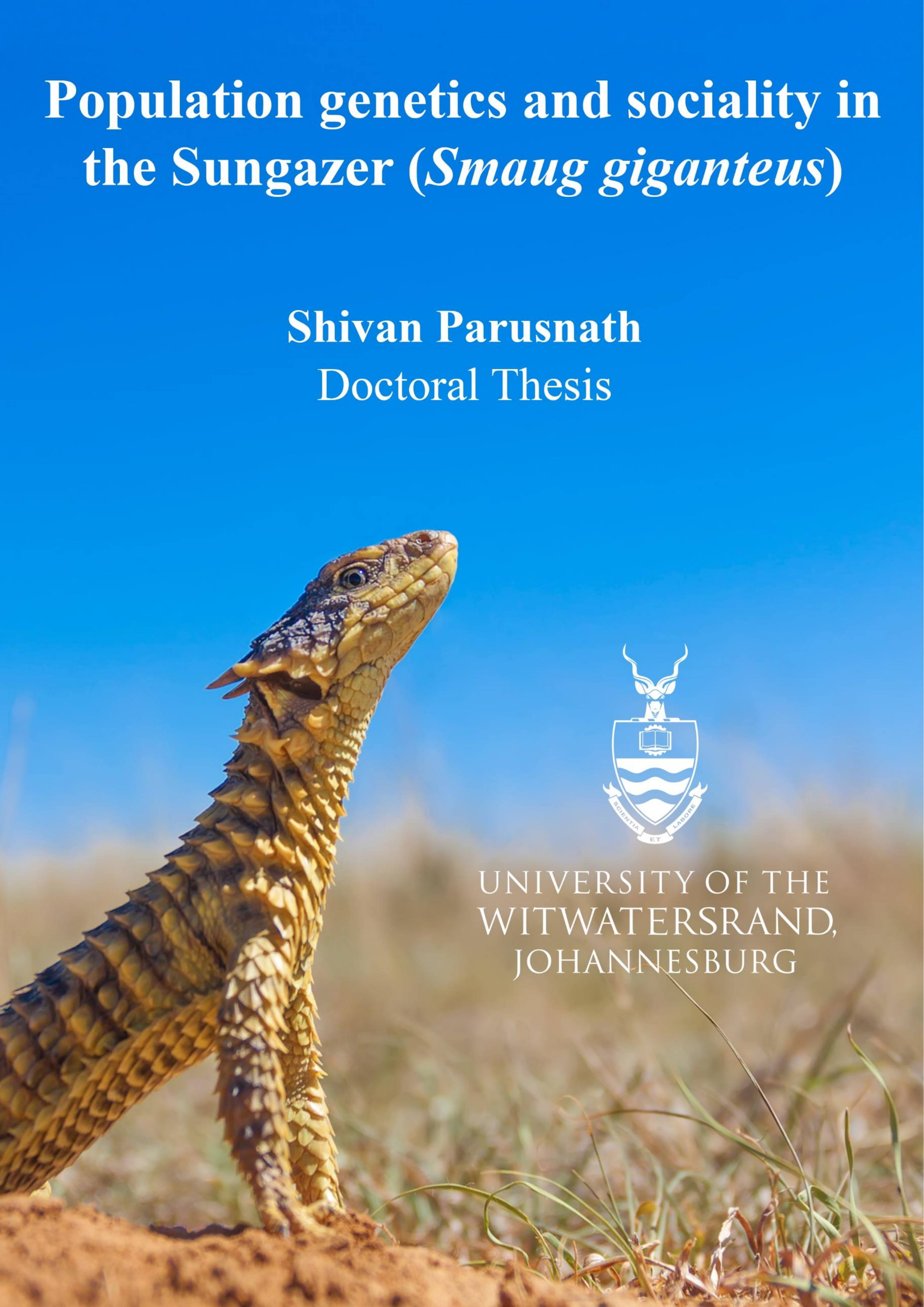
Population genetics and sociality in the Sungazer (*Smaug giganteus*)

Shivan Parusnath

Doctoral Thesis



UNIVERSITY OF THE
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Prof. Graham Alexander
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Prof. Antoinette Kotze

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

30th October 2020

“My philosophy is basically this, and this is something that I live by, and I always have, and I always will: Don't ever, for any reason, do anything, to anyone, for any reason, ever, no matter what, no matter where, or who, or who you are with, or where you are going, or where you've been, ever, for any reason whatsoever.”

- Michael G. Scott

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

All protocols were approved by the University of the Witwatersrand Animal Ethics Screening Committee (2014/56/B; 2016/06/30/B), and the National Zoological Garden, South African National Biodiversity Institute (NZG SANBI) Research, Ethics and Scientific Committee (P14/18). Permits to work with Threatened or Protected Species were granted by the South African Department of Environmental Affairs (TOPS59). Permits to collect samples in the Free State and Mpumalanga provinces were provided by Department of Small Business Development, Tourism and Environmental Affairs (01/34741; JM938/2017) and Mpumalanga Tourism and Parks Agency (MPB. 5519) respectively. Research approval in terms of Section 20 of the Animal Diseases Act (35 of 1984) was granted by the South African Department of Agriculture, Forestry and Fisheries (12/11/1/1/18). All samples used in this project were stored at the National Zoological Garden Biobank in Pretoria.

Shivan Parusnath
30th Day of October 2020

ABSTRACT

Establishing links between the evolution and ecology of a species is one of the central facets of organismal biology. Quantifying the past and present flow of genetic material between populations can provide insight into the landscape features and associated environmental variables that impede or facilitate this process. An integrative approach utilising a) genetic markers that evolve at different evolutionary rates, b) ecological niche models that indicate niche suitability across a landscape, and c) knowledge of the life-history, social system, and morphology of a species can allow for the formulation and testing of hypotheses of scenarios of genetic structure in a species.

The Sungazer (*Smaug giganteus*) is a large cordylid lizard endemic to the Highveld grasslands of South Africa. It is unique amongst the Cordylidae family because of its adherence to primary grassland, reliance on self-excavated burrows as long-term refuge sites, and organisation of multiple Sungazer burrow systems into spatially discrete colonies. Young Sungazers are often seen sharing burrows with adults, and because of this behaviour and the unique spatial organisation of colonies, the species has long been touted as potentially exhibiting kin-based sociality. Little is known about the dispersal ability of Sungazers, the levels of genetic differentiation within and between colonies, the processes that influence genetic structure in the species, and the existence of kin-based sociality in the species. The primary aim of this thesis was to investigate the population and social structure of *S. giganteus* at multiple spatial and evolutionary scales, using mitochondrial DNA, nuclear DNA, and species-specific microsatellites.

Smaug giganteus occurs in five distinct genetic populations across its distribution (Western 1, Western 2, Central, Eastern 1, Eastern 2), that belong to three divergent phylogenetic clades (Western, Central, Eastern). The genetic differentiation of these clades correlate spatially and temporally with geological features that arose during the most recent uplift of the eastern Great Escarpment, ~900 m into the Highveld grasslands, ~2.5-1.8 MYA. Within these clades, a combination of barriers such as mountains, rivers, and isolation by distance appear to be the primary drivers of population structure. The average Sungazer body size (snout-vent-length and mass) in each clade was significantly different, with an increasing size gradient from west to east. This size gradient correlates with an increase in elevation and precipitation, and decrease in temperature, and may result from local adaption to different climatic niches in these vicariant populations.

Sungazer colonies comprise primarily of extended family members, and Sungazers are significantly more related to individuals within the same colony than to those in other colonies. Sungazers that share burrows tend to be immediate family members. Juveniles, in most cases, occupied burrows with either their mother or father, and full-siblings. The behaviours of refuge sharing by adults and their offspring, combined with delayed juvenile dispersal, are indicative of parental care in the species. Besides the sharing of burrows with nuclear family members, social structure within colonies appears to be relatively homogenous, likely due to the frequent moves between burrows by adults during mating periods. This is the first evidence of kin-based sociality in an African lizard.

The new insights into genetic structure in *S. giganteus* presented in this thesis have ramifications for how long-term conservation and translocation strategies for *S. giganteus* are managed, such that the genetic structure and health of populations are preserved. This study extends the evidence of lizard family living to a new taxonomic group, and a new continent, elucidating the reality that kin-based sociality may be more widespread in lizards than has long been thought. This opens the door to studies of kin-based sociality in other cordylids and African lizards, as well as more detailed research on the complex system of sociality in *S. giganteus*.

ACKNOWLEDGMENTS

The research presented in this thesis would have not been possible without the assistance, cooperation and friendliness of many, many landowners across the distribution of the Sungazer. The respect and interest that these individuals maintain for Sungazers gives me a lot of hope that the species is in good hands. Some of my finest memories from the past decade have been driving their dirt roads and walking their open fields in search of Sungazers.

This project came with considerable financial costs of fieldwork, labwork, and keeping me alive. Project funding provided by the National Zoological Gardens (previously under NRF, now under SANBI), the Rufford Foundation (Second Grant: 13956-2), the National Geographic Society (Young Explorer Grant: C288-14), and the Alexander Herp Lab, allowed for the collections of hundreds of samples from the field, developing and testing microsatellite markers, and processing these samples in the lab. Funding through the NRF Professional Development Plan allowed for me to pay for rent and food, and live a comfortable life for the first three years of my PhD. It became a hustle after that, but my parents, Vinesh and Sashi Parusnath (and my third parent, Prianka), were always there to help pay bills when times got tough. I cannot be appreciative enough of their support, long after the normal duties of parenthood had passed. I owe an immeasurable thank you to the Carnegie Corporation of New York for a grant awarded to University of the Witwatersrand for the Next Generation of African Scholars, which covered in full, my final year of PhD fees. To complete this PhD without fee debt is truly a rare gift. Thank you.

My supervisors, Graham Alexander, Krystal Tolley, Desire Dalton, and Antoinette Kotze were vital mentors to me on my journey of genetics, ecology and evolution. Each of you has imparted valuable life lessons onto me in multifarious ways. In particular I would like to thank Krystal Tolley and Graham Alexander who make for a supervisory dream team. Any student to have either of you supervising them can consider themselves lucky, but to land the pair is indeed great fortune.

I think it was Tolkien that said, “in the company of friends, peril becomes adventure”. Wade Stanton-Jones, Thilo Beck, Mareike Dinberger, Hiral Naik, Bevan Dell, Ashadee Miller, and Mimmie Kgaditse all assisted in collecting tissue samples and making difficult decisions in the field. Thanks for the adventures. Wade in particular, who also spent several years doing research on Sungazers, was always a welcome field companion. Not only for the assistance, but for the great conversations about Sungazers, and the many memories made in the field together. Special thanks must also be given to Allan Vorster, who has welcomed me to Welkom on many occasions, to show friends, colleagues and film crews the colony of Sungazers that he watches over. He has also shared many interesting observations and photographs of Sungazers with me that have stimulated enthralling discussion.

While labwork may appear to be more comfortable and simple than fieldwork to the uninitiated, this could not be further from the truth. It can in fact be far more terrifying. Many skilled students and technicians at the National Zoological Gardens taught me the ropes, and were always available to help solve problems. Almero Oosthuizen, Thabang Madisha, Antonie Kloppers, and Clearance Mnisi in particular made labwork more accessible. My labmates at the Alexander Herp Lab at Wits provided support in various ways throughout the PhD. In particular, Dr. Hanlie Engelbrecht went far beyond her duties as a post-doc in assisting me with the complex statistical analyses involved in this study. Her fervour to teach and upskill others is something that I endeavour to match. I hope to in turn pass these lessons and skills on to others. Other staff members at Wits have also served as important mentors to me, Stuart Sym, Dave Mycock, Marcus Byrne, and Carol Still chief among them. A special thanks also to Rose Sephton-Poultney, who proofread my thesis before submission.

Hannes Van Wyk, Le Fras Mouton, Martin Whiting, Ed Stanley, Niels Jacobsen, Trevor McIntyre – not only has your work been an inspiration to me, but our conversations on Sungazers over the past several years have been invaluable in shaping my research.

Working on the Sungazer has also meant a long and difficult battle against the illegal trade of the species, and many people from various institutes share my passion and dedication in the conservation of these special animals. Key among these individuals are Ian Little, Bradley Gibbons, Ian Rushworth, Chris De Beer, Zwelakhe Zondi, Hannes Botha, Andre Schlemmer, Luke Verbugt, Chris Cooke, Gary Kyle Nicolau, Vince Egan, Michele Pfab, and Krystal Tolley. With as many decent, caring people as these dedicated to the conservation and protection of the Sungazer in its habitat, surely the outlook is more positive than we sometimes think it is.

I have had many mentors that have helped me tell stories of Sungazers in popular articles, photographs, videos, interviews, and public talks. Pragna Parsotam-Kok, Noel Kok, Chloé Cipoletta, Jannes De Villiers, Schalk Mouton, Peter Lamberti, Greg McGruder, Beata Nas and many people at the National Geographic Society have been important allies in this endeavour. Anyone who has the opportunity to work with these talented humans can consider themselves lucky – I certainly do.

I could not have dealt with the trauma of completing a PhD without The Office. Michael, Dwight, Angela, Jim, Pam, Toby, Kelly, Ryan, and the rest of the Dunder Mifflin team, made anxious, unbearable days and nights survivable. Your craft pervades realms far beyond that which you may think. You are still the only reason that I can fall asleep most nights. Thank you. Similarly, I have accompanied Rambalac on hundreds of hours of quiet, peaceful walks through Japan. Your dedication to what you do is legendary. On days when the anxiety was so intense that I could hardly breathe, your videos calmed me down and opened up my lungs. How can I thank you enough for something that most people need medication to deal with? Thank you for every adventure that you have taken me on. Through the incredible voice of Rob Inglis, I experienced J. R. R. Tolkien's 'The Lord of the Rings' several times over whilst in the field. It often felt that my adventures in the wild country paralleled that of Frodo Baggins and his companions on their journey to Mordor to destroy the One Ring. The poem, 'A Walking Song' from the 'Fellowship of the Ring' was heartening to listen to after many nights moving from town to town:

"Home is behind, the world ahead,
And there are many paths to tread
Through shadows to the edge of night,
Until the stars are all alight.
Then world behind and home ahead,
We'll wander back and home to bed.
Mist and twilight, cloud and shade,
Away shall fade! Away shall fade!"

On companions, Bianca Fizzotti, Danielle Durbach, Graham Stansell, Geoffrey Mulder, Nick Quarta, Jolene Fisher, Joshua Weiss, Ryan van Huyssteen, Anusha Shankar, Nasreen Peer, Jason Klinkert and Viktor Radermaker, you were a worthy Fellowship. Thank you for the support along every step of the journey.

Finally, to my Sam. Celine Klinkert, your unwavering support and love is something that I thought existed only in storybooks. You have shown me that reality is in fact gloriously more wonderful than fiction. May your every hope and dream come true.

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PREFACE: A PERSONAL PERSPECTIVE ON THE PHD

My interest in the Sungazer has always surpassed scientific research. The charismatic lizard captured my attention long before I engaged in studies on its conservation, ecology and evolution. To have a species as iconic and unique as the Sungazer occurring only in my country has long been a point of pride for myself and many other South Africans. This was evident in talking to hundreds of landowners over the past several years. Sungazers only inhabit about 12% of the pristine grassland remaining across its distribution, and landowners therefore regard the presence of Sungazers on their land as an indication of the quality of their grasslands. Another point of national pride in the Sungazer is reflected in that the species has at least eight names in four languages that I know of. Sungazer or Giant Dragon Lizard in English, Sonkyker, Ouvolk, Karkoerhotnot or Skurwejantjie in Afrikaans, Mbedhla in Zulu, and Pathagalle in Sotho. These names are not generic for any other lizard, reserved just for the Sungazer. I am not sure how many other lizards throughout the world have been given so many names from so many different communities, or are so well known across their distribution.

Habitat loss and fragmentation are clear threats to the species, and this is apparent when searching for Sungazers in the field, or looking at the grasslands that they inhabit through satellite imagery. But through many years of research it became very clear that one of the most nefarious threats is not the transformation of grassland to grow crops, which in its own way is an honest endeavour in supplying our country with food. Instead, it is the greed of humans. It is a disheartening experience to revisit a field site and follow a GPS point to the location of a burrow, only to find the burrow dug up, and the Sungazers that once lived inside it, gone. CITES export numbers and frequent posts on social media of Sungazers for sale correlated with the disappearances of Sungazers and their burrows, and it became evident to me, the risk that poaching and laundering of wild Sungazers has on the species and its longevity in the wild. With its late age of first reproduction and low fecundity, Sungazers can’t keep up with persistent pilfering of their populations for the pet trade.

The microsatellite markers designed in chapter 2 of this study were developed for analysing the fine-scale genetic structure of wild Sungazer populations and their sociality, but came to play perhaps a bigger role in their function as tools in regulating trade. Their ability to test the relatedness of an individual to other individuals in a population meant that they could be used to assess whether Sungazers were captive bred before CITES permits were granted for their exportation. So before they were ever used for the purpose that they were designed for, the microsatellite markers were used to assess whether F2 breeding (captive bred grandchildren of wild Sungazers) could be verified in a group of about 60 Sungazers destined for the pet trade. As might be suspected of a group of lizards plundered from their homes, some lizards were with a parent, but for most individuals, a set of parents could not be assigned to them from within the 'captive breeding colony'. No grandparents could be assigned to any of the individuals, meaning that F2 generation captive breeding could not be verified. This resulted in the first case that I know of, in which a CITES export permit for a reptile was rejected in South Africa based on a lack of genetic evidence of captive breeding to the second generation.

With these cases going on throughout my Masters and Doctoral years, the battle against the illegal trade of the Sungazer and South African reptiles in general became very personal to me. I was lucky to be granted funding by the National Geographic Society for my PhD work, but I was considerably luckier for their interest in me and my stories of Sungazers. I became a part of their first Young Explorer Leadership and Development Programme in 2017, and amongst many other valuable skills and experiences, this gave me an international stage on which I could talk about the illegal reptile trade and the damage it is doing to our wild populations. A short TV insert about this research on Sungazers was recorded for broadcast across Africa by National Geographic and still plays to this day. A dream came true in having an article about the Sungazer and the genetic tools used to regulate its trade was published in three international editions of the National Geographic magazine in 2019. Having these stories emanate from the NGS brand without a doubt allowed the story to go further, and be heard by more people than would have been possible without the society and their support.

Between 2017 and 2018, I also embarked on my first filmmaking endeavour. With funding and support from Pragna Parsotum-Kok and Noel Kok from the Nature Environment and Wildlife Filmmaking Congress (NEWF), I produced a short documentary about the illegal trade in South African reptiles (https://youtu.be/v3TmKQ5dzKM). Producing an eight-minute documentary turned out to be a much larger task than I had envisioned, and deadlines for the documentary often clashed with deadlines for my PhD. Despite the stress of this experience, going through the process of producing a documentary, even though it is only eight minutes in length, was a very important learning curve for me. This documentary has been entered into several festivals and competitions and aired on South Africa's longest running TV show – 50|50 – which endeavours to tell stories of humans and our interactions with nature. In the end, I think that the film was successful in its aim – to get conversations started about the impacts that the pet trade has on reptiles.

Ultimately, the photographs, videos, radio interviews, popular articles, and public talks about the Sungazer and how it is affected by the illegal reptile trade relate little to the aims and findings of this thesis, but the victories against the illegal reptile trade would not have been possible without it. Many of the endeavours in battling the trade and telling its story took time away from my PhD, but I hope that in time, the collective storytelling through media endeavours, and the scientific work of this thesis tell one cohesive story of the Sungazer and the lengths that people went through to conserve it.

GLOSSARY OF ABBREVIATIONS, ACRONYMS, TERMS & UNITS

$A_{260/280}$	ratio of absorbance at 260 nm and 280 nm
AMOVA	analysis of molecular variance
ANOVA	analysis of variance
AOO	area of occupancy
asl	above sea level
AUC	area under curve
BI	Bayesian Inference
bp	base pairs
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
ddH_2O	double distilled water
df	degrees of freedom
DNA	deoxyribonucleic acid
EOO	extent of occurrence
g	grams
GIS	Geographic Information Systems
Highveld	short temperate grasslands in the high elevation regions of southern Africa
IBD	isolation by distance
IUCN	International Union for the Conservation of Nature
F_{ST}	proportion of the total genetic variance contained in a subpopulation (the S subscript) relative to the total genetic variance (the T subscript).
km	kilometre
ML	Maximum Likelihood
mm	millimetre
mtDNA	mitochondrial DNA
MYA	million years ago
NDF	Non-detriment finding
nDNA	nuclear DNA
ng	nanogram
PCR	polymerase chain reaction
r^2	coefficient of determination
SAMOVA	spatial analysis of molecular variance
SANBI	South African National Biodiversity Institute
SVL	snout-vent length
µl	micro litres

STRUCTURE OF THE THESIS

This thesis is organised into five chapters. It is bookended by general introduction and conclusion chapters that contextualise the research of this thesis. The three central data chapters employ the use of several molecular genetic techniques, some of which are used multiply between chapters. The sampling methods and laboratory protocols are therefore repeated to some extent in the methods sections of these data chapters. The thesis is structured as follows:

Chapter 1. General introduction: Theoretical framework, problem statement and study species
The theoretical framework of the thesis is outlined, and the problem statement and overarching aims of the thesis are provided. The study species, *Smaug giganteus*, is introduced and aspects of the life-history, ecology, conservation status, and research history of the species are detailed.

Chapter 2. Development of microsatellite loci for the threatened Sungazer lizard (*Smaug giganteus*), and the efficacy of non-invasive sampling
I detail the development of polymorphic microsatellite markers that are integral to the investigations of contemporary population and social structure in *S. giganteus* in the subsequent chapters. I quantify DNA extracted from different tissue types and compare their success rates in microsatellite profiling to assess whether non-invasive sampling can successfully be used in genetic studies on the species.

Chapter 3. Population structure of the Sungazer (*Smaug giganteus*)
I investigate the population structure of the Sungazer and identify historical and current events and landscape features that influence gene flow between populations. A variety of techniques are employed including phylogenetic analysis using mitochondrial and nuclear gene sequences, population structure analyses using microsatellite markers, ecological niche modelling, and an investigation of morphological variation between populations.

Chapter 4. Kin-based sociality in the Sungazer (*Smaug giganteus*)
The social system within three well-sampled Sungazer colonies is investigated. In particular, the relatedness between Sungazers sharing burrows, between nearest neighbours, and of Sungazers within and between colonies is quantified.

Chapter 5. Conclusion: Synthesis, future directions and conservation recommendations
I synthesise and contextualise the findings of this thesis, and highlight avenues of future research. The applications of the findings of this thesis to conservation management are detailed, and a final conclusion is reached.

DETAILS OF PUBLICATIONS AND PRESENTATIONS

Manuscripts in preparation

Parusnath, S., Tolley, K.A., Dalton, D., Kotze, A., Alexander, G. J. Development of microsatellite loci for the threatened Sungazer lizard (*Smaug giganteus*), and the efficacy of non-invasive sampling for the species (In preparation for Journal of Genetics)

Parusnath, S., Tolley, K.A., Dalton, D., Kotze, A., Alexander, G. J. Phylogeography and landscape genetics of the Sungazer lizard (*Smaug giganteus*) (In preparation for Ecology and Evolution)

Parusnath, S., Tolley, K.A., Dalton, D., Kotze, A., Alexander, G. J. Kin-based sociality in the African Sungazer lizard (*Smaug giganteus*) (In preparation for Behavioural Ecology)

Conference presentations delivered

Parusnath, S., Cunningham, M.J., Little, I. T., Jansen, R., Alexander, G. J. 2015. Saving Smaug: Conservation of the Sungazer (*Smaug giganteus*). Southern African Rufford Small Grants Conference. Kirstenbosch Gardens, South Africa.

Parusnath, S., Dalton, D., Kotze, A., Alexander, G. J. 2016. How to save your dragon: Conservation genetics of the Sungazer (*Smaug giganteus*). 7th Annual National Zoological Gardens Research Symposium. Pretoria, South Africa (Best PhD student award)

Parusnath, S., Dalton, D., Kotze, A., Alexander, G. J. 2017. Conservation genetics of the Sungazer (*Smaug giganteus*). 13th Conference of the Herpetological Association of Africa. KwaZulu-Natal, South Africa (Best student award – second place)

Parusnath, S., Tolley, K.A., Dalton, D., Kotze, A., Alexander, G. J. 2019. Complex sociality in the Sungazer (*Smaug giganteus*). 14h Conference of the Herpetological Association of Africa. Cape St. Francis, South Africa (Best PhD student award)

A NOTE ON LOCATIONS

Smaug giganteus is a highly sought-after species in the illegal pet trade, and is frequently poached from the wild for this purpose. Precautions have therefore been taken to protect the locations of Sungazer colonies sampled in this study. The precise location of sample sites on maps in this thesis have been altered such that they cannot be used by poachers to pinpoint a specific colony. The popular citizen science website, iNaturalist, obscures records of sensitive species by placing the record within a 0.2 x 0.2 degree grid, and randomising the displayed location within this grid (see www.inaturalist.org/pages/geoprivacy for more information). I have used the same system here, so that the reader still has a visual reference of the sampling regime and approximate locations from where the genetic stock originates. As it stands, the maps presented in this thesis do not present any novel, detailed locations of Sungazer colonies above what is readily available online or in field guides.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Theoretical Framework

A fundamental challenge in the study of organismal biology is establishing a link between the evolution of species and their environments (Bohonak 1999). Species evolve alongside, and as a result of changes in geology, climate, and other environment variables within their fundamental niche (Darwin 1859). The partitioning and distribution of a species into its constituent populations within a landscape are in turn determined by the processes of vicariance and dispersal (Rosen 1978, Wiley 1988, Ronquist 1997). As these processes act on a species and its populations through time and space, so changes the genetic makeup of individuals within populations through selection, drift, and random mutations (Lande 1976, Allendorf 1986). As populations form, expand and isolate, these processes affect their innate genetic diversity and structure (Prugh et al. 2008, Pinho and Hey 2010). Measures of genetic diversity and structure within and between populations therefore indicate the interaction that a specific population has had with other populations, and how the composition of the landscape has influenced these interactions. Examining genetic structure and differentiation between populations can provide insight into the biotic and abiotic factors that have influenced population structure in a species, in the past and present.

The scale at which spatial genetic variation occurs is typically proportional to the dispersal distance of individuals (Schweizer et al. 2007, Heath et al. 2012). As geological, climatic, and anthropogenic processes drive changes in the composition of a landscape over time, the capability of individuals to reach and inhabit the entirety of the landscape changes. The respective ability or inability of individuals to reach the constituent populations of a species results in an intricate network of genetic exchange within and between these populations (Bohonak 1999, Manel et al. 2003, Avise 2009). The dispersal ability of individuals can vary greatly both intra- and interspecifically (Slatkin 1985, Bowler and Benton 2005, Garant et al. 2007) and can range from highly sedentary (resulting in low gene flow and strong population structuring) to highly mobile (resulting in high gene flow and weak population structuring) (Peterson and Denno 1998, Habel and Schmitt 2009, Avise 2009). The ability of dispersing individuals to inhabit and navigate their environment in the past results in the various forms of genetic structure inherent in extant populations in the present.

The interruption of dispersal patterns and therefore gene flow by means of barriers results from the process of vicariance (Rosen 1978, Wiley 1988, Ronquist 1997). Typically, vicariance arises as the result of geological changes such as the formation of mountain ranges, bodies of water, islands (Macey et al. 2000, Veith et al. 2003, Hayes and Sewlal 2004, Lores and Raya 2006, Poulakakis et al.

2012), or geoclimatic processes such as climate change or climatic gradients (Matthee et al. 2004, Douglas et al. 2006, Bryson et al. 2012). Theoretically, if a panmictic population experiences fragmentation through vicariance, the resulting subpopulations will be genetically identical. However, over time, these vicariant subpopulations may diverge as a result of local adaptation or the injection of new alleles through migration events (Whitlock and McCauley 1999, Lenormand 2002). The changes in these subpopulations are fixed over time by genetic drift, selective pressures, and the accumulation of different, often novel mutations in the gene pools of these populations. Eventually, the lack of exchange of genetic information between populations, and local adaptation and fixation leads to divergence. If divergence is sufficient, this may result in genetically differentiated populations, and ultimately, speciation (Dobzhansky 1940, Pinho and Hey 2010, Nosil and Feder 2012). With an understanding of vicariance events within a species distribution, and proposed dates of vicariance, testable hypotheses can be formulated in order to investigate the effects of these events on species population differentiation (Strange and Burr 1997, Macey et al. 2000, Van Veller et al. 2002).

For many species, genetic exchange is restricted to small distances because of the short-range dispersal ability of individuals, even within a completely continuous and homogenous landscape (Slatkin and Maddison 1990, Slatkin 1993, Epperson 1995). This purely spatial limitation to the extent of gene flow between populations can lead to genetic differentiation. This mechanism is known as isolation by distance (IBD) (Mayr 1942, Wright 1943). Isolation by distance patterns are typically strongest for modestly mobile species, whereas species that are either highly mobile or highly sedentary tend to exhibit weaker IBD patterns owing to higher or lower levels of differentiation respectively (Peterson and Denno 1998). Species with lower dispersal ability therefore tend to exhibit fine-scale spatial genetic structure (Schweizer et al. 2007, Heath et al. 2012, Pabijan et al. 2012).

In the modern era, anthropogenic landscape features such as roads (Andrews et al. 2008, Shepard et al. 2008, Jackson and Fahrig 2011), agricultural lands (Glor et al. 2001, Kay et al. 2016, Veach et al. 2017), and urban developments (Noël et al. 2007, Krawiec et al. 2015, French et al. 2018) may also impede gene flow between animal populations. Dispersal is typically reduced in transformed habitats, due to the inability or unwillingness of individuals to manoeuvre between fragments (Saunders et al. 1991, Debinski and Holt 2000, Couvet 2002, Quesnelle et al. 2013). This increases geographical isolation in populations that experience limited rates of gene flow (Slatkin 1987, Hellberg 2009, Sexton et al. 2014), predisposing them to a loss of genetic diversity (Lacy 1987, Garner et al. 2005, Pinsky and Palumbi 2014). As a result, many animal populations living in and around urban or other heavily transformed areas have lower genetic diversity than population in untransformed areas (Delis et al. 1996, Delaney et al. 2010, Munguia-Vega et al. 2013, Beninde et al. 2016). In most cases, the effects of anthropogenic landscape changes are more perceptible in the genetic health of species with short generation times, because the proliferation of homozygous alleles that result from inbreeding and lowered genetic diversity occurs more rapidly in these populations (Ewers and Didham 2006). Conversely, species with long generation times may experience genetic lag, where the effects of disrupted gene flow are only detected many years or decades after the cause (Metzger et al. 2009, Uezu and Metzger 2016).

Losses in genetic variation can also occur through population bottlenecks after colonisation, through genetic drift which causes loss of alleles and changes in allele frequencies, limited migratory options, and inbreeding (Chakraborty and Nei 1977, Allendorf et al. 2007). Inbreeding results from the aggregation of related individuals within isolated fragments (Benirschke 2006, Frankham 2006, O'Grady et al. 2006), and reduces individual and population fitness. In turn, inbreeding may result in supressed evolutionary responsiveness in the face of changing environmental conditions, and increased population extinction (Lande 1993, Frankham and Ralls 1998, Crispo et al. 2011). Inbreeding depression can be alleviated with very small amounts of gene flow, and an understanding of population processes can therefore provide valuable input for conservation management strategies that aim to aid the long-term survival of a species in its habitat (Segelbacher et al. 2010, Van Strien et al. 2014, Bertorelle et al. 2015).

Historical patterns of gene flow between populations also affect extant inter- and intrapopulation genetic structure to an extent. The nature of these historical scenarios, such as the number and location of populations, and mode and pace of colonization are associated with characteristic patterns of extant spatial genetic variation (Emerson and Hewitt 2005, Himes et al. 2008, Chiucchi and Gibbs 2010, Epps and Keyghobadi 2015). Populations that have been colonised from multiple source areas typically show strong genetic structure, with unique alleles or haplotypes confined to distinct regions (Nagy et al. 2003, O'Brien et al. 2009, McHugh et al. 2014). In contrast, populations that originate from a single source typically lack genetic structure, and have geographically widespread, shared haplotypes or alleles and high diversity (Filardi and Moyle 2005, Pulgarín-R and Burg 2012, Bradler et al. 2015). Founding populations are typically less genetically diverse than the populations that their constituent members originate from, having a limited representation of alleles due to the limited subset of individuals involved in the introduction event (Nei et al. 1975, Leberg 2002, Excoffier et al. 2009). Quantifying the genetic diversity of populations can therefore provide insight into the events that resulted in the formation of a particular population or set of subpopulations.

An effective way to study the events and mechanisms that drive the genetic differentiation of populations is to combine various knowledge bases for a species – geography, ecology, life-history, and genetics – in the integrative discipline of phylogeography (Avise 1987, 2000, Emerson and Hewitt 2005). Phylogeographic studies aim to explicitly link genealogy and geography, and investigate the historical factors that have influenced the contemporary geographic distributions of gene lineages (Kuchta and Meyer 2001, Wiley and Lieberman 2011). Phylogeographic analyses of spatial genetic structuring can also be used to identify cryptic species which are morphologically indistinguishable (Rissler and Apodaca 2007, Hughes et al. 2018), and make inferences about the broader historical patterns for a given geographic region through comparative phylogeographic analyses between species (Rosen 1978, Bermingham and Moritz 1998, Arbogast and Kenagy 2001).

Landscape genetics is another modern research tool that allows for the incorporation of detailed spatial information into genetic analyses (Spear et al. 2005, Storfer et al. 2007, Wagner and Fortin 2013). This spatially-explicit technique combines the use of fast-evolving microsatellite markers with novel statistical analyses, enabling the identification of landscape features (e.g. geological, climatic, or anthropogenic variables) that affect population genetic structure (Wang et al. 2009, Wagner and Fortin 2013). As such, landscape genetics can be used as an alternative to or in conjunction with phylogeography, especially in cases where the evolutionary changes being examined are most recent or fine scale (Wang 2010, Lind et al. 2011, Rissler 2016). Spatial genetic patterns can be visualised through the parallel use of geographical information systems (GIS), whereby genetic data is overlaid onto landscape variables (e.g. specific climatic datasets, landcover, vegetation type, or soil or underlying rock type) to interrogate hypotheses of the causes of these patterns (Kozak et al. 2008, Gurrutxaga et al. 2010, Chan et al. 2011). Identifying landscape features that facilitate or constrain gene flow can assist in identifying priority areas for conservation (Neel 2008) through corridor design that maximises functional connectivity (Mech and Hallett 2001, Storfer et al. 2007, Epps et al. 2007, Braunisch et al. 2010, Christie and Knowles 2015).

Molecular genetics analyses have become a valuable tool in conservation management (Greig 1977, Milligan et al. 1994, Crandall et al. 2000, Hoban et al. 2013, Vernesi and Bruford 2015), particularly for threatened species (Frankham et al. 2014, Whiteley et al. 2015, Willoughby et al. 2015, Grosser et al. 2017). The extinction risk of threatened populations and species was in the past assumed to be related solely to environmental and demographic changes, but genetic structure and diversity are now understood also be significant factors that influence the short and long term destiny of populations (Lande 1993, Spielman et al. 2004, Frankham 2010, Kyriazis et al. 2019). Taxa that exhibit small, declining population sizes may be prone to increased genetic drift and inbreeding, resulting in a loss of genetic diversity and individual fitness over time (Willi et al. 2007, Frankham 2010, Haag et al. 2010, Rivera-Ortíz et al. 2015). In turn, this can reduce the ability of a population to adapt to changing environmental conditions, subjecting the population to heightened extinction risk (Burkey 1989, Lande and Shannon 1996, Spielman et al. 2004, Frankham 2010).

The detection of decreased genetic diversity at species or population level allows for mitigation through, for example, augmented genetic exchange or the maintenance of corridors for gene flow (Tewksbury et al. 2002, Christie and Knowles 2015, Thatte et al. 2016). A species level study of genetic structure can also allow for the identification of evolutionary significant units that should be prioritised for conservation (Moritz 1994, Fraser and Bernatchez 2001, Mee et al. 2015, Carlson et al. 2016). Consideration of evolutionary distinctiveness from a systematic level can identify priority species for conservation based on their evolutionary distinctiveness (Vane-Wright et al. 1991, Cadotte and Jonathan Davies 2010, Tonini et al. 2016). Genetics can also serve an important function in translocations. For example, individuals can be selected based on their genetic make-up for translocations to minimise the probability of inbreeding and reduced genetic variation, which would render the translocated population vulnerable to extirpation (Allendorf et al. 2007, Weeks et al. 2011, Averill-Murray and Hagerty 2014). Additionally, based on gene flow patterns between populations, reserves or protected areas can be established or enhanced to ensure the continued exchange of migrants, and thus genes, between populations, or to repopulate depleted populations (Tewksbury et al. 2002, Rouget et al. 2006, Christie and Knowles 2015). Knowledge of the population genetic structure and diversity of species are therefore important sources of information to feed into effective conservation management plans (Pertoldi et al. 2007, Bertorelle et al. 2015, Taylor et al. 2017).

Modern molecular techniques also offer a wide range of options in answering questions of evolutionary changes over time (Bertorelle et al. 2015, Vieira et al. 2016, Charlesworth and Charlesworth 2017). Depending on the scale of the intended investigation, different types of molecular markers can quantify the relatedness between individuals, connectivity between populations, or relatedness between species and higher level taxa. The level of detail and precision at which genetic studies are conducted are no longer limited by technology, but rather access to the database of genetic material and demographic information to make them possible. Mitochondrial and nuclear gene sequences have long been used in phylogenetic studies, and are still widely utilised in investigations of inter- and intraspecies analysis (Springer et al. 2001, Jansa et al. 2006, Lecompte et al. 2008). Mutations occur more frequently in mitochondrial genes, accumulating 10-20 faster than in nuclear genes (Vawter and Brown 1986, Zhao 2017). This makes mitochondrial genes more suitable for use in resolving comparatively young evolutionary relationships (Lin and Danforth 2004, Galewski et al. 2006, Omote et al. 2015), whereas nuclear genes may be more appropriate for the investigation of deeper temporal divergences (Friedlander et al. 1992, Amrine-Madsen et al. 2003, Avila et al. 2013). Ideally, a combination of mitochondrial and nuclear genes selected for a particular situation is best for assessing phylogenetic relationships within a study system (Yoder and Yang 2000, Overton and Rhoads 2004, Wuster et al. 2018).

Whereas mitochondrial and nuclear gene sequences are typically employed for broad scale investigations of genetic structure, rapidly evolving markers such as microsatellites allow for investigations of fine-scale, contemporary patterns of genetic exchange (Schlotterer 2000, Guichoux et al. 2011, Flanagan and Jones 2019). Microsatellites are short regions (10s – 100s of base pairs) of DNA composed of tandem repeats of nucleotide repeats (Wright and Bentzen 1994). The rapidly mutative and polymorphic nature of microsatellites make them ideal markers for investigations of fine-scale population structure, (Fredsted et al. 2005, Wiens et al. 2010, Ribout et al. 2019), assessments of relatedness between individuals (Holderegger and Wagner 2006, Wagner et al. 2006, Hedrick et al. 2015), and parentage analysis (Dakin and Avise 2004, Flanagan and Jones 2019). Microsatellites have also been useful in identifying poaching hotspots by isolating the geographic origin of confiscated wildlife contraband (Mondol et al. 2015, Ogden and Linacre 2015, Wasser et al. 2015). Because microsatellites are tolerant to low quality samples and have high efficacy and specificity with the species they were designed for (Selkoe and Toonen 2006, Schoebel et al. 2013), they have been widely utilised over the past several decades (Hodel et al. 2016, Vieira et al. 2016). Combining data from a combination of appropriate mitochondrial and gene sequences with microsatellite markers can allow for the investigation of the evolutionary history of a species at different temporal scales (Eggert et al. 2008, Charruau et al. 2011, Hui et al. 2017, Serrao et al. 2018)

1.2. Reptiles and genetics

Molecular genetic techniques are particular useful in studies of genetic population differentiation in species that are rare, elusive, and difficult to study using traditional long-term ecological or behaviour studies (Shaffer et al. 2015). Although molecular genetic research of wild animal populations began to proliferate in the 1980s, these studies focused primarily on mammals and birds (Mack et al. 1986, Sage and Wolff 1986). Genetic research on reptiles lagged behind, only gaining momentum in the late-2000s (Shaffer et al. 2015). Reptiles are a diverse class with more than 11 000 species described to date (Uetz et al. 2020), and occupy oceans, fresh water, and terrestrial habitat across six continents. Reptiles range vastly in form and niche - from large apex predators, to miniscule organisms that do not break water tension when traversing it (Seigel et al. 1987, Pianka and Vitt 2003). Home range size and dispersal ability amongst reptile species are therefore extremely variable (Stickel and Cope 1947, Rose 1982), and various landscape features and environmental variables may therefore influence population structure among species differently. For example, rivers have been recognised as significant barriers to gene flow in many reptile species (Pounds and Jackson 1981, Pellegrino et al. 2005, Paquette et al. 2007, Ratsoavina et al. 2012, Souza et al. 2013), but can also serve as important conduits for gene flow in others (Burbrink et al. 1998, Casper 2012, McCartney-Melstad et al. 2012), in some cases even facilitating range expansion (Smissen et al. 2013). The system of sociality within a species can also affect the overall genetic structure of the species and its constituents populations, since dispersal, mating systems, and group living influence the spatial distribution of genotypes (see Pearson et al. 2020 and references within). This can in turn lead to genetic differentiation at a local scale. Consideration of the life-history, social system, and ecological niche of a reptile species is therefore integral in understanding how a specific vicariant event may influence its population structure.

A commonality amongst reptiles however, is that as an ectothermic group, climate variability spatially and temporally are significant in determining where reptile populations do and do not occur within a landscape (Vitt and Caldwell 2013). As such, the dispersal ability of reptiles and range of climes that they can inhabit are often significantly different from birds and mammals within the same regions (Tingley and Dubey 2012, Smissen et al. 2013, Rolland et al. 2018, Saladin et al. 2019). The reliance of most species of reptiles on suitable basking spots for conductive heat transfer (Black et al. 2019) has also been found to influence population structure in some species (Clark et al. 2008, Blair et al. 2013, Nelson-Tunley et al. 2016). Past (Matthee et al. 2004, Douglas et al. 2006, Muñoz-Mendoza et al. 2017), present (Clark et al. 2011), and future scenarios (Araújo et al. 2006, Tolley et al. 2009, Bickford et al. 2010, Boyle et al. 2016) of climate change will continue to be important factors in understanding and predicting genetic structure in reptile populations.

Reptile species also vary in their response to human-mediated landscape change. Some species are more vulnerable to becoming road kill than others (Colino-Rabanal and Lizana 2012), and this is largely dependent on the size and mobility of the species (Shine et al. 2004, Andrews and Gibbons 2005). Roads have therefore served as important barriers to gene flow in some, leading to higher genetic differentiation in areas isolated by roads than in continuous habitat species (Clark et al. 2010, Griffin 2015). Agricultural areas are comprised of complex matrices of pristine habitat interspersed with unsuitable transformed landscapes. The persistence of gene flow between populations depends on the ability of individuals to move through this matrix (Hoehn et al. 2007, Watling et al. 2011, Munguia-Vega et al. 2013, González-Fernández et al. 2018). For many reptile species, agricultural land transformation results in heightened genetic differentiation between populations because of compromised genetic exchange (Smith et al. 2009, Row et al. 2010, Sunny et al. 2015). In other species, genetic differentiation is prevented by gene flow if patches of suitable habitat are interconnected (Sumner et al. 2004, Meister et al. 2010). The effects of habitat transformation may also manifest differently in species at variable temporal scales (Richmond et al. 2009). While some long-lived species show signs of increased genetic structure in response to recent habitat modification (Moore et al. 2008), other species may experience delayed genetic effects of habitat fragmentation (Mccoy et al. 2010). It is therefore important to consider that contemporary levels of genetic structuring of species surviving in heavily fragmented areas may not necessarily reflect the ultimate fate of these populations.

It is clear that investigations into the drivers of population structure in reptiles require knowledge and consideration of many aspects of a species life-history. The integrative use of ecological niche models with genetic studies on reptiles can be useful in understanding how specific environmental variables may correlate with genetic structure for a given species (Clostio 2010, Beninde et al. 2016, Kaliontzopoulou et al. 2018), and how these changing variables may affect a species in future scenarios (Fuentes et al. 2010, Penman et al. 2010, Sillero and Carretero 2013). Knowledge of a species' genetic structure and spatial spread of genetic diversity are also important considerations when planning conservation management strategies for threatened reptile species (Branch et al. 2003, Thomassen et al. 2011, Vasconcelos et al. 2012). Ultimately, knowledge of genetic differentiation between reptile populations and the factors that drive them will surpass the scope of understanding the links between evolution and ecology for the species, and contribute towards their effective conservation in the future.

1.2.1. ***Sociality in reptiles***

Systems of social structure and behaviour have been widely studied in mammals and birds, but reptiles have received significantly less attention in this regard (Bonnet et al. 2002, Stahlschmidt 2011). Several reasons have been put forward to explain this: social behaviours may be cryptic in reptiles and not as openly observable as in birds and mammals (Doody et al. 2013), a bias may exist among researchers against reptiles because of the perceived difficulty in studying them (Pawar 2003), or researchers avoid research on reptiles simply because of a fear or dislike of them (Kellert 1993). Despite the apparent long-standing historical bias, the last two decades have seen a burgeoning trend towards research of social behaviour in reptiles.

Social associations in reptiles range from transient interaction between individuals, to stable aggregations of kin groups across generations (Halliwell et al. 2017b). While egalitarian sociality (aggregations of non-related individuals) in a wide variety of reptiles has long been acknowledged (Kearney et al. 2001, Schutz et al. 2007, Qi et al. 2012, Strickland et al. 2014), research on fraternal social groups has focused primarily on lizards (Gardner et al. 2016, Whiting and While 2017, While et al. 2019). In particular, a large body of research has emerged on kin-based sociality in the Melanesian-Australian Egerniiae subfamily (Family: Scincidae) (consisting of eight genera: *Egernia, Liopholis, Lissolepis, Bellatorias, Cyclodomorphus, Tiliqua, Tribolonotus* and *Corucia;* While et al. 2019), hereafter referred to as the *Egernia* group.

Research on sociality in the *Egernia* group was pioneered by Michael C. Bull, whose seminal body of work began in the 1980s and spanned more than three decades (Godfrey and Gardner 2017). Over this period, Bull and colleagues detailed social monogamy (Bull 1988, 2000, Bull et al. 1998, Gardner et al. 2002), kin recognition (Main and Bull 1996, Bull et al. 2001), site fidelity (Bull and Freake 1999), inbreeding avoidance (Bull and Cooper 1999, Gardner et al. 2012), family structure (Fuller et al. 2005), and stable aggregations (Bull et al. 1998, Gardner et al. 2001, 2007, Duffield and Bull 2002) among other topics in the *Egernia* group. This body of work catalysed many more studies on sociality in more than 20 species in the *Egernia* group over the next several decades (Chapple 2003, O'Connor and Shine 2003, 2004, 2006, Stow and Sunnucks 2004, Chapple and Keogh 2005, Langkilde et al. 2007, Duckett et al. 2012, Whiting et al. 2018). The lowered cost of genetic techniques allowed for empirical evidence to be collected on the long-term population dynamics of related individuals in these species, and together, have brought about a new understanding of the level of complex sociality present within lizards.

The first evidence of kin-based sociality in a species outside of the *Egernia* group was reported in the North American species *Xantusia vigilis* (Family: Xantusidae) (Davis et al. 2011). Kin-groups in this species form through delayed offspring dispersal as in *Egernia striolata, E. stokesii*, and *Liopholis whitii* (Duffield and Bull 2002, Duckett et al. 2012, Halliwell et al. 2017a) amongst others. Although the Xantusidae falls into the Scincoidae superfamily along with the Scincidae (Pyron et al. 2013), this finding resulted in the formulation of the hypothesis that kin-based sociality may arise convergently in lizard species with similar key life-history traits, namely viviparity, delayed offspring dispersal, and limited refuge availability (Chapple 2003, Davis et al. 2011, While et al. 2015, Halliwell et al. 2017b). Kin-based sociality has also since been documented within the Liolaemidae (*Liolaemus leopardinus;* Brito 2017) and Agamidae (*Intellagama lesueurii;* Piza-Roca et al. 2019) families. Although evidence of kin-based sociality has only been found in a single species within each family, these findings speak to the potential presence of these behaviours outside of the Scincoidae superfamily. At least 60 other lizard species in 16 families show some evidence of aggregative behaviour (Gardner et al. 2016), and it is a likelihood that future research employing genetic investigations of relatedness between individuals will continue to unveil systems of kin-based sociality in other lizard species and families.

1.2.2. *Sociality in African lizards*

Research on social structure and sociality in African lizards lags far behind the comprehensive body of research on this topic in the *Egernia* group. Research has focused primarily on the Cordylidae family, which is a sister taxon to the Scincidae and Xantusidae (Pyron et al. 2013). Species in the Cordylidae are highly variable morphologically, but several species are characterised by large body sizes, viviparity and aggregative behaviour (Stanley et al. 2011). *Ouroborus cataphractus* has received the majority of attention, due to the easily observable aggregations of these heavily armoured lizards that form in rock crevices. Research over the past three decades has detailed grouping behaviour (Mouton et al. 1999, 2014b, Visagie et al. 2002, Hayward and Mouton 2007, Mouton 2011, Shuttleworth et al. 2013), and group fidelity (Costandius et al. 2006) in the species. Aggregative behaviour in the species has been ascribed primarily as clustering in proximity to termitaria of their main prey species, the Harvester Termite (*Microhodotermes viator*) (Shuttleworth 2006, Shuttleworth et al. 2013), but the advantages of vigilance behaviour (Mouton et al. 1999, Hayward and Mouton 2007, Mouton 2011) and reduced mate search effort (Effenberger 2004) have also been suggested.

Aggregative behaviour in the family has also been investigated in *Cordylus macropholis*, *Namazonurus peersi*, *Platysaurus broadleyi*, and *Karusosaurus polyzonus* (Nieuwoudt et al. 2003a, Visagie et al. 2005, Schutz et al. 2007, Mouton 2011). In each of these species, individuals did not aggregate in experimental scenarios where refuges were plentiful, and it is therefore possible that aggregative behaviour *in situ* is simply due to limited refuge availability. *Cordylus cordylus* and *C. macropholis* both display aggressive behaviour towards conspecifics, particularly males towards other males (Wirminghaus 1990, Nieuwoudt et al. 2003b). This aggressive behaviour further lends credence to the notion that these species do not preferentially aggregate, since they appear to exhibit territoriality over refuges.

Smaug giganteus (commonly known as the Sungazer), the largest species of the Cordylidae, exhibits a unique form of aggregation in the family. Sungazers live in self-excavated burrows in Highveld grassland that form part of a larger colony. While burrows are often occupied by individual lizards, they are more frequently occupied by small groups (1-7 lizards) that are frequently seen basking and foraging together near the burrow entrance (De Waal 1978, Stoltz and Blom 1981, Jacobsen 1989, Jacobsen et al. 1990, Van Wyk 1992). FitzSimons (1943) suggested that newborns frequently seen with adults were their offspring. Sungazers show high site-fidelity between years (Ruddock 2000), and some individuals have been recorded in the same burrow after more than 20 years (pers. obs., McIntyre 2006). Despite the observations of preferential aggregative behaviour in *S. giganteus* and *O. cataphractus*, no research has investigated the relatedness within aggregations using genetic techniques, precluding further questions on kin-based sociality in these species.

1.3. Problem statement of the thesis

The Sungazer is unique amongst the Cordylidae in its inhabitation of grasslands, reliance on self-excavated burrows for shelter, and spatial organisation into spatially discrete colonies. Despite extensive research on the life history, ecology, physiology, and conservation status of the species, little is known about the factors that determine how Sungazers occupy their distribution. Various natural and anthropogenic landscape features are interspersed across the distribution of the species, but their influences on the genetic structure of the species are unknown. The social behaviour of Sungazers sharing burrows and colonies has long been recognised, but studies on the relatedness between individuals in these interactions have never been done. Key aspects of the life history, ecology and evolution of the Sungazer therefore remain unanswered, precluding a deeper understanding of how the species has evolved to life in the grasslands.

1.4. Aims and objectives

In this thesis, I aim to utilise a combination of molecular, ecological, and morphological analytical approaches to investigate the genetic structure of *S. giganteus* – from the relatedness of individuals sharing burrows, to the characterisation of distinct genetic populations and the processes that have led to the formulation of these populations. In detail, my aims and objectives are as follows:

1) **Assess the use of non-invasive tissue sampling in genetic research on *Smaug giganteus* (Chapter 2)**
    a) Quantify DNA concentration and purity in different tissue types from Sungazers
    b) Test the efficacy of DNA extracted from each tissue type in microsatellite profiling

2) **Investigate the population genetic structure of *S. giganteus* (Chapter 3)**
    a) Develop species-specific microsatellite markers
    b) Conduct a phylogenetic analysis on Sungazers from across the distribution
    c) Conduct population genetic analyses using several models and algorithms
    d) Identify the number of unique genetic populations

3) **Identify how natural and anthropogenic landscape features influence gene flow in *S. giganteus* (Chapter 3)**
    a) Run an ecological niche model for *S. giganteus*
    b) Identify concordance between genetic discontinuities, niche suitability, and landscape features

4) **Investigate morphological differences between Sungazers from different genetic clades (Chapter 3)**
    a) Measure snout-vent length and mass for adult Sungazers in three different clades
    b) Quantify trends in morphology across the distribution of the species

5) **Investigate the presence of kin-based sociality in *S. giganteus* (Chapter 4)**
    a) Investigate the relatedness of Sungazers sharing burrows

b) Identify the parents of juvenile Sungazers and their location in relation to their offspring
c) Investigate the relatedness of candidate parent pairs
d) Investigate the spatial organisation of genetic structure within Sungazer colonies
e) Compare the relatedness of Sungazers within and between colonies

1.5. Study species

Smaug giganteus is an ideal model species in which to investigate population structure and sociality in the Cordylidae for the following reasons:

1. **Grassland habitat -** The majority of the Cordylidae are rupicolous (Branch 1988). *Smaug giganteus* is one of few cordylids to inhabit grasslands, and the only species to create and live in burrows. It therefore presents a unique system to study in order to further understand the adaptations that this diverse family has employed in order to inhabit different parts of sub-Saharan Africa. Grasslands are also one of the most threatened biomes globally (Rouget et al. 2004, Ceballos et al. 2010), and an investigation of the drivers of genetic structure in *S. giganteus* will provide insights into how the loss of grasslands may affect the species.

2. **High site fidelity -** Sungazer burrows represent significant investments of time and energy and therefore appear to be utilised by the same inhabitant(s) over long periods of time (at least 20 years; pers. obs., McIntyre 2006). This makes the collection of tissue samples from a known individual possible over time simply by returning to the same burrow. Samples can also be collectively non-invasively and the GPS location of the Sungazer taken without having to capture or disturb the individual.

3. **Colony structure -** An investigation of social groupings in lizards requires an understanding of what defines a social group for a particular species. This may be complicated in situations where delineating where one group starts and other ends can be difficult. Sungazer burrows and the colonies that they belong to may represent two levels at which social groups may form, and the permanent location of burrows makes this easy to quantify spatially.

4. **Life history -** Sungazers have numerous life-history traits in common with lizard species that are known to exhibit kin-based sociality. Key among these traits are live birth, late age of first reproduction, small litter size, and long lifespan. The species also occurs in an environment in which refuges are limited, which may promote stable aggregations. This makes it an ideal candidate species from the Cordylidae to facilitate comparisons with other social lizard species.

5. **Conservation status -** The Sungazer is a Vulnerable species (Alexander et al. 2018) due to the severe levels of habitat and population decline resulting from crop farming, and poaching for the pet trade. This provides a strong motivation to investigate the species' genetic structure and the influence of different landscape features on it, since understanding these factors may contribute towards more effective conservation management planning for the species.

1.5.1. ***Classification and biology***

The Sungazer (*Smaug giganteus*) is the largest (snout-vent length (SVL) > 183 mm) species of the Cordylidae - a family of lizards endemic to sub-Saharan Africa (Branch 1988, Van Wyk 1992, Stanley et al. 2011). A taxonomic revision of the family in 2011 assigned the species to the novel genus *Smaug*, along with seven other large-bodied species previously belonging to the genus *Cordylus* (Stanley et al. 2011). *Smaug giganteus* is estimated to have diverged from the *Smaug warreni* complex approximately 20.5 MYA (Stanley and Bates 2014), and has been ranked in the top 20 most evolutionarily-distinct threatened squamate species (top 0.5% of all assessed squamate species; Tonini et al. 2016).

The common name of 'Sungazer' is derived from the distinctive posture that individuals adopt when basking, in an anterior body up position with their heads pointing upwards as if looking at the sun (Branch 2001). One of the few cordylids to inhabit grasslands, Sungazers live in self-excavated burrows in hard Highveld clay soil and generally restrict movements for foraging and thermoregulation to a 1 m^2 area adjacent to the entrance of their burrows (Van Wyk 1992, Ruddock 2000). Sungazers are heavily-armoured, their dorsal surfaces, head, limbs and tail covered with ossified keeled spines (Branch 1988, Van Wyk 1988). Their limbs are relatively short and stocky compared to other species in the family, and their morphology appears to have evolved for mechanical defence rather than fleeing (Losos et al. 2002, Broeckhoven et al. 2015, 2018). When approached by a predator, Sungazers descend into their burrows headfirst and anchor themselves in the burrow using their dorsal and occipital spines, and lash out at the predator with their thick muscular spiked tails (Marais 1984, Fogel 2000, Losos et al. 2002).

1.5.2. ***Distribution***

Smaug giganteus is endemic to the Highveld grasslands of the northern Free State and southern Mpumalanga provinces of South Africa, forming two geographically disjunct populations (Fig. 1.1). The species has previously been reported to occur in the KwaZulu-Natal Province (Bourquin 1993, 2004, Lambiris and Bourquin 1993), but Armstrong (2011) suggests that this is incorrect. Furthermore, Armstrong (2011) reported that records of *S. giganteus* in KwaZulu-Natal were the result of translocations from other provinces, and not based on indigenous populations. Records from western Lesotho (Ambrose 2006) have also been considered to be doubtful (Mouton 2014).

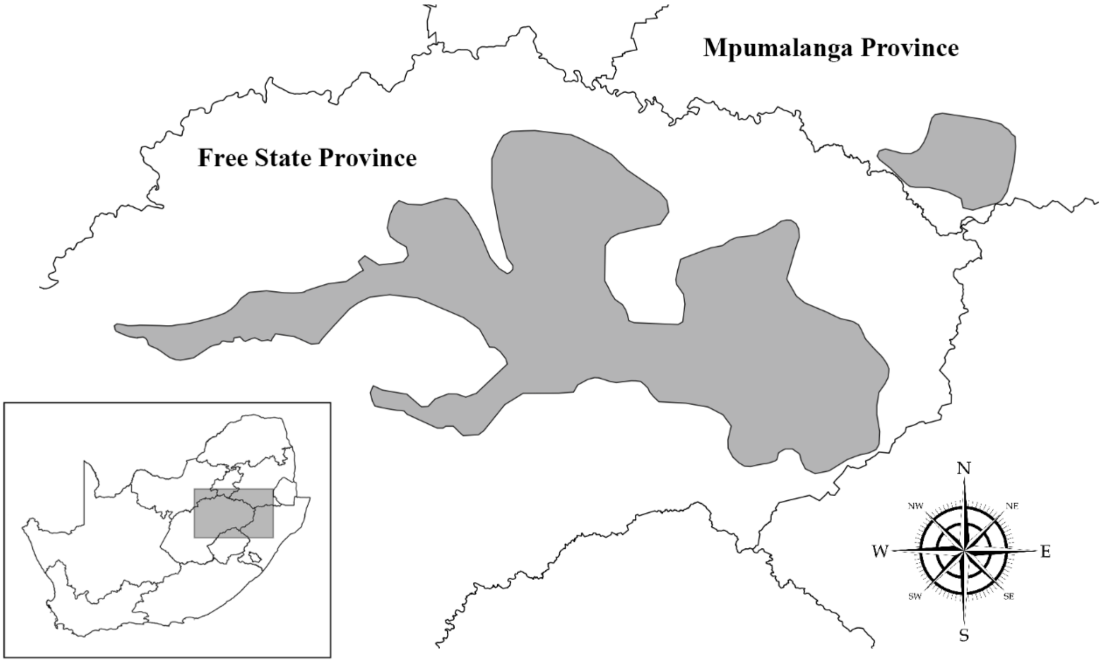


Figure 1.1. Interpreted distribution of *Smaug giganteus* (grey shading) (adapted from Parusnath et al. 2017) within the Free State and Mpumalanga provinces of South Africa.

1.5.3. *Environmental niche*

Smaug giganteus occurs between the elevations of 1260 m and 1840 m, with the highest population densities occurring between 1550 m and 1750 m (Parusnath 2014). Sungazers are typically found on flat or gently sloping land (De Waal 1978, Van Wyk 1992), and 80% of the distribution is comprised of level land with a gradient lower than 30% (Parusnath 2014). The range of the species is underlain by sandstones, mudstones and shales of the Beaufort series (Van Wyk 1992). Dolerite intrusions form ridges and escarpments across most of the area (Scheepers 1975, Newbery and Jacobsen 1994). The soil is generally brown and loamy, dominated by Avelon, Escort, Kroonstad and Longlands soil groups (Soil Classification Working Group 1991).

The distribution of *S. giganteus* falls across 31 vegetation groups within the Highveld grassland described by Mucina and Rutherford (2006), yet the species occupies only 10 of the vegetation types within its extent of occurrence (EOO) (Parusnath 2014). More than half of these vegetation types are classified as Vulnerable or Endangered, yet less than 1% of the total area of these vegetation types is formally protected (Parusnath 2014). The region of the Highveld grassland in which *S. giganteus* occurs is characterised by summer rainfall and winter frost. Mean annual precipitation across the distribution ranges from 500 mm in the west to 800 mm in the east, and 600-700 mm is typical for the central area (Van De Wal 1976, Van Wyk 1992). Maximum rainfall (70%) occurs between November and March (Van Wyk 1992). Mean temperature ranges from 18 °C in the east to 24 °C in the west during austral summer (January), from 7 °C in the east to 9 °C in the west during austral winter (July) (Van Wyk 1992).

1.5.4. *Life-history*

Sungazers are visually-orientated, extreme ambush (sit-and-wait) foragers (Jacobsen 1989, Perry 1995, Cooper et al. 1997, Van Wyk 2000). The majority of feeding behaviour occurs near the entrance of the burrow that the lizard occupies, seldom venturing further than a few metres from the burrow to capture a prey item. They feed during eight months of the year (September – April), with Coleoptera, Diplopoda, Hemiptera, Hymenoptera, Orthoptera, and Lepidoptera making up the six major taxa present in their diet (Van Wyk 2000). Cannibalism of neonates of conspecific females has been recorded in wild (Van Wyk 1992, 2000) and captive (Marais 1984) Sungazers.

Sungazers are active from austral spring to austral autumn and appear to brumate in their burrows during the winter period (De Waal 1978, Van Wyk 1988). Breeding is seasonal, although females only reproduce every two or three years, depending on resource availability (Van Wyk 1992). Reproducing females generally give birth to 1-3 live young in the austral autumn (Van Wyk 1992). Males and females reach sexual maturity at four to five years of age, at an average SVL of 165 mm (Van Wyk 1992). Growth is relatively slow and lizards may only reach maximum length in their eleventh year (Van Wyk 1992). Longevity records for Sungazers in captivity show that they can live for up to 25 years (HAGR, 2014). Anecdotal observations from various landowners, some of whom have kept Sungazers in enclosures in the natural environment within the distribution of the species, have claimed maximum longevity of 35 years. The slow reproductive rate, age to maturity and long lifespan are typical of a K-selection life-history strategy (MacArthur and Wilson 1967, Van Wyk 1992).

1.5.5. *Threats*

1.5.5.1. Habitat transformation

Just over forty percent of the primary grassland within the distribution of *S. giganteus* has been transformed, predominantly for crop farming (Parusnath et al. 2017). Although Branch and Patterson (1975) noted that Sungazer burrows reach a depth that would leave them unharmed during ploughing, this is unlikely to be the case since the burrows tend to extend only 50 cm below ground level (Van Wyk 1992). Branch and Patterson (1975) suggested that Sungazers might migrate into fallow fields, but this is also unlikely to be true since there has been no evidence of Sungazer colonies inhabiting anything other than primary grassland. Crop farming is regarded as an irreversible loss of habitat for the species (Van Wyk 1992). Additionally, many Sungazer colonies and pristine grassland have been lost to the development of roads, urban built-up areas, plantations and artificial water bodies (Van Wyk 1992, Parusnath et al. 2017). As a result, 48% of the original population of the species has been lost due to habitat loss, and at least 39% of the remaining population lost due to the effects of habitat fragmentation and illegal harvesting (Parusnath et al. 2017).

Translocation attempts to relocate Sungazers from areas marked for development have been largely unsuccessful (Groenewald 1992). Groenewald (1992) reported that burrow occupancy rates as low as 10% were reported 49 days after a population of Sungazers were translocated to the Golden Gate Highlands National Park. It was argued that this was due to predation by Yellow Mongoose (*Cynictis penicillata*), Suricate (*Suricata suricatta*) and Secretary Bird (*Sagittarius serpentarius*), but is also likely to be a result of the lizards not having suitable burrows in which to live. This latter contention is supported by the fact that lizards generally did not make use of the artificial burrows created for them with soil augers and travelled as far as 1000 m from the initial point of translocation (Groenewald, 1992), exposing themselves to predation. Although formal scientific studies have not been conducted on the Golden Gate Highlands National Park since 1992, it is estimated that less than 2% of Sungazers survived the translocation over the next two years (Groenewald, pers. comm.).

1.5.5.2. Illegal harvesting for the pet trade

Sungazers have been recorded in the international pet trade since at least 1897 (Bateman 1897) and the species remains popular and highly sought after in the trade to this day (Auliya et al. 2016, Parusnath et al. 2017). Species such as *S. giganteus* that have a restricted distribution, low reproductive rate and high level of protection are uncommon in the reptile pet trade and command premium prices due to their high demand (Böhm et al. 2013, Auliya et al. 2016). The high prices demanded for Sungazers serve as an incentive for their illegal capture and exportation (Wulf Haacke pers. comm. in McLachlan 1978, Van Wyk 1988, Auliya 2003), with cases of poaching for the European and American trades being reported since the 1970s (Branch 1988, Van Wyk 1988). Concerns about the effects of over collection of Sungazers for the pet trade led to the announcement of a total ban on exports of *Smaug giganteus* (previously *Cordylus giganteus*) from South Africa (TRAFFIC 1980). This ban was not enforced, although the species was listed on CITES Appendix II in 1981 (UNEP 2020), and permits for the export of Sungazers are supposedly strictly regulated and issued only under exceptional circumstances (Branch 1990).

Despite these efforts to restrict the exportation of wild-caught Sungazers, large numbers of individuals have been exported from South Africa in the subsequent four decades (Parusnath et al. 2017). Japan, the United States of America and Germany are the primary import countries of Sungazers, collectively making up 74.8% of imports over the past three decades (Parusnath et al. 2017). The vast majority of these Sungazers are reported as produced in captivity, although no verified Sungazer breeding facilities exist in South Africa or elsewhere in the world to supply these numbers (Loehr et al. 2016). It is therefore widely suspected that the vast majority of Sungazers that leave South Africa are in fact wild-caught individuals that have been laundered as captive-bred (Loehr et al. 2016, Auliya et al. 2016, Parusnath et al. 2017).

In response to concerns that excessive poaching of Sungazers for the pet trade may be affecting population declines in the species, a non-detriment finding (NDF) was conducted by the Scientific Authority of South Africa in 2015 (SANBI 2015). The Scientific Authority concluded that harvest of wild Sungazers for the pet trade would be detrimental to the species' longevity in the wild, based on its threatened status, level of population and habitat decline, slow reproductive rate, and low fecundity (SANBI 2015). The ratification of this NDF by the Scientific Authority mandated the CITES recommendation that *S. giganteus* may only be exported from South Africa if individuals are F2 generation captive bred. Application for CITES permits to export *S. giganteus* from South Africa have since required verification of F2 captive breeding, and to my knowledge, no permits of this nature have been granted since 2015. Sungazers still regularly appear for sale in the trade (Auliya et al. 2016, pers. obs.), and it is unclear whether these are individuals that were exported prior to 2015, or individuals that have been smuggled out of the country since. Sungazers have been found in the luggage of European nationals travelling back from South Africa as recently as 2016 (Dutch News 2016).

1.5.5.3. Illegal harvesting for indigenous traditional medicine

Sungazers have historically been harvested for use in traditional Sotho and Zulu medicine across the distribution of the species (Petersen et al. 1985). They are purchased by Sangomas (witch-doctors) who powder dried Sungazer carcasses to make potions that purportedly allow a man to achieve harmonious consent from his wife or girlfriend to have multiple partners (pers. obs., Peterson *et al.*, 1985). These potions are sold in small quantities (50 g) and sell for R200 per 'treatment'. McLachlan (1978) reported that three collectors have been known to collect 200 Sungazers in a day. In an investigation of use of animal parts in traditional medicine Cunningham and Zondi (1991) noted that the traditional medicinal trade in South Africa presented a major threat to *S. giganteus*. Ngwenya (2001) subsequently reported that few traders in KwaZulu-Natal Province (outside the range of the species, but historically known to utilise it) mentioned the use of the species, but did note that it was in high demand. Ngwenya (2001) concluded however, that this trade could pose a problem to the conservation of the species, given its restricted geographical distribution and threats posed by habitat destruction.

In recent times however, Sungazers have been found in traditional medicine markets outside of the distribution of the species, in the neighbouring provinces of KwaZulu-Natal and Gauteng (pers. obs., Whiting et al. 2013). Whiting et al. (2013) found that 21.9% of traders at the Faraday market in Johannesburg, (South Africa's second largest traditional medicine market; at least 100km from the closest Sungazer population) had cordylid species for sale. Five traders were recorded selling Sungazer body parts and whole Sungazers, and recently killed Sungazers were observed on sale. Quantifying the large-scale effects of harvesting of Sungazers for the traditional medicine trade is difficult because of the unwillingness of traders to reveal the sources of their stock, and the turnover rate of lizards (Whiting et al. 2013). The plight of the Sungazer is exacerbated by the fact that females breed only biennially or triennially, depending on resource availability (Van Wyk 1992). McKinney (1997) suggests that species with these life-history characteristics are the most at risk from unsustainable harvesting.

1.5.6. ***Conservation status***

Smaug giganteus was first classified as Vulnerable in the first South African Red Data Book - Amphibians and Reptile (RDB-AR) McLachlan (1978), due to the scale of habitat destruction across the distribution of the species, collection of animals for the pet trade, and for use in laboratory dissections. The status was retained in the subsequent RDB-AR (Van Wyk 1988), chiefly because the reassessment was based on the original data with few additions (Van Wyk 1992). The species has since been re-listed as Vulnerable in the first assessment for the IUCN Red Data List (Groombridge 1994), and a subsequent update (Baillie and Groombridge 1996), based on criteria A2cd (suspected > 30% population reduction in the past three generations based on habitat quality and actual or potential levels of exploitation; IUCN 2001 Red Data List Category and Criteria Version: 2.3). The conservation status of the species was reassessed in the Atlas and Red Data List of the Reptiles of South Africa, Lesotho and Swaziland (Mouton 2014), and the Vulnerable status for the species based on a population reduction of 30% over the last 27 years, was inferred from habitat destruction across the Grassland Biome.

A comprehensive conservation assessment of the species by Parusnath et al. (2017) classified the species as Vulnerable under IUCN Red List Criteria A2acd and B2ab (ii–v), based on the small AOO (1 149 km^2), population decline of 39 % over three generations, irreversible impact of habitat transformation, and the perceived level of exploitation for the pet trade. Under new IUCN Red List Categories and Criteria: Version 3.1 (IUCN 2012), this assessment was reclassified as Vulnerable under IUCN Red List Criteria A2bcd and 4bcd (Alexander et al. 2018). Although the species has retained its Vulnerable classification since the original assessment in 1978 (McLachlan 1978), the threats that face the species have now been quantified empirically (Parusnath et al. 2017). If current trends in habitat loss and fragmentation continue, *S. giganteus* may reach the Endangered category by ~ 2050 (Parusnath 2014)

1.6. The history of research on *Smaug giganteus*

The Sungazer is an iconic species in South Africa. Its charismatic appearance, threat status, and unique morphology and habitat within the Cordylidae family have made it a popular study species over the last half century. Subsequent to the description of the species description in 1844 by Andrew Smith, the primary body of research on *S. giganteus* related to quantifying its distribution (De Waal 1978, Jacobsen 1989), and minor observations on the ecology and behaviour of the species (Branch and Patterson 1975, Marais 1984). The conservation concerns of the species were highlighted early on in the development plan for the conservation of South African reptiles and amphibians (McLachlan 1978, Van Wyk 1988, Dodd Jr 1989), and was classified as Vulnerable in its first International Union for Conservation of Nature (IUCN) Red List assessment (McLachlan 1978).

Large-scale land transformation for the construction of power stations within the distribution of the species in the 1980s necessitated the translocation of thousands of Sungazers to new areas. The process of excavating Sungazers burrows provided opportunities to assess several aspects of the species ecology for the first time (Petersen et al. 1985, Jacobsen et al. 1990, Groenewald 1992). Although the translocation attempts proved to be largely unsuccessful long term, the first records of Sungazer burrow density, burrow occupancy, and population demographics were established.

The first significant body of comprehensive research on *S. giganteus* was by Hannes Van Wyk in the late 1980s and early 1990s. His doctoral thesis (Van Wyk 1992) and subsequent publications (Van Wyk 1991, 1994, 1995, 2000) on the life history and physiology of *S. giganteus* were seminal to our current understanding of the species. Through Van Wyk’s research, the feeding ecology, male and female reproductive systems and cycles, growth rates, population dynamics, and thermoregulatory physiology were detailed for the first time. Following on from Van Wyk’s observational studies on Sungazer behaviour, Ruddock (2000) investigated aspects of the social structure of the Sungazer. In particular, Ruddock reported on the movement and interactions between wild-living Sungazers, the chemical composition of their generation glands, and behavioural responses of individuals to the chemical signal of their own glands, and those of their conspecifics. The body of work produced by Van Wyk and Ruddock continues to be highly influential to researchers to this day.

Concerns over the species longevity in the wild as a result of habitat transformation prompted more conservation driven research. McIntyre (2006) investigated the effects of heavy metals on the physiology of Sungazers in mining areas, and found that Sungazers in areas characterised by mining runoff had poorer body conditions and skewed sex ratios compared to Sungazers in undisturbed areas. Parusnath (2014) conducted a comprehensive conservation assessment for the species, amid concerns that no new empirical evidence on changes in Sungazer populations and habitat had been collected since the initial assessment in 1978 (McLachlan 1978). The species retained its Vulnerable status in this assessment, but empirical evidence of the threats facing the species through habitat loss and poaching for the pet trade was quantified for the first time.

Over the past decade, a plethora of studies focused on the Sungazer and its family have produced an updated phylogeny of the Cordylidae (Stanley et al. 2011), insight into the evolution of body armour (Broeckhoven et al. 2015, 2017, 2018), non-invasive methods for monitoring stress (Scheun et al. 2018), thermoregulatory behaviour (Stanton-Jones et al. 2018), a system for remote monitoring of Sungazer activity patterns (Stanton-Jones 2018), and further investigation of the enlarged generation glands in the species (Louw et al. 2007, 2011, Mouton et al. 2014a, 2018).

Despite the significant body of work on this species, several key questions about the evolution, ecology, and social system of *S. giganteus* remain unanswered. The limited use of molecular techniques in investigating these aspects of the species has meant that the genetic structure of Sungazer colonies and populations across the landscape are unstudied. This precludes us from understanding how the species utilises its landscapes, and the processes that drive the formation and maintenance of genetic populations. The lack of use of genetic tools has also precluded empirical investigations of the complex social structure that appears to be present in the species. The research in my thesis aims to address these gaps in our knowledge of the species. Although I do not claim to definitively put these topics to rest, I hope that like the seminal studies on *S. giganteus* before me, my research can contribute to solving a part of the complex puzzle of the biology of the Sungazer.

CHAPTER 2

DEVELOPMENT OF MICROSATELLITE LOCI FOR THE SUNGAZER (*SMAUG GIGANTEUS*), AND THE EFFICACY OF NON-INVASIVE SAMPLING

2.1. Introduction

Genetic studies of species-level systematics and population structure can employ a wide array of molecular tools depending on the scale and scope of the investigation. Modern genetic markers can be used to examine genetic structure and diversity of a species at many resolutions – from characterising and identifying individuals and the relatedness between individuals, gene flow within and between populations, to the phylogenetic relationships of divergent clades. The integrative use of molecular markers that evolve at different rates over time can provide insight into the temporal changes in genetic structure of a species and its populations through its history of vicariance and dispersal events. Mutations occur 10-20 faster in mitochondrial genes than in nuclear genes (Vawter and Brown 1986, Firth and Hurst 2017), and conserved nuclear genes are therefore utilised when older relationships between genetic populations or clades are suspected (Zhang and Hewitt 1996, Lin and Danforth 2004), whereas mitochondrial genes are used for resolving younger evolutionary relationships. However, for more contemporary, fine-scale investigations of genetic patters, more rapidly evolving markers are required (Beaumont 1999, Schlotterer 2000, Vieira et al. 2016). Polymorphic microsatellite markers have emerged as one of the most ubiquitous genetic markers over the past two decades due to the declining costs of developing species-specific marker sets, and their power in detecting contemporary genetic patterns (Ellegren 2004, Selkoe and Toonen 2006, Guichoux et al. 2011).

Microsatellites are short tandem repeats of nucleotide DNA motifs that are 1-6 base pairs in length (Wright and Bentzen 1994). These regions have high mutation rates due to polymerase slippage, and since mutations are heritable between generations, analyses of relatedness between individuals and populations are possible at a fine-scale (Ellegren 2004). Microsatellites have therefore been employed for myriad uses in studies of population and social structure: investigations of fine-scale genetic structure and connectivity between populations (Fredsted et al. 2005, Wiens et al. 2010, Ribout et al. 2019), relatedness between individuals (Blouin et al. 1996, Van De Casteele et al. 2001, Wagner et al. 2006), parentage analysis (Dakin and Avise 2004, Hodel et al. 2016, Flanagan and Jones 2019) and elucidating evidence of genetic structure resulting from human-mediated landscape changes (Cunningham and Moritz 1998, Moore et al. 2008, Goossens et al. 2016). Microsatellites have also found important use in wildlife trades matters: assessing parentage to confirm multigenerational captive breeding for CITES listed species (Lyons and Natusch 2011, Hogg et al. 2018), isolating the geographic origin of confiscated animals or animals products, and identifying poaching hotspots in threatened species (Mondol et al. 2015, Ogden and Linacre 2015, Wasser et al. 2015). Since microsatellites are also effective when used with low amounts of DNA (Beebee 2008, Ford et al. 2016), they are ideal markers in studies where high-quality samples are difficult to obtain.

Genetic studies on reptiles have typically relied on the collection of blood, tissue clippings (e.g. scale, toe, or tail clips), or whole animals (Beebee 2008, Herrel et al. 2012, Maigret 2019) for genetic source material. Although these tissue collection techniques tend to provide large quantities of high-quality DNA, the collection of tissue samples directly from live animals can be technically and logistically challenging, and carry ethical and animal welfare concerns (Parris et al. 2010, McMahon et al. 2012, Lefort et al. 2019). Drawing blood from the caudal vein of reptiles for example yields large quantities of pure DNA, but can result in damage to muscles and the spinal cord if done improperly (Prival 2015). The effects of toe-clipping in reptiles varies amongst species, with no significant effects observed in some (Hudson 1996, Paulissen and Meyer 2000, Borges-Landáez and Shine 2003) but reduced mobility and lower survival rates resulting in others (Bloch and Irschick 2005, Olivera-Tlahuel et al. 2017).The collection of whole animals as specimens can provide a multitude of uses, since the specimen can be deposited in museum collections for additional uses after tissue material has been collected for genetic analysis (Rocha et al. 2014). This practise is ideally avoided however, (Taberlet et al. 1999, Powell and Proulx 2003, Pauli et al. 2010, Smith and Wang 2014) particularly when working with species of elevated conservation status (Russo et al. 2017) since wanton collection of individuals may lead to local extirpation or extinction (Norton et al. 1994, Rodríguez-Estrella and Moreno 2006, Tournant et al. 2012).

Genetic research using non-invasive sampling has become more commonplace over the last two decade, as less harmful and invasive approaches have been tested and utilised in mainstream research (Jones et al. 2008, Ruiz-González et al. 2013, Du Toit et al. 2017, Antognazza et al. 2019). Methods of DNA collection are defined as non-invasive if “the source of the DNA is left behind by the animal and can be collected without having to catch or disturb the animal” (Taberlet and Luikart 1999, Taberlet et al. 1999). Non-invasive sampling typically involves the collection of material such as faeces, hair, or feathers (Piggott and Taylor 2003, Russello et al. 2015, Lefort et al. 2019). Since the animal does not need to be captured to be sampled, it is possible to collect a higher number of non-invasive samples if the material is ubiquitous and easy to find, thereby also potentially reducing sampling costs (Pearson et al. 2014, Russello et al. 2015, Andrews et al. 2018). In reptiles, non-invasive sampling is facilitated primarily through the collection of shed skin. DNA derived from the shed skin of lizards and snakes has been proven to be of sufficient quality and quantity for polymerase chain reaction (PCR) based genotyping (Bricker et al. 1996, Clark 1998, Fetzner 1999, Horreo et al. 2015, Tawichasri et al. 2017).

While there are merits to employing non-invasive sampling, the use of these samples in population genetic studies can also present challenges (Russello et al. 2015, Andrews et al. 2018). For example, unless the sample has been retrieved directly from an animal, the genetic sample cannot be linked to a particular individual. This may not necessarily present a problem for many types of genetic studies – for instance when investigating wide-scale genetic structure of a species or population – but can be problematic when trying assess the relatedness between the individuals in a population. Non-invasive samples may also provide DNA of poor quality and lower quantity relative to more traditional starting materials such as tail clippings or blood. DNA in non-invasive samples begins to degrade shortly after the material is deposited into the environment, typically due to exposure to high temperatures, moisture and UV radiation. This can result in lower DNA yields and fragmentation of template DNA from these samples (Poinar et al. 1996, Jones et al. 2008), and ultimately lower success rates in downstream laboratory protocols (Brinkman et al. 2010, Demay et al. 2013, Lonsinger et al. 2015). Degradation may also be exacerbated by improper storage after collection, since the enzymes that catalyse the degradation of DNA continue to operate if steps are not taken to inhibit them (Seutin et al. 1991, Murphy et al. 2002, Roeder et al. 2004). Testing the efficacy of non-invasive samples in downstream laboratory applications is therefore imperative on a per species basis, since the quantity and quality of different sizes and forms of samples such as shed skins are likely to differ between species.

The Sungazer (*Smaug giganteus*) is a Vulnerable (Alexander et al. 2018), CITES Appendix II (UNEP 2020) listed species of cordylid lizard endemic to the Highveld grasslands of South Africa (De Waal 1978, Jacobsen 1989). The taxonomic status of *S. giganteus* within its genus and family has been detailed using mitochondrial and nuclear gene sequences (Stanley et al. 2011, Stanley 2013), however studies of fine-scale population genetic structure in the species have been precluded because of the lack of availability of rapidly evolving genetic markers. Aside from understanding of how various natural and anthropogenic landscape features influence gene flow between Sungazer populations, insight into the genetic structure of populations would also prove important in considering effective conservation managements for this threatened species (Segelbacher et al. 2010, Keller et al. 2015). The lack of such genetic markers has also impeded empirical studies on sociality in the species (Gardner et al. 2016, Whiting and While 2017). Adult Sungazers are often seen sharing burrows with juveniles, although the relatedness of these interactions are unknown. Similarly, the colonies that Sungazers live in appear to be spatially discrete from other colonies, and the familial nature of these groups remain uncertain. Finally, as a CITES Appendix II listed species, only F2 generation captive-bred Sungazers should be exported from South Africa. Without genetic markers available to conduct relatedness analyses, hundreds of wild-caught Sungazers are believed to have been exported from South Africa falsely labelled as captive-bred individuals (Loehr et al. 2016, Auliya et al. 2016).

The primary aim of this chapter was to develop and test a suite of species-specific polymorphic microsatellite markers for *S. giganteus* for use in studies of contemporary population genetic structure, fine-scale social structure, and verification of F2 generation captive breeding in the species. Since *S. giganteus* is a Vulnerable species with declining populations (Parusnath et al. 2017), non-invasive tissue sampling might prove to be an effective approach in rapidly collecting tissue samples from wild living Sungazers with minimal disturbance, stress or mortality to individuals. I tested the feasibility of utilising shed skin samples collected from their burrows in genetic research. I quantified and compared the quantity and purity of blood, tail clippings, and shed skin samples, and their relative success rates in microsatellite profiling. Since shed skin samples were collected over a long period of time and varied in the period of time in which they were stored before processing, I also compared the success rates of shed skins in microsatellite profiling after 12, 28, 20, and 22 months of storage.

2.2. Materials and methods

2.2.1. *Microsatellite primer development*

Blood samples were collected from two captive Sungazers at the National Zoological Gardens by a veterinarian. These samples were used to prepare a microsatellite enriched library by following the fast isolation by Amplified Fragment Length Polymorphisms (AFLPs) of sequencing containing repeats (FIASCO) protocol (Zane et al. 2002) using the probes: $(AGGG)_4$, $(GTG)_5$, $(GTA)_5$, $(AC)_5$, $(AAAT)_5$, $(ATA)_5$, $(CT)_5$, and $(TGC)_5$. The microsatellite enriched library was then sequenced on the Illumina HiSeq2500 ® platform by Inqaba Biotech (Pretoria, Gauteng, South Africa), generating 6 357 278 paired reads. The enriched DNA was pooled and end repaired, and Illumina compatible adapters were ligated to all fragments. The sample was indexed and size selected using AMPure XP magnetic-bead purification (Agencourt Bioscience, Beverly, USA). The sample was quantified using a NanoDrop™ 3300 Fluorospectrometer (ThermoFisher, Waltham, MA, USA) and the fragment distribution was checked on an Agilent 2100 BioAnalyzer (Agilent, Santa Clara, USA). The library was sequenced on Illumina's® MiSeq platform, following the standard manufacturer's protocol. A 600-cycle kit was used to generate 2GB of 2x300 base pair (bp) paired end reads. I searched the resulting reads for microsatellite motifs between 2 and 6 bp and ≥ 8 repeats in length using MSATCOMMANDER 1.0.8 (Faircloth 2008), and identified a total of 65 536 reads containing microsatellite repeats. I designed primers using PRIMER3 0.4.0 (http://frodo.wi.mit.edu/primer3/; Koressaar and Remm 2007, Untergasser et al. 2012) to amplify a target region of ~250 bp including both primers, with an optimal primer length of 20 bp (range of 18-22 bp), and optimal melting temperature of 60 °C (range of 58-62 °C).

I tested and optimised 40 microsatellite primer pairs on blood samples collected from 28 captive Sungazers by a veterinarian at a private facility in South Africa. I extracted DNA using the ZR Genomic DNA II Kit (Zymo Research) following the standard protocol for blood and hair samples. Polymerase chain reaction amplification was conducted using a 25 µl reaction volume consisting of 12.5 µl Invitrogen Platinum Taq Mastermix (Invitrogen Life Technologies), 2.5 µl Invitrogen GC Enhancer, 2.5 µl bovine serum albumin (BSA), 4 µl ddH2O, 0.5 µl of forward and reverse primers, and 2.5 µl (~50 ng) genomic DNA. Polymerase chain reaction amplification was conducted under the following conditions: denaturation for 2 min at 95 °C, 30 cycles for 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, extension at 72 °C for 40 min, in a T100TM Thermal Cycler (Bio-Rad Laboratories). I pooled and analysed PCR products on an ABI 3130 Genetic Analyzer (Applied Biosystems Inc.), using GenescanTM 500 LIZ® as a size standard. Samples were genotyped using GENEMAPPER 4.0 (Applied Biosystems Inc.) and the incidence of genotyping errors, allele dropout and null alleles were assessed using MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004). The number of alleles per locus, observed heterozygosity (Ho) and expected heterozygosity (He) were calculated with GENALEX 6.5 (Peakall and Smouse 2006, 2012), while deviations from Hardy-Weinberg (HW) proportions and gametic disequilibrium in loci were tested for using GENEPOP 4.2 (Rousset 2008). I corrected probability values for multiple comparisons using Bonferroni adjustment for a significance level of 0.05.

2.2.2. *Testing suitability of tissue types for downstream applications*

I collected shed skin samples (n = 298) and tail clippings (n = 168) from Sungazers in 69 colonies across the distribution of the species between March 2012 and March 2018 (Fig. 2.1). Shed skin samples were collected from the area surrounding the entrance of Sungazer burrows, and kept dry in 1.5 ml Eppendorf tubes (Eppendorf GmbH, Hamburg, Germany) following Fetzner (1999). For tail clippings, I captured Sungazers using standard noosing techniques (Van Wyk 1992, McIntyre and Whiting 2012) and clipped ~3 mm sections of tissue from the large caudal spines of adults, or the terminal tail scute in juveniles with a nail clipper. Tail clippings were stored in 99% ethanol in 1.8 ml Nunc™ Cryogenic Tubes (ThermoFisher, Waltham, MA, USA. The area of incision on the caudal spines were disinfected with F10SC Veterinary Disinfectant spray (Health and Hygiene (Pty) Ltd., South Africa) before clipping the spine, and F10 Germicidal Barrier Ointment (Health and Hygiene (Pty) Ltd., South Africa) afterwards, before releasing the animal at the burrow it was captured from.

Blood samples (n = 28) were collected from captive Sungazers at a private facility in South Africa by a veterinarian. Blood samples were collected from the caudal vein of Sungazers using a 23 gauge needle and 5 ml syringe, and then stored in BD Vacutainer® tubes (BD Vacutainer Systems, Becton Dickinson and Company, Franklin Lakes, NJ) containing an anticoagulant

(Ethylenediaminetetraacetic acid). Equipment and surfaces used during tissue collection were sterilised with 10% bleach (sodium hypochlorite; Kemp and Smith 2005) and rinsed with sterile double-distilled water (ddH2O) between the sampling of each individual. Tissue clippings and shed skin samples were stored at room temperature, and blood samples were stored at -4 °C.

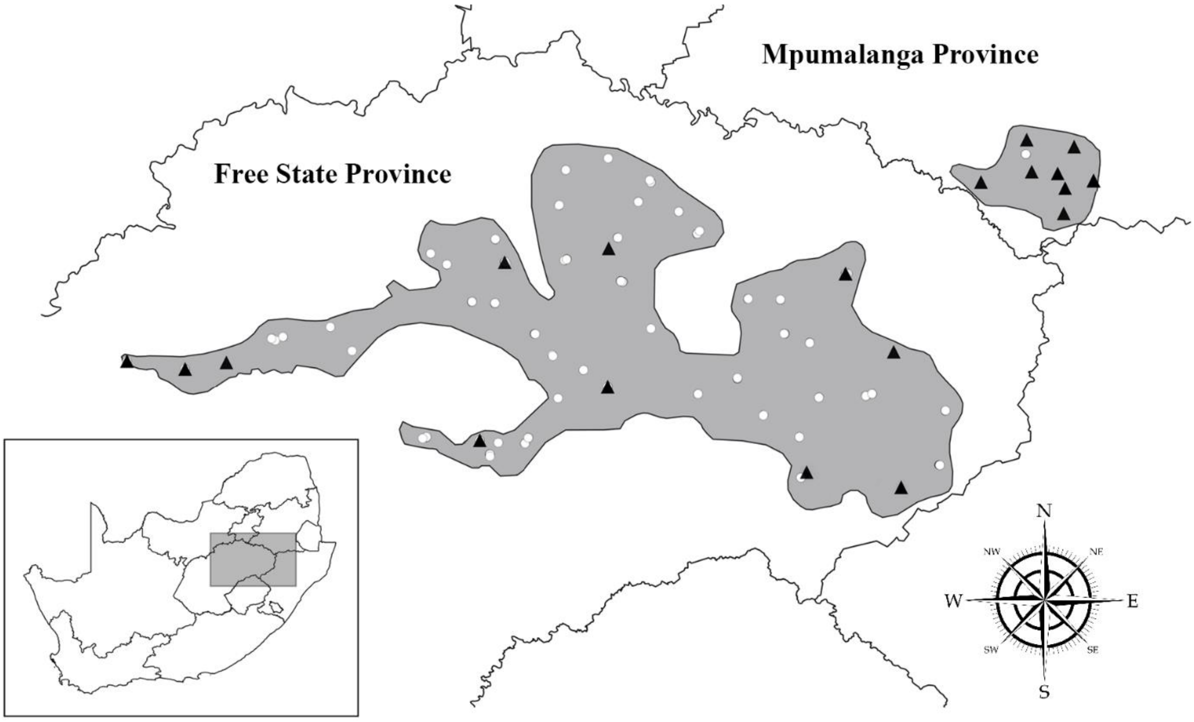


Figure 2.1. Interpreted distribution showing 69 tissue collection sites for *Smaug giganteus*. White circles represent sites where shed skin samples were collected non-invasively, and black triangles represent sites where tissue clippings were collected through active sampling of Sungazers.

Tail clippings and blood samples were processed within three months of collection. Shed skin samples were collected over a longer period of time, and batches of shed skin were processed either within 12, 18, 20, or 22 months of being collected. DNA was extracted from all tissue types using the ZR Genomic DNA II Kit (Zymo Research Corporation, Irvine, Calif., USA), following the manufacturer's protocol for blood and hair. Shed skin samples and tail clippings were homogenised using a Qiagen™ TissueRuptor handheld rotor–stator homogenizer (Qiagen Inc., Valencia, CA, USA) to allow for faster and more efficient digestion of samples, and rinsed with ddH2O to remove soil and other potential contaminants present in the samples. Extracted DNA was stored in 1.5 ml Eppendorf Tubes (Eppendorf GmbH, Hamburg, Germany) at -20 °C. The concentration (ng/µl) and purity (260/280 absorbance ratio ($A_{260/280}$)) of selected DNA samples (shed skin, n = 73; tail clippings, n = 31; blood, n = 27) were quantified using a NanoDropTM 3300 Fluorospectrometer (ThermoFisher, Waltham, MA, USA). I compared the concentration and purity of DNA from different tissue types using one-way analysis of variance (ANOVAs). All samples were then processed using the 18 polymorphic microsatellite markers designed in this study using the amplification and sequencing protocols outlined in section 2.2.1, to assess the success rate of each tissue type in profiling *S. giganteus* microsatellite markers. Since the profiling percentage success rates were not normally distributed across tissue types, I compared success rates for each tissue type using a non-parametric Kruskal-Wallis H test, and used Dunn's multiple comparisons post-hoc test to investigate the differences between each pair compared in the analysis.

2.3. Results

2.3.1. *Microsatellite loci characterisation*

A total of 40 microsatellite loci were tested, of which 18 loci were polymorphic. The number of alleles per loci ranged from 2 to 18. The average observed heterozygosity (Ho) across the 18 loci was 0.644 ± 0.029 (SE) while the expected heterozygosity (He) was 0.669 ± 0.028 (SE) (Table 2.1). The average Shannon diversity index was 1.323 ± 0.0979 (SE). Two loci (SunPen2, SunTet8) showed evidence for null alleles, however no significant deviations from Hardy-Weinberg and linkage disequilibrium following Bonferroni correction were observed for any of the markers tested.

2.3.2. *DNA concentration and purity*

DNA was successfully extracted from all tissue types. The DNA extracted from each tissue type was significantly different in concentration (one-way ANOVA; $F_{1,3} = 37.563$, df = 130, $p < 0.01$) and purity (one-way ANOVA; $F_{1,3} = 15.079$, df = 130, $p < 0.01$). Tail clippings yielded the highest concentrations of DNA (mean 34.99 ng/µl), followed by blood (mean 22.87 ng/µl) and shed skins (mean 7.93 ng/µl) (Table 2.2, Fig. 2.2). Blood however yielded purer DNA ($A_{260/280} = 2.07$) than tail clippings ($A_{260/280} = 1.88$) or shed skin ($A_{260/280} = 1.52$) (Table 2.2, Fig. 2.2).

2.3.3. *Success in downstream application*

The microsatellite profiling success rates of DNA extracted from different tissue types in this study were significantly different (Kruskal-Wallis H test; $H(6) = 271.78$, $p < 0.01$) (Fig. 2.3). Of the possible 21 pairwise comparisons, 16 were significantly different (Dunn's test; $p < 0.01$). The following pairs were not significantly different in profiling success: blood/shed skin 12 months, tail clippings/shed skin 12 months, shed skin overall/shed skin 18 months, shed skin 20 months/shed skin 22 months, shed skin 18 months/shed skin 20 months. Success rates ranged from as low as 13% in shed skin samples processed 22 months after collection, to 82% in blood samples. Shed skin samples improved considerably in their sequencing success rate when the time between collection and processing was reduced to below 12 months. Shed skin samples that were processed with a year of being collected were comparable to tail clippings in profiling success rates.

Table 2.1. Characterization of 18 species-specific microsatellite loci for *Smaug giganteus*. The letters F and R at the end of primer names indicate forward and reverse primers respectively. The nucleotide sequence, fluorescent dye label, size range of primer sequence in base pairs, repeat unit, observed heterozygosity (Ho), and expected heterozygosity (He) are given for each primer.

Name	**Sequence (5'–3')**	**Fluorescent dye label**	**Size range (bp)**	**Repeat unit**	**Ho**	**He**
SunDi1F	TCTTTGGAAAGGAAGAAAACCA	VIC	100–160	(AC)12	0.464	0.631
SunDi1R	ATTGCCTGTGGACAGAAATCTT					
SunDi2F	GGTTGTTTGCTGTGTGGTAAAC	VIC	180–210	(AG)12	0.500	0.408
SunDi2R	TAGGGACATGGGAAGCTATGTT					
SunTri1F	ATTCTCAACTTCAGCAGCTTCA	PET	120–160	(ACG)12	0.679	0.665
SunTri1R	GAGTTTGGTTTTGTGGCTGACT					
SunTri2F	GGCAACACAAAACAACAATTTC	NED	130–210	(ATC)12	0.464	0.458
SunTri2R	CTGAGTGCAAAGCAGATGCTAC					
SunTri3F	AGCAAAAGCTATACGAAGGCAC	NED	160–220	(ACT)12	0.643	0.596
SunTri3R	CAGCCTAACCTACTTCACAGGG					
SunTri8F	TGTACTGCTTGGCTTACTGCAT	NED	130–170	(AAT)19	0.929	0.809
SunTri8R	TCATGATAACTTTTCCATGCC					
SunTet1F	ATGAAAGGTGGCGTATAAGTGG	FAM	100–230	(ACAT)12	0.607	0.516
SunTet1R	GCTGTATTGCCTCCATCTTCTT					
SunTet2F	CCACCCAGAGATGTAAGTGTGA	FAM	160–220	(AAGG)12	0.750	0.755
SunTet2R	ATGCTTTCCAAGAAATCCCA					
SunTet3F	CAGAGACAGAAGTTTGGGGC	FAM	180–230	(ACAG)12	0.464	0.491
SunTet3R	ATGGCAGGAACAGGTAAAGAAA					
SunTet4F	CAGTTTCCTATACTGCTTTTCA	VIC	160–190	(AGAT)12	0.643	0.750
SunTet4R	ATTTGCGACAAATATGCATGAG					
SunTet5F	AGACAGATCACCCTGGAGAGAA	FAM	190–230	(AACC)13	0.679	0.594
SunTet5R	CCACAATAAGTTGTAATGCCCA					
SunTet6F	TACTTTGTGCTGATCTATGACC	NED	190–220	(ATCC)11	0.714	0.765
SunTet6R	TCAATAAATAAGTCCCCCACCC					
SunTet7F	ATGAACCTGAGCCTGATACCTC	PET	140–180	(AAAC)11	0.679	0.581
SunTet7R	TATTACAAGGGGGTCTGCAACT					
SunTet8F	ACCATGTAGAATGTTCTGGGCT	FAM	120–170	(AAAG)13	0.571	0.828
SunTet8R	TTATTGACCAGCTTCCAAAAGG					
SunPen2F	GTAAGAGCAGCTTCTCCTGCTG	VIC	160–220	(AACCT)12	0.429	0.671
SunPen2R	CACCTTTGGGAAACAGAAAGAC					
SunPen3F	CGTTTACCACTGTTTGATGGTG	PET	170–210	AAGAG (9)	0.643	0.739
SunPen3R	AAAGGTGGAGAACCAGAATGTG					
SunPen4F	ATCAAGTGAGTCCATCCACCTT	VIC	180–230	(AAAGG)13	0.786	0.755
SunPen4R	GCCTATGGTCCAGAGTTGTTTT					
SunPen5F	GAATCCAAGGCCTGTTCTCATA	PET	160–180	(ACACC)14	0.893	0.782
SunPen5R	ACAATGTTTGGCGACTTGAGAG					

Table 2.2. Average, range, and standard deviation of concentrations (ng/µl) and purity ($A_{260/280}$) of DNA extracted from *Smaug giganteus* blood, tail clippings, and shed skin samples

Tissue type	**No. of samples**	**DNA concentration (ng/µl)**			**DNA purity ($A_{260/280}$)**		
		Average	**Range**	**Std. Dev.**	**Average**	**Range**	**Std. Dev.**
Blood	27	22.87	3.83 – 54.49	13.69	2.07	1.53 – 2.82	0.38
Tail clipping	31	34.99	2.41 – 82.25	17.18	1.88	1.28 – 2.4	0.52
Shed skin	73	7.93	0.63 – 22.11	5.81	1.52	0.58 – 3.28	0.32

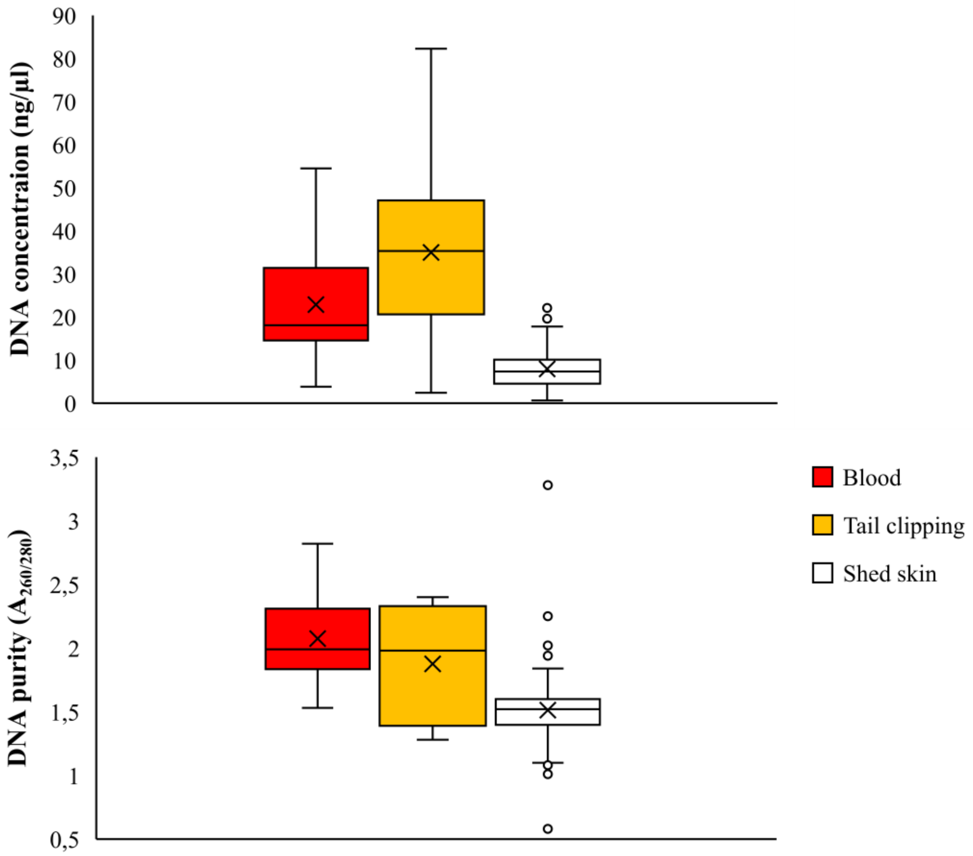


Figure 2.2. Concentration (top) and purity (bottom) of DNA extracted from *Smaug giganteus* blood, tail clippings, and shed skin samples. Box plots show the median, first and third quartile, X indicates the mean, whiskers extend to 1.5 times the interquartile range. Circles indicate outliers, with values greater than 1.5 times the interquartile range.

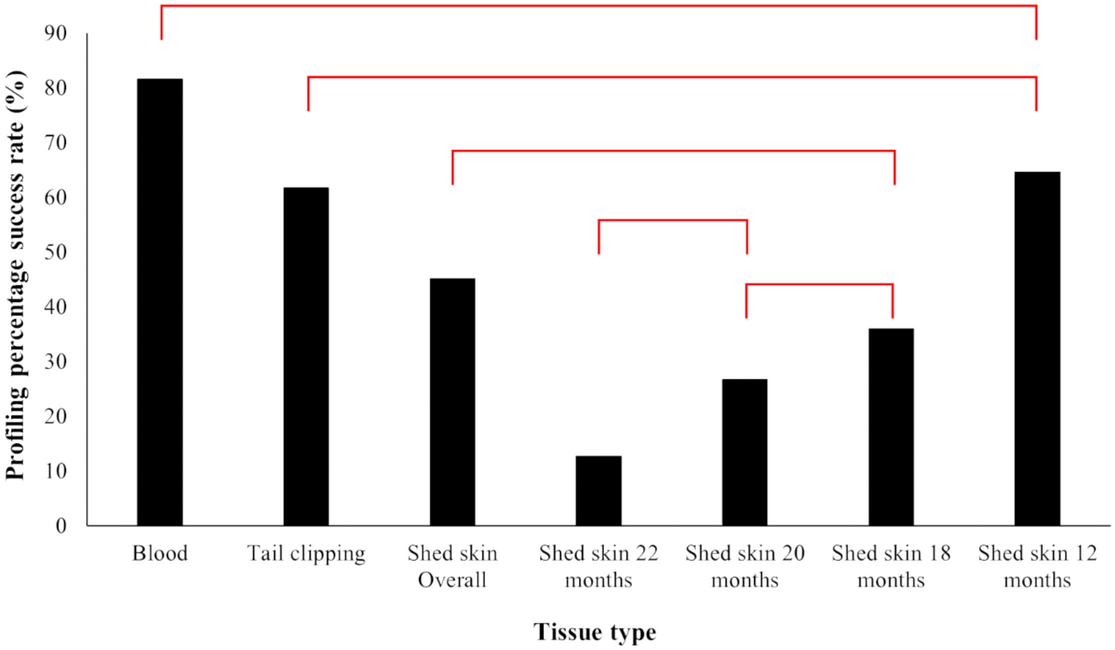


Figure 2.3. Average percentage microsatellite profiling success rate using DNA extracted from *Smaug giganteus* blood, tail clippings, and shed skin samples. An overall percentage success rate is given for shed skin, as well as the success rates of batches of shed skin samples that were processed within 12, 18, 20, and 22 months of collection. Red bars indicate pairs that were not significantly different. All other pairs were significantly different (Dunn’s test; $p < 0.01$).

2.4. Discussion

The 18 microsatellite loci isolated in this study were polymorphic and were suitable for investigations of genetic diversity, population structure, and relatedness in *S. giganteus*, and are the first microsatellite markers developed for a cordylid. The concentration and purity of DNA extracted from Sungazer blood, tail clippings, and shed skin differed significantly, with blood and tail clippings yielding higher levels of DNA concentration and purity on average than shed skin. Shed skin samples generally yielded the lowest success rates in microsatellite profiling, and showed a clear decline in sequencing success as time between collection and processing increased. Shed skin samples that were processed within 12 months of collection, however, showed comparable sequencing success to tail clippings, despite the significantly lower concentrations of DNA yielded. Non-invasive sampling can therefore be used as an effective sampling approach for *S. giganteus* if shed skins are utilised shortly after collection.

Since the red blood cells of reptiles are nucleated, blood samples would be expected to yield higher concentrations of DNA than shed skin samples (Burbrink and Castoe 2009, Arikan and Çiçek 2014). Tail clippings contain a small portion of blood along with muscle tissue and skin, and therefore have higher concentrations of DNA than shed skin samples, but lower levels of purity than blood because of potential contaminants present on the surface of the skin at the time of sampling. The purity values of DNA from blood and tail clippings were within the optimal range ($A_{260/280} =$ ~1.8-2.0; Horreo et al. 2015, Lucena-Aguilar et al. 2016) for pure DNA. DNA from shed skin was below the optimal range, however $A_{260/280}$ values below ~1.6 are not necessarily indicative of unsuitable DNA, rather that there are likely to be more contaminants in the DNA. DNA from shed skin had purity values that were significantly below (0.58) or above (3.28) the optimal value (~1.8-2), and large deviations in either direction are indicative or contaminants in the DNA sample. The lower purity of shed skin samples is most likely due to the contaminants present in the environment from which the samples were collected. Although the samples were rinsed with sterile double-distilled water (ddH2O) prior to DNA extraction, contaminants such as soil particles, microbes, or other proteins may have remained on the tissue. The presence of outlying samples in the boxplots for concentration and purity of DNA from shed skin samples is likely due to the potentially high differences in time these samples were exposed to environmental conditions that degrade DNA prior to collection in the field.

The concentration of DNA extracted from reptile shed skin in my study was similar to (mean 6.15 ng/µl; Brekke et al. 2019) or higher than (mean ~1 ng/µl; Tawichasri et al. 2017, mean 1.3 ng/µl; Horreo et al. 2015) other studies when using commercial extraction kits, but significantly lower than yields when using a Chelex extraction kit (mean 31.2 ng/µl; Horreo et al. 2015). The DNA from Sungazer shed skins in my study were purer than those collected by Horreo et al. (2015) ($A_{260/280} =$

1.2), even though the shed skins collected by Horreo were collected directly from the animal. It is important to note that the shed skin samples in my study were not collected directly from Sungazers, whereas most other studies that use shed skin either collect from captive animals, or removed sheds from a wild caught lizard. As a result, the samples collected in other studies would likely have experience less exposure to the elements that degrade DNA in the field. Shed skin samples collected directly from either captive or wild reptiles tend to yield very high concentrations of DNA that have high amplification success rates (Feldman and Spicer 2002, Dubey et al. 2010, Horreo et al. 2015, Ford et al. 2017, Brekke et al. 2019), even after storage for several months or years (Clark 1998, Fetzner 1999, Jones et al. 2008). Although DNA inevitably degrades over time, storage under the optimal conditions and using optimised laboratory procedures can reduce the rate of degradation so that tissue samples can be successfully processed long after collection. Samples can be stored in media that inhibit the enzymes and processes that cause the degradation of DNA. For example, storage in media that reduce moisture (ethanol, silica gel), at lower temperatures to inhibit nuclease activity, or in media that remove cations (EDTA, chelex) (Thomas and Gilbert 2006, Beja-Pereira et al. 2009). Similarly, testing of different extraction methods and kits may yield significantly different levels of amplification success using the same samples (Bhagavatula and Singh 2006). Horreo et al. (2015) achieved significantly higher DNA concentrations using a Chelex-resin based protocol (24.2-48 ng/µl) (Estoup et al. 1996) than DNeasy Blood & Tissue Kit (0.4-3.8 ng/µl) (Qiagen, Verlo, Netherlands), even eleven months after initial tissue collection.

There are several factors to consider in understanding the differences in the purity and concentration of DNA extracted from different sample types, and their respective downstream success rates. Firstly, the exposure of each tissue type to conditions that degrade DNA prior to collection. The age of shed skin collected from Sungazer burrows was difficult to assess since Sungazer skin is thick and keratinous and appears to retain structural integrity long after being shed. These samples may have been exposed to a combination of high temperature, rainfall and UV radiation prior to collection – all of which are known to degrade DNA. As DNA degrades, it also becomes fragmented, decreasing the chance of the necessary nucleotide sequencing bonding with the appropriate primer sequence during PCR. Furthermore, extended exposure to environmental conditions may expose these samples to soil particles, microbes and other proteins that inhibit PCR. Tail clippings may also have experienced exposure to contaminants prior to collection. By comparison, blood samples were collected directly from Sungazers and injected directly into sterile containers, minimising exposure to contaminants. This may explain the comparatively lower success of shed skin and tail clippings in profiling compared to blood.

Secondly, the difference in the period of time between collection and processing samples. Shed skin samples were collected over years of fieldwork and stored for different periods of time before being processed, simply due to the time gap between the fieldwork and labwork components of my study. Blood and tail clippings by comparison were processed within less than three months, since they were collected towards the end of my fieldwork period. DNA degrades in quality and quantity over time (Gamble 2014), and shed skin samples were in some cases processed almost a year after collection from the field. The enzymes that degrade DNA may likely have continued the degradation process once collected. Shorter periods of time between collection and processing of shed skin samples yielded purer DNA and higher profiling success rates. This is an important consideration to build in to the planning of fieldwork and labwork periods, since samples can not always be processed immediately after collection.

Finally, the medium and conditions in which different tissue types were stored. Blood samples were stored in tubes containing Ethylenediaminetetraacetic acid, which inhibits proteins such as nucleases that degrade DNA (Nevarez 2009, Gamble 2014), and kept at -4 °C. Cooler temperatures are integral in preserving DNA, since chemical and biological processes occur at slower rates as temperature drops (Lindahl 1993, Taylor 2006). Tail clippings were stored in 99% ethanol at room temperature. Although ethanol is a proven fluid preservative of DNA, the comparatively lower profiling success of tail clippings may be due in part to the temperature that these samples were stored at. Storage of tail clippings at -4 °C or lower temperatures may reduce DNA degradation even over relatively short periods of time (Gamble 2014). Shed skins were kept dry at room temperature following Fetzner's (1999) protocol for shed skin samples from reptiles. The samples that Fetzner used were shed skins from captive individuals and therefore likely experienced less degradation prior to collection than my field-collected samples. Tissue samples that are likely to have been exposed to environmental conditions and contaminants should therefore ideally be stored in media that reduce DNA degradation, and at as low temperatures as possible until processing.

While the collection of non-invasive DNA samples have several advantages over traditional sampling techniques, one of the primary downsides to the non-invasive approach is the variability in DNA concentration and quality in these samples. Non-invasive samples such as faeces, hair, and shed skin are prone to degrade in the field prior to collection for genetic analysis. Time in the field prior to collection, exposure to moisture, and temperature are the primary conditions that impact DNA amplification success rates (Fernando et al. 2000, Murphy et al. 2007, Brinkman et al. 2010). For sensitive sample types such as faeces, amplification success declines rapidly after ±3 days in the field (Fernando et al. 2000), and continues to decline in efficacy until ±12 days, when the amplification success rate plateaus at ±20% (Murphy et al. 2007). Similarly, DNA samples collected from wet environments showed significantly lower amplification success than samples from dry environments, and were not successfully genotyped after seven days of exposure to moisture (Brinkman et al. 2010). Reptile shed skins are keratinous (Rutland et al. 2019) and degrade less quickly than faeces (Horreo et al. 2015), however environmental conditions in the Highveld conditions can reach high temperatures and high levels of precipitation and may be prone to similar levels of DNA degradation. The age of shed skin samples collected in the field can also be difficult to ascertain, since shed skin may retain its physical form after several months in the field.

Conclusion

The microsatellite markers designed and presented in this chapter show promise as tools to investigate fine-scale contemporary patterns of genetic structure in the Sungazer by quantifying the relatedness between individuals and colonies. The ability of these markers to conduct relatedness and parentage analyses also makes them suitable for verifying the F2 status of Sungazers in trade cases. Previous studies have reported high levels of transferability of species-specific microsatellite markers across closely related taxa (Barbará et al. 2007, Song et al. 2017) and the utility of these loci on other *Smaug* species should be investigated. Non-invasive sampling through the collection of Sungazer shed skin proves to be an effective sampling method with amplification and microsatellite sequencing, but is less predictable for DNA quantity and quality than tail clippings and blood samples. Although shed skin samples that were stored for more than 12 months before processing had high failure rates, this may be mitigated through several steps: utilising careful sample collection procedures to ensure minimal exposure of samples to moisture and high temperatures, storage in media and temperatures that inhibits DNA degradation, and the optimisation of laboratory protocols that maximise DNA extraction from low quality sample and reduce PCR inhibitors during amplification. With these points taken into account, the utilisation of non-invasive sampling of Sungazer shed skins can allow for the collection of higher number of samples at reduced field effort compared to the collection of tail clips or blood over the same period of time.

CHAPTER 3

PHYLOGEOGRAPHY AND POPULATION GENETICS OF THE SUNGAZER (*SMAUG GIGANTEUS*)

3.1. Introduction

The spatial spread of a species across its distribution and the corresponding pattern of genetic exchange are determined by vicariance and dispersal (Rosen 1978, Wiley 1988, Ronquist 1997). Historical vicariant events such as the formation of geological features (Macey et al. 2000, Veith et al. 2003, Hayes and Sewlal 2004, Lores and Raya 2006, Poulakakis et al. 2012), and changes in climactic variables over time (Matthee et al. 2004, Douglas et al. 2006, Bryson et al. 2012) may have altered the distribution of populations into their current extant pattern. The ability of individuals to disperse between populations dictates how genetically differentiated these populations are (Peterson and Denno 1998, Habel and Schmitt 2009, Avise 2009). Isolation of populations over long periods of time can lead to divergence and speciation (Dobzhansky 1940, Pinho and Hey 2010, Nosil and Feder 2012). Contemporary landscape features also influence the distribution and genetic structure of populations (Saunders et al. 1991, Debinski and Holt 2000, Couvet 2002, Martinez-Cruz et al. 2004). Modern anthropogenic landscape transformation through agricultural and urban development processes further isolate populations, leading to lower genetic diversity, increased genetic structure (Slatkin 1987, Frankham et al. 2002, Reed et al. 2002), and the extinction of sub populations (Wilcox and Murphy 1985, Fahrig 2003, Templeton et al. 2015).

Significant vicariant features may influence species in different ways. The dispersal ability of individuals dictates to a large extent whether specific landscape features affect genetic exchange between populations (Fahrig 1998; Walkup et al. 2017). Rivers, crop fields, and roads result in severe genetic isolation in some species (Jackson and Fahrig 2011, Veach et al. 2017, French et al. 2018), whereas dispersal is undisturbed in other species in the same environment (Burbrink et al. 1998, Meister et al. 2010, Casper 2012). Ectothermic individuals may be more affected by climactic changes than birds or mammals because of their sensitivity to thermal clines (Tingley and Dubey 2012, Smissen et al. 2013, Rolland et al. 2018, Saladin et al. 2019). The spatial occupation of a habitat by ectothermic species may also depend on the availability of basking sites that allow for the effective thermoregulation of individuals in order to maintain a target temperature (Clark et al. 2008, Blair et al. 2013, Nelson-Tunley et al. 2016). The specificities of a species response to environmental changes are therefore important to consider in organismal biology, and conservation management (Betts et al. 2014).

Quantification of the genetic structure of populations in a species can allow for insights in the evolutionary history of a species, and how anthropogenic landscape features influence these patterns in the present. An integrative approach to understanding a species occupation of a landscape can be more impactful than the use of a single technique (Diniz-Filho et al. 2009, Chan et al. 2011, Pavón-Vázquez et al. 2018). A plethora of molecular genetic approaches are currently available that allow for the interrogation of the genetic relatedness at various spatial and temporal scales. Slower evolving nuclear DNA used in conjunction with faster evolving mitochondrial DNA and rapidly evolving microsatellite markers can provide insight into how different vicariant events in a species evolutionary history have affected gene flow between populations (Tollefsrud et al. 2009, Raeymaekers et al. 2012, Hui et al. 2017, Kheng et al. 2018). The integrated use of geographic information systems (GIS) and ecological niche models can also provide visual representations of genetic structure across space (Spear et al. 2005, Clostio 2010, Hobbs et al. 2011), whether specific landscape features present suitable areas for a species to occupy, and the utilisation or avoidance of these areas impede or allow gene flow. Through this integrative approach, phylogeographic hypotheses can be formulated (Strange and Burr 1997, Macey et al. 2000, Van Veller et al. 2002).

Investigations of genetic structure and diversity are particularly pertinent for threatened species, since the extirpation of populations and reduced population density can result in the loss of rare alleles and genetic diversity. This is particularly important for species that inhabit areas that have undergone heavy human-mediated land transformation which result in habitat loss and fragmentation (Lindenmayer and Fischer 2007). For many species, poor dispersal ability between discontinuous patches that are separated by roads, crop fields or urban areas has resulted in lowered genetic diversity and increased genetic structure (Fahrig 2003, Andrews and Jochimsen 2007, Noël et al. 2007, Kay et al. 2016). Isolated populations are prone to inbreeding, population bottlenecks and lower effective population size as a result of restricted genetic exchange with other populations (Nei et al. 1975, Moore and Ali 1984, Allendorf 1986, Hedrick and Kalinowski 2000). This may lead to a supressed evolutionary response to environmental changes within populations, and eventually the extinction of these populations (Lande 1993, Frankham and Ralls 1998, Crispo et al. 2011). Studies of the patterns of genetic exchange between populations in fragmented areas is therefore important in understanding the extinction risk of a species and its populations, and the development of conservation plans that take genetic structure and diversity into account (Branch et al. 2003, Thomassen et al. 2011, Vasconcelos et al. 2012).

The Sungazer (*Smaug giganteus*) is a Vulnerable (Alexander et al. 2018) species of Cordylidae endemic to the Highveld grasslands of the Free State and Mpumalanga provinces of South Africa (De Waal 1978, Jacobsen 1989). The high elevation grassland (1260-1840 m asl; Parusnath 2014) that the species occupies represent a fairly homogenous structural matrix, interspersed with perennial and non-perennial rivers and floodplains. The northern reaches of the Drakensberg Mountain Range introduce dolerite intrusions and low lying ridges and escarpments along the south-eastern edge of the species distribution (Scheepers 1975). The region is characterised by high annual rainfall and a temperate climate, with relatively hot summers and cool winters (Van Wyk 1992). A strong climactic gradient extends from west to east, with cooler temperatures and greater annual precipitation correlated with an increasing elevational gradient (Scheepers 1975, Van De Wal 1976). The species distribution falls across six vegetation bioregions (Mucina and Rutherford 2006) that harbour different assemblages of plant species, all of which are dominated by the pioneer grass species *Themeda triandra* (Mucina and Rutherford 2006)**.** The species distribution appears is bordered by mountains and rivers, which may present significant barriers to the dispersal of individuals and thereby limit the expansion of the species from this area (Parusnath et al. 2017).

Sungazer populations in the Free State and Mpumalanga provinces have been noted as being geographically disjunct (Petersen et al. 1985, Branch 1990, Parusnath et al. 2017). Anecdotal reports have also suggested that Sungazers from the Free State Province may be considerably larger in body size than Sungazers from the Mpumalanga Province (Stoltz and Blom 1981). The reason for the lack of occurrence of Sungazers in the intermediate area, and differences in body size between populations is unknown. Apart from this gap in occurrence, Sungazers occur ubiquitously but sparsely across their distribution, occupying only 12% of the available habitat (Parusnath et al. 2017). Sungazers only dig burrows in flat or low inclination primary grasslands in hard clay soil (Soil Classification Working Group 1991) that is not underlain with surface-level rocks. These conditions are also ideal for crop production, and much of the primary grassland within the distribution of the species has therefore been transformed for the monoculture of maize, wheat, sugar, sorghum, and sunflowers (Parusnath et al. 2017). As a result, just over half of the primary grassland and the Sungazer colonies that they support remain intact (Parusnath et al. 2017). Many colonies now exist within disconnected islands of grassland in a matrix of unsuitable habitat.

The effects of the strong gradient of climate and elevation, and geological and anthropogenic landscape features on the genetic construct of *S. giganteus* and its constituent populations are unstudied. This precludes an understanding of how the species has evolved across its landscape through various dispersal and vicariance events, and how modern changes in the landscape may affect gene flow and therefore the genetic structure of populations. In this chapter I investigated the phylogenetic and population genetic structure of *S. giganteus* across its distribution. I utilised a combination of mtDNA and nDNA gene sequences and rapidly evolving microsatellite markers so that the evolutionary history of the species could be explored at various temporal scales. Additional to exploratory population genetic analyses, I tested the hypotheses that allopatric Sungazer populations from the Free State and Mpumalanga provinces are genetically distinct, and that genetic structure corresponds with vegetation bioregions. I compared the various scenarios of population genetic structure using estimates of confidence to validate the most parsimonious scenario of population differentiation in the species. I employed the use of an ecological niche model and topographic and landcover GIS layers to assess whether genetic differentiation between populations corresponds with landscape features. Finally, I investigated body size differences of Sungazers across phylogenetic clades (based on the results of the phylogenetic analysis in section 3.3.1).

3.2. Materials and methods

3.2.1. *Tissue sample collection*

I collected tissue samples from 161 Sungazers in 19 colonies across the distribution of the species (Fig. 3.1; Table 3.1) between March 2015 and March 2017. I defined a colony as an aggregation of Sungazers clearly spatially isolated from the neighbouring aggregation by a minimum distance of 1 km. I captured Sungazers using standard noosing techniques (Van Wyk 1992, McIntyre and Whiting 2012), and clipped ~3 mm sections of tissue from the large caudal spines of adults, or the terminal tail scute in juveniles using a sterilised pair of nail clippers. Caudal spines and tail tips were disinfected with F10SC Veterinary Disinfectant spray (Health and Hygiene (Pty) Ltd., South Africa) before being clipped. Blood samples were collected on FTA® filter paper (Whatman, Clifton, NJ, USA) as backup samples from the clipped area on the tail of each Sungazer. I applied F10 Germicidal Barrier Ointment (Health and Hygiene (Pty) Ltd., South Africa) to the area of incision on the tail after ensuring that bleeding had ceased. I released Sungazer at the burrow from which they were captured. Tail clippings were stored in 99% ethanol in 1.8 ml Nunc™ Cryogenic Tubes (ThermoFisher, Waltham, MA, USA) at room temperature. Blood samples on FTA® filter paper cards were stored dry in 0.75 ml Eppendorf Tubes (Eppendorf GmbH, Hamburg, Germany) at room temperature. Equipment and surfaces used during tissue collection were sterilised with 10% bleach (sodium hypochlorite; Kemp and Smith 2005) and rinsed with sterile double-distilled water (ddH2O) between the sampling of each individual.

3.2.2. *Morphometric data collection*

I collected snout-vent length (SVL) and mass (g) for 152 adult Sungazers (adults are individuals with SVL >165 mm; Van Wyk 1992) across the distribution of the species. Snout-vent length was measured to the nearest 1 mm using a ruler, and mass to the nearest 1 g using a digital scale. The sex of all individuals was established by examining lizards for the presence of generation glands in the ventral femoral and forearm regions. Generation glands on the forearms are only present in male lizards (Van Wyk 1992). There was no significant difference in SVL between adult male ($n = 66$) and female ($n = 86$) Sungazers (two-sample t-test; $t = 0.7$, $df = 151$, $p < 0.01$), and so data from both sexes were pooled for morphometric analyses. Based on the findings of genetic structure in *S. giganteus* in this chapter (section 3.3.1), I compared SVL and mass between the Western clade (98 individuals, 11 colonies), Central clade (5 individuals, 1 colony), and Eastern clade (31 individuals, 6 colonies) populations using one-way analysis of variance (ANOVA). Data for mass and SVL across all three clades were normally distributed.

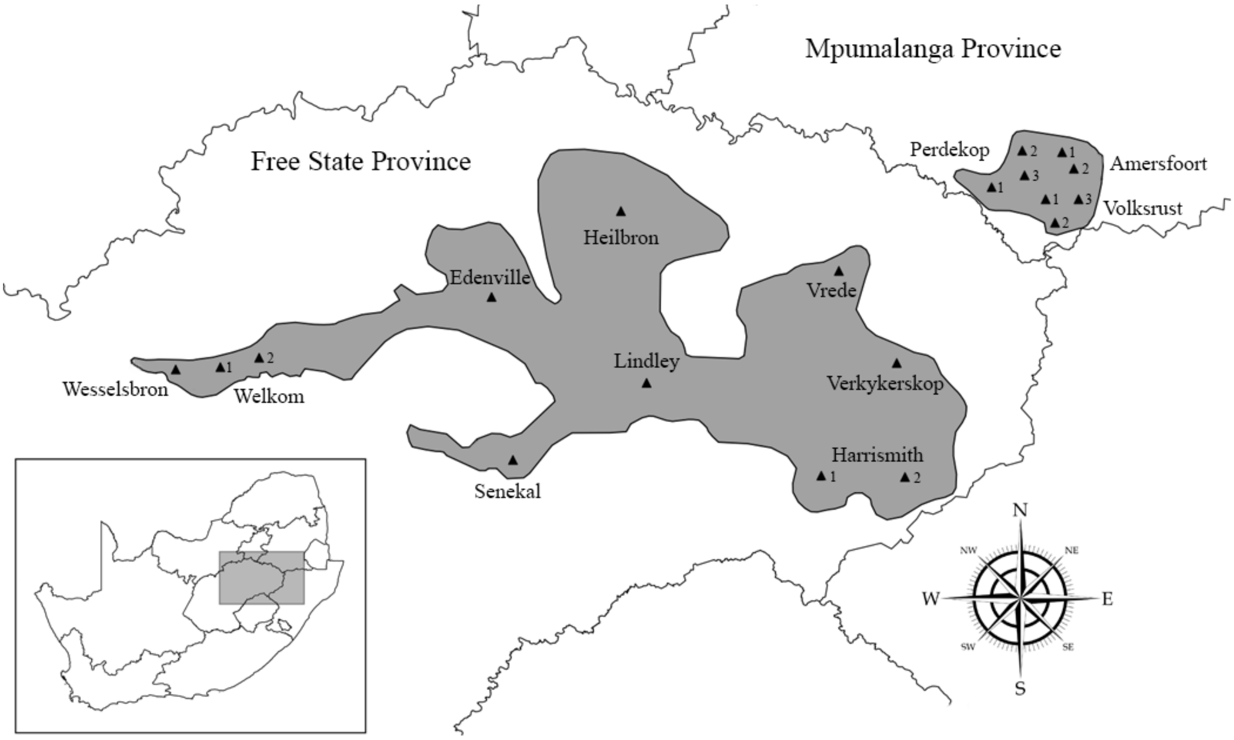


Figure 3.1. Interpreted distribution of *Smaug giganteus* shaded in grey (Parusnath *et al*. 2017) across the Free State and Mpumalanga provinces of South Africa. Triangles show the location of 19 *S. giganteus* colonies that tissue samples were collected from.

Table 3.1. Sungazer colonies sampled, and number of individuals sampled per colony

Colony name	Number of individuals sampled
Amersfoort 1	3
Amersfoort 2	32
Edenville	3
Harrismith 1	2
Harrismith 2	3
Heilbron	35
Lindley	10
Perdekop 1	2
Perdekop 2	5
Perdekop 3	1
Senekal	7
Verkykerskop	5
Volksrust 1	2
Volksrust 2	8
Volksrust 3	2
Vrede	8
Welkom 1	2
Welkom 2	29
Wesselsbron	2

3.2.3. *DNA extraction, PCR, and sequencing*

DNA was extracted from tissue clippings and blood samples using the ZR Genomic DNA II Kit (Zymo Research Corporation, Irvine, Calif., USA), following the manufacturer's protocol for blood and hair. Extracted DNA was stored in 1.5 ml Eppendorf Tubes (Eppendorf GmbH, Hamburg, Germany) at -20 °C. A total of two mitochondrial and one nuclear gene sequences (Table 3.2), and 18 species-specific polymorphic microsatellites markers (developed in Chapter 2 of this thesis) were used to genotype all individuals. The partial sequences of the mitochondrial genes 12S (Stanley et al. 2011), and 16S (Simon et al. 1994), and the nuclear gene Kinesin Family Member 24 (KIF24) (Portik et al. 2010) were utilized in previous studies of cordylids (Frost et al. 2001, Odierna et al. 2002, Ding 2008, Stanley et al. 2011) and were included in this study to facilitate comparisons with prior analyses. Mitochondrial DNA evolves at rates of 5-10 times that of nuclear DNA (Brown et al. 1979, Vawter and Brown 1986), and is reflected in the comparatively higher nucleotide sequence variation in mtDNA gene sequences. Using both mitochondrial and nuclear DNA in combination allows for an investigation of population structure in *S. giganteus* at different evolutionary time-scales (Avise 2009).

Table 3.2. Primer name, gene name, nucleotide sequence, and source of genes utilised in this study

Primer	Gene	Sequence (5' – 3')	Source
16Sa	16S	CGCCTGTTTATCAAAAACAT	Simon et al. 1994
16Sb	16S	CCGGTCTGAACTCAGATCACGT	Simon et al. 1994
12SA700	12S	AAACTGGGATTAGATACCCCACTAT	Stanley et al. 2011
12sr600	12S	GAGGGTGACGGCGGTGTGT	Stanley et al. 2011
Kif24f	Kif24	WGGCTGCTGRAAYTGCTGGTG	Portik et al. 2010
Kif24r	Kif24	SAAACGTRTCTCCMAAACGCATCC	Portik et al. 2010

3.2.3.1. Mitochondrial and nuclear gene sequencing

Polymerase chain reaction was conducted with Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Odense M, Denmark) in 15 µL reaction volumes containing 1x Taq 2x Master Mix, 1.0-2.0 mM $MgCl_2$, 0.2 µM each forward and reverse primer, and 2.5 µl (~20 ng/µl) template DNA. The conditions for PCR amplification were: denaturing at 95 °C for 5 min, annealing with 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min in a T100^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc. Hercules, CA). Successful PCR products were purified with Exonuclease I and FastAP (Thermo Fisher Scientific Inc., Waltham, MA, USA). Gene fragments were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and visualised on 3130xl or 3500 Genetic Analysers (Applied Biosystems).

3.2.3.2. Microsatellite genotyping

Polymerase chain reaction was conducted with Invitrogen Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California), in 10 µL reaction volumes containing 1 x PCR buffer (Invitrogen), 1.0-2.0 mM $MgCl_2$, 0.02 mM each forward and reverse primer, 0.1mM dNTP mix, 0.25 U Invitrogen Platinum Taq DNA polymerase, and 1 µL template DNA (~10 ng/µL). The conditions for PCR amplification were: 5 min at 95 °C initial denaturation, 30 cycles for 30 s at 95 °C, 30 s at 45 °C, and 30 s at 72 °C, followed by a final extension at 72 °C for 40 min. The PCR was carried out in a BOECO TC-PRO Thermal Cycler (Boeckel + Co., Hamburg, Germany). Polymerase chain reaction products were pooled together and run against a Genescan 500 LIZ (Applied Biosystems, Foster City, California) internal size standard on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, California). Samples were visualized using GENEMAPPER 4.0 software (Applied Biosystems, Foster City, California) and bins were assigned to alleles and calls were then checked by eye.

3.2.4. ***Phylogenetic analyses***

To assess the overall genetic structure of the species and investigate potential cryptic diversity within *Smaug giganteus,* a genus-level phylogeny for *Smaug* was run using published data (Table 3.2; Stanley and Bates, 2014), with *Cordylosaurus subtessellatus* and *Platysaurus capensis* as outgroup species. Stanley and Bates (2014) included only four *S. giganteus* samples from three localities (Free State Province, n = 3; Mpumalanga Province, n = 1) in their phylogeny, precluding any examination of cryptic diversity within the species. Therefore, sequences from 14 *S. giganteus* individuals from across the distribution of the species were included to investigate additional structuring within the species. This subset was selected so that the number of *S. giganteus* samples per site matched that of Stanley and Bate's (2014) phylogeny, to avoid false signals stemming from high sample density for *S. giganteus*. The phylogenetic analysis was run using 12S (Stanley et al. 2011), 16S (Kohli et al. 2014), Kinesin Family Member 24 (KIF24) (Portik et al. 2010) partial gene sequences.

Sequences were aligned in GENEIOUS 8.1.9 (http://www.geneious.com, Kearse et al. 2012) using the Clustal W alignment model (Thompson et al. 1994), and screened for base ambiguities. The best-fitting evolutionary model for each gene fragment was identified using PARTITIONFINDER 1.1.1 (Lanfear et al. 2014). Phylogenetic trees were constructed in maximum likelihood (ML) and Bayesian inference (BI) frameworks for three datasets: mitochondrial dataset, nuclear dataset, and the concatenated mitochondrial and nuclear dataset. A partition homogeneity test (1 000 replicates) was implemented in PAUP∗ 4.0a163 (Swofford 2002) on the concatenated dataset prior to analysis, to assess congruence among individual topologies. The test indicated that there was no conflict in phylogenetic signals (P = 0.11).

Maximum likelihood analyses were performed in RAxML 8.0 (Stamatakis 2014) with a GTRGAMMA model of nucleotide evolution for all genes and partitions, with 100 random addition replicates and partition branch lengths and parameters estimated independently for each partition.

Nodal support of the ML tree was assessed by 1 000 bootstrap pseudoreplications (Felsenstein 1985). Bayesian inference analyses were conducted in MRBAYES 3.2 (Ronquist et al. 2012). Four Markov chain Monte Carlo (MCMC) chains were run in parallel for 10 000 000 generations with random starting trees, and Markov chains were sampled every 1 000 generations. PARTITIONFINDER 1.1.1 (Lanfear et al. 2014) indicated that the best partitioning strategy and substitution models were: 12S (F81), 16S (HKY), and Kif24 (HKY + I + G). Data were therefore portioned by gene and the appropriate model of evolution for each gene was coded in the MRBAYES block in the input nexus file.

TRACER 1.7 (Rambaut et al. 2018) was used to assess run length sufficiency by confirming adequate chain mixture, convergence, and stationarity, noting if the effective sample size (ESS) values were sufficient (>200). The ESS value indicated that a burn-in of 10% was sufficient and therefore 1 000 trees were discarded as burn-in. A 50% majority-rule consensus tree was generated from the trees retained. Clades/nodes with bootstrap values of ≥ 70 % for ML, and posterior probability values of ≥ 0.95 for BI, were considered highly supported (Huelsenbeck and Rannala 2004). The phylogenetic tree for the total evidence dataset tree was visualised in FIGTREE 1.4.3 (Rambaut 2017).

3.2.5. *Population genetic structure analysis*

A multitude of software packages are available for use in population genetic analyses. The models employed in these packages may use different algorithms and methodological principles to detect barriers and assign individuals to unique genetic populations. The resultant number and configuration of genetic populations detected may therefore differ between models. The use of multiple models, followed by statistical model selection is therefore advised in order to confirm which model may provide the most parsimonious pattern of genetic structure (François and Durand 2010). The results from each analysis should also be interpreted in the context and scale of the study, and the life history of the species being investigated.

I used GENELAND, STRUCTURE, and SAMOVA software packages to assign individual Sungazers from 19 different sampled colonies (Fig. 3.1) to genetic populations. I compared the genetic structure identified in each analysis with the clustering of colonies into populations as predicted by my hypotheses. Finally, I used analyses of molecular variance (AMOVAs) in ARLEQUIN to estimate the level of confidence in each scenario of genetic structure to confirm which scenario is the most parsimonious explanation of genetic structure in the species. Analyses performed in STRUCTURE and GENELAND used only microsatellite data, whereas the combined mitochondrial and nuclear gene dataset (12S, 16S, and Kif24) were used in SAMOVA, and both microsatellite and the combined gene dataset were used in ARLEQUIN to test the various scenarios of genetic structure.

3.2.5.1. Bayesian clustering analyses

STRUCTURE 2.3.4 (Pritchard et al. 2000) and GENELAND 4.0.5 (Guillot et al. 2005) are two of the most widely used Bayesian clustering software packages in population genetic studies (François and Durand 2010). Bayesian methods infer population based on allele frequency and cluster individuals based on their genetic values (Bolstad 2004). Each model has its own strengths and limitations. GENELAND's spatially explicit model allows for the detection of weaker population structure than is detectable with STRUCTURE'S non-spatial model (Françọis et al. 2006, Olsen et al. 2014). STRUCTURE offers the option of admixture models which are more flexible and robust than models without admixture, such as in GENELAND (Durand et al. 2009, François and Durand 2010).

GENELAND 4.0.5 (Guillot et al. 2005) uses a Reversible Jump Markov Chain Monte Carlo (RJMCMC) algorithm for the inference of number of populations, which is implemented using a spatially explicit model that assumes the geographical location of the samples (Guillot et al. 2005). I used the spatial model with uncorrelated allele frequencies to infer the number of populations (K) in my dataset. I performed 10 independent runs for each value of K ranging from 1 to 10. For each simulation, parameters were set to 10 independent runs with 500 000 MCMC iterations, thinning of 50, no filtering of null alleles, and the delta coordinate (representing the potential error for spatial coordinates) set at 0, given that each sample was accurately recorded to the nearest 3 m with a GPS. The 10 runs were post-processed with a burn-in of 100 iterations in order to obtain posterior probabilities of population membership for each individual and each pixel of the spatial domain (200 pixels along the X and Y axes). These runs were checked for convergence and used to establish the modal value of K. Based on this value, I then fixed K at 5 to estimate the other parameters included in the model, including population membership probability of individuals, map of population membership and posterior density of the model.

STRUCTURE 2.3.4 (Pritchard et al. 2000) infers the optimal number of populations (K) using a Markov Chain Monte Carlo (MCMC) algorithm (Pritchard et al. 2000). I performed 10 independent runs for each value of K ranging from 1 to 10. The parameter settings specified use of the admixture model, with allele frequencies correlated and location information not given a priori. Each run consisted of 1 000 000 MCMC iterations, with an additional 100 000 generations discarded as burn-in. All other settings were left as default. The K value with the greatest increase in posterior probability (ΔK; Evanno et al. 2005) was identified as the optimum number of populations using STRUCTURE HARVESTER 0.6.94 (Earl and von Holdt 2012).

The mathematical model used in STRUCTURE has received criticisms for assigning individuals into too few populations, and the strong susceptibility of being influenced by sample size (Kalinowski 2011).

To avoid these potential problems, I employed an approach of progressive partitioning of populations (*sensu* Hobbs et al. 2011) to further scrutinise the dataset using STRUCTURE. This method operates on the assumption that hierarchical clustering algorithms produce a nested series of partitions (Jain et al. 2007). Populations identified in the first round of analysis are expected to be the most differentiated, with subsequent populations having progressively lower levels of genetic differentiation. The progressive partitioning technique potentially allows for the identification of otherwise cryptic populations when looking for populations at lower levels of genetic differentiation, although it has the disadvantage of explicitly ignoring the likelihood values for the different values of K. Progressive partitions produced by Bayesian techniques can be displayed using GIS software and compared to identify consistent patterns of sub-structuring at different degrees of differentiation. I therefore ran STRUCTURE first on the complete microsatellite dataset, and then again individually on the two initial populations identified by STRUCTURE.

3.2.5.2. Spatial analyses of molecular variance

Spatial analyses of molecular variance (SAMOVA) were used to investigate geographical structure with the software package SAMOVA 1.0 (Dupanloup et al. 2002).
SAMOVA uses geographical coordinates and corresponding sequence data to identify maximally differentiated groups of populations in geographical space, without a priori assumptions of population groupings. The procedure uses a simulated annealing procedure to investigate the composition of groups (K), and maximise the proportion of genetic variation resulting from differences between groups (F_{CT}) (Dupanloup et al. 2002, Excoffier and Lischer 2010). The program was run for 2 to 10 differentiated populations using 100 000 permutations from 100 random initial conditions. The actual number of populations was determined by plotting the relationship between F_{CT} (the proportion of total genetic variance representing differences among groups of populations) and K (number of populations) to another, with the peak increase corresponding to the number of populations with the highest likelihood (Dupanloup et al. 2002).

3.2.5.3. Hierarchical partitioning of molecular variance (AMOVA)

I used hierarchical analysis of molecular variance (AMOVA) in ARLEQUIN 3.5 (Excoffier and Lischer 2010) to assess the significance of the genetic partitioning of colonies into genetic populations in each scenario of population structure. The AMOVA framework uses a priori groupings of individuals and sample sites to assess how genetic variation is partitioned between groups. I grouped the 19 sampled Sungazer colonies into the these a priori groups based on the results of the population structure from STRUCTURE, GENELAND, SAMOVA and phylogenetic analysis, as well as groupings under the hypotheses that populations from the Free State and Mpumalanga provinces are genetically distinct, and that genetic structure of populations is correlated with the spatial organisation of vegetation bioregions (Table 3.3). AMOVAs were performed using pairwise differences and 10,000 random permutations; other settings were left as defaults. The level of genetic differentiation among populations was quantified for the three groups in scenario III (Table 3.3) by the estimation of the pairwise F_{ST} values and their statistical significance.

Table 3.3. Groupings of 19 *Smaug giganteus* colonies used to test the significance of six competing scenarios of genetic structure in AMOVA analysis in Arlequin. Letters A-F represent the genetic population that each colony was assigned to for each scenario.

	Scenarios of population structure					
Colony	**I**	**II**	**III**	**IV**	**V**	**VI**
	Two populations: Free State Province, Mpumalanga Province	**Two populations:** Western population, Eastern population (Based on Structure results (K = 2))	**Three populations:** Western, Central, Eastern clades (based on phylogenetic analysis and SAMOVA results)	**Five populations:** Western 1, Western 2, Central, Eastern 1, Eastern 2 populations (Based on Geneland results (K = 5))	**Five populations:** Western 1, Western 2, Central, Eastern 1, Eastern 2 populations (Based on Structure Results (K = 5))	**Six populations:** Vegetation bioregions (Western Free State Clay Grassland, Central Free State Grassland, Eastern Free State Clay Grassland, Eastern Free State Sandy Grassland, Soweto Highveld Grassland, Amersfoort Highveld Clay Grassland)
Harrismith 1	A	A	A	B	B	D
Harrismith 2	A	A	A	B	B	D
Heilbron	A	A	A	B	B	C
Edenville	A	A	A	B	B	B
Lindley	A	A	A	B	B	C
Senekal	A	A	A	A	A	B
Verkykerskop	A	A	A	B	B	D
Welkom 1	A	A	A	A	A	A
Welkom 2	A	A	A	A	A	A
Wesselsbron	A	A	A	A	A	A
Vrede	A	B	B	C	C	D
Perdekop 1	B	B	C	D	D	F
Perdekop 2	B	B	C	D	D	F
Perdekop 3	B	B	C	D	D	F
Amersfoort 1	B	B	C	D	D	E
Amersfoort 2	B	B	C	D	E	E
Volksrust 1	B	B	C	D	E	E
Volksrust 2	B	B	C	D	E	E
Volksrust 3	B	B	C	E	E	E

3.2.6. *Isolation by distance*

I conducted a Mantel test (Mantel 1967) in Alleles in Space 1.0 (Miller 2005) to investigate whether Sungazer populations experience genetic differentiation through isolation by distance (IBD). IBD is anticipated when populations have been stable over a long period and gene flow occurs more often among geographically neighbouring populations (Wright 1943). The Mantel test evaluates isolation by distance by testing the correlation between genetic and geographic distance matrices (Mantel 1967, Nekola and White 1999). The genetic distance implemented in Alleles in Space is an analogue of Nei's distance (Nei et al. 1983) applied to pairs of individuals. I first conducted the test on the dataset for all sample sites. The analysis was then run separately for the sites within the Western, Central, and Eastern clades identified in phylogenetic and SAMOVA analyses (section 3.3.2). Mantel tests were run with 1 000 permutations.

3.2.7. *Ecological niche model and visualisation in GIS*

To investigate how various scenarios of population genetic structure in *S. giganteus* correlate spatially with the ecological niche of the species, I constructed an ecological niche model using the maximum entropy method implemented in MAXENT 3.4.1 (Phillips et al. 2006). I used 1 517 GPS coordinates recorded at Sungazer burrows across the distribution as occurrence records to input into the model. These consisted of records that I recorded during this study, and records from previous studies (Parusnath et al. 2017, Stanton-Jones et al. 2018) and field surveys conducted by the Endangered Wildlife Trust between 2011-2018. *Smaug giganteus* is a habitat specialist, and the presence of the species is known to be closely associated with distinct vegetation types and soil profiles, as well as falling within a well-defined altitudinal and climatic envelope (Van Wyk 1988, 1992, Jacobsen 1989). A total of 24 environmental GIS layers representing important aspects of the species' known niche requirements were selected to model the fundamental niche of the species in geographic space. This included 19 bioclimatic variables (Hijmans et al. 2005), elevation (Hijmans et al. 2005), vegetation type (Mucina and Rutherford 2006), soil type (Dijkshoorn et al. 2008), landcover type (DEA 2015), and underlying geology (AGIS 2007) (Table 3.4).

Table 3.4. Climatic and environmental variables used in MAXENT model for *Smaug giganteus*.

Code	Environmental variable layer
1	Annual Mean Temperature
2	Mean Diurnal Range
3	Isothermality
4	Temperature Seasonality
5	Max Temperature of Warmest Month
6	Min Temperature of Coldest Month
7	Temperature Annual Range
8	Mean Temperature of Wettest Quarter
9	Mean Temperature of Driest Quarter
10	Mean Temperature of Warmest Quarter
11	Mean Temperature of Coldest Quarter
12	Annual Precipitation
13	Precipitation of Wettest Month
14	Precipitation of Driest Month
15	Precipitation of Seasonality (CoVar)
16	Precipitation of Wettest Quarter
17	Precipitation of Driest Quarter
18	Precipitation of Warmest Quarter
19	Precipitation of Coldest Quarter
Elevation	Elevation
Vegetation	Vegetation Type
Soil	Soil Type
Landcover	Landcover Type
Geology	Geology

Environmental variable layers were converted into ASCII format in ARCMAP 10.2 (ESRI Inc., Redlands, CA, USA) for input into MAXENT. A cell size of 0.016 (1 arc minute) was used. In order to increase the precision of the model, the layers were cropped to a rectangle that included that species' distribution and a surrounding buffer zone. The model was run with 10 000 background points, 5 000 iterations and 15 replicates. The selected output grid format was 'logistic', in which pixel values ranged from 0 to 1. To assess model performance, I used Receiver Operating Characteristic (ROC) curves (Fielding and Bell 1997, Phillips et al. 2004). The main advantage of ROC analysis is that the area under the ROC curve (AUC) provides a measure of model performance, independent of any choice of threshold (Phillips et al. 2006). To estimate the importance of environmental variables in predicting Sungazer distribution, a jack-knife analysis was also carried out in MAXENT. In this procedure, each variable is excluded in turn, and a model is created with the remaining variables, and with each variable in isolation (Phillips et al. 2006).

Genetic groups identified in different analyses were plotted on a map of the species interpreted distribution in geographic information software QGIS 3.6 (Open Source Geospatial Foundation Project, 2019) to assess the spatial spread of these populations. The interpreted distribution showing the population structure identified in GENELAND was also overlain onto maps showing terrain and landcover use so that the relationship between genetic structure and landscape features could be visualised.

3.3. Results

3.3.1. *Phylogenetic analysis*

The phylogenetic analysis recovered all *S. giganteus* samples as monophyletic for both ML and BI analyses. Within *S. giganteus*, there is strong support for two clades: Eastern (Amersfoort, Volksrust), and Central (Vrede). All other samples were from the Free State Province and were unresolved, forming a large polytomy with the Eastern and Central clades. The basal node joining the Eastern and Central clades is supported by Bayesian analysis, but not ML. Nevertheless, it is clear that all other Free State colonies are not part of the clades encompassing the Eastern and Central populations. While the sample sites from the Free State do not form a monophyletic clade, for ease of terminology, these groups will be referred to as: A) Western clade (Wesselsbron, Welkom, Senekal, Edenville, Heilbron, Lindley, Harrismith, Verkykerskop), B) Central clade (Vrede), and C) Eastern clade (Amersfoort, Volksrust, Perdekop) (green, orange, and blue clades respectively in Fig. 3.2 and Fig. 3.3).

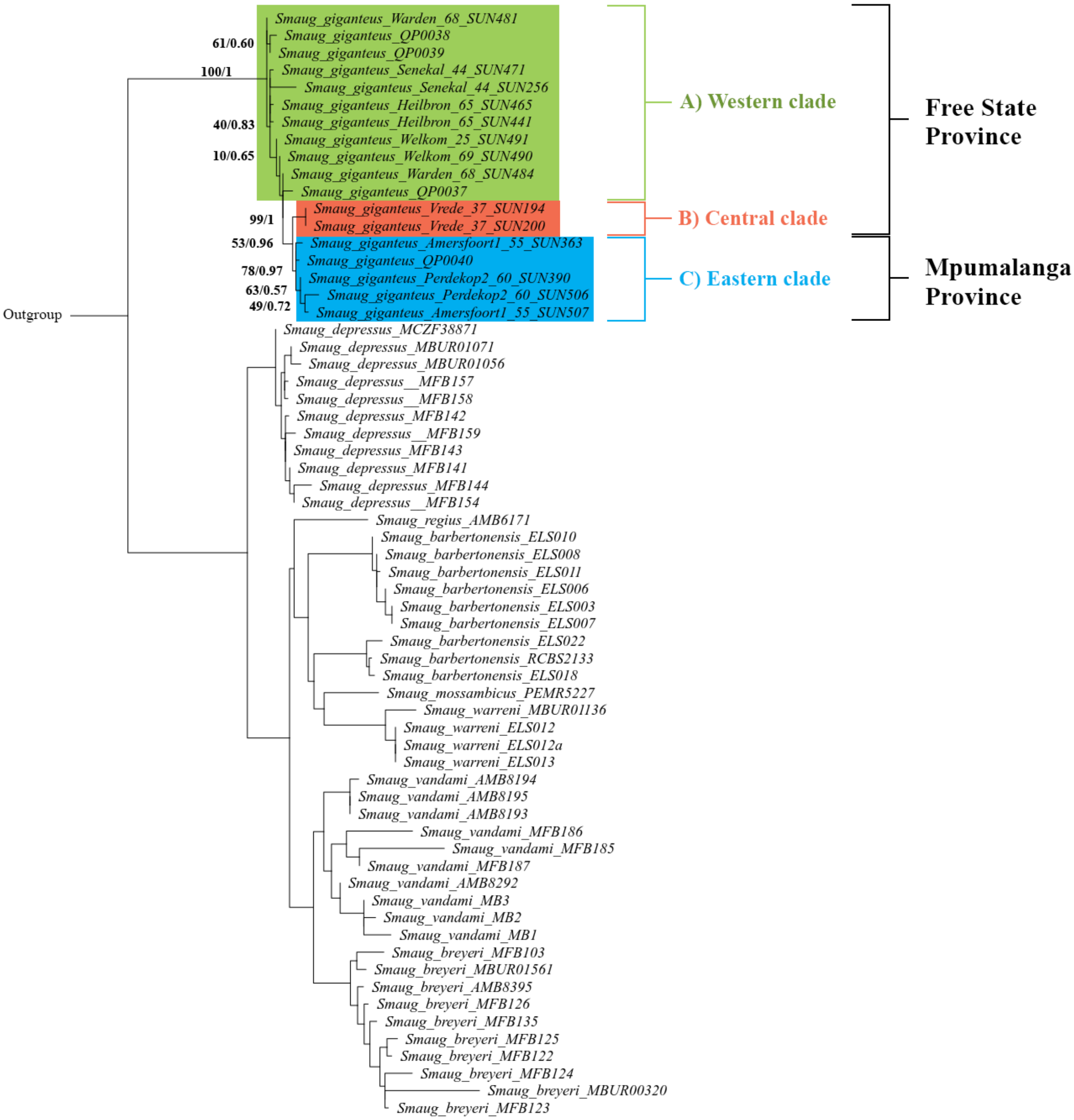


Figure 3.2. Phylogenetic tree of *Smaug giganteus* inserted into genus level *Smaug* phylogeny from Stanley and Bates (2014). The tree is based on the concatenated dataset of 12S, 16S, and Kif24 gene regions, and inferred by BI and ML (Bayesian topology shown). Support values for ML bootstrap and BI posterior probability are provided at each node (ML/BI). Green, orange, and blue shading denote the Western, Central, and Eastern clades (section 3.3.1) respectively.

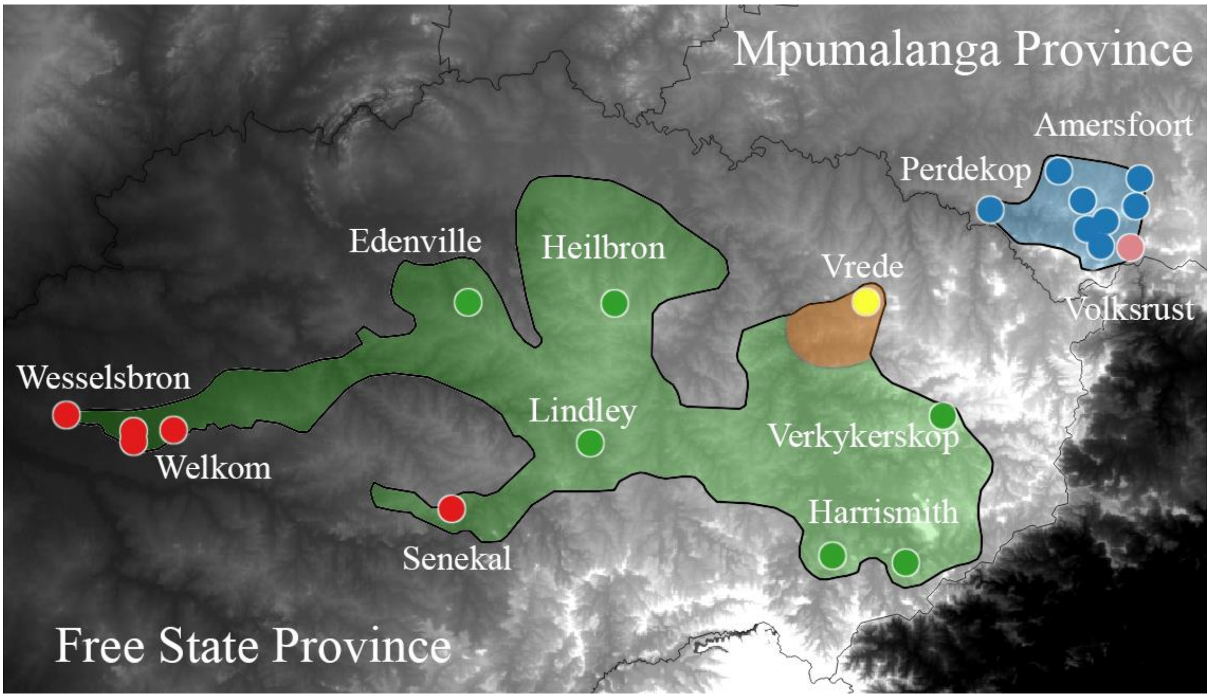


Figure 3.3. *Smaug giganteus* interpreted distribution showing population structure as indicated by phylogenetic and microsatellite analysis. Polygon colours correspond with phylogenetic clades (Fig. 3.2) and SAMOVA analysis (Fig. 3.6). Different coloured circles indicate further populations identified within each clade by GENELAND software.

3.3.2. *Population genetic structure*

3.3.2.1. Bayesian clustering analysis

The Bayesian clustering algorithm in GENELAND identified five genetic populations (Fig. 3.3, Fig. 3.4, Table 3.6). The clustering arrangement in this analysis is similar to the phylogenetic and SAMOVA analyses, but with further divisions within the Western and Eastern clades/populations (Table 3.6). The non-spatial algorithm implemented in STRUCTURE detected that the largest ΔK (Evanno et al. 2005) occurred at K = 2 (Fig. 3.4), grouping all Mpumalanga and Vrede colonies into one population (Eastern population), and all other Free State samples into another population (Western population) (Fig. 3.5). Progressive partitioning of these populations in STRUCTURE resulted in a near identical arrangement to GENELAND'S clustering analysis (Fig. 3.5), with the exception that GENELAND placed the south-eastern most Mpumalanga colony (Volksrust 3) into its own population (Western 2), whereas STRUCTURE also placed the adjacent colonies of Volksrust 1, Volksrust 2, Amersfoort 2 into this population (Table 3.6, Fig. 3.5). While the majority of individuals in the STRUCTURE analysis had high member coefficients (0.8-1.0) to a population for all examined K values, some individuals showed high levels of admixture between different populations (Fig. 3.5).

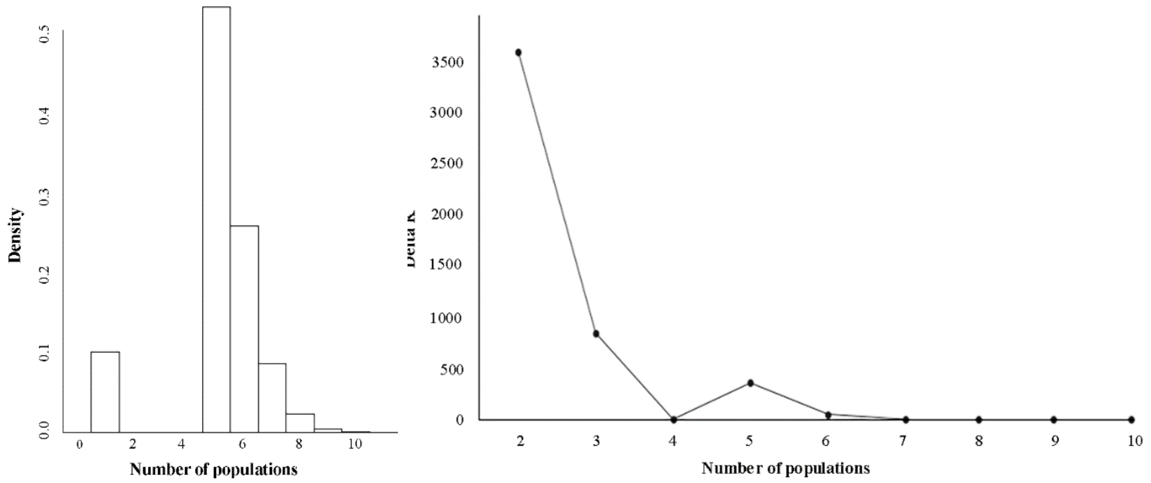


Figure 3.4. Number of unique *Smaug giganteus* genetic populations (K) estimated in Bayesian clustering programmes GENELAND (left) and STRUCTURE (right). The highest Density and Delta K values for each graph respectively indicates the most likely number of *S. giganteus* populations (GENELAND, K = 5; STRUCTURE, K = 2).

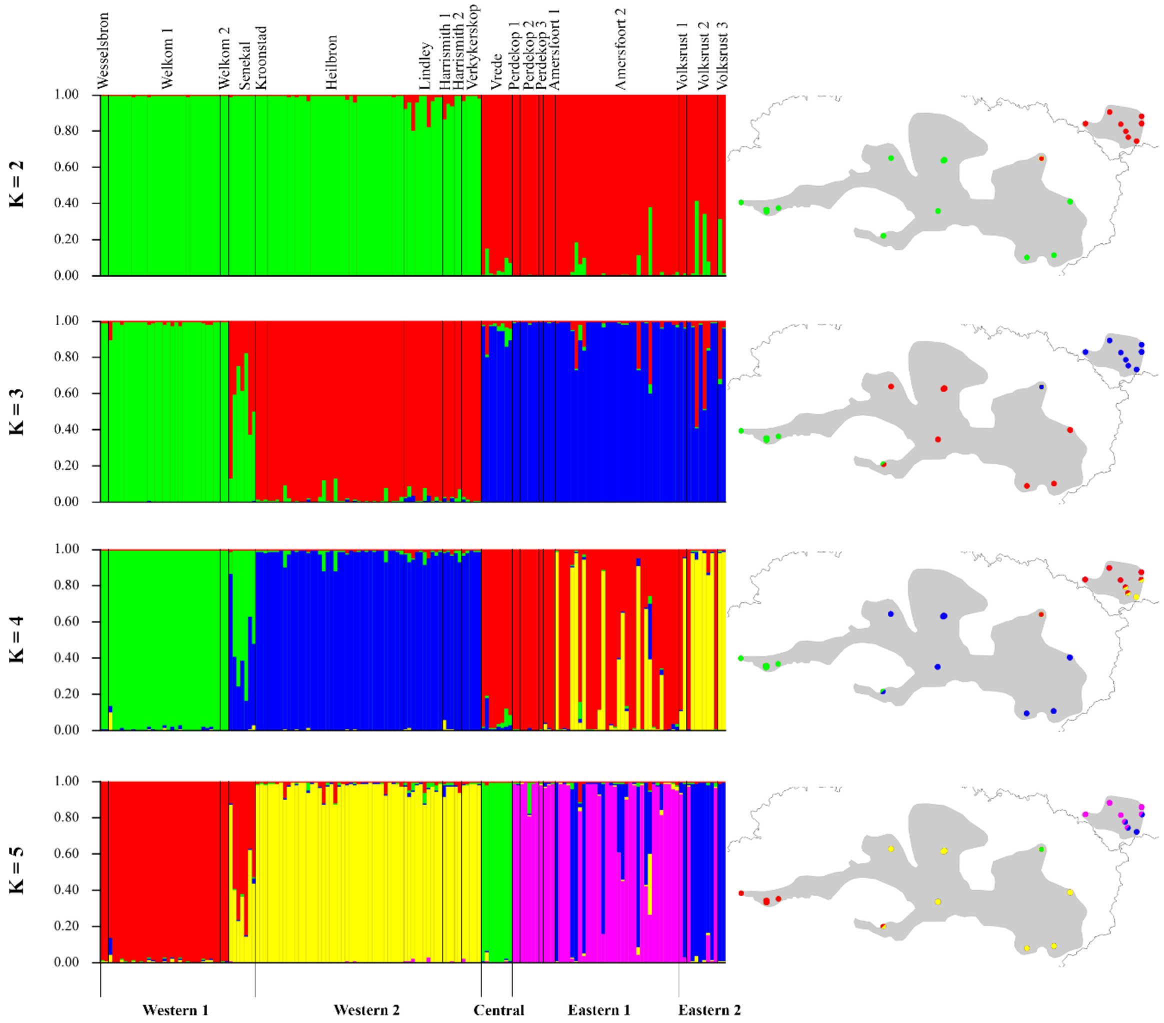


Figure 3.5. Coloured bars represent assignment of individuals within 19 *Smaug giganteus* colonies to different populations as identified in STRUCTURE and STRUCTURE HARVESTER (K = 2-5). The accompanying map shows the spatial spread of each genetic population. Coloured circles respond with the majority assignment of individuals within a colony to a population. Colonies which had a high level of mixed assignment of individuals to different populations are represented by circles filled with colours of the two colonies that they are split between.

The GENELAND analysis had a higher F_{IT} score than the STRUCTURE analysis in the comparative ARLEQUIN AMOVA (Table 3.5). Though very similar, the population genetic structure identified by GENELAND is therefore regarded as more accurate than the genetic structure identified in STRUCTURE, and is therefore the model of genetic structure displayed on subsequent illustrations of population genetic structure for *S. giganteus* (Fig. 3.12, Fig. 3.14, Fig. 3.15).

3.3.2.2. Spatial analyses of molecular variance

Analysis of population structure in SAMOVA showed that the largest increase in F_{CT} values was observed when the geographic sampling area was partitioned into three populations (Fig. 3.6). The genetic populations identified by the SAMOVA analysis is identical to grouping of populations in the phylogenetic analysis (Fig. 3.1): A) Western clade (Wesselsbron, Welkom, Senekal, Lindley, Edenville, Heilbron, Harrismith, Verkykerskop), B) Central clade (Vrede), and C) Eastern clade (Perdekop, Amersfoort, Volksrust).

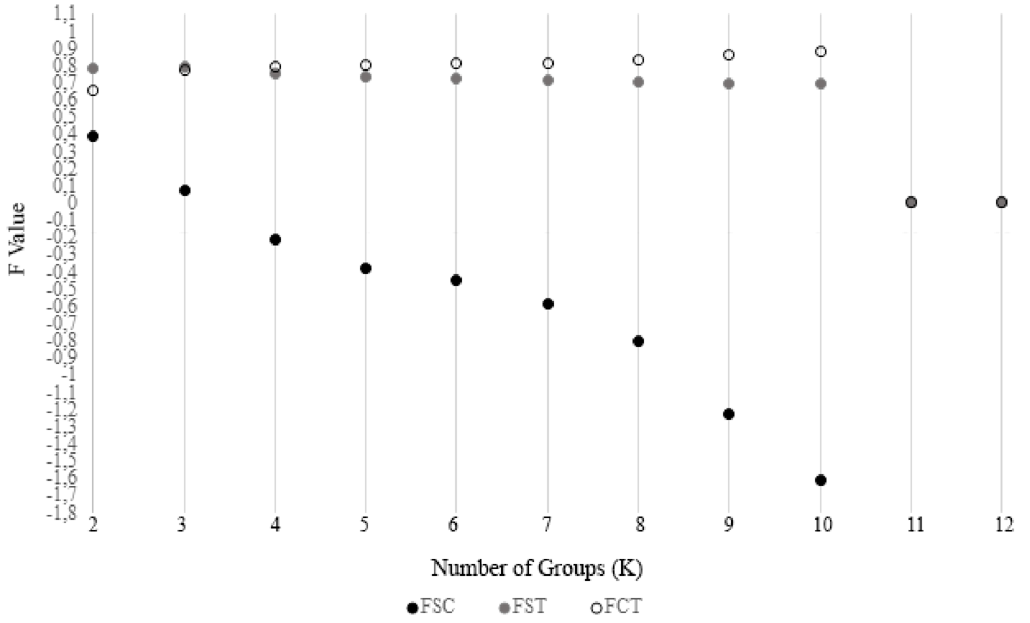


Figure 3.6. Number of unique *Smaug giganteus* genetic populations estimated in SAMOVA. The position at which the greatest increase in FCT values occur indicates the most likely number of genetically unique *S. giganteus* populations.

3.3.2.3. Analysis of molecular variance

The same patterns of genetic structure were favoured in AMOVA analysis using the concatenated gene dataset and the microsatellite dataset. SAMOVA/phylogenetic analysis (K=3) (scenario III) had the highest F_{CT} score, followed by GENELAND (K=5) (scenario IV) and STRUCTURE (K=5) (scenario V) (Table 3.5). Pairwise F_{ST} / F_{IT} values (all p values < 0.01) (Table 3.5) between populations for each of these scenarios indicates significant genetic differentiation between populations for both gene sequences and microsatellites.

Table 3.5. Results of analysis of molecular variance (AMOVAs) run in ARLEQUIN for six different grouping scenarios of population structure for *Smaug giganteus*. Grouping scenarios were based on different hypotheses of genetic structure and findings from prior analyses within this chapter. F_{CT} is the proportion of total genetic variance attributable to the differences between groups of sample sites; F_{SC} represents the variation between sample sites within groups; F_{ST} / F_{IT} is the genetic variation between sample sites relative to the total sample.

	Scenarios of population structure					
	I	**II**	**III**	**IV**	**V**	**VI**
	Two populations: Free State Province, Mpumalanga Province	**Two populations:** Western population, Eastern population (Based on STRUCTURE results (K=2))	**Three populations:** Western, Central, Eastern clade (Based on phylogenetics and SAMOVA results)	**Five populations:** Western 1, Western 2, Central, Eastern 1, Eastern 2 populations (Based on GENELAND results (K=5))	**Five populations:** Western 1, Western 2, Central, Eastern 1, Eastern 2 populations (Based on STRUCTURE results (K=5))	**Six populations:** Vegetation Bioregions
	Combined gene sequences (12S, 16S, Kif24)					
F_{SC}	0.503	0.390	0.069	-0.068	-0.068	0.549
F_{ST}	0.792	0.788	0.796	0.734	0.734	0.699
F_{CT}	0.589	0.652	0.777	0.750	0.750	0.331
	Microsatellites					
F_{SC}	0.159	0.139	0.125	0.106	0.103	0.127
F_{IT}	0.188	0.198	0.200	0.175	0.171	0.162
F_{CT}	0.084	0.116	0.131	0.123	0.123	0.089

F_{ST} values between clades were relatively high, indicating significant divergence between populations in each clade (Table 3.7). The Western and Central clades showed the highest pairwise differentiation for the combined gene sequence dataset, indicating that these populations may have lower gene flow between them compared to the other populations. Western and Eastern clades showed the lowest pairwise F_{ST} value despite the greater geographic distance between them.

Table 3.6. Geographic spread of *Smaug giganteus* genetic populations identified in population genetic and phylogenetic analyses. The type of analysis, genetic dataset used, number of unique genetic populations (K), assignment of 19 *S. giganteus* colonies to genetic populations, and a visual representation of geographic genetic structure on a map of *S. giganteus* interpreted distribution are provided.

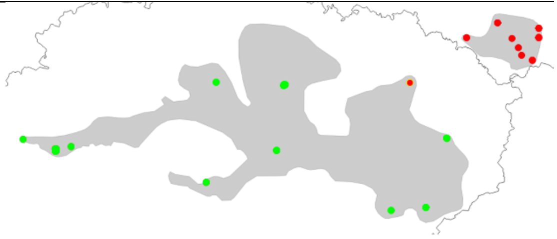
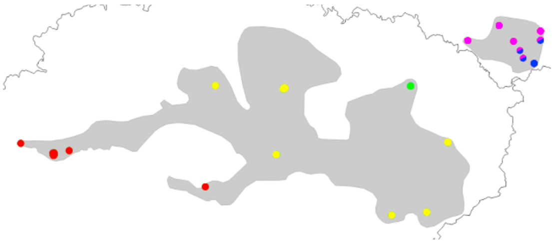
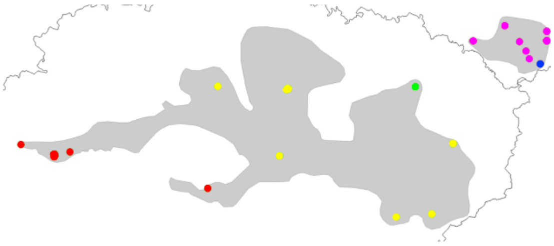
Analysis	**Dataset used**	**K**	**Genetic populations and constituent colonies**	**Map showing location and assignment of colonies to different genetic populations**
STRUCTURE	Microsatellites	2	**1) Western population** (Edenville, Harrismith 1, Harrismith 2, Heilbron, Lindsey, Senekal, Verkykerskop, Welkom 1, Welkom 2, Wesselsbron) **2) Eastern population** (Amersfoort 1, Amersfoort 2, Perdekop 1, Perdekop 2, Perdekop 3, Volksrust 1, Volksrust 2, Volksrust 3, Vrede)	
SAMOVA & phylogenetic tree	Gene sequences	3	**1) Free State Province population** (Edenville, Harrismith 1, Harrismith 2, Heilbron, Lindsey, Senekal, Verkykerskop, Welkom 1, Welkom 2,Wesselsbron) **2) Vrede Province population** **3) Mpumalanga Province population** (Amersfoort 1, Amersfoort 2, Perdekop 1, Perdekop 2, Perdekop 3, Volksrust 1, Volksrust 2, Volksrust 3, Vrede)	
STRUCTURE	Microsatellites	5	**1) Western population 1** (Senekal, Welkom 1, Welkom 2, Wesselsbron) **2) Western population 2** (Edenville, Harrismith 1, Harrismith 2, Heilbron, Lindsey, Verkykerskop) **3) Central population** **4) Eastern population 1** (Amersfoort 1, Perdekop 1, Perdekop 2, Perdekop 3,) **5) Eastern population 2** (Amersfoort 2, Volksrust 1, Volksrust 2, Volksrust 3)	
GENELAND	Microsatellites	5	**1) Western population 1** (Senekal, Welkom 1, Welkom 2, Wesselsbron) **2) Western population 2** (Edenville, Harrismith 1, Harrismith 2, Heilbron, Lindsey, Verkykerskop) **3) Central population** **4) Eastern population 1** (Amersfoort 1, Amersfoort 2, Perdekop 1, Perdekop 2, Perdekop 3, Volksrust 1, Volksrust 2) **5) Eastern population 2** (Volksrust 3)	

Table 3.7. Pairwise F_{ST} values for the Western, Central, and Eastern clades of *Smaug giganteus* under population structure scenario III (estimated using combined gene dataset; Table 3.6) (p values for each pairwise F_{ST} comparison < 0.01).

	Eastern clade	**Western clade**
Western clade	0.137	-
Central clade	0.290	0.198

3.3.3. *Isolation by distance*

The Mantel test performed for samples across the distribution of *S. giganteus* revealed a positive correlation between the two distance measures ($r^2 = 0.055$, $p < 0.01$; Fig. 3.7), indicating that with increasing geographical distance, individuals are increasingly genetically differentiated. Mantel tests run separately on the Western, Central, and Eastern clades found that while the Western clade experiences significant isolation by distance ($r^2 = 0.44$; $p < 0.01$), the Central ($r^2 = -0.09$; $p = 0.63$) and Eastern ($r^2 = -0.13$; $p = 0.98$) clades did not (Fig. 3.8). This indicates that genetic variation found within the Western clade can be explained by the geographic distance between sites. This is contrary to the genetic structure found within the Western clade by GENELAND and STRUCTURE analyses (Table 3.6). The lack of IBD for the Eastern clade indicates that populations in that clade may be panmictic, or that genetic differentiation between sites result from geographic barriers to gene flow. This corresponds with GENELAND and STRUCTURE analyses that suggest further genetic structure within the Eastern clade (Table 3.6). It is not possible to point to a cause for the lack of IBD for the Central clade given the limited number of individuals sampled from that area.

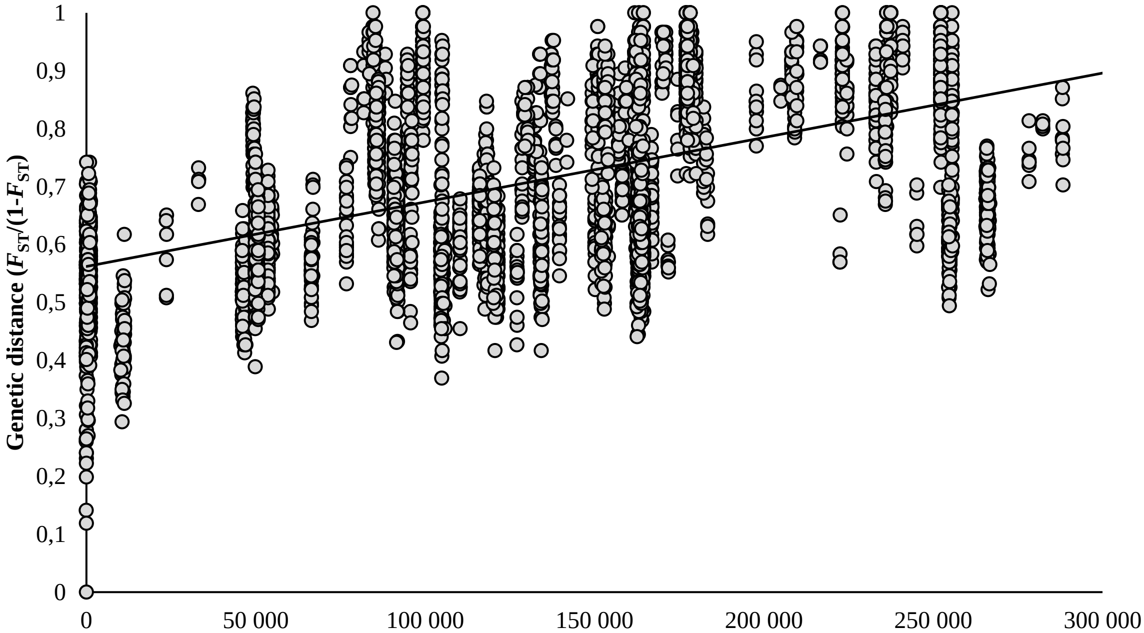


Figure 3.7. Results of the Mantel test for genetic distance ($F_{ST}/(1-F_{ST})$) and geographic distance (m) for *Smaug giganteus* across the distribution of the species. Regression coefficient (r^2) = 0.055, p < 0.01.

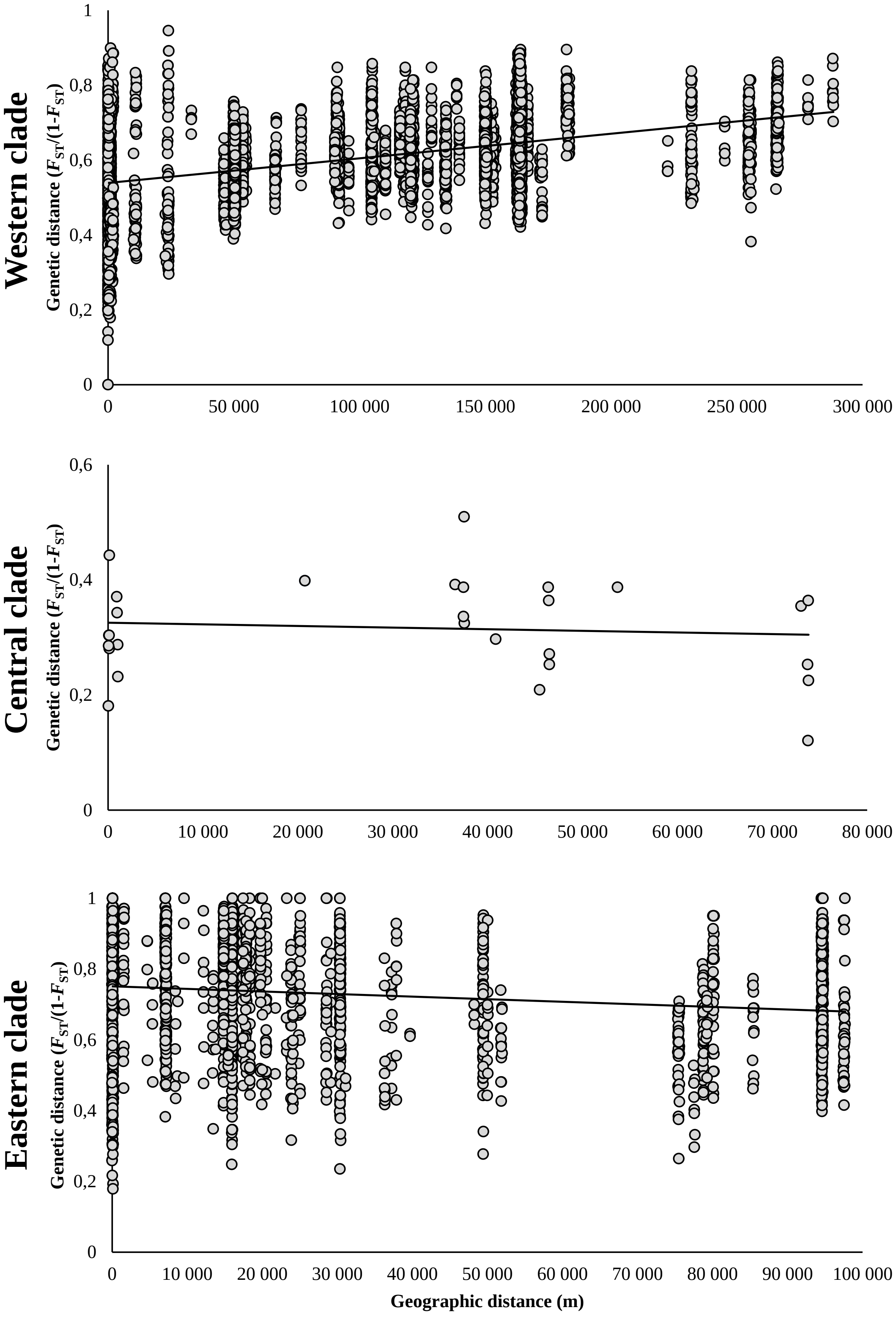


Figure 3.8. Results of the Mantel test for genetic distance (F_{ST}/(1-F_{ST})) and geographic distance (m) for the Western ($r^2 = 0.44$; $p < 0.01$) (top), Central ($r^2 = -0.09$; $p = 0.63$) (middle), and Eastern ($r^2 = -0.13$; $p = 0.98$) (bottom) *Smaug giganteus* clades identified in the phylogenetic analysis (Fig 3.1).

3.3.4. ***Morphometric comparison***

Sungazers from the Western, Central and Eastern clades are significantly different in SVL (one-way ANOVA; $F_{2,126} = 18.78$, df = 151, $p < 0.01$) and mass (one-way ANOVA; $F_{2,126} = 13.94$, df = 151, $p < 0.01$) (Fig. 3.9). Further pairwise post-hoc analyses revealed that despite the overall signal from the ANOVA, only the Western and Eastern clades are significantly different from each other in both SVL and mass (Tukey test; $p < 0.01$). Sungazers from the Western clade are on average 6% longer, and 18% heavier than Sungazers from the Eastern clade, while Sungazers from the Central clade are intermediate, but closer in size to the animals from the Eastern clade. This gradient in mass and length corresponds with the genetic structure of the species across these three clades, as well as a gradient in mean elevation of populations in each clade. The elevational gradient ranges from 1291-1806 m (average = 1567 m) in the Western clade, to 1591-1763 m (average = 1692 m) in the Eastern clade.

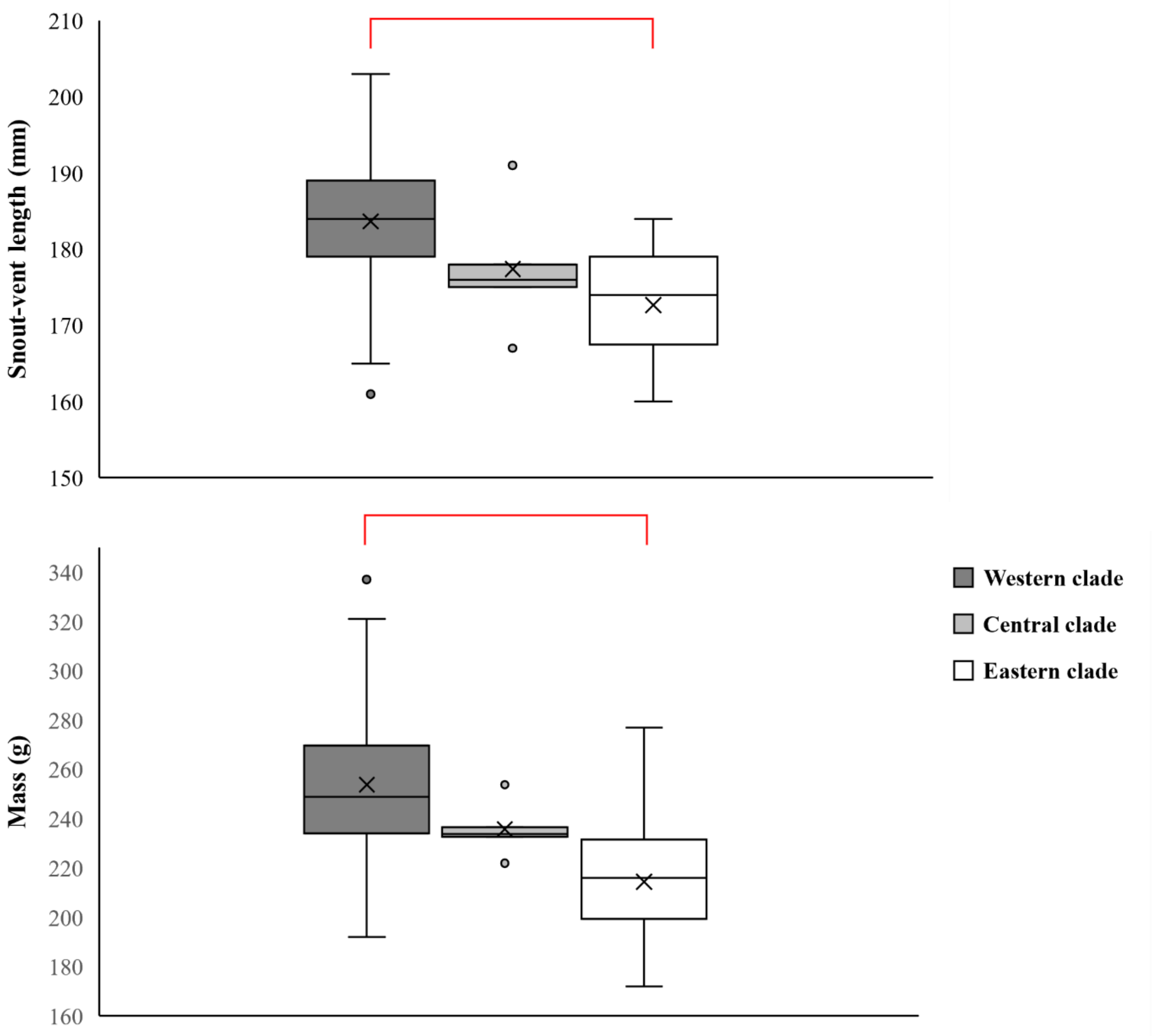


Figure 3.9. Snout-vent length (mm) (top) and mass (g) (bottom) of *Smaug giganteus* in the Western, Central, and Eastern clades. Box plots show the median, first and third quartile, X indicates the mean, whiskers extend to 1.5 times the interquartile range. Circles indicate outliers, with values greater than 1.5 times the interquartile range. Red bars indicate pairs that were not significantly different. All other pairs were significantly different (Tukey test; $p < 0.01$).

3.3.5. *Ecological niche model and visualisation in GIS*

The Area Under Curve (AUC) value of 0.92 corresponding with the ecological niche model (Fig. 3.10) reflects a high predictive ability of *S. giganteus* presence in geographic space. Values of AUC range from 0.5 (i.e. random) for models with no predictive ability, to 1.0 for models that provide perfect predictions (Swets 1988, Elith et al. 2006). The model identified soil profile, geological profile, and vegetation type as the most important predictor variables for *S. giganteus* niche suitability (Fig. 3.11). In addition, jack-knife tests showed these variables to have the greatest test gain when used in isolation, with AUC values over 0.83, indicative of these variables being most informative when used in isolation. Precipitation of the wettest quarter, landcover type, and precipitation of wettest month were the three variables that had the lowest predictive power.

The niche model identifies several areas of extremely low niche suitability within the distribution of the species that correspond with the presence of rivers, wetlands, and mountain ranges (Fig. 3.12). In particular, key landscape features that correspond with low niche suitability are the non-perennial Middelspruit River, the perennial Wilge and Vaal Rivers, the Seekoeivlei Wetland, and the Drakensberg Mountain Range. This makes sense, given that soil and geological profiles, and vegetation type had the highest predictive power for *S. giganteus* niche suitability. Rivers, wetlands, and mountain ranges are likely the main features within the landscape that significantly alter these variables, and therefore the ability of Sungazers to colonise an area.

Satellite imagery of topography (Fig. 3.12B) illustrates that the genetic disjunct between the populations of Sungazers in the Western, Central, and Eastern clades correlates with intense geological features in the region of the highest genetic differentiation. This map also illustrates how the Drakensberg Mountain Range serves as a limiting factor to the species distribution in the east. The landcover map (Fig. 3.12C) illustrates that genetic structure in the species does not appear to be correlated with habitat loss or fragmentation, since the most affected areas of the distribution fall within the genetically homogenous Western 2 population. The intermediate areas between the Western, Central, and Eastern clades are comprised primarily of intact grassland (Fig. 3.12C).

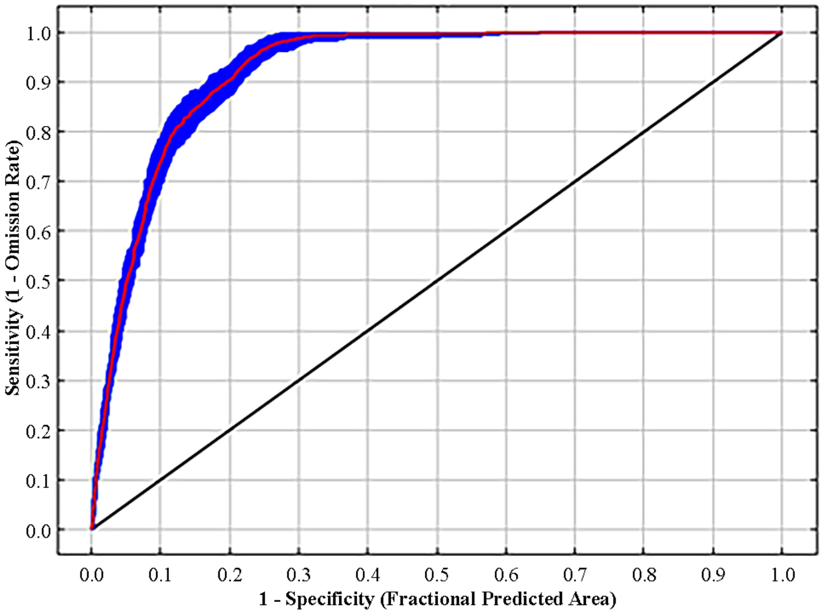


Figure 3.10. The average Receiver Operating Characteristic (ROC) plot for the ecological niche model for *Smaug giganteus* created in MAXENT. The red curve represents the mean of the average sensitivity vs 1 – specificity curve for the model, the blue line on either side of the mean is mean +/- one standard deviation, and the black horizontal bar represents a random prediction. The Area Under Curve (AUC) mean value for the model is 0.924 +/- 0.009.

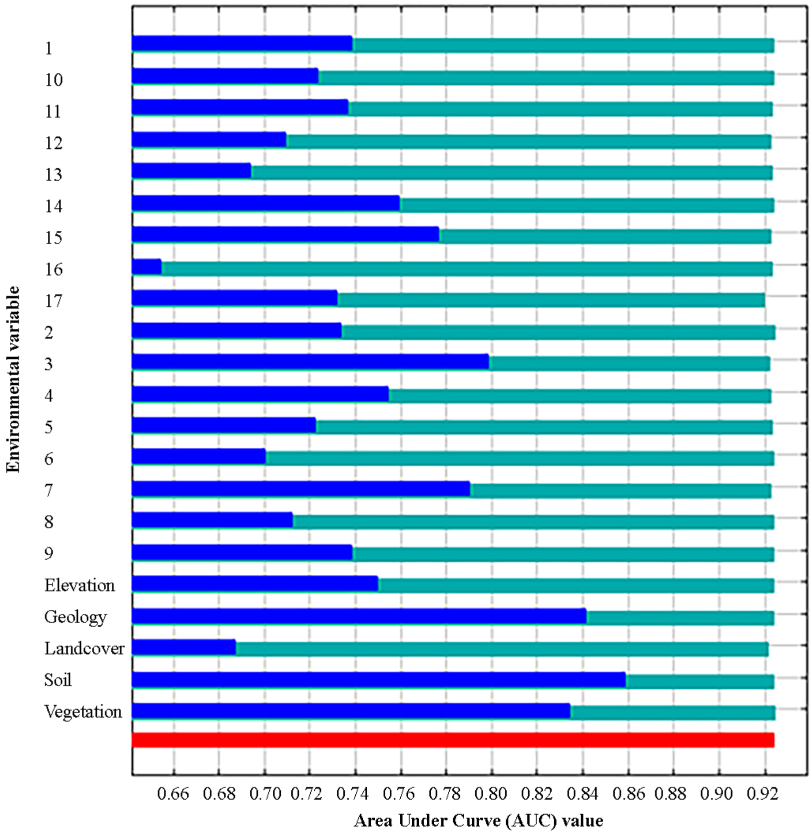


Figure 3.11. Results of jack-knife evaluations of relative importance of predictor variables for *Smaug giganteus* in MAXENT. Higher Area Under Curve (AUC) values indicate higher relative importance of each variable. The dark blue bars indicate the MAXENT model performance using the variable only, turquoise bars indicate model performance without the variable, and the red bar indicates model performance with all variables.

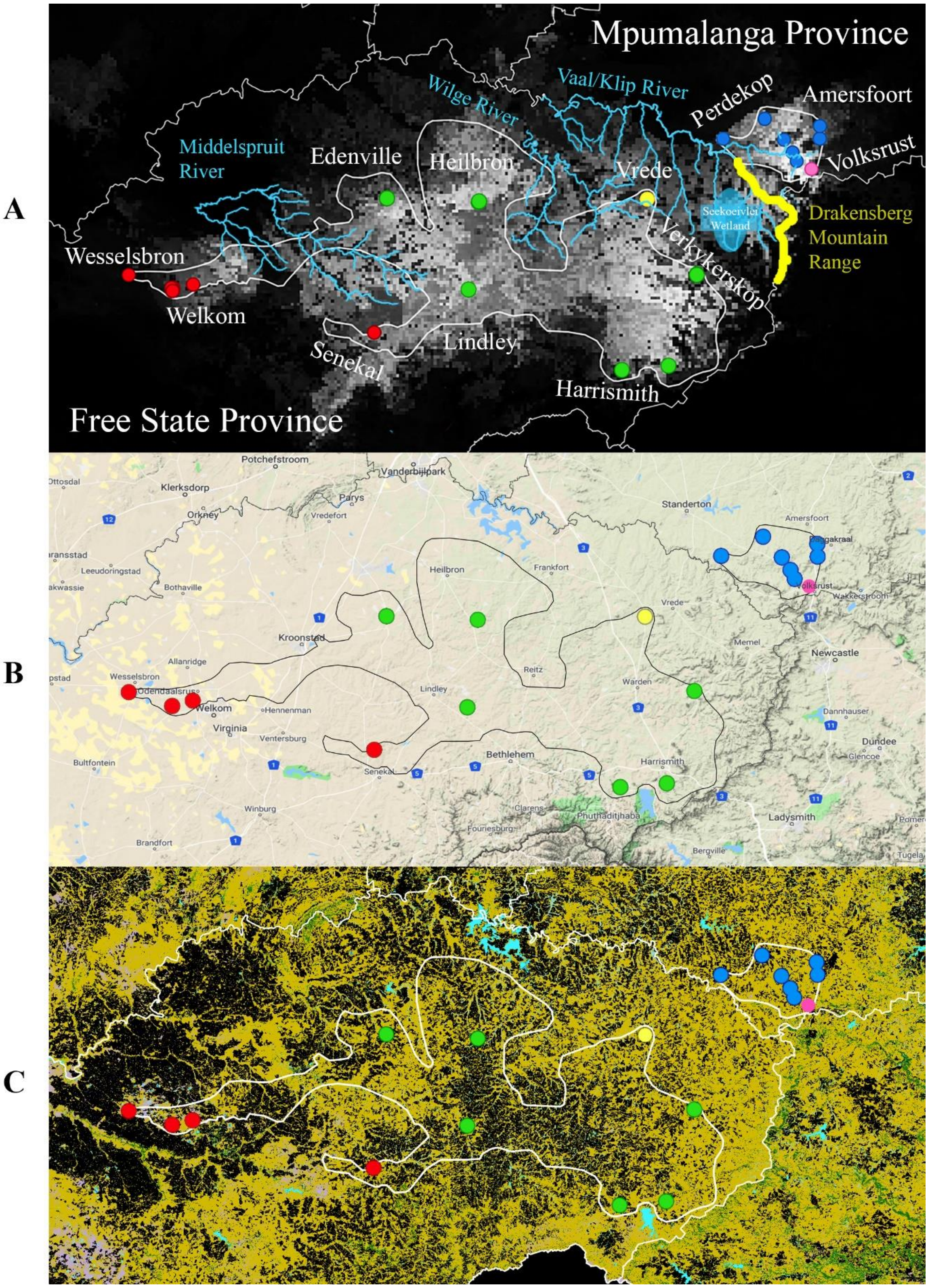


Figure 3.12. Interpreted distribution of *Smaug giganteus* overlaid onto A) graphical output of the MAXENT model showing *S. giganteus* niche suitability. The black to white colour gradient corresponds with a gradient of least to most suitable *S. giganteus* niche suitability. Rivers are indicated in turquoise, and mountain ranges in yellow, B) satellite imagery of topography (Google Maps: Terrain ©2020 AfriGIS (Pty) Ltd), and C) national data for landcover type (DEA 2015). Dark yellow = grassland, black = cultivated fields, pink = low shrubland, green = thicket/dense bush, turquoise = water bodies. Coloured circles within *S. giganteus* interpreted distribution for A-C indicate the assignment of sampled colonies to genetic populations identified in GENELAND (K = 5) (section 3.3.2).

3.4. Discussion

Smaug giganteus occurs in five genetically differentiated populations, belonging to three divergent phylogenetic clades. Phylogenetic analysis using mitochondrial and nuclear gene sequences showed that the Central (Vrede) and Eastern (Amersfoort, Perdekop, Volksrust) clades are strongly supported, whereas the Western clade (Wesselsbron, Welkom, Senekal, Edenville, Heilbron, Lindley, Harrismith, Verkykerskop) forms a large unsupported polytomy. Population genetic analyses using microsatellite markers indicated that the Western and Eastern clades are each comprised of two further genetically differentiated populations – Western 1 and Western 2, and Eastern 1 and Eastern 2, respectively. Isolation by distance, water bodies and mountains appear to be the primary drivers behind this genetic structure. A series of separate vicariant events over time may have caused the differentiation of clades and populations as evidenced in the detection of different numbers of populations by molecular markers with different evolutionary rates. There is a significant and directional difference in body size of Sungazers between the three clades. Sungazers in the cooler high elevation eastern clade are smaller in body size than the warmer lower elevation western clade, while animals in the central clade are intermediate in size.

Although I did not date the phylogenetic tree in this study, Stanley and Bates (2014) found divergence between Sungazer samples belonging to the Western clade (n = 3) and the Eastern clade (n = 1) in the early-mid Pleistocene. This timing corresponds with the most recent uplift of the eastern region of the Great Escarpment ~900 m into the Highveld grasslands during that period approximately 2.5-1.8 MYA (Partridge and Maud 1987). This event resulted in the elevation of the northern most regions of the Drakensberg Mountain Range, along which the political boundary between the Free State and Mpumalanga provinces is delineated. The formation of these mountains also appears to have altered the course of the Wilge River, and lower stretches of the Vaal River and its tributary, the Klip River, which runs longitudinally just west of the Free State/Mpumalanga boundary. The Klip River feeds into the Seekoeivlei wetland – the largest floodplain wetlands in the South African highland – which is also situated just west of the provincial boundaries. The temporal and spatial correlation of phylogenetic divergence in *S. giganteus* clades and the formation of these significant geological features is highly suggestive that these mountains, rivers and wetlands have served as a barrier to gene exchange between populations on either side of them since the late Pliocene/early-mid Pleistocene.

The Drakensberg Mountain Range and surrounding doleritic intrusions in the eastern Free State and western Mpumalanga province (De Oliveira and Cawthorn 1999, Holmes and Barker 2006) may all serve as physical barriers to dispersing Sungazers. Sungazers are large-bodied, heavily armoured lizards with relatively short limbs, making them well-adapted for defending themselves from predators within their burrows (Broeckhoven et al. 2015, Arbour and Zanno 2018), but not particularly well suited for fleeing from predators (Losos et al. 2002). This may also make them poor dispersers. This morphological build viewed in combination with the sedentary lifestyle of Sungazers suggests that they would not be able to traverse structurally complex landscapes. Thus, the eruption of these physical barriers into the homogenous grasslands is likely to have impeded gene flow between populations on either side of them. The low niche suitability for Sungazers in these montane, heterogenous environments can clearly be observed in the spatial output of the ecological niche model conducted in this study. Soil, geology, and vegetation profiles which are the most important predictors of Sungazer niche suitability are likely to be extremely different from the grasslands in these environments. Thus, even in the scenario that some individual Sungazers successfully navigate these complex montane landscapes, these areas are unsuitable for Sungazers to dig burrows into, and are therefore unlikely to survive for extended periods of time without the safety of a burrow to retreat into.

Although Stanley and Bates (2014) did not include samples from the Central clade in their dated phylogeny, the genetic differentiation between the Western, Central, and Eastern clades could all possibly be explained by the same vicariant event, since the spatial partitioning of these genetic clades are all associated with geological intrusions in that region. Similarly, the genetic differentiation detected between the Eastern 1 and Eastern 2 genetic populations with the Eastern clade appears to be correlated with a structurally complex landscape in that region. The escarpments and ridges that occur between the Eastern 1 and Eastern 2 populations are at a much lower elevation than those between clades, but may nevertheless impede gene flow between the colonies that surround it. GENELAND, which is spatially explicit in its analysis, showed that the Eastern 2 population contains only the Volksrust 3 colony, whereas STRUCTURE which is not spatially explicit, also included the Amersfoort 2, Volksrust 1, and Volksrust 2 colonies into this population. Several individuals in the Amersfoort 2, Volksrust 1, and Volksrust 2 colonies showed partial assignment to both the Eastern 1 and Eastern 2 populations in the STRUCTURE analysis. This indicates that while these three may experience admixture between the two genetic groups, the colonies on the northwest and southeast borders of the Eastern clade do not exchange genetic material. The relative complexity of the landscape in the Eastern clade compared to the Western clade suggests that high levels of genetic differentiation may occur even across relatively short distance in the Eastern clade.

Mountain ranges are commonly serve as genetic barriers to reptiles (Jacobsen and Pienaar 1983, Turner et al. 2003, Gholamifard 2011). The physical terrain may be difficult for individuals to traverse, and the microclimate at high elevations may be unsuitable for many species to inhabit. The majority of the Cordylidae however, are rupicolous, and in most cases mountain ranges do not necessarily impede flow. In rupicolous species, divergence may be related to other geoclimactic processes. Cladogenesis in the *Smaug warreni* complex is suggested to have resulted primarily from climactic cooling during the Miocene (Stanley and Bates 2014). The drop in climate may have resulted in an increase in suitable habitat for the species complex, resulting in expansion and dispersal into new areas. Radiation in the *Platysaurus* is traditionally thought to have resulted from the eastern migration of the Kalahari sands in the Plio-Pleistocene, fragmenting populations (Broadley 1978). Although (Scott et al. 2004) suggest that speciation events in Platysaurus occurred prior to the ingression of the Kalahari sands and that speciation may rather be aligned with the formation of major river systems. In *Hemicordylus capensi*s and *Cordylus cordylus*, river valleys appear to serve as a physical barriers to dispersal (Stanley 2013, Diedericks and Daniels 2014). The TweeRivier and Breede River valleys both contain microclimate and ecological conditions that are unfavourable to these species, and are therefore unlikely to be traversed by them, impeding gene flow. *Hemicordylus capensi*s and *C. cordylus,* like most other cordylid species, have adapted to montane habitats, and gene flow in these species is facilitated by rather than impeded by mountains. In each of these cases, it is clear that montane habitat does not appear to inhibit gene flow, but rather may facilitate it. The mountain barriers detected for *S. giganteus* in my study are therefore likely to be rare amongst cordylids.

The thickset heavy bodies of Sungazers also appear to limit their movements across water bodies. The Middelspruit River, Wilge River, Vaal River (and its tributary, the Klip River), and Seekoeivlei Wetland in particular all correlate with low niche suitability, and genetic differentiation of populations that occur on either side of them. Loveridge (1944) noted that Sungazers appear to not be able to swim, sinking and drowning when introduced to a body of water. Field observations suggest that during heavy rainfall, Sungazers remain weighted at the bottom of the burrow, and do not float to the surface even when the burrow is fully flooded (pers. obs). This is likely due to their heavily ossified spines that serve as armour, but also make them heavy. This apparent negative buoyancy of Sungazers might prevent them from crossing even small water bodies. The Wilge and Vaal/Klip are wide, perennial rivers, and therefore unlikely to be traversed by Sungazers. The Seekoeivlei Wetland is also permanently inundated, and its 12 000 hectares represent an unhabitable environment to Sungazer in its entirety. Each of these three latter water bodies also occur in the montane habitat in the east of the distribution, and their influence on gene flow between Sungazer populations is difficult to isolate from the surrounding montane areas. It is likely however, that these water bodies in combination with the montane habitat that they are associated with, present strong barriers to dispersing Sungazers.

The non-perennial Middelspruit River flows between the Western 1 and Western 2 populations in the Western clade. The river is associated with Highveld Alluvial Vegetation (Aza 5; Mucina and Rutherford 2006), which is seasonally flooded and underlain by coarse sand which is an unsuitable substrate for the construction of Sungazer burrows. The area surrounding the Middelspruit River has been dominated by alien plants in recent times (Mucina and Rutherford 2006), and has also been heavily transformed for use as crop fields. The genetic disjunct between the Western 1 and Western 2 populations was not detected in the phylogenetic analysis using mitochondrial and nuclear gene sequences, only with the use of rapidly evolving microsatellite markers. This may indicate that the non-perennial nature of this river could allow for limited dispersal of individuals over periods when the river does not flow, such that long-term genetic isolation of populations on either side of the river is not experienced. A significant isolation by distance signal was detected within the Western clade, and the correlation of genetic differentiation of populations with the location of the river may not reflect the true cause of this genetic structure. The interpretation of the Middelspruit River as a putative barrier to gene flow therefore needs to be taken with caution.

Rivers serve as barriers to dispersal in many reptiles, both within and between species (Pounds and Jackson 1981, Rodrigues and Juncá 2002, Pellegrino et al. 2005, Ratsoavina et al. 2012, Souza et al. 2013, Somers et al. 2017). The level of genetic differentiation between populations is often linked to the size of the river and therefore its effectiveness as a barrier (Pounds and Jackson 1981, Hayes and Sewlal 2004). Nevertheless, even fairly narrow rivers (~20 m wide), can serve as a significant barriers to gene flow in species that are not adept at traversing large bodies of water (Pounds and Jackson 1981, Ratsoavina et al. 2012). Rivers appear to have resulted in several vicariant events in the Cordylidae. Stanley and Bates (2014) suggest that the increased volume of the Limpopo River in the late Miocene correlates with *Smaug regius* and *S. mossambicus* splitting from the rest of the *Smaug warreni* species complex. Similarly, Scott et al. (2004) found that genetic differentiation between *Platysaurus mitchelli* and *P. m. maculatus* from the rest of the Platysaurus family to be concomitant with the flow of the Zambezi River system. In *Hemicordylus capensi*s and *Cordylus*, river valleys appear to serve as a physical barriers to dispersal (Stanley 2013, Diedericks and Daniels 2014). As opposed to the rivers themselves, population differentiation in both species is suggested to occur due to unfavourable microclimate and ecological conditions within the valley that reduce the likelihood of individuals traversing them (Stanley 2013, Diedericks and Daniels 2014). It is clear that rivers and their associated microclimates are important barriers to species in the Cordylidae. More focused studies on the differentiation between Sungazer colonies on either side of the various rivers within the species distribution may elucidate the specific effect that each river has on genetic structure.

Isolation by distance was detected across the distribution of *S. giganteus*. Further tests on each phylogenetic clades identified in my study indicate that isolation by distance is only present in the Western clade, and not the Central and Eastern clades. The geographic area covered by the Western clade populations is significantly larger than the other two clades, and much more homogenous in the complexity of the landscape. Sungazers in this clade therefore appear to have few significant barriers to dispersal, and the geographic distance alone between colonies may serve to limit dispersal. Therefore the limited dispersal ability of Sungazers also lends to the genetic structuring identified within the Western clade using population genetic analyses. Isolation by distance signals are stronger in species that are small in body size (Jenkins et al. 2007), or poor dispersers (Pinto et al. 2019). Ectotherms are also twice as likely to exhibit IBD patterns that endotherms, suggesting the influence of metabolic processes on gene flow in ectothermic species (Jenkins et al. 2010). The familial nature of Sungazer colonies (detailed in Chapter 4), high-site fidelity of individuals (Ruddock 2000, Stanton-Jones 2018), low mobility of individuals (Losos et al. 2002), and IBD signal in the homogenous Western clade all indicate the Sungazers are poor dispersers, and prone to the emergence of genetic structure through distance alone.

Vegetation type was identified as an important predictor variable for Sungazer niche suitability, but vegetations bioregions did not correlate with genetic differentiation between populations. Although the vegetation type changes from the dryer and warmer Dry Highveld Grassland bioregion in the west into the wetter, cooler Mesic Highveld Grassland in the east – both these vegetation types are dominated by the grass species *Themeda triandra* with which *S. giganteus* is closely associated. It is possible that the soil conditions and invertebrate prey assemblages associated with *T. triandra* are beneficial to *S. giganteus*. The presence or absence of other plant species in these assemblages is therefore less likely to have any effect of significance on the species' distribution or genetic structure.

The level of genetic differentiation between the Western, Central, and Eastern clades identified in my study are relatively high ($F_{ST} = 0.137\text{-}0.290$) (Wright 1965). The Central and Eastern clades showed the highest genetic differentiation, despite the small geographic distance between them. Results from population level SAMOVA analyses show that there is almost no mixing of haplotypes between these major clades, indicating that the putative mountain, river, and wetland barriers between them may be absolute. The genetic differentiation between the Western and Eastern clades is lower than that between the Western and Central clades, despite the greater geographic distance between the former two clades. This may indicate that Sungazers in the Central are the most genetically distinct from the three clades. This population is the smallest of the three clades, and few individuals were sampled from this area, in part due to the rarity of the species in the region. The small size of the population, isolation from other populations, and putative age of isolation may have resulted in increased genetic drift and lower genetic diversity in the Central clade Sungazers. Eventually, such cases may result in population bottlenecks and local extirpation. Due to the small sample sizes, analyses of a population bottleneck was not possible for the Central clade population. It therefore remains unknown whether the population experiences any negative genetic effects as a result of its small population size and apparent isolation from other populations. More concerted sampling efforts within the Central clade may elucidate whether Sungazers in this clade are more at risk of extinction than those in the other clades.

There is no evidence to suggest that the genetic structure detected in my study is caused by human-mediated processes such as habitat fragmentation. In fact, areas with the highest degree of habitat loss and fragmentation such as the region between Heilbron, Lindley, and Harrismith had no discernible genetic structure between them. The intermediate area between the highly differentiated Western, Central, and Eastern clades are montane, and therefore not as intensely utilised for crop production as the adjacent low lying grassland areas. This was corroborated with results from the ecological niche model, which showed that landcover type had very low predictive power of niche suitability. This was surprising since landcover change is one of the factors that has most affected Sungazer distributions over the past century. This can be explained however, in that Sungazer colonies persist in small patches of remaining grassland even within heavily fragmented areas. Soil and geological profiles that are good predictors for Sungazer niche quality are also good predictors of suitability for crop farming. This indicates the extent of optimal Sungazer habitat that has been irreversibly transformed for crop production in the area.

The lack of genetic structure corresponding with the high level of fragmentation does not necessarily reflect that habitat transformation does not affect gene flow in the species. Allele frequencies respond to changes in gene flow on an evolutionary time frame rather than an ecological one, and there is often a time-lag in the population genetic signature expected from fragmented populations (Varvio et al. 1986, Keyghobadi 2007, Epps and Keyghobadi 2015). The large-scale agricultural land transformation within the distribution of *S. giganteus* began around 1840 with the arrival of the first Afrikaner farmers (Thompson and Berat 2014), although this only intensified around 1960 with the introduction of tractors that revolutionised ploughing (Pingali et al. 1988). The generation length of Sungazers is 15 years (Parusnath et al. 2017), and these two periods (1840-2018 and 1960-2018) equate to only 12 and 4 generations respectively. Reduction in genetic diversity resulting from genetic drift in isolated may only be detectable after 10-20 generations (Lacy 1987, Landguth et al. 2010, Blair et al. 2012). Given the long generation time, low dispersal ability, and low fecundity of Sungazers, increased genetic structure and lower genetic diversity resulting from habitat loss and fragmentation may therefore only be detected in Sungazer populations within the next 8-16 generations (120-240 years). It is possible that as with other long-lived slow reproducing lizards (Krawiec et al. 2015), high local abundance of Sungazers may cause genetic inertia that slows the genetic impact of fragmentation from being detected above the natural effects of historical patterns of isolation by distance. It is therefore imperative to continue monitoring the genetic structure of

Sungazer populations within this time period, such that the manifestation of human-mediated habitat change is recognised in future. Preventative measures should also be considered in conservation management plans, such as restoration of fallowed grassland and the maintenance of dispersal corridors.

Delayed temporal responses to habitat responses appear to be common in long-live species in fragmented areas. *Egernia cunninghami* populations in fragmented habitats did not show signs of increased inbreeding or lowered heterozygosity compared to natural habitats after 70+ years/14 generations (Stow and Sunnucks 2004) despite altered dispersal patterns (Stow et al. 2001). Inbreeding avoidance behaviour is cited as an explanation of the lack of the negative genetic effects on *E. cunninghami* populations in fragmented habitats (Stow and Sunnucks 2004), although it is possible that these effects have not yet fully manifested in extant *E. cunninghami* populations. Other studies on lizard species in agricultural areas have found that after 20 years/10 generations (*Ctenotus fallens*, Krawiec et al. 2015), 60 years/12 generations (*Eulamprus heatwolei*, Lyndon-Gee et al. 2018), 60 years/15 generations (*Plestiodon reynoldsi*, Mccoy et al. 2010), 90 years/30-38 generations (*P. reynoldsi*, Richmond et al. 2009), no significant effects on genetic structure are reported.

An important consideration in these studies is the type of marker used, and the scale at which the study is done. *Gnypetoscincus queenslandiae* showed no clear impact of forest clearing on genetic structure after 70 years/11 generations using mtDNA (Cunningham 1993, Cunningham and Moritz 1998), but similar analysis using rapidly evolving microsatellite markers found signs of declining genetic diversity and smaller effective population sizes in fragmented areas after 80 years/12 generations (Sumner et al. 2004). Even in the latter study however, the effects of forest clearing on genetic structure in *G. queenslandiae* were much lower than expected. *Gnypetoscincus queenslandiae* may also be experiencing a time-lag effects. Moore et al. (2008) reported genetic differentiation amongst populations of *Sphenodon punctatus* as a result of recent habitat modification after only 2-3 generations (110 years). The study on this long-lived species was conducted a finer geographic scale than most other comparable studies on reptiles and highlights the danger of only assessing genetic impacts of habitat fragmentation at a broad-scale. Further analyses that statistically interrogate the link between habitat fragmentation (through variables such as road, crop and human population density) and genetic structure in *Smaug giganteus* may reveal further insight into how anthropogenic factors influence gene flow in the species.

Sungazers from the Western and Eastern clades are significantly different in body size. Individuals of both sexes from the Western clade being longer and heavier than those from Eastern clade. The Central clade individuals are intermediate in both length and mass, and not significantly different from the Western or Eastern clades. Populations in the Eastern clade are 140 m higher in elevation than populations in the Western clade on average, mean temperatures are lower than the west by 25% in summer, and 23% in winter, and experience 60% more annual precipitation than the Western clade (Van Wyk 1992). This clinal trend from west to east appears to indicate that *S. giganteus*, like some other squamate species, follow the inverse of Bergmann's rule, i.e. decreasing body size within a species with increasing elevation or possibly decreasing environmental temperature (Ashton and Feldman 2003).

Smaller body sizes in cooler climates may be the result of selection for increased surface area to volume ratio to allow more rapid heating and cooling (Cowles 1945, Bogert 1949, Stevenson 1985a, 1985b). Because optimal body temperature may be constant throughout the geographic range of a species (Bogert 1949), smaller individuals in cooler environments may be able to more finely control their body temperature behaviourally and be active for a greater proportion of the day. Such thermoregulatory abilities may be critically important for digestion and development in squamates that inhabit cooler areas, since many squamates swallow food items whole or in large pieces, and retain young for long periods (Pianka and Vitt 2003, Pincheira-Donoso et al. 2008). In warmer climates, on the other hand, thermoregulation may not be such an important factor (Shine and Madsen 1996), thereby relaxing selective pressure on an organism's surface area to mass ratio, and permitting squamates to attain larger sizes (Arnold and Peterson 1989).

Bergmann's rule (and its inverse) however, is a controversial and much debated concept when applied to ectotherms (Angilletta, Jr., and Dunham 2003, Angilletta, Jr. et al. 2004, Sears and Angilletta, Jr. 2004). An intercontinental analysis of 65 squamate species found that 6% of species supported Bergmann's rule, 9% follow its inverse, and the remaining 85% showed no generalised trend between body size and climate or elevation (Pincheira-Donoso and Meiri 2013). It is prudent therefore to also consider other explanations for the difference in body sizes of Sungazers in different clades, since although Sungazers show a clear correlation between body size and climate/elevation, this not necessarily imply causation in a genetic sense. Prey availability is another factor that may directly influence body size. For example, snakes fed a high-energy diet are heavier at maturity than those fed a low-energy diet (Ford and Seigel 1989, 1994). Sungazers in cooler areas may experience fewer food resources, resulting in smaller body sizes. Studies of captive rearing of Sungazers from different clades with the same diet may elucidate whether body size is in fact determined by its genotype, or simply a morphologically plastic trait (Stillwell 2010). In the case that body size is linked to genetic differentiation between clades, this phenotypic change may result from random genetic drift and mutation, and not necessarily local adaptation. Further studies are required to elucidate the link or lack thereof, between these variables.

The findings of my study have implications for the management of this species and its habitat. The Highveld grasslands that the species occupy are already heavily transformed, and further utilisation of this land for agricultural, urban, or infrastructural developments threaten existing populations. Efforts to translocate Sungazers from areas earmarked for development should take the genetic structure of the species reported in this study into account. Translocations among populations may dampen local adaptation or habitat-specific evolutionary potential and is not advisable (Goldberg et al. 2005). If inter-population translocations are deemed necessary, a careful cost-benefit analysis of the results of outbreeding versus inbreeding depression are required (Weeks et al. 2011).

Similar to other grassland species, *S. giganteus* is susceptible to loss of genetic diversity with increasing habitat fragmentation and isolation (Herkert 1994, Collinge 2000, Masterson et al. 2009, Ceballos et al. 2010). Despite the use of rapidly evolving microsatellite markers in my study, no effects of the severe fragmentation across the species distribution was detected. While this may be a result of a time-lag due to the long generation time and low fecundity of Sungazers, it may also be an issue of resolution. My study considered genetic structure at species-scale, and may be too broad to detect fine-scale genetic structure between populations. More focused, fine-scale investigations of the genetic structure and diversity of populations within heavily fragmented areas are necessary to elucidate the current level of genetic structure caused by habitat fragmentation in the species.

Conclusion

My results suggest that *Smaug giganteus* occurs in three phylogenetic clades, separated by mountain and river barriers that limit dispersal between them. Cladogenesis is likely to have occurred as a result of the most recent uprising of the northern reaches of the Drakensberg mountains and the associated geological changes during the late Pliocene. Sungazers from these clades are different in body size, with body size decreasing with higher elevations and lower temperatures. The Western and Eastern clades are further divided into two more recent genetic populations, detected through use of rapidly evolving microsatellite markers. The genetic break between the Western 1 and Western 2 genetic population corresponds geographically with the non-perennial Middelspruit River, although an IBD signal was detected within the Western clade. Further and more fine-scale research is required to elucidate whether the river or IBD is the effector of genetic structure within that clade. Despite intense fragmentation of primary grasslands in some areas of the species distribution, no clear effect of these processes were detected in my analyses. This suggests that like many other species with long generation length and low fecundity, *S. giganteus* may experience a temporal lag between reduced dispersal and gene flow between populations and genetic structure. The genetic structure of the species and the potential future manifestation of habitat fragmentation are therefore important factors to consider in conservation management plans for the species.

3.5. Supplementary material

Table S3.1. Species and GenBank accession numbers of species used in the phylogeny of the *Smaug* genus by Stanley and Bates (2014)

Species	**GenBank accession number**
Smaug barbertonensis	ELS010
Smaug barbertonensis	ELS008
Smaug barbertonensis	ELS011
Smaug barbertonensis	ELS003
Smaug barbertonensis	ELS006
Smaug barbertonensis	ELS007
Smaug breyeri	MBUR01561
Smaug breyeri	MFB103
Smaug breyeri	AMB8395
Smaug breyeri	MFB126
Smaug breyeri	MFB135
Smaug breyeri	MBUR00320
Smaug breyeri	MFB122
Smaug breyeri	MFB125
Smaug breyeri	MFB123
Smaug breyeri	MFB124
Smaug depressus	MCZF38871
Smaug depressus	MFB157
Smaug depressus	MFB158
Smaug depressus	MBUR01056
Smaug depressus	MBUR01071
Smaug depressus	MFB159
Smaug depressus	MFB142
Smaug depressus	MFB143
Smaug depressus	MFB144
Smaug depressus	MFB154
Smaug depressus	MFB141
Smaug giganteus	QP0037
Smaug giganteus	QP0038
Smaug giganteus	QP0039
Smaug giganteus	QP0040
Smaug mossambicus	PEMR5227
Smaug regius	AMB6171
Smaug vandami	AMB8292
Smaug vandami	MB1
Smaug vandami	MB2
Smaug vandami	MB3
Smaug vandami	AMB8194
Smaug vandami	AMB193
Smaug vandami	AMB195
Smaug vandami	MFB186
Smaug vandami	MFB185
Smaug vandami	MFB187
Smaug warreni	MBUR01136
Smaug warreni	ELS012
Smaug warreni	ELS012a
Smaug warreni	ELS013
Smaug swazicus	ELS018
Smaug swazicus	ELS022
Smaug swazicus	RCBS2133

CHAPTER 4

KIN-BASED SOCIALITY IN THE SUNGAZER (*SMAUG GIGANTEUS*)

4.1. Introduction

Sociality in animal populations comprises of a complex suite of behaviours that range from rare, transient interactions between unrelated individuals, to long-term monogamy, stable aggregations, and parental care (Ward and Webster 2016, Lukas and Clutton-Brock 2018, Kramer and Meunier 2019). Complex social behaviours such as fraternal, kin-based sociality have traditionally been associated with vertebrate classes such as mammals and birds (Stahlschmidt 2011, Doody et al. 2013). Reptiles, on the other hand, have long been thought to align closely with the traditional “r-selected” strategy – characterised by early age of first reproduction, large litter size, and minimal parent-offspring interaction (see Dunham 1982). However, research on reptile behaviour and social structure, particularly over the past two decades, has illuminated the level of complex kin-based sociality present in a variety of reptile species (Gardner et al. 2016, Whiting and While 2017, While et al. 2019). In fact, recent phylogeny-based research has estimated that up to 10% of squamate reptile species may exhibit some form of complex kin-based sociality (Halliwell et al. 2017b).

Kin-based sociality in squamates can consist of long-term stable groups, refuge sharing, pair monogamy, and prolonged parent-offspring interaction (Bull 2000, Lanham and Bull 2000, Gardner et al. 2001, 2016, Duffield and Bull 2002, Whiting and While 2017, Alexander 2018). While group-living may present disadvantages to the individual such as competition for resources and inbreeding (Leu et al. 2011), the many potential benefits of group-living may supersede these disadvantages, and drive the evolution of complex sociality. Advantages of group-living in squamates include increased vigilance for predator avoidance, increased offspring survival through parental care or parental tolerance, social learning, inheritance of territory, and a reduction in conspecific aggression (Langkilde et al. 2007, McAlpin et al. 2011, Munch et al. 2018, Riley et al. 2018, Whiting et al. 2018). Species that display kin-based sociality often share similar life-history traits, including late age of first reproduction, long life-span (and/or generation length), large body size, live birth, and small litter size (Pianka and Vitt 2003, Halliwell et al. 2017b, Whiting and While 2017). Although aggregations have been reported in almost 100 squamates species (Gardner et al. 2016), this trait appears to be more prevalent in lizards than in snakes (Halliwell et al. 2017b). This is likely a result of the higher prevalence of live birth and stable social groupings in lizards than in snakes. Live birth in particular appears to be an ancestral state in most squamates with stable social groups, and may be an important precursor to the evolution of kin-based sociality in reptiles (Halliwell et al. 2017b).

Although numerous studies have detailed the systems of egalitarian sociality in lizards (eg. Qi et al. 2012, Strickland et al. 2014), the body of work investigating fraternal sociality in lizards has been significantly biased towards the *Egerniiae* subfamily (consisting of eight genera: *Egernia, Liopholis, Lissolepis, Bellatorias, Cyclodomorphus, Tiliqua, Tribolonotus* and *Corucia;* While et al. 2019), hereafter referred to as the *Egernia* group. Aside from this group, detailed studies on kin-based sociality utilising a combination of genetic evidence and long-term population monitoring have only been conducted on three other species in three families – *Xantusia vigilis* (Xantusiidae; Davis et al. 2011, Rice and Edward 2017), *Liolaemus leopardinus* (Liolaemidae; Brito 2017), and *Intellagama lesueurii* (Agamidae; Piza-Roca et al. 2019) . Several other species of lizard that live in stable aggregations may also potentially exhibit kin-based sociality, however, without genetic investigations into the relatedness of the individuals in these groups, it is challenging to ascertain the true nature of these relationships (Gardner et al. 2016).

The Cordylidae family of lizards endemic to Africa contains several species that display aggregative behaviour, ranging from ephemeral groups that temporarily co-occupy refuge sites, to what appear to be long-term stable groups (Gardner et al. 2016). Studies investigating social behaviour in the family have ranged from observations of antagonistic and territorial behaviour of lizards in captivity (*Cordylus* - Wirminghaus 1990, *Cordylus macropholis* - Nieuwoudt et al. 2003), aggregation behaviour in captivity (*C. cordylus, C. macropholis, Ouroborus cataphractus* - Visagie et al. 2005), aggregation behaviour in wild-living lizards (*O. cataphractus* - Effenberger 2004), social interactions in wild-living lizards (*Smaug giganteus* - Ruddock 2000), as well as the role of scent glands in social behaviour (*S. giganteus* - Ruddock 2000). While these studies have illuminated several aspects of sociality in this family, key traits of kin-based sociality, such as parent-offspring associations, are only identifiable using molecular techniques (Gardner et al. 2016). Given that most members of the family share many life-history characteristics (late age of first reproduction, long life-span (and/or generation length), live birth, and small litter size) with other highly social lizard species, detailed genetic work on this group may reveal the presence and extent of kin-based sociality in the taxon.

The Sungazer (*Smaug giganteus*) is a species of Cordylidae that appears to display many signs of kin-based sociality analogous with other highly social lizard species. *Smaug giganteus* is an obligate burrowing species endemic to the Highveld grasslands of South Africa (De Waal 1978, Jacobsen 1989). Individuals live in self-excavated burrow systems that form part of a colony – a discrete aggregation of burrows spatially isolated from adjacent colonies by several kilometres (Jacobsen et al. 1990, Parusnath 2014). These burrows are significant investments in both time and energy, exposing the animal to predation risk throughout the digging process. Sungazers of both sexes aggressively defend their burrows against Sungazers from other burrows (Ruddock 2000), and have been recorded as occupying the same burrow for over a decade (pers. obs.). Branch and Patterson (1975) reported that Sungazers live in ‘family groups’, with adult females living with offspring in their first few years. However, without genetic evidence or direct observations of reproduction and birth, it is not possible to confirm whether these burrows are in fact occupied by family groups, or random aggregations of unrelated individuals. Given the evidence of aggregative behaviour and other life history traits shared with highly social lizard species, *Smaug giganteus* represents an ideal candidate system to investigate kin-based sociality in African lizards.

In this study, I investigated the system of sociality in *S. giganteus* using spatial and genetic data from 15 colonies across the distribution of the species. I hypothesised that Sungazers sharing burrow systems are immediate family members (parent-offspring, sibling-sibling), and in particular, that juveniles and adults sharing burrows are parent-offspring dyads, regardless of the sex of the adult. I tested whether Sungazers genotyped from shed skin samples found at burrows differed in their level of relatedness with other burrow co-inhabitants compared to Sungazers that were captured at the time of sampling. This was to gain perspective on whether the non-present lizards were unrelated Sungazers visiting for mating purposes, or close relatives that had since dispersed. I conducted a parentage analysis to identify the most likely candidate parent-pair of each juvenile Sungazer in the study. I calculated the relatedness of candidate parent-pairs to each other, to assess whether parent-pairs are less related to each other than would be expected by chance (i.e. inbreeding avoidance).

To understand the spatial organisation of related individuals within a colony (extra-burrow system), I firstly investigated the relatedness of nearest-neighbour pairs - hypothesising that nearest neighbour pairs are extended family (aunts, uncles, cousins, half-siblings). Secondly, I conducted an isolation by distance analysis for three well-sampled colonies to investigate if there is any spatial structuring between burrows, within a colony. I hypothesised that relatedness of Sungazers within a colony is higher than the relatedness of Sungazers between colonies, signifying that colonies are essentially family groups, consisting of immediate and extended family members (1st and 2nd degree relatives). I estimated inbreeding coefficients for three well-sampled colonies to investigate whether the high levels of relatedness recorded in the study are a result of inbreeding, or the natural life-history of the species. Finally, I compared the relatedness of Sungazers at different spatial scales – within burrows, between nearest neighbours, within colonies, and between colonies, to assess whether there are spatial effects of distance on relatedness at the level of the species distribution.

4.2. Materials and methods

4.2.1. *Tissue sample collection*

I collected tissue samples from 149 Sungazers in 15 colonies across the distribution of the species, between March 2015 and March 2017. Sungazers are inactive during the coldest of the austral winter months (June-August), and so sampling efforts were focused on summer months. I defined a colony as an aggregation of Sungazers clearly distinct from the neighbouring aggregations by a minimum distance of 1 km between the closest burrows. I sampled between 2-6 individuals per colony for 13 of the 15 colonies, and sampled the remaining three colonies more extensively (Heilbron (n = 35), Welkom (n = 27), and Volksrust (n = 17)). Sungazers were captured using standard noosing techniques directly at their burrow entrance (Van Wyk 1992, McIntyre and Whiting 2012). To ensure that individuals sharing burrows at the time of sampling were all captured, I retained captured individuals in a cotton bag, and used a Bosch® GIC 120 C cordless inspection camera (Robert Bosch GmbH, Germany) to inspect the interior of the burrow for remaining lizards. All individuals that were captured were checked for previous evidence of tissue sampling to avoid resampling.

I clipped ~3 mm sections of tissue from the large caudal spines of adults, or the terminal tail scute in juveniles with a nail clipper. I collected any blood that resulted from the incision on FTA® filter paper cards (Whatman, Clifton, NJ, USA) as duplicate samples. The area of incision on the caudal spines or tail tips were disinfected with F10SC Veterinary Disinfectant spray (Health and Hygiene (Pty) Ltd., South Africa) before clipping, and F10 Germicidal Barrier Ointment (Health and Hygiene (Pty) Ltd., South Africa) afterwards. All captured Sungazers were weighed, sexed, and snout-vent length (SVL) and tail-length measured. Lizards were classed by sex and age following (Van Wyk 1992): neonates (60-90 mm SVL), juveniles (90-169 mm SVL), adult males (168-200 mm SVL), and adult females (169-200 mm SVL). Adult males and females were differentiated by the presence or absence of generation glands which are highly pronounced in males (Van Wyk 1992). In some cases, freshly shed Sungazer skin was found at the entrance of burrows. These exuvia were collected for tissue analysis in the case that they were from lizards that were not present in the burrow at the time of sampling. Sungazers were released at their site of capture once the incision had dried, and it was confirmed that no further bleeding would occur from the wound.

Tissue samples were stored in 99.9% ethanol in 1.8 ml Nunc™ Cryogenic Tubes (ThermoFisher, Waltham, MA, USA) and stored at -20 ºC, while shed skin samples were stored dry in 0.75 ml Eppendorf tubes at room temperature. Equipment and surfaces used during tissue collection were sterilised with 10% bleach (sodium hypochlorite; Kemp and Smith 2005) and rinsed with sterile double-distilled water (ddH2O) between the sampling of each individual.

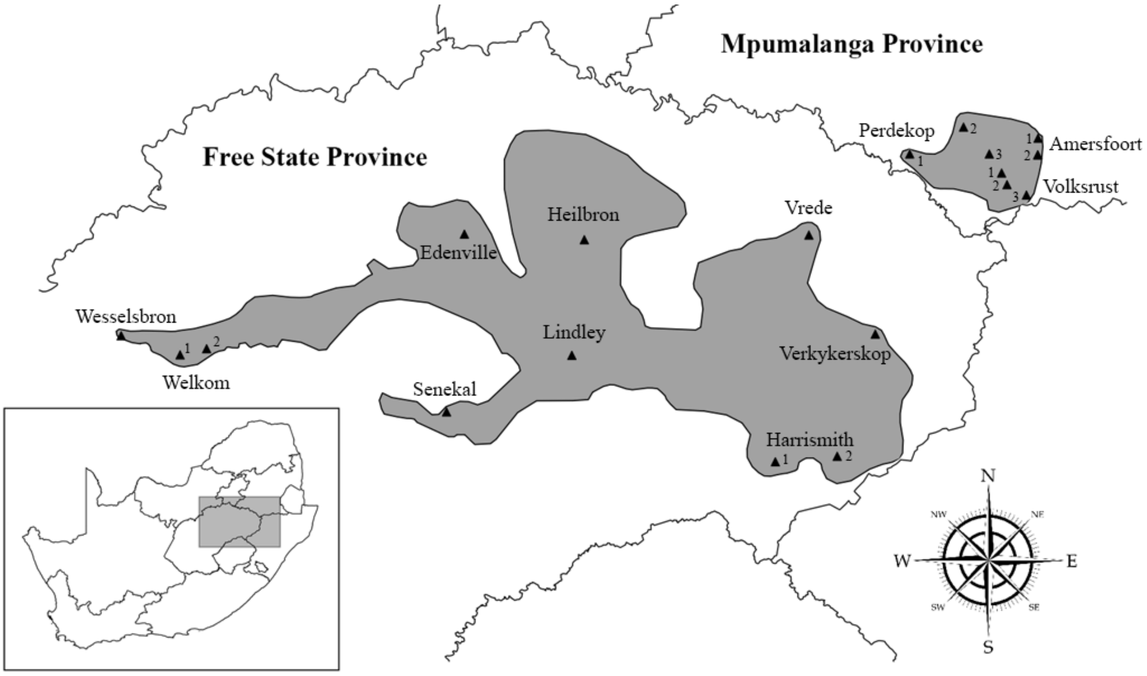


Figure 4.1. Interpreted distribution for *Smaug giganteus* (grey shading; adapted from Parusnath et al. 2017) across the Free State and Mpumalanga provinces of South Africa showing the location of 15 colonies (black triangles) where tissue samples were collected. Numbers next to triangles denote the identity of the colony if there was more than one colony from a specific region.

4.2.2. *DNA extraction, PCR, and sequencing*

DNA was extracted from tissue samples using the PrepFiler® BTA Forensic DNA Extraction Kit (Applied Biosystems, part of Life Technologies, Foster City, California), using the standard protocol for bone and teeth. Extracted DNA was stored in 1.5 ml Eppendorf Tubes (Eppendorf GmbH, Hamburg, Germany) at -20 °C. A total of 18 species-specific polymorphic microsatellites markers (developed in Chapter 2 of this thesis) were used to genotype all 149 sampled Sungazers. Polymerase chain reaction was conducted with Invitrogen Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California), in 10 µL reaction volumes containing 1 x PCR buffer (Invitrogen), 1.0-2.0 mM MgCl2, 0.02 mM each forward and reverse primer, 0.1 mM dNTP mix, 0.25 U Invitrogen Platinum Taq DNA polymerase, and 1 µL template DNA (~10 ng/µL). The conditions for PCR amplification were: 5 min at 95 °C initial denaturation, 30 cycles for 30 s at 95 °C, 30 s at 45 °C, and 30 s at 72 °C, followed by extension at 72 °C for 40 min. The PCR was carried out in a BOECO TC-PRO Thermal Cycler (Boeckel + Co., Hamburg, Germany). Polymerase chain reaction products were pooled together and run against a Genescan 500 LIZ (Applied Biosystems, Foster City, California) internal size standard on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, California). Samples were visualized using GENEMAPPER 4.0 (Applied Biosystems, Foster City, California). Bins were assigned to alleles and calls were checked by eye.

4.2.3. *Calculation of pairwise relatedness coefficients*

Pairwise relatedness coefficients (R) were estimated for all possible combinations of the 149 sampled Sungazers using the GENALEX 6.5 (Peakall and Smouse 2006, 2012) add-on in Microsoft Excel. The QGM estimator (Queller and Goodnight 1989) was selected because it is one of the most widely used estimators of relatedness across studies of reptile sociality (e.g. Stow and Sunnucks 2004, Fuller et al. 2005a, Moore et al. 2008, Qi et al. 2012). Values of relatedness coefficients for the QGM estimator range between -1 and +1, and are indicative of the proportion of shared alleles which are identical by descent between pairs of individuals. Values between -1 and 0.125 indicate 3rd degree relatives/unrelated or distantly-related dyads, 0.125 - 0.375 indicates 2nd degree relatives/extended family members (e.g. cousins, aunts, uncles, half-siblings), while values between 0.375 and 0.625 indicate 1st degree relatives/immediate or nuclear family members (e.g. full siblings, parents, offspring).

4.2.4. *Within burrow relatedness*

To test the hypothesis that burrows are shared by immediate family members, I calculated the pairwise relatedness of all burrow inhabitants in burrows shared by multiple individuals. The relatedness of Sungazers sharing burrows with juveniles was given special attention, to test the hypothesis that juveniles share burrows with at least one parents, regardless of the sex of the parent (indicating parental care). I conducted an independent sample t-test by groups in STATISTICA 8.0 (Statsoft Inc., USA), to investigate whether Sungazers genotyped from shed skin alone had significantly different pairwise relatedness values with burrow co-inhabitants compared to Sungazers that were present in the burrow during sampling. This was to understand whether these Sungazers were close relatives of the individuals whose burrows they were visiting, or unrelated individuals that were visiting these for mating or other purposes.

4.2.5. *Parentage estimates and relatedness of breeding partners*

Parentage assignment for each juvenile sampled was estimated in CERVUS 3.03 (Kalinowski et al. 2007). CERVUS estimates the log-likelihood of each candidate parent as the true parent relative to an arbitrary individual, and then calculates the difference between the two most likely parents (Delta) (Kalinowski et al. 2007). Using the observed Sungazer population data for allele frequencies as a baseline measure, simulation parameters were set at 10 000 offspring, with 80% of candidate parents sampled and a total proportion of loci typed over all individuals of 0.99, mistyping error rates = 0.01 and likelihood calculation error rates = 0.01, permitting two unscored loci. Strict confidence was set to 95% while the relaxed confidence level was 80%. After the initial run, CERVUS was re-run, assigning each adult sharing a burrow with a juvenile as a known parent (if the pair had a relatedness value greater than R = 0.375). Using this procedure, the software assumes the identity of one known parent, allowing for a more accurate estimation of the identity of the other parent. This was only applicable to 8 juvenile-adult pairs. I compared the relatedness of candidate parent-pairs to each other, to assess whether parent-pairs are less related to each other than would be expected by chance as a measure of inbreeding avoidance. I conducted a regression analysis to investigate if there is a distance effect on the relatedness of candidate parent-parent pairs. The frequency of pairwise relatedness classes of Sungazers sharing burrows with juvenile Sungazers was plotted in a frequency diagram.

4.2.6. *Nearest-neighbour relatedness*

I compared the relatedness of each nearest neighbour pair across three well-sampled Heilbron, Welkom, and Volksrust colonies, to investigate the relatedness of individuals occupying adjacent burrows. I used the Distance Matrix tool in QGIS 3.6 (Open Source Geospatial Foundation Project, 2019) to calculate the identity and distance of the nearest neighbour to each Sungazer in each colony. The frequency of different categories of relatedness coefficients (1st, 2nd, or 3rd degree) is expressed in a frequency diagram. I then correlated relatedness coefficient with distance between pairs, to evaluate whether distance between nearest neighbours had an effect of relatedness between the pair.

4.2.7. *Within-colony social structure*

To investigate the relationship between burrow distance and genetic relatedness, I conducted Mantel tests (Mantel 1967) in ALLELES IN SPACE 1.0 (Miller 2005) separately for the Heilbron, Volksrust, and Welkom colonies. The Mantel test was run using values for genetic and geographic distance with 1000 permutations. This allowed me to test whether populations conformed to isolation by distance (IBD) by exhibiting a significant positive correlation between genetic and geographical distance.

4.2.8. *Pairwise relatedness – within and between colonies*

To test the hypothesis that Sungazer colonies consist of family groups (immediate and extended family members), I estimated the average pairwise relatedness of Sungazers within and between 15 colonies. A regression was used to investigate the relationship between relatedness and geographic distance between colonies. I also conducted a regression analysis to assess if colony size has an effect on the average relatedness within a colony, for each of the 15 colonies. This analysis provides an insight into whether smaller colonies have higher levels of relatedness due to the smaller selection of mate choices.

4.2.9. *Inbreeding analysis*

I estimated inbreeding coefficients in INEST 2.2 (Chybicki and Burczyk 2009) individually for the Heilbron, Welkom, and Volksrust colonies. This analysis accounts for the presence of null alleles in the dataset, which can bias inbreeding estimates (Chybicki and Burczyk 2009). I used the Interesting

Itemset Miner (IIM) Bayesian model, because this approach is suitable for small sample sizes (Chybicki 2017). INEST simultaneously estimates the relative contribution of the inbreeding coefficient (F), null allele frequencies (n) and random genotyping failure (b), and should be more accurate than independent parameter estimations. I ran the model twice for each colony – first with the ‘nfb’ model (null alleles, inbreeding coefficients, genotyping failures), and then with the ‘nb’ model (null alleles, genotyping failures), in order to facilitate direct comparisons between models to infer the effect that inbreeding has on each model. Each model was run for 500 000 cycles (MCMC iterations) with a burn-in of 50 000. The deviance information criterion (DIC) was used to ascertain the best model. In cases where the difference in DIC (Δ DIC) exceeded a value of 2, the model with the lower Wright’s F_{IS} score was considered a better fit (Spiegelhalter et al. 2002).

4.2.10. *Comparison of relatedness at different spatial scales*

I conducted an analysis of variance (ANOVA) to assess whether there is a significant difference in relatedness between Sungazers sharing burrow systems, Sungazer pairs in nearest neighbouring burrows, Sungazers within a colony, and Sungazers between colonies. The ANOVA was run in STATISTICA 8.0 (Statsoft Inc., USA), using R (relatedness) as the dependent variable and colony as the independent variable.

4.3. Results

4.3.1. *Within burrow relatedness*

Sungazers sharing burrow systems had an average relatedness coefficient of R = 0.346, indicating that burrow co-inhabitants are likely to be immediate or extended family members (Table 4.1). Approximately half (49%) of all pairs of Sungazers sharing burrows were 1st degree relatives, 28% were 2nd degree relatives, and the remaining 23% were distant relatives (Table 4.1). Occupancy of burrows containing multiple Sungazers ranged from 2-5 Sungazers/burrow, with an average of 2.5 Sungazers/burrow. Fifteen burrows contained only two individuals, two burrows contained five individuals, and a single burrow contained three individuals. Sungazers genotyped from shed skin samples had significantly lower pairwise relatedness scores with burrow co-inhabitants (average R = 0.187) than Sungazers that were present in the burrow during sampling (average R = 0.464) (independent sample t-test, $t = -3.76$, $df = 37$, $p < 0.01$). Further, the level and type of relatedness that these Sungazers had with burrow co-inhabitants was significantly different from those present in the burrows (Fig. 4.2). They had far fewer 1st and 2nd degree relations with Sungazers in the burrows they were visiting, and far more 3rd degree relations – in general, they were likely to be unrelated to the other Sungazers in the burrow.

Table 4.1. Pairwise relatedness of *Smaug giganteus* within each of 19 unique burrow systems. Columns display the identity, sex, and age class of each individual, relatedness coefficient between pairs of lizards, and assumed relationship between each pair. Some individuals were genotyped based on shed skin samples and do not have associated demographic data. These individuals were also not assigned relationships to other individuals sharing burrows, as indicated by N/A in the assumed relationship column. Burrows Sun126, Sun316, Sun318, Sun320, Sun325, Sun331 and Sun468 had both shed skins and individuals with demographic data. All other burrows contained only individuals with associated demographic data. Individuals that were too young to visibly sex were demarcated with N/A in the sex column.

Burrow	**Sungazer**	**Sex**	**Age**	**Sungazer**	**Sex**	**Age**	**Relatedness**	**Assumed relationship**
Sun119	Sun119A	M	Adult	Sun119B	N/A	Juvenile	0.703	Father-offspring
Sun127	Sun127A	F	Adult	Sun127B	F	Juvenile	0.543	Mother-daughter
Sun235	Sun235A	F	Adult	Sun235B	F	Juvenile	0.487	Mother-daughter
Sun236	Sun236A	F	Juvenile	Sun236B	F	Juvenile	0.327	Half-siblings
	Sun236A	F	Juvenile	Sun236C	M	Adult	0.118	Distant relatives
	Sun236B	F	Juvenile	Sun236C	M	Adult	0.576	Father-daughter
Sun239	Sun239A	F	Adult	Sun239B	M	Juvenile	0.596	Mother-son
Sun259	Sun259B	N/A	Neonate	Sun259C	N/A	Neonate	0.468	Full-siblings
Sun333	Sun333	F	Adult	Sun334	N/A	Neonate	0.054	Distant relative
Sun372	Sun372	F	Juvenile	Sun373	F	Adult	0.351	Aunt-niece
Sun467	Sun467A	F	Adult	Sun467B	N/A	Juvenile	0.621	Mother-daughter
Sun482	Sun482A	M	Adult	Sun482B	N/A	Neonate	0.704	Father-offspring
Sun485	Sun485A	M	Adult	Sun485B	F	Juvenile	0.791	Father-daughter
Sun491	Sun491	M	Adult	Sun492	F	Adult	0.433	Full-siblings
Sun126	Sun126A	N/A	N/A	Sun126B	M	Juvenile	0.521	N/A
Sun316	Sun316	N/A	N/A	Sun317	F	Adult	-0.005	Distant relative
Sun318	Sun318	N/A	N/A	Sun319	M	Adult	-0.16	Distant relative
Sun320	Sun320	N/A	N/A	Sun321	F	Juvenile	0.02	Distant relative
	Sun320	N/A	N/A	Sun323	F	Adult	-0.07	Distant relative
	Sun320	N/A	N/A	Sun322	N/A	N/A	-0.083	Distant relative
	Sun320	N/A	N/A	Sun324	N/A	Neonate	-0.147	Distant relative
	Sun321	F	Juvenile	Sun322	N/A	N/A	0.452	N/A
	Sun321	F	Juvenile	Sun323	F	Adult	0.354	Aunt-niece
	Sun321	F	Juvenile	Sun324	N/A	Neonate	0.299	Half-siblings
	Sun322	N/A	N/A	Sun323	F	Adult	0.387	N/A
	Sun322	N/A	N/A	Sun324	N/A	Neonate	0.317	N/A
	Sun323	F	Adult	Sun324	N/A	Neonate	0.641	Mother-offspring
Sun325	Sun325	N/A	N/A	Sun329	N/A	Neonate	0.244	N/A
	Sun325	N/A	N/A	Sun327	F	Juvenile	0.181	N/A
	Sun325	N/A	N/A	Sun326	F	Adult	0.179	N/A
	Sun325	N/A	N/A	Sun328	M	Juvenile	0.066	N/A
	Sun326	F	Adult	Sun327	F	Juvenile	0.566	Mother-daughter
	Sun326	F	Adult	Sun328	M	Juvenile	0.522	Mother-son
	Sun326	F	Adult	Sun329	N/A	Neonate	0.324	Aunt-Niece
	Sun327	F	Juvenile	Sun328	M	Juvenile	0.597	Full-siblings
	Sun327	F	Juvenile	Sun329	N/A	Neonate	0.246	Half-siblings
	Sun328	M	Juvenile	Sun329	N/A	Neonate	0.186	Half-siblings
Sun331	Sun331	N/A	N/A	Sun332	F	Adult	0.605	N/A
Sun468	Sun468A	N/A	N/A	Sun468B	F	Juvenile	0.481	N/A
Average							**0.346**	

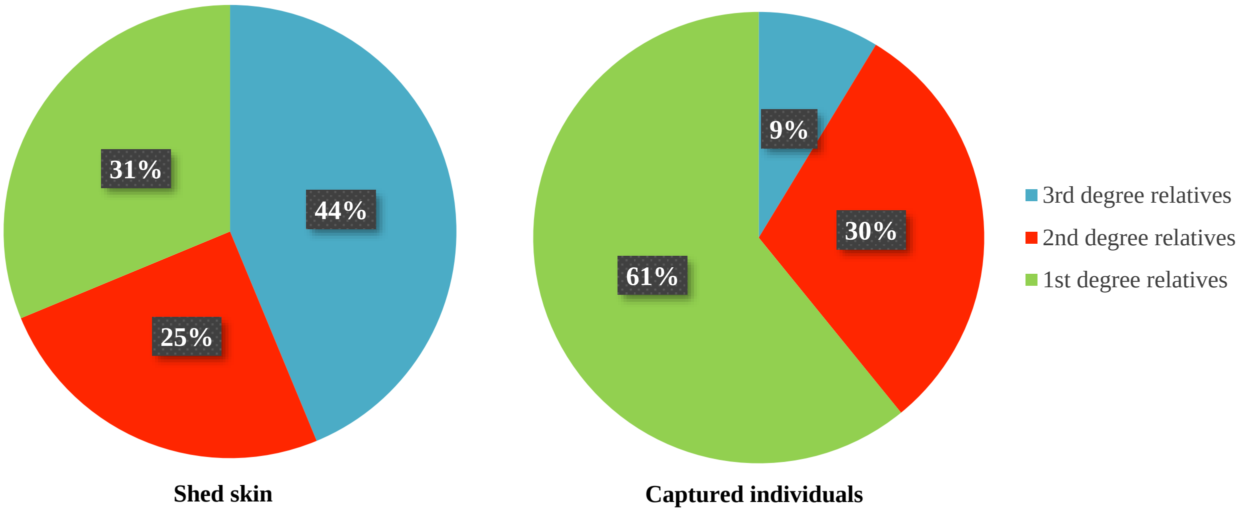


Figure 4.2. Proportion of different categories of relatedness between *Smaug giganteus* burrow co-inhabitants and individuals genotyped from shed skin samples (left), and individuals present in the burrow during sampling (right). Pairwise relatedness scores with burrow co-inhabitants were grouped into categories of relatedness.

Just under half (9/21) of all Sungazer pairs occupying the same burrow systems were adult females with juveniles. A quarter of cases (5/21) were adult males with juveniles, and a quarter (6/21) were juveniles with other juveniles. There was a solitary case of adults sharing a burrow (one male and one female; Table 4.2). Each pair type (except juvenile-juvenile, R = 0.354) had an average relatedness score greater than R = 0.375, indicating that in most cases, these pairs were immediate family members. In the cases of adult females and adult males sharing burrows with juveniles, only 22% and 20% of pairs respectively had relatedness scores lower than R = 0.375. Juveniles sharing burrows with juveniles were more likely to be extended family members, with only 33% of pairs being full-siblings, and the remaining 67% possibly being half-siblings. No juvenile-juvenile pairs were distant relatives, however both adult male and adult females each had a single instance of sharing a burrow with a distantly related juvenile.

Table 4.2. Summary of different unique pair combinations of *Smaug giganteus* occupying individual burrows. Columns display the sex and age class of each individual in each pair combination, as well as number of cases, average relatedness, and range of relatedness values for each pair combination.

Sungazer 1		**Sungazer 2**		**Number of cases**	**Average relatedness (R)**	**Relatedness (R) Range**
Sex	**Age class**	**Sex**	**Age class**			
Female	Adult	N/A	Juvenile	9	0.460	0.054 - 0.641
Male	Adult	N/A	Juvenile	5	0.578	0.118 - 0.791
Male	Adult	Female	Adult	1	0.433	N/A
N/A	Juvenile	N/A	Juvenile	6	0.354	0.186 - 0.597

Burrows containing a single adult female and a juvenile are likely to be mother-offspring (Average R = 0.442; n = 6). The same is true with a single adult male adult and a juvenile (Average R = 0.733; n = 3). Cases in which an adult of either sex lives with several offspring, the average relatedness within the burrow is lower (adult male – R = 0.340; n = 1; adult female – R = 0.407 - 431; n = 5) than in cases with single parent-offspring dyads. In a single instance, an adult male and adult female Sungazer were found together in a burrow, and were 1st degree relatives (R = 0.430). Although there are no population demographic data over several years to verify the nature of their relationship, they are similar in body size (181 and 180 mm respectively). Given that maximum adult body size is approximately 200 mm, these individuals probably are not fully grown. The similar body size therefore suggests they might have been in the same cohort of offspring, and that they are full-siblings.

Table 4.3. Summary of cases of different combinations of *Smaug giganteus* burrow inhabitants age and sex classes. Each row represents a different potential combination of inhabitants. Columns display the number of *S. giganteus* per burrow, number of cases of each, and the average within-burrow relatedness of each case.

Adult males	**Adult females**	**Juveniles**	**Total in burrow**	**Number of cases**	**Average relatedness (R)**	**Range of relatedness (R)**
1	0	1	2	3	0.733	0.703 - 0.791
0	1	1	2	6	0.442	0.351 - 0.621
1	0	2	3	1	0.340	0.118 - 0.576
0	0	2	2	1	0.468	N/A
1	1	0	2	1	0.433	N/A
0	1	2	3	1	0.431	0.299 - 0.641
0	1	3	4	1	0.407	0.186 - 0.597

4.3.2. *Parentage estimates and relatedness of breeding partners*

Juvenile Sungazers in this study tended to share a burrow with either a male or female parent, with the other parent being in a burrow between 10 m and 1271 m away. Fathers were likely to be closer (186 m away) than mothers (333 m away) on average. Candidate parent pairs showed high levels of pairwise-relatedness (range: R = 0.116-0.545, mean: R = 0.327). These values were slightly lower than the average within-colony relatedness (R = 0.345), but not significantly so (t = 0.34; p = 0.735; df = 37). The Volksrust colony had the lowest overall relatedness level between candidate parent pairs of R = 0.243, however some pairs (58%) were still 1st or 2nd degree relatives. In contrast, candidate parent pairs in the two other colonies both had average relatedness scores indicative of 1st degree relations (Welkom, r = 0.380) or 2nd degree relations (Heilbron, R = 0.356). Overall, 35% of candidate parent pairs were 1st degree relatives, 47% were 2nd degree relatives, and the remaining 18% were 3rd degree relatives. The regression analysis showed no significant trend between the relatedness of candidate parent pairs and distance between each member of the pair, however, it does appear that candidate parent pairs from burrows further apart had slightly higher levels of relatedness (Fig. 4.3).

Table 4.4. Identity and relatedness (R) of candidate mothers and fathers of juvenile *Smaug giganteus* in three sampled colonies (Heilbron, Welkom, Volksrust). Distance (m) between each candidate parent and offspring, distance (m) between each candidate parent of a juvenile from each other, and the relatedness (R) between candidate parent pairs are provided.

Offspring ID	Candidate mother			Candidate father			Pairwise relatedness (R) between candidate parents	Distance (m) between candidate parents
	Sungazer ID	Distance (m)	Pairwise relatedness (R) with offspring	Sungazer ID	Distance (m)	Pairwise relatedness (R) with offspring		
				Heilbron				
Sun442	**Sun438**	217.9	0.270	**Sun465**	16.2	0.438	0.248	231.76
Sun421	**Sun431**	1271.8	0.306	**Sun437**	17.6	0.693	0.319	1254.63
Sun435	**Sun436**	48.2	0.454	**Sun466**	268.3	0.349	0.130	309.04
Sun439	**Sun452**	1087.8	0.503	**Sun427**	1043.2	0.400	0.706	47.70
Sun444	**Sun456**	678.1	0.337	**Sun437**	227.1	0.384	0.377	852.95
Average		771.5	0.374		314.5	0.453	0.356	539.22
				Welkom				
Sun119B	**Sun467A**	15.0	0.588	**Sun119A**	0	0.703	0.341	14.95
Sun126B	**Sun492**	779.1	0.501	**Sun119A**	379.2	0.621	0.538	1032.89
Sun127B	**Sun125**	65.7	0.585	**Sun120**	260.4	0.509	0.271	323.75
Sun467B	**Sun467A**	0	0.621	**Sun119A**	15.0	0.575	0.408	149.39
Sun468B	**Sun127A**	10.2	0.597	**Sun461**	156.2	0.448	0.341	14.95
Average		174	0.579		162.1	0.571	0.380	307.19
				Volksrust				
Sun315	**Sun332**	138.8	0.445	**Sun330**	114.7	0.335	0.063	35.26
Sun321	**Sun326**	86.6	0.586	**Sun330**	133.1	0.466	0.034	56.32
Sun327	**Sun326**	0	0.566	**Sun319**	82.1	0.486	0.363	82.10
Sun328	**Sun332**	60.9	0.489	**Sun319**	82.1	0.453	0.440	140.09
Sun324	**Sun323**	0	0.641	**Sun314**	51.9	0.470	0.545	63.59
Sun329	**Sun337**	57.9	0.187	**Sun319**	82.1	0.265	0.139	113.80
Sun334	**Sun337**	33.1	0.170	**Sun330**	19.6	0.005	0.116	14.35
Average		53.9	0.44		80.8	0.35	0.243	72.22
Overall		333.1	0.46		185.8	0.46	0.327	306.21

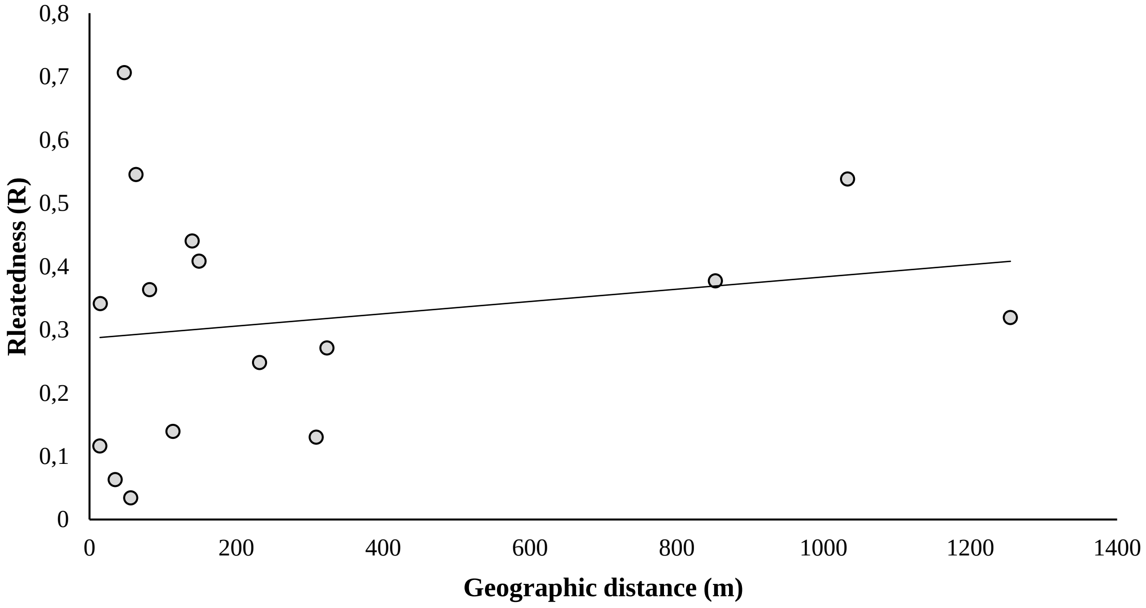


Figure 4.3. Regression showing the relationship between the relatedness (R) of candidate parent pairs of 17 juvenile *Smaug giganteus*, and geographic distance (m) between pairs. Regression coefficient (r^2) = 0.0401, p = 0.457.

Juvenile Sungazers share their burrows with 1st degree relatives (R = 0.39; n = 21) in 57% of cases (Fig. 4.4). Based on the demographics of Sungazers sharing burrows with juveniles, burrow co-inhabitants are likely to consist of mothers (7/21, 33% of cases), fathers (4/21, 19% of cases), full-siblings (1/21, 5% of cases), and half-siblings or cousins (7/21, 33% of cases) (Table 4.5). Only 10% of cases (2/21) were juveniles sharing burrows with unrelated individuals.

Table 4.5. Relatedness (R) and assumed relationship between neonate and juvenile *Smaug giganteus* and other burrow inhabitants in 18 individual burrows. Burrow number, identity, sex, and age class of each Sungazer are provided. Each line represents one pair. Relatedness between each pair and the assumed relationship between the pair is given.

Burrow No.	**Sungazer 1**			**Sungazer 2**			**Relatedness (R)**	**Assumed relationship**
	Sungazer ID	Sex	Age Class	Sungazer ID	Sex	Age Class		
Sun119	Sun119A	M	Adult	Sun119B	N/A	Juvenile	0.703	Father
Sun127	Sun127A	F	Adult	Sun127B	F	Juvenile	0.543	Mother
Sun235	Sun235A	F	Adult	Sun235B	F	Juvenile	0.487	Mother
Sun236	Sun236A	F	Juvenile	Sun236C	M	Adult	0.118	Not father
	Sun236A	F	Juvenile	Sun236B	F	Juvenile	0.327	Half-Sibling/Cousin
	Sun236B	F	Juvenile	Sun236C	M	Adult	0.576	Father
Sun239	Sun239A	F	Adult	Sun239B	M	Juvenile	0.596	Mother
Sun320	Sun321	F	Juvenile	Sun324	N/A	Neonate	0.299	Half-Sibling/Cousin
	Sun321	F	Juvenile	Sun323	F	Adult	0.354	Half-Sibling/Cousin
	Sun323	F	Adult	Sun324	N/A	Neonate	0.641	Mother
Sun325	Sun328	M	Juvenile	Sun329	N/A	Neonate	0.186	Half-Sibling/Cousin
	Sun327	F	Juvenile	Sun329	N/A	Neonate	0.246	Half-Sibling/Cousin
	Sun326	F	Adult	Sun329	N/A	Neonate	0.324	Half-Sibling/Cousin
	Sun326	F	Adult	Sun328	M	Juvenile	0.522	Mother
	Sun326	F	Adult	Sun327	F	Juvenile	0.566	Mother
	Sun327	F	Juvenile	Sun328	M	Juvenile	0.597	Full sib
Sun333	Sun333	F	Adult	Sun334	N/A	Neonate	0.054	No relation
Sun372	Sun372	F	Juvenile	Sun373	F	Adult	0.351	Half-Sibling/Cousin
Sun467	Sun467A	F	Adult	Sun467B	N/A	Juvenile	0.621	Mother
Sun482	Sun482A	M	Adult	Sun482B	N/A	Neonate	0.704	Father
Sun485	Sun485A	M	Adult	Sun485B	F	Juvenile	0.791	Father
Average							**0.457**	

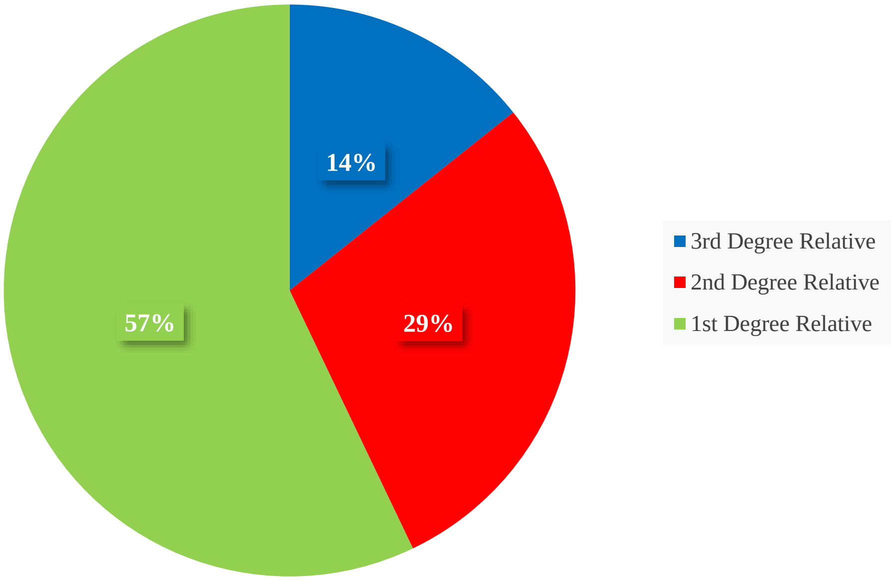


Figure 4.4. Frequency of pairwise relatedness classes of *Smaug giganteus* individuals sharing burrows with *S. giganteus* juveniles.

4.3.3. *Nearest neighbour analysis*

The average relatedness coefficient of R = 0.29 between nearest neighbour pairs across all three colonies indicates that nearest neighbours on average are likely to be extended family members. Just under a quarter (23%) of all nearest neighbour pairs were 1st degree relatives (parents or full-siblings), just under half (47%) were 2nd degree relatives, and the remaining 30% were unrelated individuals. There were notably more male-female nearest neighbours (57%) than male-male (24%) or female-female (20%) nearest neighbour pairs (Fig. 4.5). Several burrows were relatively far from the nearest neighbouring burrow (257-557 m), however pairwise relatedness scores between individuals in these burrows had relatedness scores of R = 0.142-0.342, indicating that despite the distance, their nearest neighbours were still 2nd degree relatives. The regression analysis of the relationship between geographic distance (m) and relatedness (R) between nearest neighbour pairs confirmed that there is no significant effect of distance on relatedness between nearest neighbour pairs ($r^2 = 0.0011$, $p = 0.833$; Fig. 4.6).

Table 4.6. Relatedness of nearest neighbour pairs in three sampled *Smaug giganteus* colonies from South Africa (Heilbron, Welkom, Volksrust). Individual identification number and sex are provided for each individual per pair, and distance between individual burrows, relatedness coefficient (R) and degree of relation are given.

Sungazer 1	Sex	Sungazer 2	Sex	Distance (m)	Relatedness (R)	Degree of relation
				Heilbron		
Sun438	F	Sun423	F	7.1	0.216	2nd degree
Sun427	M	Sun428	F	12.1	0.057	3rd degree
Sun449	M	Sun450	M	12.4	0.045	3rd degree
Sun421	F	Sun423	F	13.0	0.086	3rd degree
Sun440	F	Sun427	M	13.4	0.175	2nd degree
Sun444	M	Sun465	M	13.9	0.078	3rd degree
Sun426	F	Sun449	M	14.0	0.344	2nd degree
Sun422	F	Sun423	F	14.2	0.148	2nd degree
Sun454	F	Sun466	M	14.5	0.169	2nd degree
Sun442	F	Sun444	M	15.0	0.653	1st degree
Sun437	M	Sun421	F	17.6	0.483	1st degree
Sun433	M	Sun436	F	19.2	-0.024	3rd degree
Sun453	M	Sun431	F	19.2	0.168	2nd degree
Sun434	F	Sun435	F	21.7	0.187	2nd degree
Sun424	M	Sun425	F	22.7	0.239	2nd degree
Sun451	F	Sun450	M	24.8	0.005	3rd degree
Sun452	F	Sun429	M	30.26	0.264	2nd degree
Sun445	M	Sun466	M	54.1	0.176	2nd degree
Sun448	M	Sun456	F	67.6	-0.028	3rd degree
Sun441	F	Sun430	M	87.3	0.098	3rd degree
Sun446	M	Sun466	M	95.8	0.196	2nd degree
Sun439	F	Sun444	M	103.6	0.105	3rd degree
Sun455	M	Sun446		120.2	0.177	2nd degree
Average				**34.6**	**0.374**	
				Welkom		
Sun491	M	Sun492	F	1.5	0.407	1st degree
Sun468B	F	Sun127A	F	10.2	0.545	1st degree
Sun468B	F	Sun127B	F	10.2	0.403	1st degree
Sun124	M	Sun125	F	18.7	0.446	1st degree
Sun457	M	Sun458	F	19.3	0.419	1st degree
Sun463	F	Sun120	M	19.9	0.112	3rd degree
Sun496	F	Sun491	M	25.1	0.513	1st degree
Sun499	M	Sun492	F	257.2	0.301	2nd degree
Sun462	M	Sun464	F	28.9	0.622	1st degree
Sun497	F	Sun499	M	328.6	0.15	2nd degree
Sun459	F	Sun458	F	37.3	0.15	2nd degree
Sun126B	M	Sun124	M	40.3	0.208	2nd degree
Sun500	F	Sun497	F	452.0	0.142	2nd degree
Sun502	F	Sun500	F	547.7	0.204	2nd degree
Sun498	M	Sun497	F	557.9	0.342	2nd degree
Sun460	F	Sun461	M	6.3	0.321	2nd degree
Average				**133.9**	**0.360**	
				Volksrust		
Sun332	F	Sun335	F	10.1	0.299	2nd degree
Sun317	F	Sun319	M	12.1	-0.024	3rd degree
Sun330	M	Sun337	F	13.8	0.122	3rd degree
Sun315	M	Sun317	F	17.0	0.008	3rd degree
Sun314	M	Sun315	M	39.1	0.247	2nd degree
Average				**18.4**	**0.149**	
Overall				**62.3**	**0.294**	

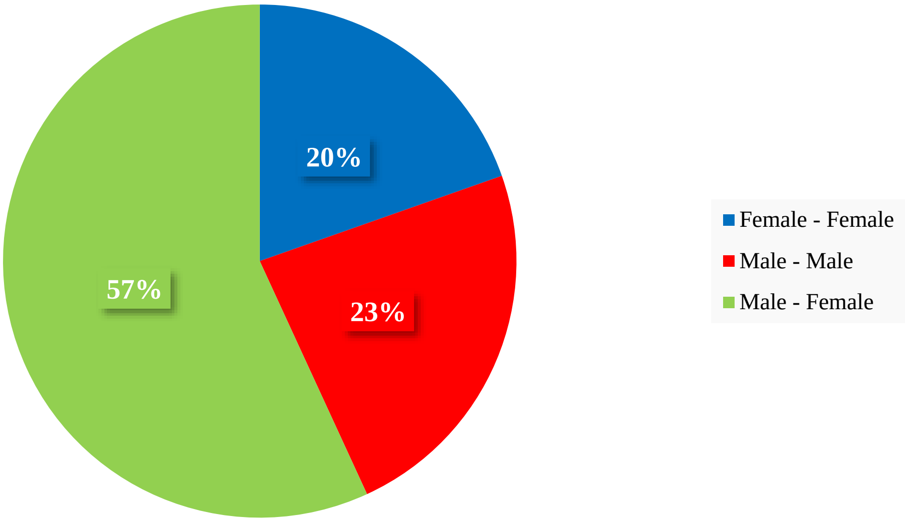


Figure 4.5. Summary of number of cases of different nearest neighbour pair combinations in three sampled *Smaug giganteus* colonies from South Africa (Heilbron, Welkom, Volksrust).

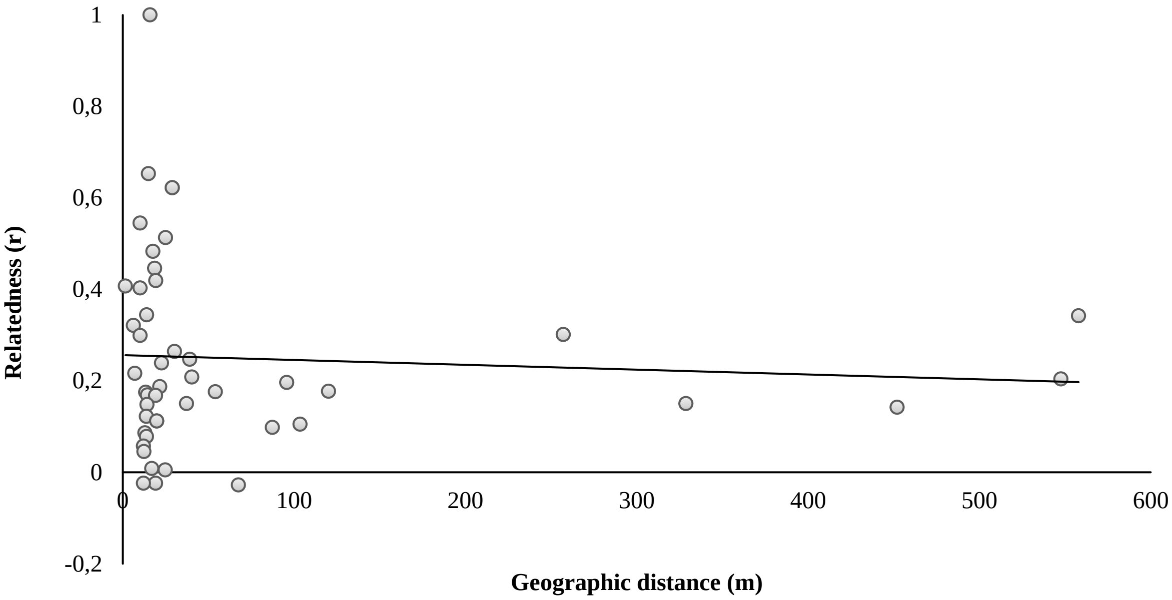


Figure 4.6. Regression showing the relationship between geographic distance (m) and relatedness (R) between 44 nearest neighbour pairs in three sampled *Smaug giganteus* colonies from South Africa (Heilbron, Welkom, Volksrust). Regression coefficient (r^2) = 0.0011, p = 0.833.

4.3.4. *Within-colony social structure*

None of the three well-sampled colonies showed evidence of isolation by distance in Mantel tests (Fig. 4.7). The Welkom colony showed the strongest interaction between genetic and geographic distance within a colony, however the regression coefficient indicated a very poor fit. This suggests that there is no significant distance effect on genetic relatedness on individuals within a colony, and that the population of Sungazers within a given colony is relatively homogenous.

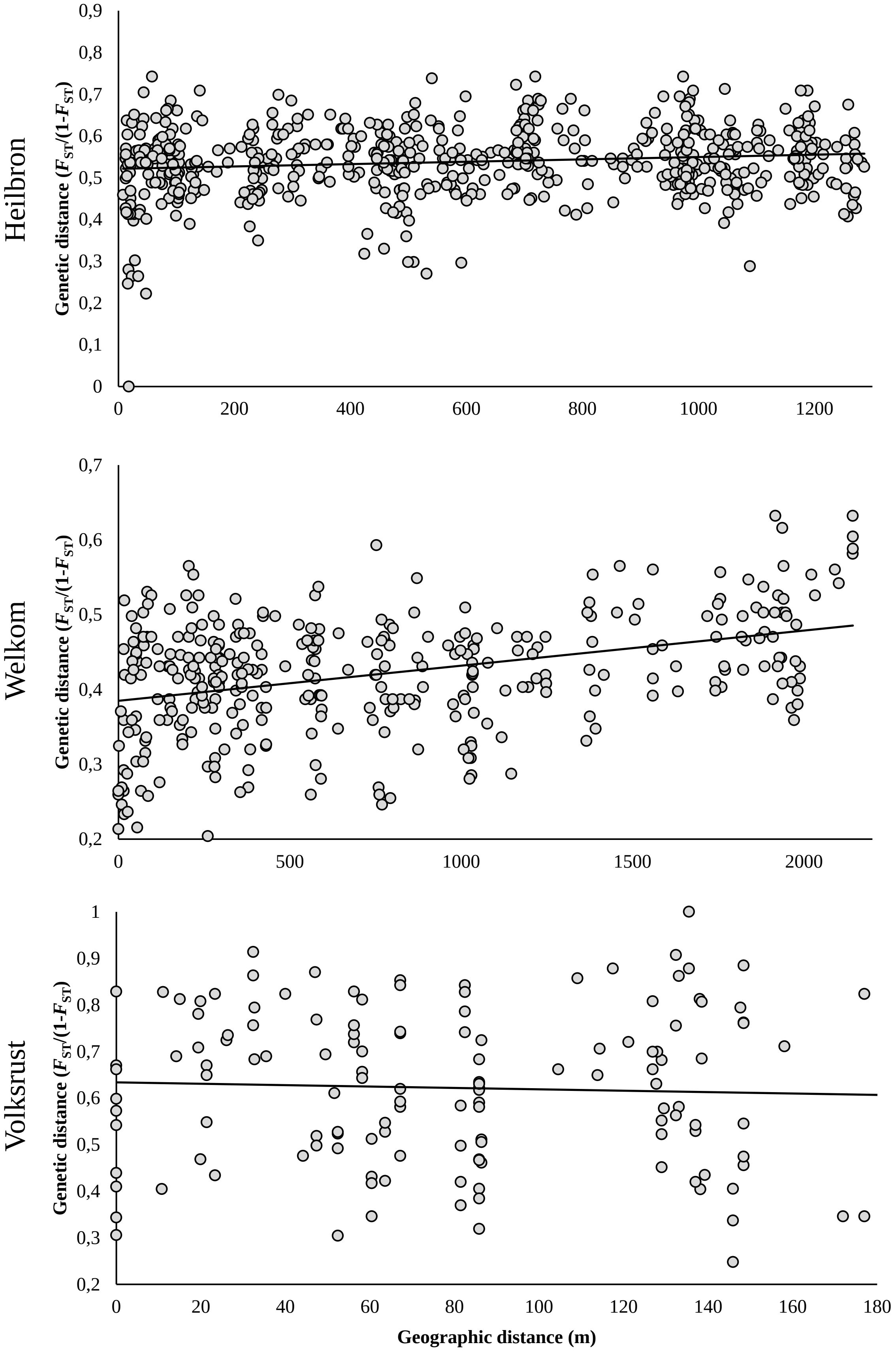


Figure 4.7. Results of the Mantel test for genetic distance ($F_{ST}/(1-F_{ST})$) and geographic distance (m) for *Smaug giganteus* from Heilbron ($r^2 = 0.019$, $p < 0.01$) (top), Welkom ($r^2 = 0.145$, $p < 0.01$) (middle), and Volksrust ($r^2 = 0.002$, $p = 0.616$) (bottom) colonies.

4.3.5. *Pairwise relatedness – within and between colonies*

The average relatedness within a Sungazer colony is R = 0.345, ranging from R = 0.168 in Volksrust 2 to R = 0.813 in Kroonstad (Table 4.7). The majority of colonies (70%) had average within-group relatedness scores between R = 0.125 and R = 0.375, indicative of the colony consisting primarily of extended family members. The remaining 30% of colonies had average within-group scores above R = 0.375, indicating that the colony was made up primarily of immediate family members. None of the colonies sampled had an average relatedness score below R = 0.125. Sungazers were significantly more related to other Sungazers within their own colony than to individuals in other colonies (one-way ANOVA; $F_{1,14} = 10.64$, df = 146, $p < 0.01$), and the regression analysis showed a clear trend of greater genetic distance between colonies that were geographically further apart ($r^2 = 0.351$, $p < 0.01$) (Fig. 4.9) There was a weak and non-significant correlation between group size and relatedness ($r^2 = 0.092$, $p = 0.151$; Fig. 4.8), however, the trend of colonies of small sizes having higher average relatedness scores within the colony might indicate that smaller colonies are more prone to inbreeding.

Inter-colony relatedness was R = -0.043 on average and ranged from R = -0.33 between the most geographically distance colonies, Wesselsbron and Volksrust 3 (345 km), to R = 0.323 between the relatively close Welkom 1 and Welkom 2 colonies that were 11 km apart. Just under 20% of pairs of colonies had high average inter-colony relatedness values between R = 0.125 and R = 0.375, indicating that their members were extended family members. These colonies were between 5 and 255 km apart. The majority of the lowest levels of relatedness between colonies were colony pairs with one colony in the Free State province, and the other in the Mpumalanga province.

Table 4.7. Average intra-colony relatedness (R) and number of lizards per colony in 15 *Smaug giganteus* colonies across the species distribution in South Africa.

Colony	Average intra-colony relatedness (R)	Number of lizards per colony
Volksrust 2	0.168	7
Heilbron	0.174	35
Amersfoort	0.219	31
Volksrust 3	0.246	2
Volksrust 1	0.263	2
Harrismith 2	0.267	2
Lindley	0.294	10
Verkykerskop	0.330	5
Senekal	0.340	7
Welkom 1	0.347	29
Wesselsbron	0.378	2
Welkom 2	0.419	2
Harrismith 1	0.503	2
Vrede	0.608	8
Kroonstad	0.814	3
Average	**0.356**	-

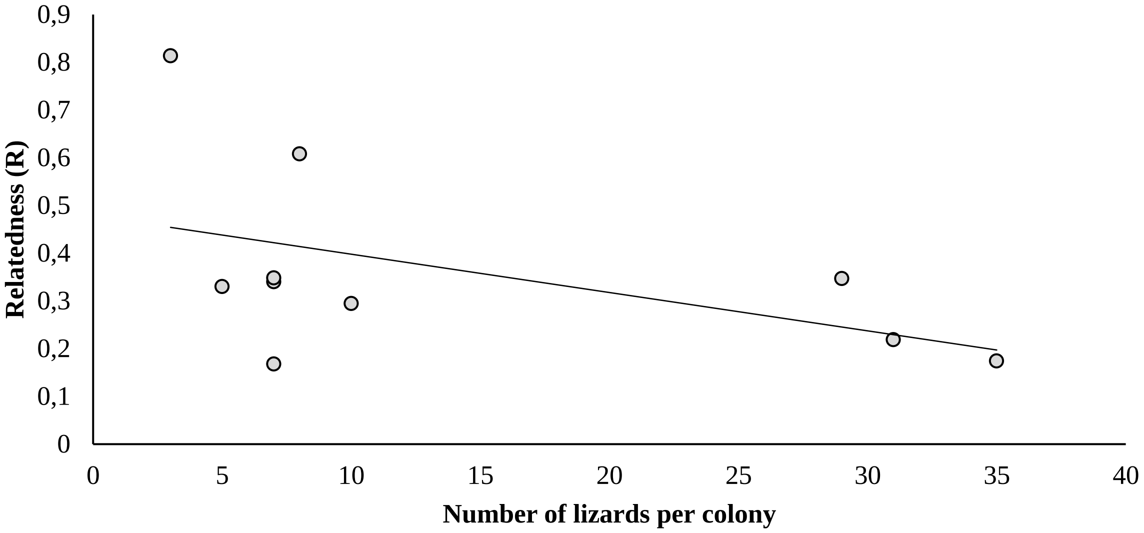


Figure 4.8. Regression showing the relationship between average intra-colony relatedness (R) and colony size for 10 *Smaug giganteus* colonies across the species distribution in South Africa. Colonies comprising of 3 or fewer individuals were removed from this analysis. Regression coefficient (r^2) = 0.24, p = 0.151

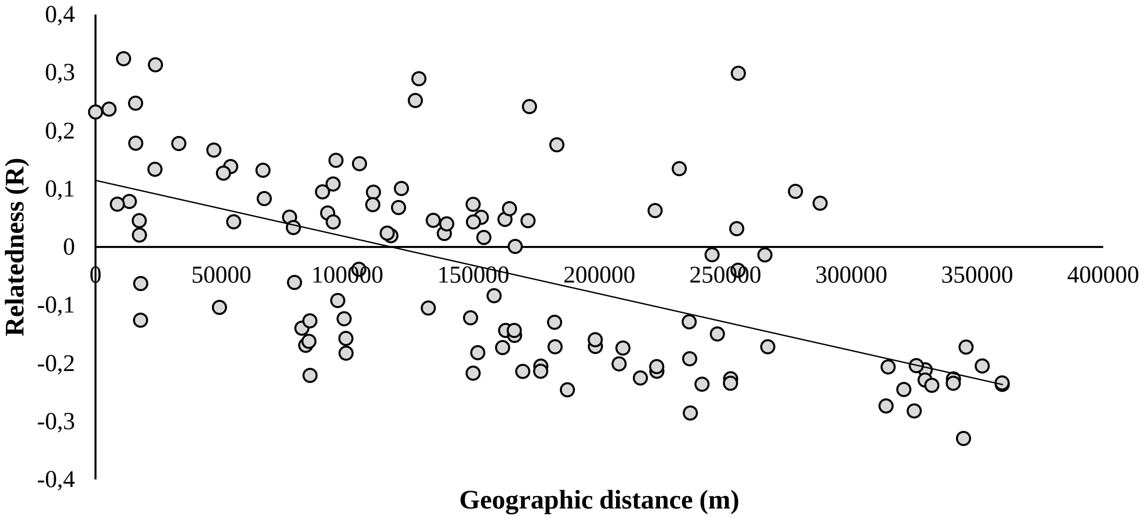


Figure 4.9. Regression showing the relationship relatedness coefficients (R) between *Smaug giganteus* colonies and distance (metres) for 15 colonies (115 pairwise comparisons) across the species distribution in South Africa. Regression coefficient (r^2) = 0.351, p < 0.01.

4.3.6. *Inbreeding analysis*

Deviance information criterion (DIC) values for the ‘nb’ model were lower than the ‘nfb’ model in both the Heilbron and Welkom colonies, indicating that the models without inbreeding are a better fit than models that include the presence of null alleles (Table 4.8). The Volksrust colony had lower DIC values for the ‘nfb’ model than the ‘nb’ model, suggesting that inbreeding is a significant component of the model in this colony. This is further supported in that the Volksrust colony had the highest Average (F_{IS}) values of the three colonies - almost double that of the Heilbron colony, and almost triple that of the Welkom colony.

Table 4.8. Results of Bayesian inbreeding models run in INEST for the Heilbron, Welkom, and Volksrust *Smaug giganteus* colonies. INEST was run using different combinations of inbreeding (f), null alleles (n), and random genotyping errors (b) model parameters. The deviance information criterion (DIC) was used to determine the best model for each colony (shown in bold). Avg (F_{IS}) is average inbreeding coefficient for each population, HPD is the highest probability density.

	Heilbron				**Welkom**				**Volksrust**			
Model	DIC	ΔDIC	Avg (F_{IS})	95% HPD	DIC	ΔDIC	Avg (F_{IS})	95% HPD	DIC	ΔDIC	Avg (F_{IS})	95% HPD
Nb	**3358.92**	0.519	NA	NA	**1900.64**	1.597	NA	NA	1686.10	3.519	NA	NA
Nfb	3359.44		0.016	0.000 – 0.041	1902.24		0.010	0.000 – 0.033	**1682.58**		0.029	0.000 – 0.065

4.3.7. *Comparison of relatedness at different spatial scales*

There is a significant difference in relatedness between Sungazers sharing burrows, nearest-neighbour pairs, Sungazers within a colony, and Sungazers between colonies (one-way ANOVA; $F_{(3, 170)} = 33.36$; $p < 0.01$). Bonferroni post-hoc comparison indicated that all four groups differed significantly from one another (all p-values < 0.001).

4.4. Discussion

Smaug giganteus exhibits kin-based sociality. Individuals share burrows with immediate family members, and live in colonies with extended family members. Sungazer colonies have among the highest within-group relatedness scores of any social lizard species studied to date. Parental care appears to be present in both male and female Sungazers, and includes refuge sharing with offspring, delayed dispersal, and prolonged tolerance of offspring. Young Sungazers may benefit from enhanced survival as a result of these behaviours. *Smaug giganteus* is the first African lizard species known to exhibit family-based social organisation, and only the fourth species outside of the Melanesian-Australian *Egernia* group to my knowledge confirmed to show this behaviour. Unlike most other highly social lizard species, Sungazers have a tiered social organisational pattern. Small groups (2-5 individuals in my study, but as many as seven per burrow based on field observations (pers. obs.)) occupy a burrow that forms part of a larger family colony that is geographically isolated and unrelated to other colonies. Although it is common to find a Sungazer living on its own within a burrow, the average occupancy of a Sungazer burrow (1.83 burrows/ha; Parusnath, 2014) indicates that it is typical for more than one Sungazer to occupy a given burrow. Together, these findings expand our understanding of sociality in lizards, extend the taxonomic range of lizards with social organisation based on family groups to a new lineage within the Scincoidea superfamily (*sensu* Pyron et al. 2013), and present a unique variation in the type of kin-based social organisation that has been studied in lizards thus far.

The sharing of burrows by parent-offspring Sungazer groups appears to be a result of delayed dispersal of juveniles. Refuge sharing and delayed juvenile dispersal together are an important tenet of parental care in lizards (Whiting and While 2017). Similar levels of refuge sharing and high natal philopatry of juveniles are a hallmark of parental care in many species of the *Egernia* group of lizards (e.g. Gardner et al. 2001, O'Connor and Shine 2004, Stow and Sunnucks 2004, Sinn et al. 2008, McAlpin et al. 2011). Ruddock (2000) reported that adult male Sungazers shared burrows with juveniles more frequently than adult females did, however the opposite was true in this study (females with offspring (33%), males with offspring (19%), siblings only (5%)). The ratios in which different combinations of sex and age classes of Sungazers occupy burrows is similar to *X. vigilis,* in which adult males are also found living alone with offspring, but at lower proportions than females and their offspring (females with offspring (27%), males with offspring (9%), siblings only (11%); Davis et al. 2011). It is possible that in *S. giganteus,* there is high plasticity in terms of which parent associates with offspring across years, since Sungazers appear to utilise more than one burrow across seasons and years (Ruddock 2000, Stanton-Jones 2018), and male and females that mate in a season occasionally swap burrows with each other for short periods (Ruddock 2000).

Aside from the sharing of burrows by immediate family members, there were also several cases in this study where adults shared burrows with juveniles that were not their own offspring, and juveniles sharing burrows with other juveniles that were not their full-siblings (often without adults present). I suggest that in both of these scenarios, these cases arise as a result of high mobility of Sungazers between burrows within a colony. Ruddock (2000) described the behaviour of burrow swapping, whereby an adult Sungazer of either sex swaps burrows with an individual of the opposite sex for up to two days before returning to their home burrows, after spending a brief period together in the burrow. Ruddock (2000) observed this behaviour across multiple years by a large proportion of Sungazers in his study, and suggested this as possibly representing mating behaviour. Further, Ruddock (2000) and Stanton-Jones (2018) both reported on the frequent movements between burrows by adult Sungazers across multiple years. Ruddock proposed that Sungazers utilise several burrows within a colony that they visit, but occupy one primary burrow that they most frequently return to. Both burrow swapping with mates, and frequently moving between burrows can clearly result in an adult sharing a burrow with a juvenile to which it is not related.

The other mechanism to aid in understanding the system of burrow occupancy by extended family members is the mating system in *S. giganteus*. Ruddock (2000) reported on male and female Sungazers visiting the same burrows belonging to the opposite sex frequently across years, hinting at a system of long-term pair monogamy or potentially polygny, since some males visited more than one female across years. While monogamy or polygyny with social group members provides a mating advantage in guaranteeing mating success over several years (Graves and Duvall 1995), consistent mating with close relatives can lead to inbreeding depression. As an inbreeding avoidance mechanism, several fraternal lizard species that exhibit long-term pair monogamy with partners in the same social group employ the tactics of 1) mating with other individuals from within the colony between mating with the long-term partner (Olsson et al. 1994), 2) mating with extra-group individuals that arrive in a colony through dispersal (Amos et al. 1993, Sillero-Zubiri et al. 1996), 3) mating with other males during the same season, resulting in multiple paternity of offspring (*Tiliqua rugosa*, Bull et al. 1998, *Egernia stokesii*, Gardner et al. 2002, *E. saxatilis*, O’Connor and Shine 2003, *E. cunninghami*, Stow and Sunnucks 2004, *Liopholis whitii*, While et al. 2014). All three mechanisms can result in higher heterozygosity of offspring, and counter the potentially deleterious effects of intragroup breeding (Brooker et al. 1990, Coltman and Slate 2003, Eimes et al. 2005, Chapman et al. 2009, Whittingham and Dunn 2010, Michalczyk et al. 2011). The shed-skin samples belonging to Sungazers that could not be found in the colony at the time of sampling were generally unrelated to the adults occupying the burrows where the shed-skins were found. These animals may have been dispersing adults from another colony that had since moved on from the time of sampling. These mechanisms serve as potential explanations of the occurrence of both full-siblings and half-siblings sharing a burrow with an adult of either sex, although long-term social studies are required to fully understand the complexities of this system and which if any of these mechanisms are employed by *S. giganteus*.

Reproductive adults of many social lizard species (*T. rugosa*; Bull and Cooper 1999, *E. stokesii*; Gardner et al. 2001, *E. cunninghami*; Stow and Sunnucks 2004, Chapple and Keogh 2005) tend to choose mates within a group that are less related to them than would be expected by chance, given the average level of relatedness within a given social group. In contrast, Sungazers that were identified as candidate parent pairs of offspring in this study were less related, but not significantly so, than would be expected by chance. This could be a result of the comparatively high level of relatedness within Sungazer colonies compared to social groups in other species. Despite the high level of relatedness between mate pairs, inbreeding was not detected in any of the three well-sampled colonies in this study. In addition to the mating strategies that are employed for inbreeding avoidance, cooperative breeding animals are known to exhibit high levels of inbreeding tolerance, and it therefore may not be possible to identify strong inbreeding costs in systems where inbreeding avoidance mechanisms have evolved simply because inbreeding levels, and hence statistical power, are low (see Foerster et al. 2006).

Pre-copulatory inbreeding avoidance behaviour in many of the *Egernia* group species is attributed to olfactory-based kin recognition (Main and Bull 1996, Michael Bull and Cooper 1999, Bull et al. 2000, 2001). This is a functional mechanism that may be employed in *S. giganteus,* since the species has highly developed generation glands that have been suggested to play a role in chemical signalling between conspecifics (Ruddock 2000, Louw et al. 2007, 2011, Mouton et al. 2018). Mate choices of pairs of Sungazers in my study are likely due to potential constraints on a female's choice of social partner within a colony, driven by limited or non-random dispersal resulting in a fine-scale genetic structure (*sensu* Szulkin and Sheldon 2008, Szulkin et al. 2009). One potential benefit of increased relatedness in a colony is the promotion of kin-selected behaviour, decreasing aggression towards other group members, which can potentially increase survival (Kokko and Ots 2006). When coupled with the greater relatedness of social partners compared with random mates within the population, these results may actually suggest selection for mating with kin (e.g. via positive effects on the inclusive fitness of the parents; Kokko and Ots 2006, Szulkin et al. 2013)

The relatedness of Sungazers in nearest neighbouring burrows was significantly different from the average relatedness of Sungazers within a colony – although both groupings were extended family members on average. High natal philopatry appears to lead to clustering of immediate family within burrows, with no further discernible structure within the colony, as there was no pattern of isolation by distance pattern detected within colonies. The clustering among related individuals in a population observed in this study is suggested to be a first indicator of cohesive social aggregations (Fuller et al. 2005). The lack of discernible structure within a colony is likely attributed to the aforementioned mechanisms of burrow swapping, and utilisation of several burrows by individual Sungazers. Ruddock (2000) found that 68% of Sungazers moved between different burrows over a single month, and 75% moved between burrows across seasons. Stanton-Jones (2018) similarly found that 60% of Sungazers moved burrows across a season, with both studies finding that males move around significantly more than females. Although Sungazers appear to show high-fidelity and have been recorded occupying the same burrow for as long as 20 years (pers. obs., McIntyre 2006), it is clear that Sungazer social behaviour is characterised by frequent movement between burrows. This high level of mobility and utilisation of several burrows within a colony by individual Sungazers provides a likely explanation for the lack of obvious structure within colonies, outside of immediate family members sharing burrows.

The within-group relatedness scores recorded for Sungazer colonies in my study (R = 0.345) are amongst the highest recorded for a social lizard species. Species belonging to the *Egernia* group typically have within-group relatedness scores indicative of primarily extended family membership (e.g. *E. stokesii,* R = 0.166 (Gardner et al. 2001); *L. whitii,* R = 0.199 (Chapple and Keogh 2005); *Bellatorias frerei,* R = 0.208 (Duckett et al. 2012); *E. cunninghami,* R = 0.253-0.264 (Stow et al. 2001)). By comparison, the relatedness scores within colonies in my study for Sungazers indicate a higher composition of immediate family members than most species in the *Egernia* group. *Xantusia vigilis* groups containing juveniles had an average relatedness score of R = 0.137 (Davis et al. 2011), which is generally lower than values found in the *Egernia* group and the present study, although still representative of a group made up primarily of extended family. By comparison, none of the *S. giganteus* colonies in this study had an average relatedness score lower than R = 0.137 (as in *Xantusia vigilis,* Davis et al. 2011), and only a third had average relatedness scores lower than R = 0.264 (as in *Egernia cunninghami,* Stow et al. 2001). The high level of within-group relatedness recorded in these species are generally attributed to high levels of natal philopatry and delayed juvenile dispersal that results in high levels of relatedness between adults and offspring within a social group .The results of my study indicate the same is likely true for *S. giganteus*. The higher levels of within group relatedness in *S. giganteus* can possibly be attributed to the nature of the species' refuges. Whilst most social species of the *Egernia* group use rock crevices, trees, and fallen logs as refuges (Whiting and While 2017), Sungazers depend on self-excavated burrows as refuges. These burrows are the only available shelters from predation and a harsh climate, and are also important in daily maintenance of optimal body temperatures (Stanton-Jones et al. 2018). The reliance on these burrows may facilitate a high level of high site fidelity over time – leading to more closely related family members living together over long periods.

Obligate burrowing is uncommon among highly social species, with only a few cases in the *Egernia* group following a similar life-history. *Liopholis kintorei* is one such species, but differs in that multiple individuals construct elaborate multi-tunnelled burrows that are occupied for up to seven years by multiple generations of cohorts, generally with both parents (McAlpin et al. 2011). These burrow systems are suggested to serve an important function in protecting juveniles from predation and intraspecific aggression (McAlpin et al. 2011). Sungazer burrows are aggressively defended by adult Sungazers of both sexes (Ruddock 2000), and aside from the protection of this valuable resource, it is possible that the defence of a burrow is also related to the direct protection of offspring from intraspecific aggression and cannibalism (Van Wyk 1992). Neonate Sungazers are especially vulnerable to predation by mesopredators, since their protective spikes have not yet ossified and are therefore more palatable to predators (Parusnath 2012). Sungazer burrows are remarkably uniform in dimension (Van Wyk 1992), and are most likely dug only by adults, since young Sungazers are unlikely to be able to construct burrows of such size in the hard clay-like soil that the species burrows in. These burrows are likely constructed over several months in the wet austral summer season (Branch and Patterson 1975), and young Sungazers would expose themselves to significant risk while digging such a burrow over this period. Finally, although the dispersal system is not well studied in *S. giganteus*, it is possible that offspring may benefit from the inheritance of territories as a result of prolonged parental care (sensu Gardner et al. 2001). Together, these behaviours associated with

Sungazers self-excavated burrow systems are likely to signifantly reduces infant mortality, and may provide some insight into the evolution of kin-based sociality in the species.

Smaug giganteus, similar to all species of the *Egernia* group that have been studied this far, has significantly higher levels of relatedness within colonies (‘family groups’ in *Egernia*) than between colonies (Gardner et al. 2001, Stow and Sunnucks 2004, Fuller et al. 2005, Chapple and Keogh 2005, McAlpin et al. 2011, While et al. 2014) – further supporting the hypothesis that Sungazers preferentially group with family members. *Smaug giganteus*, however, appears to be unique amongst the social lizards studied to date, since colonies appear to be discrete units that are separated by at least ~1km, and the placement of colonies in the environment appears to be serendipitous and not necessarily associated with any discernible features. In other social lizard species, distances between social groups tends to be dependent on the abundance and availability of niches, and can range from several metres to several kilometres between trees (*B. frerei*, Duckett et al. 2012), or several metres to several kilometres between rock outcrops (*E. stokesii*, Gardner et al. 2001; *L. whitii*, Godfrey et al. 2006). Nevertheless, the average between-colony relatedness in *S. giganteus* (R = -0.043) is similar to other social lizard species (e.g. *E. cunninghami*, R = -0.064 - 0.028 Stow et al. 2001; *E. stokesii*, R = -0.024 Gardner et al. 2001; *B. frerei*, R = -0.004 Duckett et al. 2012; *L. whitii*, R = -0.001 (Chapple and Keogh 2005)).

As expected, there was a significant negative relationship between colony relatedness and geographic distance in *S. giganteus*. The trend of increasing genetic differentiation with genetic distance indicates the low dispersal ability of Sungazers (also corroborated by the IBD in areas devoid of significant physical barriers), and mirrors similar trends found in the *Egernia* group (Whiting and While 2017). Some pairs of *S. giganteus* colonies, however, showed higher than expected levels of relatedness given the geographic distance between them (eg. Wesselsbron – Harrismith; R = 0.299). While this may suggest a high level of gene flow between these populations, it may also suggest the presence of similar common alleles in these populations. Both colonies occur within the relatively homogenous Western clade, in which conditions between colonies at the western and eastern edges of the clade may not be significantly different enough to induce local adaptation. The colonies may also not have been separated for long enough periods to experience high levels of genetic drift. Nevertheless, the high pairwise relatedness of some pairs of colonies towards the southern extreme of the species distribution might indicate that within particular areas, there is a greater degree of gene flow among those colonies than is typical. Further investigation into gene flow in these areas may elucidate the factors that serve to facilitate higher levels of genetic exchange between these populations.

Conclusion

This is the first study to provide genetic evidence of kin-based sociality in an African lizard species. Additionally, it is only the second case of kin-based sociality in lizards outside of the Melanesian-Australian *Egernia* radiation of lizards. These findings open the door to further investigations of sociality in other species in the Cordylidae family, as well as the Gerrhosauridae, the fourth and final family within the Scincoidae superfamily in which kin-based sociality has been suspected to occur (Whiting and While 2017). Several of these species share similar life-history traits with other social lizards species, such as late age of first reproduction, low litter size, aggregative behaviour, and high site-fidelity – traits that combined with low refuge availability may lead to the convergent emergence of kin-based sociality in these species. Key among these species are *O. cataphractus* which has long been known for its stable aggregative behaviour in rock crevices (Mouton et al. 1999, Visagie et al. 2005, Mouton 2011), and *Matobosaurus validus* and *Gerrhosaurus skoogi*, which may form loosely structured colonies (Bates et al. 2013). Frequent observations of adult *M. validus* with juveniles may indicate the presence of familial living in the species (Martin Whiting pers. obs.). *Smaug giganteus* remains unique however, in that it is the only burrowing species within the Cordylidae, and as such likely exhibits behaviours unique to its burrow-dependent life-history. Despite the value of these findings, our understanding of the social structure system in *S. giganteus* is still in its infancy, and lags several decades behind research on the *Egernia* group of lizards. As such, studies focused on investigating particular aspects of the species social structure such as inbreeding avoidance mechanisms, long-term population dynamics and the intricacies of social networks within colonies will continue to unravel the complex puzzle of kin-based sociality in lizards.

CHAPTER 5

SYNTHESIS & DIRECTIONS FOR FUTURE RESEARCH, CONSERVATION RECOMMENDATIONS, AND FINAL CONCLUSION

5.1. Synthesis and directions for future research

5.1.1. *Understanding drivers of genetic structure in Smaug giganteus*

Smaug giganteus is unique amongst its family and genus. Most species of Cordylidae, and all other species of *Smaug,* are rupicolous – inhabiting rocky outcrops and taking refuge in narrow crevices (Branch 1988, Mouton and Van Wyk 1997). Sungazers, in contrast, are dependent on flat, primary grasslands, where the self-excavated burrows that they are reliant on are their only refuge from predators and a harsh climate (Branch 1988, Stanton-Jones et al. 2018). Although the other species within *Smaug* are large in body size, *Smaug giganteus* is by far the largest. Stanley (2009) suggests that the Cordylidae originated in east Africa and migrated southwest. In this scenario, *Smaug giganteus* has migrated furthest south east amongst the *Smaug* species, ostensibly from mountain ranges in the north east of its current range. The grassland habitat that the species has evolved to occupy to may therefore be the most novel amongst the genus, requiring equally novel adaptations to thrive within it.

The large body size of Sungazers suggest several adaptations to life in the grasslands. The requirement of having to excavate a ~1.5 m long burrow in hard clay soils may have conferred selective advantages onto Sungazers with larger and more heavily armoured bodies, since Sungazers are most vulnerable to predation when out of their burrows over extended periods of time (Jacobsen et al. 1990, Groenewald 1992). Thick thoracic armour typically evolves in small animals that increase in body size and move into more open habitats (Stankowich and Campbell 2016). Living in a more exposed habitat may increase predation risk, thus spurring rapid changes in morphology and behaviour in order to adapt (Vamosi 2005, Nosil and Crespi 2006, Broeckhoven et al. 2015). Sungazers are also free from the constraints of rock crevices in their grassland habitat, which to an extent restrict the body sizes that rupicolous species can adopt (Mouton and Van Wyk 1997, Stanley 2009). Finally, the short muscular limbs, and thick spinose tail that the species have evolved appear to play a significant role in the defensive behaviour that Sungazers exhibit from within their burrows. When threatened, Sungazers retreat headfirst into their burrows, and thrust their heads and dorsal surfaces against the upper surface of the burrow. The large occipital and thoracic spines are anchored in place in the roof of the burrow by the force produced by their forelimbs (Losos et al. 2002). If antagonised, the Sungazer will either swing its tail laterally (Marais 1984), or induce a sigmoidal motion with its tail (pers. obs.), forcing its ossified spines into the hand/snout of the researcher/predator that is trying to capture it. The weaponization of tails in amniotes typically evolves in response to adaptation to an open environment, or one where a single entry burrow can be defended - both of which are characteristic of that the habitat that Sungazers occupy (Stankowich and Campbell 2016, Arbour and Zanno 2018).

These morphological and behavioural adaptations that have allowed for Sungazers to successfully colonise grasslands also appear to significantly limit their mobility, particularly in structurally complex habitats. The species has not been recorded as inhabiting landscapes other than flat or gently sloping primary grassland (Parusnath 2014). It is very likely therefore, that the rocky outcrops and escarpments that erupted into the eastern Highveld grasslands approximately 2.5-1.8 MYA (Partridge and Maud 1987), presented significant barriers to genetic exchange between what were once connected populations. The high levels of genetic differentiation between the Western, Central, and Eastern *S. giganteus* clades identified in my study suggest that these barriers may be absolute between clades. Although mountains do not seem to serve as barriers to gene flow for most species within the Cordylidae, rivers commonly do (Diedericks and Daniels 2014, Stanley and Bates 2014), even in the most agile members of the Cordylidae, the Platysaurinae (Scott et al. 2004). Aside from the physical barriers of rivers, the microclimates within river valleys are also generally unsuitable for the cordylid species that occur on either side of them, precluding movement and therefore genetic exchange across these valleys (Stanley 2013, Diedericks and Daniels 2014). It is evident from analysing the spatial spread of niche suitability for *S. giganteus* that the same is true for this species. It is difficult to ascertain the specific effect of rivers on dispersal and gene flow in the species, since most of the putative river barriers are closely associated with geological structures that also appear to serve as barriers. The comparatively homogenous landscape of the Western clade is the only region in which isolation by distance was detected, indicating that in areas devoid of physical barriers, the low mobility of dispersing individuals may still result in genetic structuring across a population through distance alone.

The ecological niche model conducted for *S. giganteus* in this study provides some insight into the parameters that correlate with niche suitability for the species. Soil profile, geological profile and vegetation type were the strongest predictor variables for high niche suitability, and are perhaps the variables most linked with the ability of Sungazers to dig burrows within a particular area. Given that Sungazers are reliant on their own self-excavated burrows as refuges from predation, regions within and outside of the distribution that differ significantly from optimal soil and geological profiles preclude the expansion of Sungazers into those areas. The low mobility and agility of individual Sungazers also suggests that without expansion over a landscape through the construction of burrows over generations, it is unlikely for Sungazers to move great distances over unsuitable habitat without being exposed to great predation risk. The habitat specialist nature of *S. giganteus* and the strict adherence to its ecological niche are therefore also important factors that restrict gene flow across unsuitable habitats.

Transformed habitat can present as significant a barrier to dispersing reptiles as geological features, but affect gene flow over a much more recent, and shorter temporal scale. The effects of reduced dispersal therefore do not necessarily reflect in contemporary patterns of gene flow between populations. Sungazers have long generation lengths and slow reproductive rates, which may make the species particularly prone to a time-lag effect, in which current effects of a landscape feature on dispersal only manifest genetically in future generations. The impacts of habitat transformation that began in the distribution of the species in the early 1900s (representing fewer than 10 generations) appear to not yet be evident in patterns of genetic structure in the species. Habitat fragmentation is already believed to be the causal factor behind population declines in the species however, believed to be due to edge effects such as drought stress, flooding, prey shortages, and exposure to pollution and pesticides that are associated with small patch size (Parusnath et al. 2017). Sungazers may also be exposed to high stochastic risk when navigating crop fields and fallow lands, since these environments are not partial to burrow construction and may be more vulnerable to predation. It is therefore to imperative to 1) further investigate the comparative ability of Sungazers to traverse agricultural matrices vs natural matrices, 2) conduct more focused, fine-scale studies of gene flow in areas particularly marred by agricultural landcover change.

Differences in body size between Sungazers from the west and east of the distribution correlate with elevational and climactic differences. Squamates tend to be smaller in size at higher elevations and cooler temperatures (Ashton and Feldman 2003), possibly as the result of selection for increased surface area to volume ratio to allow more rapid heating and cooling (Cowles 1945, Bogert 1949, Stevenson 1985a, 1985b). While it may be that selection favours smaller body sizes at higher elevation, as per the inverse of the controversial Bergmann’s rule, it is also plausible that the observed differences in average body size across clades result from differences in food availability, or random genetic drift. It is difficult to definitively assign a cause to the differences in Sungazer body size between clades, and there are several theories that require further examination before a conclusion can be reached. Whether local adaptation, genetic drift, or food availability, understanding the driver behind intraspecific body size differences in *S. giganteus* will provide further insight the adaptive ability of cordylids in colonising different environments and habitats.

5.1.2. *Sociality in lizards*

In this thesis I report the first quantitative evidence of kin-based sociality in *S. giganteus*. This is the first confirmation of kin-based sociality in an African lizard species, and only the fourth species outside of the Australian *Egernia* group (Davis et al. 2011, While et al. 2019) known to exhibit such behaviour. Kin-based sociality has been studied in detail in 17 species belonging to the *Egernia* group (While et al. 2019), and a single species from North America (*Xantusia vigilis*, Davis et al. 2011). While *S. giganteus*, *X. vigilis*, and the *Egernia* group all belong to the Scincoidae superfamily, the presence of kin-based sociality in each of these species is believed to result from convergent evolution (Davis et al. 2011, While et al. 2015, 2019). Indeed, all known species with kin-based sociality share very similar life-history traits including high site-fidelity, late age of first reproduction, low reproductive rate, and live birth. These life-history traits are characteristic of the "k-selected" life-history (Dunham 1982) which is usually associated with some species of birds and mammals. Lizards that give live birth to few, large offspring per litter in environments with limited refuges may undergo selective pressure to tolerate the sharing of refuges with their offspring in an effort to increase survivorship, leading to the evolution of sociality in these species. Other lizard species with similar life history traits are therefore ideal candidates in which kin-based sociality should be investigated.

Several other species within the Cordylidae may also exhibit kin-based sociality, based on similar life history traits and anecdotal evidence of grouping behaviour. *Ouroborus cataphractus* and other species within *Smaug* are likely candidates to next investigate for the presence of kin-based social systems (Mouton et al. 1999, Mouton 2011, Visagie et al. 2005). The aggregative behaviour of *O. cataphractus* has long been recognised and studied (Mouton et al. 1999, Visagie et al. 2002, Mouton 2011), although the selective forces behind these aggregations has been ascribed to clustering because of nearby food sources (Shuttleworth et al. 2013). Besides the results of my thesis, no other genetic investigations on the family have been undertaken to assess the relatedness of individuals that cluster together. The limited use of genetic tools in investigating aspects of social behaviour in the Cordylidae is surprising, given observations of some social aspects over the years (Van Wyk 1992, Ruddock 2000, Stanton-Jones et al. 2018). It is possible that these stable aggregations of *O. cataphractus*, like *S. giganteus* colonies, may represent family groups.

The remaining family within the Scincoidae in which kin-based sociality has been suspected, but not yet investigated is the Gerrhosauridae. Many species in the Gerrhosauridae share many of the life-history traits with the social *Egernia* species and *S. giganteus*, but are oviparous rather than viviparous (Loveridge 1942, FitzSimons 1943, Broadley 1966, Branch 1998), and are active foragers, as opposed to the sit-and-wait foragers as most of the Cordylidae are. Nevertheless, *Matobosaurus validus* and *Gerrhosaurus skoogi* are believed to form loosely structured colonies (Bates et al. 2013), and observations of juveniles frequently seen associated with adults may be suggestive of family living (Martin Whiting pers. obs.). Sixty eight other species of lizards in 16 families are known to show aggregative behaviour (Gardner et al. 2016), although evidence of the long-term stability of these aggregations is limited. Genetic studies of kinship between individuals living in aggregations are even more limited in lizards. Given the extent of what appears to be independent evolutions of kin-based sociality within the Scincoidae, genetic investigations of relatedness between aggregating individuals in other lizard species may be the first step in elucidating the full extent of sociality in lizards.

The Sungazer is an ideal model species in which to expand research on sociality in African lizards. The high level of site-fidelity of Sungazers within colonies over decades means that long-term studies on population dynamics and behavioural observations can be performed over time with relative ease. New technologies such as the automated cellular PIT tag reader system (ACRS) developed by Stanton-Jones (2018) would allow for the remote monitoring of genotyped individuals and how they interact with each other. The ACRS systems have shown a 98.5% success rate in reporting the emergence and retreating activities of Sungazers. Burrow systems fitted with ACRS systems can be monitored with camera traps so that the behaviours displayed by individuals can be observed. Camera traps could also potentially record the entry of untagged Sungazers into burrows and reveal the frequency with which Sungazers from a different colony enter the study colony. Modern camera traps can be accessed remotely through mobile data connectivity, to provide a live-feed, or to access images and videos stored on the camera. Together, the technologies of the ACRS, camera traps, and microsatellite genotyping would make for a powerful, remotely-monitored system with which many questions could be answered, such as: 1) how long do juveniles stay with their parent before they disperse?, 2) how far do juveniles disperse from their home burrow?, 3) are Sungazers monogamous?, 4) is there sex-biased dispersal in Sungazers, and how far do dispersing animals travel?, 5) are the frequent movements by Sungazers between burrows for reproductive purposes?, 6) how does a male Sungazer end up in a burrow with its offspring?

The confirmation of kin-based sociality in *S. giganteus* also sheds light on social behaviours observed in the field, which allow for the formulation of hypotheses to be tested. For instance, young Sungazers are frequently observed sitting atop an adult Sungazer while basking. Based on the findings of my thesis, it can be assumed that in most cases these consist of offspring-parent dyads. This behaviour may constitute a novel form of parental care in the family. The grasslands that the species occupy are densely occupied by *Themeda triandra*, and generally devoid of objects to bask on or gain a vantage point. This may be why Sungazers have evolved the iconic ‘sungazing’ posture that the species is named for, since the elevated posture exposes their dorsal surfaces to the sunlight (Stanton-Jones et al. 2018), and allows for surveillance of predators and prey. Standing on the back of their parent may allow a young Sungazer to gain access to sunlight, from where it can reach and maintain its target temperature, and spot prey items. The dorsal surfaces of adults are also generally darker than juveniles, and young Sungazers may also benefit from conductive heating from sitting on the back of their parent. In the iguanian lizards species *Phymaturus calcogaster* and *P. flagellifer,* juveniles have been recorded sitting on the back of adults, a behaviour thought to facilitate visual crypsis from above and dissuade aerial predators that are not willing to attack an adult lizard (Eisenberg and Werning 2012, Halloy et al. 2013). This may also offer a plausible explanation of this behaviour in *S. giganteus*, since the defensive spikes on the bodies of juveniles are not yet ossified and therefore makes them an easier target for predators than an adult Sungazer.

An important benefit of social living that has only recently seen investigations in reptiles is the behaviour of social learning (Hoppitt and Laland 2013). Social learning allows for an individual to gain a skill or solve a problem without a lengthy, asocial, trial-and-error approach to learning (Munch et al. 2018). Experimental studies have revealed evidence of social, context-dependant learning in several lizard species. In these studies, individuals are able to solve a task more rapidly after observing a conspecific solving the same task prior (Kis et al 2015, Munch et al. 2018, Whiting et al. 2018). Social learning may allow for juveniles to quickly learn from conspecifics how to access resources such as food or shelter, and increase their survival rates in challenging or novel environments (Damas-Moreira et al. 2018). In environments with high predation pressure, juveniles may also learn how to detect and avoid predators through interaction with adults conspecifics (Lanham and Bull 2004, Wilkinson et al. 2010, Halliwell 2016). Juvenile Sungazers appear to associate closely with their parents/siblings when basking outside of the burrow, and may learn thermoregulatory behaviour, predator avoidance and hunting strategies through observation. Observational and experimental *in situ* studies may reveal the extent to which juveniles rely on learning from their parents and conspecifics, and provide further insight into mechanisms that have evolved in this species to enhance survival in the Highveld grasslands.

The confirmation of kin-based sociality in *S. giganteus* also poses further questions regarding several physiological and behavioural traits and observations in the species that can be considered in a new light given the findings of my thesis. The generation glands that Sungazers possess are significantly larger and have more layers than in other cordylid species (Mouton et al. 2018). Sungazers exhibit sexual dimorphism in this regard – while both sexes possess generation glands on the ventral femoral region, males possess a second set of generation glands on the ventral forearm surface (Van Wyk 1992). The chemical composition of the secretions from the forearm and femoral glands are qualitatively identical (Louw et al. 2011), and the reason for the occurrence of these glands in males and not females is unknown. Sungazers can discriminate between generation gland pheromones produced by themselves and other individuals of the same sex (Cooper et al. 1999, Ruddock 2000) and it has been suggested that the function of these glands is to transfer of pheromones to substrates, and as such are believed to function in intraspecific communication (Ruddock 2000, Mouton et al. 2010). Cooper et al. (1999) suggested that the pheromones secreted by generation glands are used to mark territories and detect the presence of transient individuals. It has also been suggested that since forearm glands cannot easily transfer pheromones to a substrate the way that femoral glands do, they may serve the purpose of marking grass surrounding their burrows (Louw et al. 2011), or their own bodies (Mouton et al. 2010). Male Sungazers visit other burrows within a colony more frequently than females do (Stanton-Jones 2018), and although it appears that Sungazers primarily occupy one burrow over time, the forearm generation glands in males may serve to mark routes to other burrows (*sensu Tiliqua rugosa*; Leu et al. 2016). These glands could also leave a chemical signal on females mated within a season. This highlights an interesting avenue of research, since the role of generation glands in intraspecific communication is still not well studied in the Cordylidae (Mouton et al. 2014a).

While living in social groups can be advantageous, an obvious downside is the heightened chance of inbreeding depression through the proliferation of deleterious homozygous alleles within a group (Keller and Waller 2002, Charlesworth and Willis 2009). Group living species therefore require mechanisms to avoid the harmful effects of inbreeding in order to maintain genetic health (Brooker et al. 1990, Keane et al. 1996, Cooney and Bennett 2000, Nelson-Flower et al. 2012). Sungazers are highly sedentary animals with high-site fidelity, and in combination with delayed dispersal of offspring from their natal area, may be highly exposed to inbreeding, particularly in highly fragmented areas. Although inbreeding was only detected in one colony and at a very low level, the majority of candidate parent pairs in my study were either 1st or 2nd degree relatives. It is likely that female Sungazers employ mechanisms to increase the heterozygosity of offspring, since individuals with higher levels of heterozygosity experience higher rates of survival (Foerster et al. 2003, Stapleton et al. 2007).

Several inbreeding avoidance mechanisms have been described in the *Egernia* group, namely multiple mating (Uller and Olsson 2008, While et al. 2009), multiple paternity (Gardner et al. 2002, O’Connor and Shine 2003, Stow and Sunnucks 2004), and extra-pair mating (in socially monogamous species) (While et al. 2014). In my thesis, the occurrence of inbreeding avoidance could not be confirmed because the assignment of candidate parents to offspring in this study was based on probability and not direct observation of mating. In order to empirically assess if multiple paternity occurs in *S. giganteus*, the following steps are required: 1) observation of in situ male-female interactions, 2) capture of gravid females and the subsequent birthing of their young in captivity, 3) genetic profiling of mothers, offspring, and all potential fathers. This process would allow for the detection of the use of this mechanism in *S. giganteus*, and serve as an explanation of how colonies are able to successfully live in social groups without significant levels of inbreeding.

5.1.3. *Non-invasive tissue sampling in S. giganteus*

I initially intended to only utilise non-invasive tissue collection protocols when collecting samples for this project. Several hundred shed-skin samples were collected from the field, but unfortunately did not reliably yield DNA suitable for the planned analyses. Preliminary tests using shed skins collected directly from captive Sungazers prior to commencing fieldwork proved to yield high quality DNA, and given the available literature on the use of shed skin in genetic analyses, I built this into my data collection for this research. I did not consider the time effects and the effects of sub-optimal storage conditions of these samples between collection and processing. Ultimately this resulted in the rejection of the large majority of these non-invasively collected tissue samples. Tissue samples were then collected through traditional sample protocols of collecting tail tips to conduct the planned analyses. In spite of this, the testing of the efficacy of various tissue types, and the effects of storage time on their efficacy were useful analyses, and will hopefully inform future studies on effective sampling and storage protocols.

Shed skin samples, when processed within 12 months of collection yielded similar levels of microsatellite profiling success to tail clippings, despite having lower quantity and purity of DNA than tail clippings. Storage of shed-skin samples in media and at temperatures that reduce DNA degradation may improve the usability of shed-skin samples stored over longer periods of time. The lower purity and concentration of DNA in shed-skin samples compared to scale-clippings or blood is likely due to both contaminants present in the environment and degradation in the field prior to collection. Nevertheless, the lower quality and quantity of DNA yielded from shed-skin samples may be worth the trade-off. The collection of shed-skin does not require capturing lizards, and is therefore less stressful to the animals, more time efficient, and allows for the collection of a greater number of samples over the same time period as active sampling.

These findings may be useful in planning similar studies of rare or threatened reptiles, since it is the first investigation to my knowledge to compare different reptile tissue types and non-invasively collected skin samples across storage periods. These implications may also extend to other groups of animals that may be difficult to collect tissue samples from directly, due to rarity, threat status, or other ethical concerns. Birds, and some mammals, shed keratinous tissue that is similar in composition to Sungazer scales, and therefore may deteriorate similarly under the conditions in which my samples were stored in this study. Although preliminary investigations comparing the usability of different tissue types in other groups prior to commencing large-scale collection efforts is advised, my findings on the efficacy of different tissue types in genetic research may prove useful in guiding sampling and lab protocols in future studies.

5.2. Conservation recommendations

5.2.1. *Conservation of genetic units*

Smaug giganteus is a Vulnerable (Alexander et al. 2018) species and has lost just under half of its natural habitat due to land transformation (Parusnath et al. 2017). The species does not naturally occur within any formal protected areas, and most colonies exist on privately owned farmland (Parusnath et al. 2017). This poses a great risk to the long-term survival of Sungazer colonies, since further land-transformation may exacerbate population decline in the species. Purchasing land specifically for the protection of the species and its habitat on a scale large enough to secure viable population sizes is economically unfeasible (Selinske et al. 2015). Biodiversity custodianship and stewardship programmes offer an alternative approach to traditional protected areas and have shown great success in focused efforts on a per species basis (Gallo et al. 2009, Cooke et al. 2012, Holmes 2013). This cost-effective community-driven approach involves the participation of land-owners that have a species of concern occurring on their property. Land-owners that enter into custodianship are required to employ management practises to the benefit of the populations on their land (e.g. burning and grazing practices), actively address threats to the populations, responsibly and minimally use chemicals on their land, educate staff on the conservation of relevant species and generally preserve these populations in a wild and unrestricted state (Little and Theron 2014).

A custodianship programme for *S. giganteus* was initiated in 2014 by the Endangered Wildlife Trust and focused on sites identified by Parusnath (2014) as containing high-density population in pristine habitat (Fig. 5.1). The aim of focusing on these sites in particular was to incorporate high numbers of Sungazers into the network of custodianship sites so that a minimum viable population could be protected (Traill et al. 2010, Flather et al. 2011, Parusnath 2014). Over the past six years, the custodianship programme has resulted in the protection of nearly 38 000 hectares of primary grassland within the distribution of the species (Bradley Gibbons/Endangered Wildlife Trust pers. comm.). This area is just under half the total area suggested for protection by Parusnath (2014) to ensure the long-term survival of wild-living Sungazer populations. The sites focused on in the custodianship programme are spread across the distribution of the species, however they do not take into account the full extent of genetic diversity detected in this thesis (Chapter 3). Quantifying the spatial pattern of genetic variation over populations of a threatened species can help in prioritizing sites and management choices that will capture and maintain that variation (Wilcox 1984, Moritz 2002, Barber et al. 2011, Toonen et al. 2011). Knowledge of diversity patterns can also reduce the number of populations necessary to commit to conservation and thus reduce costs and conflicts with competing land uses (Beger et al. 2014).

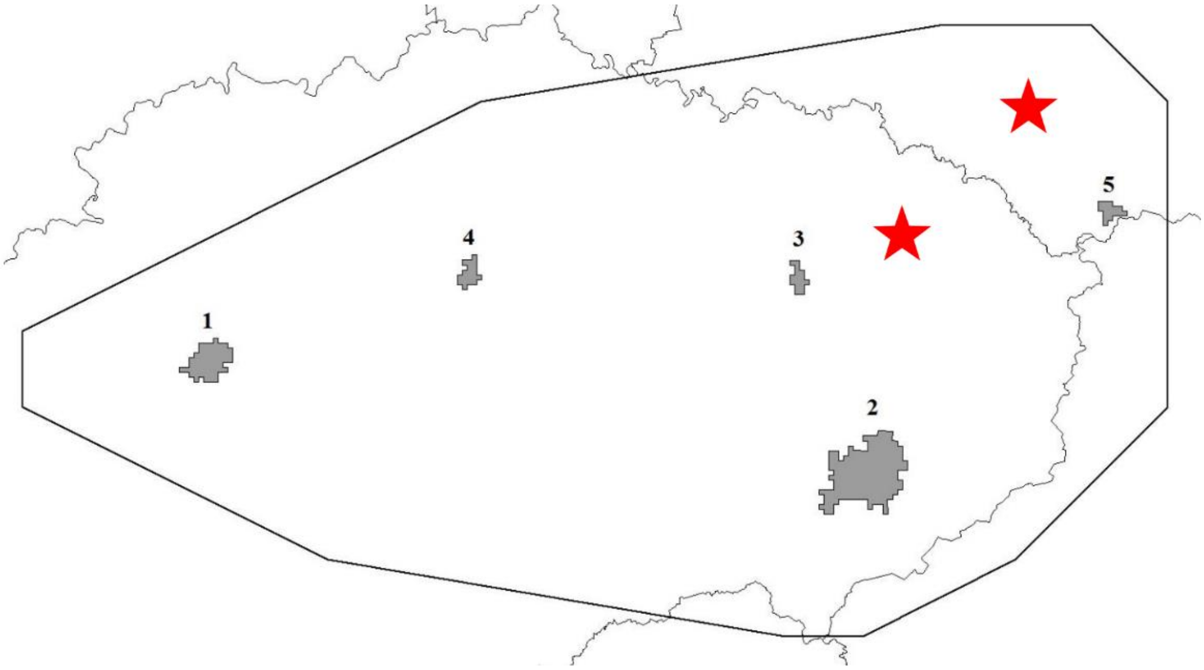


Figure 5.1. *Smaug giganteus* extent of occurrence (EOO) (minimum convex hull) showing five areas (grey shading) predicted to stock high density Sungazer populations (adapted from Parusnath (2014)). Red stars indicate the location of important sites of genetic diversity detected in Chapter 3 of this thesis (Vrede – Central clade, Perdekop – Eastern clade) that should be included into current custodianship site planning for the species.

Comparing the spatial layout of the genetic populations identified in my thesis (Fig. 3.3) to the five units of priority conservation identified by Parusnath (2014) (Fig. 5.1) allows for adjustments to be made in the planned network of custodianship sites such that the genetic diversity identified in this study is taken into account. With the addition of two further areas (red stars; Fig. 5.1), the range of genetic diversity identified in this study can be fully incorporated into the custodianship plan. Namely, a network of sites in the Vrede area, which encompass the Central clade, and sites in the north of the species range in the Eastern clade (Eastern 1 population). Three of the sites initially identified as ideal sites for custodianships (polygons 2, 3, 4; Fig. 5.1) all fall within the Western 2 population the Western clade, and genetically form a large polytomy with little evidence of further genetic structure. Nevertheless, the spatial spread of these site may be important in the future, in situations where habitat loss results in localised gene fixation due to the reduction in dispersal.

Although the custodianship programme has had immense success, custodianships are not formal agreements, and are dependent on the owner land. Custodianship sites may lose their protection with a change in land owner. Biodiversity stewardship entails formal agreements entered into by private and communal landowners by conservation authorities, and have benefits to the landowner as compensation (e.g. tax relief) for their contributions towards conservation (Paterson 2005, Van Wyk 2010). Stewardships range from 10-99 years depending on the level of participation (Barendse et al. 2016). While stewardship agreements involve a higher degree of commitment from landowners and clerical work by conservation bodies, the long-term benefits far surpass those of custodianships (Brown and Mitchell 2000, Gallo et al. 2009, Barendse et al. 2016). The high level of interest from landowners in the existing custodianship programme for Sungazers is indicative of the level of interest in the species by landowners, and perhaps efforts over the next decade can attempt to convert landowners that are currently custodians into stewards. Given that the majority of grassland within the distribution of the species is private property, privately protected areas through stewardships may pose the most feasible long-term solution to protecting the species in the wild.

5.2.2. ***The use of genetic tools in combatting illegal trade in reptiles***

During the course of my PhD, a non-detriment finding (NDF) on *Smaug giganteus* was conducted by the Scientific Authority of South Africa (SANBI 2015) (detailed in section 1.5.5.2). The ratification of this NDF mandated verification of F2 generation captive breeding of *S. giganteus* prior to receiving a CITES permit to export the species from the country. The microsatellite markers designed in my thesis (Chapter 2) have since been utilised in genetic tests to verify F2 captive breeding in the species. Without evidence of F2 captive breeding in any cases thus far, no CITES permits have been granted to export *S. giganteus* from South Africa since 2015. *Smaug giganteus* is the first South African reptile for which an NDF has been conducted, and to my knowledge, the first species in which export permits are no longer granted without genetic verification of captive breeding.

The importance of using genetic markers in reptile trade issues cannot be overlooked. South Africa is home to many highly sought-after reptile species, many of which are rare, threatened, or difficult to breed in captivity. In response to the emergence of governing bodies that seek to control and restrict unbridled exportation of wild-caught animals from their countries of origin, the laundering of wild-caught reptiles as captive-bred has become increasingly common (Lyons and Natusch 2011, Karanth et al. 2014). Some reptile breeding facilities either underhandedly supplement legitimate captive breeding with wild-caught individuals, or pose as a front for a smuggling operation (Lyons and Natusch 2011, Welton et al. 2013, Karanth et al. 2014). The legal origin of many South African reptiles in the international reptile is therefore often doubtful (Auliya et al. 2016). Online searches reveal the prevalence of South African reptiles available worldwide, despite little evidence of breeding facilities in South Africa or elsewhere that are capable of producing such numbers. The problem remains that without the mandate of genetic proof of captive F2 breeding, wild-caught South African reptiles will continue to be laundered and exported from the country as captive-bred animals.

Ideally, the following steps should be followed for South African reptile species threatened with over-exploitation: 1) collection of evidence of wild-caught South African reptiles for sale through searches of online fora, social media platforms and sales websites, 2) application of the listing of priority species on the appropriate CITES Appendix, 3) conduct NDFs in order to mandate proof of F2 breeding before granting permits for export of specimens from South Africa, 4) utilise genetic markers to verify F2 breeding within a given breeding colony upon the application of export permits. A detailed database of spatial genetic structure profiled with microsatellites or SNPs can also serve as a reference database to identify the geographic origin of confiscated reptiles, and thereby identify poaching hotspots and populations at risk from heavy exploitation pressures (eg. Ogden 2008, Ghobrial et al. 2010), Welton et al. 2013, Mondol et al. 2015, Wasser et al. 2015).

5.2.3. *The use of single nucleotide polymorphisms (SNPs)*

When my study began in 2014, microsatellites were the prevailing genetic tool used in population genetic studies. Microsatellites have several advantages in that they are tolerant to low quality samples, and have high efficacy and specificity with the species they were designed for use with (Selkoe and Toonen 2006, Guichoux et al. 2011). As a result of their specificity however, they do not necessarily amplify loci in other closely related species, limiting their applicability beyond the species they were designed for (Galbusera 2000). Scoring microsatellite alleles is a manual process and requires significant human time contribution, and is also therefore prone to human error (Kalinowski et al. 2007, Flanagan and Jones 2019).

In the intervening time, short nucleotide polymorphisms (SNPs) have emerged as the ‘next generation’ marker of choice for population genetic research, and have already been shown to overcome many limitations of microsatellites (Vignal et al. 2008, Morin et al. 2009). SNPs are generally more informative and sensitive than microsatellites (Liu et al. 2005). For example, Strickland et al. (2014) found no evidence for kin-based sociality in eastern water dragons (*Intellagama lesueurii*) using a suite of 17 microsatellite markers, but did find evidence in the same species using SNP data in a follow up study (Piza-Roca et al. 2019). SNPs can be scored semi-automatically (Guichoux et al. 2011), and are therefore less prone to errors (Coates et al. 2009). They can also cross-amplify between species within a genus more efficiently than microsatellites, making them inherently more versatile and cost-effective (Thrasher et al. 2018). The development of SNPs for further genetic research in the *Smaug* genus would allow for studies similar to my thesis without excessive additional costs per species, and make comparative studies between species more feasible. The use of SNPs also has important implications for trade issues, since a suite of SNPs developed for use with a species of concern may be suitable for use within its congeners, which would be highly advantageous for genera such as *Bitis* and *Bradypodion* that are highly targeted in the illegal reptile trade.

5.2.4. *Understanding and mitigating the effects of habitat loss and fragmentation on gene flow in S. giganteus*

The high levels of habitat loss and fragmentation caused by human-mediated land transformation within the distribution of *S. giganteus* (Parusnath et al. 2017) do not appear to have yet had a perceivable effect on gene flow in the species. Although the distances travelled by dispersing individuals is unknown, the increased distances between colonies due to land transformation, introduction of barriers to dispersal such as crop fields and roads, and increased risk of increased edge effects are all likely to decrease the success of dispersing Sungazers in reaching other colonies. As detailed in the discussion of Chapter 3, effects of reduced dispersal on gene flow and therefore genetic structure and diversity may only be detected in future decades due to the slow reproductive rate and long generation length of *S. giganteus.* Given that I did not explicitly investigate the effects of habitat fragmentation on gene flow in *S. giganteus* in my thesis, it is also possible that more focused studies may detect signals that this study did not. The following suggestions are made to detect the putative effects of fragmentation on gene flow in *S. giganteus*, and mitigate the potential current and future negative effects of habitat fragmentation and loss in the species:

1) Conduct comprehensive statistics based analyses on the effects of fragmentation on gene flow in *S. giganteus*. The ecological niche model conducted in Chapter 3 utilised environmental variables that I considered to be important in predicting the distribution of *S. giganteus*, based on what is presently known about the species' life history. Key among these is the most recent national landcover use map (DEA 2015) that details the use of land across the distribution of the species at a fine resolution. However, in order to more rigorously investigate how specific anthropogenic disturbances influence gene flow in *S. giganteus*, detailed GIS data for human population density, road density, and crop density across the distribution of the species should be incorporated into an ecological niche model. At the time of this study, such GIS layers were not available. If these data become available in future, more detailed investigations on the effects of human landcover use on gene flow in *S. giganteus* will be possible. Furthermore, statistical analyses that take both genetic diversity and habitat fragmentation/anthropogenic land use into account may reveal more comprehensive insights into how human activities affect gene flow in the species.

2) Test gene flow in heavily-fragmented vs unfragmented areas. To definitively investigate the contemporary effects of habitat fragmentation on the genetic structure of *S. giganteus*, gene flow between colonies that exist within heavily fragmented areas of the distribution such as Welkom, Lindley, and Petrus Steyn, and colonies that exist within relatively undisturbed areas such as Harrismith and Warden (Parusnath 2014) should be compared. These colonies all fall within the Western 2 genetic population identified in Chapter 3. No geological barriers were detected within this population, and significantly reduced levels of gene flow between could therefore be assigned to the effects of reduced gene flow from habitat fragmentation. Comparisons of genetic diversity and levels of inbreeding between these colonies could also provide insight into the negative genetic effects of reduced gene flow and population isolation. In Chapter 4 I found that most candidate mate pairs were extended family members on average. Since there have been no previous measures into the relatedness of mate pairs in the species, it is unknown whether this level of inbreeding is innate within colonies, or a result of reduced dispersal. Future studies could also therefore investigate whether mate pairs in less fragmented areas have lower levels of relatedness than those in more heavily fragmented areas.

3) Grassland rehabilitation. Sungazers have only been recorded colonising primary grassland (Jacobsen 1989, Newbery and Jacobsen 1994), despite earlier suggestions by Marais (1984) that they may recolonise fallow lands. The inability of Sungazers to recolonise rehabilitated lands may be due to altered soil properties that may prevent the excavation of structurally stable burrows, and reduce water drainage of burrows during high rainfall periods. The altered assemblage of vegetation types and associated changes in the diversity and abundance of invertebrate prey species (Jacobsen et al. 1990) may also reduce Sungazer survival rates in fallow land. Based on anecdotal reports from several farmers, Red Grass (*Themeda triandra),* the dominant grass species in primary Highveld grassland, takes between 60-100 years to recolonise fallowed land. The return of the natural assemblage of invertebrate prey species may follow, although the time period over which that may happen is currently unknown. Rehabilitation projects on fallow lands on which no further agricultural use is foreseen are encouraged in order to investigate the processes and periods of time that are required to rehabilitate fallowed lands such that they may once again be suitable for Sungazers. Successful rehabilitation could result in the reestablishment of Sungazer colonies, reduce the distance between colonies and improve gene flow between populations.

4) Creation of and maintenance of dispersal corridors. In addition to securing protected land for *S. giganteus*, the creation and maintenance of corridors for dispersal between Sungazer colonies may be essential in maintaining genetic diversity in the species. Dispersal corridors have been proved to be highly successful in maintaining genetic exchange between animal populations in transformed areas (Mech and Hallett 2001, Jellinek et al. 2004, Christie and Knowles 2015, Kay et al. 2016). The distribution-wide custodianship programme that is currently being operated by the Endangered Wildlife Trust presents an opportunity to build in the concept of dispersal corridors into an already existing plan. The targeted recruitment of sites that connect disparate populations may ensure that further land transformation does not occur within these areas, hopefully increasing the chances of Sungazers in reaching neighbouring colonies.

5.2.5. *Incorporating knowledge on sociality into translocations and captive breeding protocols.*

Sungazer populations have long suffered from failures to captive breed sufficient numbers to supply the demands of the international pet trade (Loehr et al. 2017; Parusnath et al. 2017), and failures of large-scale translocation efforts in the face of land development (Groenewald 1992). In both scenarios, little attention is paid to the consideration of how the complex social behaviour that the species exhibits may impact the success of these endeavours.

Animal behaviour is increasingly being considered in such approaches, as the understanding of the impacts of social behaviour on individual and group health, longevity, and reproductivity progresses (Anthony and Blumstein 2000). Consideration of the social structure within a population, social roles, and social learning may lead to populations that are more likely to persist after translocations (Goldenberg et al. 2019). In social species such as the black-tailed prairie dog (*Cynomys ludovicianus*), individuals translocated with their family members experienced significantly higher survival rates than individuals translocated without family members (Shier 2006). Detailed surveys of Sungazer burrow occupants, occupants of neighbouring burrows, distance between burrows, and colony size should be conducted in order to closely emulate the social structure in the original location, prior to translocation attempts. Given that Sungazer colonies comprise primarily of immediate and extended family members (Chapter 4), care should also be taken to avoid mixing of Sungazers from different colonies. Aside from the potentially negative genetic effects that this may have, it is possible that colonies are formed around extended family groups, and the introduction of a high number of unrelated animals may lead to lower survival rates. As future studies continue to unravel the complex sociality in this species, more knowledge can be injected into an evolving, educated translocation protocol that will hopefully result in higher success rates of established and thriving Sungazer colonies.

In the same vein, allowing for the creation and maintenance of social structure in captive Sungazer groups may be a key component to successfully breeding Sungazers in captivity. Most Sungazers in captive breeding projects, whether in private collections or zoological facilities, are not kept at population densities that emulate what they might experience in their natural environment, nor are they generally kept on substrates that are conducive to the construction of burrows. Access to a secure, defendable territory appears to be an important tenet of Sungazer behaviour and social structure, and may be key in stimulating reproductivity. In the only two verified captive breeding successes, the Sungazers were kept in outdoor enclosures that allowed for burrow digging and territory defence (Langerwerf 2001, Chris de Beer pers. comm.). In each case, it took several years for the ‘colony’ to become established, before breeding commenced. I therefore suggest that to improve the chances of captive breeding success, Sungazer captive breeding projects adhere as closely as possible to conditions that allow for a natural social structure form. For instance, an enclosure that contains a substrate in which long-term burrows can be constructed at a distance of at least two metres apart should be provided. This may provide a sense of security that Sungazers in typical, crowded terrariums may not experience. Importantly, any captive breeding endeavours should also cater to the natural dietary and climactic requirements of the species, since it is likely that the specific niche that the species occupies in its natural home contains several triggers that stimulate breeding. Attention to social structure is therefore only one of many important factors to consider in such projects. It is my hope that with the information obtained through rigorous scientific research, the species may be more successfully bred in captivity. Ultimately, this may limit, and perhaps stop, the pilfering of wild specimens to supply the pet trade.

5.3. Final conclusion

This thesis provides novel insight into the population genetic structure of the grassland specialist lizard species *Smaug giganteus*. I provide evidence for kin-based sociality in *S. giganteus* – the first for an African lizard. These findings highlight the value of investigating the presence of kin-based social systems in other species with similar life-history traits. This study represents the most comprehensive population genetic study of a cordylid species to my knowledge, and considers genetic relatedness from the scale of individuals in a burrow, to deeply divergent clades within the species. The polymorphic species-specific microsatellite markers developed in this thesis enables the possibility of numerous in-depth future studies on sociality and population structure in *S. giganteus*. These markers have also played an important role in regulating trade in the species in South Africa, and could be used in isolating the origin of confiscated Sungazers or their body parts, so that poaching hotspots can be identified. The findings of my study have implications for the long-term conservation management of the species.

The Sungazer is an iconic and enigmatic species, and is well-known globally for its unique and striking appearance. Although the species has garnered significant attention from scientists over the last century, limited research has focused on some of its most unique characteristics. The South African perspective on sociality in reptiles severely lags behind research on the Australian system, and the Sungazer is an ideal model species in which research on long-term population dynamics, family structure, parental care, and chemical signalling can be caught up. It is my hope that this thesis not only adds to the body of work on the Sungazer, but also serves as a springboard for future research into this species, and its many unique and fascinating adaptations to life in the South African grassland.

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“I can’t do this, Sam”.

“I know. It’s all wrong. By rights, we shouldn’t even be here. But we are. It’s like in the great stories, Mr. Frodo. The ones that really mattered. Full of darkness and danger they were, and sometimes you didn’t want to know the end… because how could the end be happy? How could the world go back to the way it was when so much bad happened? But in the end, it’s only a passing thing, this shadow. Even darkness must pass. A new day will come, and when the sun shines it will shine out the clearer. Those were the stories that stayed with you, that meant something. Even if you were too small to understand why. But I think, Mr. Frodo, I do understand. I know now. Folk in those stories had lots of chances of turning back, only they didn’t. They kept going, because they were holding on to something”.

“What are we holding on to, Sam?”

“That there’s some good in this world, Mr. Frodo. And it’s worth fighting for”.

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