01/2012	Prospecton	-29.97851	30.9366	Dense reedbed running parallel to N2	**Present**

Appendix E: *Afrixalus spinifrons* specimens and samples

Table 1: Specimens (sorted by specimen number) of *Afrixalus spinifrons* complex examined for morphological assessment (Chapter 5). Specimen accession number, species, location and gender is given for each specimen. Measurements: SVL = Snout–Vent Length; HW = Head Width; TL = Tibia Length.

Institute ID	Species	Location	SVL	HW	TL	TL/SV	HW/SV	HW/TL	Sex
AACRG1224	*A. s. spinifrons*	Vernon Crookes	22.00	7.35	9.73	0.44	0.33	0.76	Male
AACRG1225	*A. s. spinifrons*	Vernon Crookes	21.92	7.04	9.36	0.43	0.32	0.75	Male
AACRG1226	*A. s. spinifrons*	Vernon Crookes	23.51	7.36	10.23	0.43	0.31	0.72	Male
AACRG1227	*A. s. spinifrons*	Vernon Crookes	21.22	7.16	9.57	0.45	0.34	0.75	Male
AACRG1228	*A. s. spinifrons*	Vernon Crookes	21.76	6.91	9.96	0.46	0.32	0.69	Male
AACRG1233	*A. knysnae*	Covie	20.75	7.03	9.20	0.44	0.34	0.76	Male
AACRG1234	*A. knysnae*	Covie	22.88	7.71	9.90	0.43	0.34	0.78	Male
AACRG1235	*A. knysnae*	Covie	22.24	7.42	9.89	0.44	0.33	0.75	Male
AACRG1566	*A. s. intermedius*	Hilton	19.80	6.54	8.36	0.42	0.33	0.78	Male
AACRG1567	*A. s. intermedius*	Hilton	19.73	6.36	8.86	0.45	0.32	0.72	Male
AACRG1568	*A. s. intermedius*	Hilton	18.62	6.44	8.14	0.44	0.35	0.79	Male
AACRG1569	*A. s. intermedius*	Hilton	18.86	6.34	7.87	0.42	0.34	0.81	Male
AACRG1570	*A. s. intermedius*	Hilton	17.68	6.01	7.76	0.44	0.34	0.77	Male
AACRG1571	*A. s. intermedius*	Hilton	20.19	6.39	8.48	0.42	0.32	0.75	Male
AACRG1572	*A. s. spinifrons*	Prospecton	17.95	5.85	6.98	0.39	0.33	0.84	Male
AACRG1573	*A. s. spinifrons*	Prospecton	20.22	6.21	7.81	0.39	0.31	0.80	Male
AACRG1575	*A. s. spinifrons*	Mtunzini	18.51	6.34	7.32	0.40	0.34	0.87	Male
AACRG1576	*A. s. spinifrons*	Mtunzini	18.41	6.15	7.55	0.41	0.33	0.81	Male
AACRG1577	*A. s. spinifrons*	Mt. Moreland	21.15	7.08	8.99	0.43	0.33	0.79	Female
AACRG1579	*A. s. spinifrons*	Mt. Moreland	19.84	6.43	8.15	0.41	0.32	0.79	Male
AACRG1580	*A. s. intermedius*	Cedara Dam	23.02	6.67	9.02	0.39	0.29	0.74	Female
AACRG1581	*A. s. intermedius*	Fort Nottingham	19.88	6.46	8.23	0.41	0.33	0.79	Male
AACRG1582	*A. s. intermedius*	Fort Nottingham	21.29	6.96	8.80	0.41	0.33	0.79	Male

AACRG1583	*A. s. intermedius*	Fort Nottingham	18.37	6.31	7.77	0.42	0.34	0.81	Male
AACRG1584	*A. s. spinifrons*	Tala NR	19.38	6.61	8.22	0.42	0.34	0.80	Male
AACRG1585	*A. s. spinifrons*	Tala NR	20.16	6.52	7.98	0.40	0.32	0.82	Male
AACRG1586	*A. s. intermedius*	Rosetta	20.91	6.78	8.84	0.42	0.32	0.77	Female
AACRG1587	*A. s. intermedius*	Rosetta	21.54	7.11	9.14	0.42	0.33	0.78	Female
AACRG1588	*A. s. intermedius*	Kamberg	21.81	7.20	9.38	0.43	0.33	0.77	Male
AACRG1589	*A. s. intermedius*	Kamberg	22.29	7.22	9.35	0.42	0.32	0.77	Female
AACRG1590	*A. s. intermedius*	Kamberg	18.24	6.05	7.87	0.43	0.33	0.77	Male
AACRG1701	*A. s. spinifrons*	Coffee Bay	18.49	6.29	7.62	0.41	0.34	0.82	Male
AACRG1702	*A. s. spinifrons*	Coffee Bay	21.12	7.05	8.80	0.42	0.33	0.80	Female
AACRG1703	*A. s. spinifrons*	Coffee Bay	20.79	7.05	8.67	0.42	0.34	0.81	Male
AACRG1704	*A. s. spinifrons*	Coffee Bay	19.71	6.62	8.19	0.42	0.34	0.81	Male
AACRG1705	*A. s. spinifrons*	Coffee Bay	18.43	6.44	7.66	0.42	0.35	0.84	Male
JT160112	*A. s. intermedius*	Himeville	19.78	6.81	8.76	0.44	0.34	0.78	Male
NM 748	*A. s. spinifrons*	Unknown	17.48	5.72	7.73	0.44	0.33	0.74	Male
NM3950	*A. s. intermedius*	Nottingham Rd	17.83	5.68	7.76	0.44	0.32	0.73	Male
NM3951	*A. s. intermedius*	Dargle	20.90	6.91	8.87	0.42	0.33	0.78	Female
NM3952	*A. s. intermedius*	Cedara Dam	18.28	6.59	8.70	0.48	0.36	0.76	Male
NM3954	*A. s. intermedius*	Cedara Dam	19.27	6.65	8.89	0.46	0.35	0.75	Female
NM3956	*A. s. intermedius*	Cedara Dam	18.96	6.86	8.82	0.47	0.36	0.78	Male
NM3957	*A. s. intermedius*	Cedara Dam	18.27	6.64	8.95	0.49	0.36	0.74	Male
NM3961	*A. s. intermedius*	Cedara Dam	15.86	5.80	6.58	0.41	0.37	0.88	Male
NM3962	*A. s. intermedius*	Cedara Dam	17.47	6.66	8.13	0.47	0.38	0.82	Male
NM3963	*A. s. intermedius*	Cedara Dam	13.34	4.84	6.51	0.49	0.36	0.74	Male
NM3967	*A. s. intermedius*	Inhluzane	18.28	6.27	8.47	0.46	0.34	0.74	Male
NM3968	*A. s. intermedius*	Inhluzane	19.64	6.14	8.41	0.43	0.31	0.73	Female
NM3969	*A. s. intermedius*	Inhluzane	19.14	7.31	8.41	0.44	0.38	0.87	Female
NM3970	*A. s. intermedius*	Inhluzane	15.48	5.83	7.45	0.48	0.38	0.78	Male
NM3971	*A. s. intermedius*	Inhluzane	15.82	5.67	6.86	0.43	0.36	0.83	Male
NM3975	*A. s. intermedius*	Merthley Dam	16.71	5.55	7.45	0.45	0.33	0.75	Male
NM3976	*A. s. spinifrons*	Umvoti River	18.06	6.46	6.83	0.38	0.36	0.95	Male
NM3977	*A. s. intermedius*	Mooi River	20.20	6.09	9.24	0.46	0.30	0.66	Female
NM3978	*A. s. spinifrons*	Umvoti River	21.72	6.50	8.79	0.40	0.30	0.74	Female

NM3979	*A. s. intermedius*	Inhluzane	21.20	6.64	10.31	0.49	0.31	0.64	Female
NM3991	*A. s. spinifrons*	Mtunzini	19.64	5.72	7.88	0.40	0.29	0.73	Female
NM3992	*A. s. spinifrons*	Durban,Wentworth	15.07	4.97	5.54	0.37	0.33	0.90	Male
NM3993	*A. s. spinifrons*	Kwa–Mbonambi	17.95	5.37	7.84	0.44	0.30	0.68	Male
NM3994	*A. s. spinifrons*	Kwa–Mbonambi	17.61	5.39	7.20	0.41	0.31	0.75	Male
NM5633a	*A. s. spinifrons*	Pinetown	17.16	5.86	6.79	0.40	0.34	0.86	Male
NM5633b	*A. s. spinifrons*	Pinetown	16.33	5.58	6.38	0.39	0.34	0.87	Male
NM5633c	*A. s. spinifrons*	Pinetown	16.64	5.68	6.83	0.41	0.34	0.83	Male
NM5634a	*A. s. spinifrons*	Mtunzini	20.79	6.63	7.80	0.38	0.32	0.85	Female
NM5634c	*A. s. spinifrons*	Mtunzini	18.16	6.08	7.65	0.42	0.33	0.79	Male
NM5634d	*A. s. spinifrons*	Mtunzini	17.76	5.96	7.87	0.44	0.34	0.76	Male
NM5634e	*A. s. spinifrons*	Mtunzini	19.00	6.15	8.14	0.43	0.32	0.76	Male
NM5634f	*A. s. spinifrons*	Mtunzini	17.42	5.65	7.32	0.42	0.32	0.77	Male
NM5634g	*A. s. spinifrons*	Mtunzini	17.15	6.06	7.59	0.44	0.35	0.80	Male
NM5634h	*A. s. spinifrons*	Mtunzini	17.35	5.79	7.18	0.41	0.33	0.81	Male
NM5634i	*A. s. spinifrons*	Mtunzini	17.55	5.78	6.50	0.37	0.33	0.89	Male
NM5634j	*A. s. spinifrons*	Mtunzini	19.50	6.32	7.71	0.40	0.32	0.82	Male
NM5634k	*A. s. spinifrons*	Mtunzini	18.32	5.92	7.87	0.43	0.32	0.75	Male
NM6460	*A. s. intermedius*	Midmar Dam	17.35	6.34	7.86	0.45	0.37	0.81	Male
NM6461	*A. s. intermedius*	Midmar Dam	16.82	6.20	8.07	0.48	0.37	0.77	Male
PEMA2138	*A. s. spinifrons*	Mpande Bay	20.04	6.25	9.41	0.47	0.31	0.66	Female
PEMA2592	*A. s. spinifrons*	Mgunduli, Transkei	19.94	6.93	8.80	0.44	0.35	0.79	Female
PEMA2593	*A. s. spinifrons*	Mgunduli, Transkei	16.52	6.47	7.57	0.46	0.39	0.85	Male
PEMA2599	*A. s. spinifrons*	Coffee Bay	18.73	6.75	7.81	0.42	0.36	0.86	Male
PEMA2600	*A. s. spinifrons*	Coffee Bay	16.42	6.10	7.18	0.44	0.37	0.85	Male
PEMA2601	*A. s. spinifrons*	Coffee Bay	17.95	6.29	7.46	0.42	0.35	0.84	Male
PEMA2602	*A. s. spinifrons*	Coffee Bay	20.29	7.34	8.68	0.43	0.36	0.85	Female
PEMA2603	*A. s. spinifrons*	Coffee Bay	17.43	6.13	7.92	0.45	0.35	0.77	Male
PEMA6406	*A. s. spinifrons*	Port St. Johns	23.01	7.48	9.53	0.41	0.33	0.79	Female
PEMA6407	*A. s. spinifrons*	Port St. Johns	20.29	6.88	7.35	0.36	0.34	0.94	Male
PEMA6408	*A. s. spinifrons*	Port St. Johns	21.70	7.95	10.96	0.50	0.37	0.73	Female
PEMA6409	*A. s. spinifrons*	Port St. Johns	21.39	7.33	9.55	0.45	0.34	0.77	Female
PEMA6410	*A. s. spinifrons*	Port St. Johns	21.05	7.17	9.68	0.46	0.34	0.74	Female

PEMA6416	*A. s. spinifrons*	Mariannhill	18.93	6.25	7.75	0.41	0.33	0.81	Male
PEMA6417	*A. s. spinifrons*	Mariannhill	19.86	6.55	8.63	0.43	0.33	0.76	Male
PEMA6418	*A. s. spinifrons*	Mariannhill	18.89	6.14	7.89	0.42	0.33	0.78	Male
PEMA6419	*A. s. spinifrons*	Port St. Johns	17.96	6.44	7.56	0.42	0.36	0.85	Male
PEMA6420	*A. s. spinifrons*	Mariannhill	18.70	6.37	8.02	0.43	0.34	0.79	Male
PEMA7466	*A. s. spinifrons*	Singisis	22.39	6.80	10.11	0.45	0.30	0.67	Female
PEMA816	*A. s. spinifrons*	Coffee Bay	17.43	6.36	7.54	0.43	0.36	0.84	Male

Table 2: Tissue sample locality details for *Afrixalus spinifrons* used for molecular analysis (Chapter 5, Figure 5.5). EC = Eastern Cape; KZN = KwaZulu–Natal, WC – Western Cape.

Specimen ID	Species	Collection Date	Locality	Lat	Long	Altitude (m.a.s.l)
AACRG 1578	*A. delicatus*	07/12/2011	Mt. Moreland, KZN	–29.67946	31.08727	80
JT121011/2	*A. delicatus*	12/10/2011	Mtunzini, KZN	–28.96782	31.75322	15
AACRG 1566	*A. s. intermedius*	18/10/2011	Hilton, KZN	–29.53916	30.28625	1083
AACRG 1567	*A.s. intermedius*	18/10/2011	Hilton, KZN	–29.53916	30.28625	1083
AACRG 1568	*A.s. intermedius*	18/10/2011	Hilton, KZN	–29.53916	30.28625	1083
AACRG 1569	*A.s. intermedius*	18/10/2011	Hilton, KZN	–29.53916	30.28625	1083
AACRG 1570	*A.s intermedius*	18/10/2011	Hilton, KZN	–29.53916	30.28625	1083
AACRG 1571	*A.s. intermedius*	18/10/2011	Hilton, KZN	–29.53916	30.28625	1083
AACRG 1580	*A.s. intermedius*	13/12/2011	Cedara Dam, KZN	–29.55784	30.25541	1071
AACRG 1581	*A.s. intermedius*	15/12/2011	Fort Nottingham, KZN	–29.43200	29.90587	1455
AACRG 1582	*A.s. intermedius*	15/12/2011	Fort Nottingham, KZN	–29.43200	29.90587	1455
AACRG 1583	*A.s. intermedius*	15/12/2011	Fort Nottingham, KZN	–29.43200	29.90587	1455
AACRG 1586	*A.s. intermedius*	21/12/2011	Rosetta, KZN	–29.30417	29.96250	1398
AACRG 1587	*A.s. intermedius*	21/12/2011	Rosetta, KZN	–29.30417	29.96250	1398
AACRG 1588	*A.s. intermedius*	21/12/2011	Kamberg NR, KZN	–29.37361	29.72500	1649
AACRG 1589	*A.s. intermedius*	21/12/2011	Kamberg NR, KZN	–29.37361	29.72500	1649
AACRG 1590	*A.s. intermedius*	21/12/2011	Kamberg NR, KZN	–29.37361	29.72500	1649
JT160112/1	*A.s. intermedius*	16/01/2012	Himeville, KZN	–29.73516	29.51959	1521
AACRG 1223	*A. kynsnae*	12/2010	Covie, WC	–33.95194	23.60306	248
AACRG 1233	*A. kynsnae*	12/2010	Covie, WC	–33.95194	23.60306	248
AACRG 1225	*A. s. spinifrons*	12/2010	Vernon Crookes NR, KZN	–30.27333	30.62472	420
AACRG 1226	*A.s. spinifrons*	12/2010	Vernon Crookes NR, KZN	–30.27333	30.62472	420

AACRG 1572	*A.s. spinifrons*	30/11/2011	Prospecton, KZN	–29.98245	30.93800	6
AACRG 1573	*A.s. spinifrons*	30/11/2011	Prospecton, KZN	–29.98245	30.93800	6
AACRG 1575	*A.s. spinifrons*	05/12/2011	Mtunzini, KZN	–28.96782	31.75322	16
AACRG 1576	*A.s. spinifrons*	05/12/2011	Mtunzini, KZN	–28.96782	31.75322	16
AACRG 1577	*A.s. spinifrons*	07/12/2011	Verulum, KZN	–29.67946	31.08727	80
AACRG 1579	*A.s. spinifrons*	07/12/2011	Verulum, KZN	–29.67946	31.08727	80
AACRG 1584	*A.s. spinifrons*	16/12/2011	Tala Nature Reserve, KZN	–29.82954	30.53535	665
AACRG 1585	*A. s.spinifrons*	16/12/2011	Tala Nature Reserve, KZN	–29.82954	30.53535	665
AACRG 1701	*A.s. spinifrons*	18/11/2011	Coffee Bay, EC	–31.93496	29.08826	318
AACRG 1702	*A.s. spinifrons*	18/11/2011	Coffee Bay, EC	–31.93496	29.08826	318
AACRG 1703	*A.s. spinifrons*	18/11/2011	Coffee Bay, EC	–31.93496	29.08826	318
AACRG 1704	*A.s. spinifrons*	18/11/2011	Coffee Bay, EC	–31.93496	29.08826	318
AACRG 1705	*A.s. spinifrons*	18/11/2011	Coffee Bay, EC	–31.93496	29.08826	318
JT081210/1	*A.s. spinifrons*	08/12/2010	Prospecton, KZN	–29.98245	30.93800	6
RB10DO68	*A.s. spinifrons*	11/2011	Babanango, KZN	–28.36909	31.05787	1365
WC1	*A.s. spinifrons*	11/2011	Hluleka, EC	–31.82296	29.30773	45