Consequences of grazing resistance adaptations on *Rhodohypoxis baurii* var. *platypetala* population genetics.

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DECLARATION

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

Dylan Edmund Beukes

25 February 2019

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DEDICATION

Look at the stars

Look how they shine for you

And all the things that you do

–

Coldplay

This dissertation is dedicated to my late father Edmund Beukes.

I hope it is work he would have been proud of.

You can't always get what you want

But if you try sometimes you might find

You get what you need

–

The Rolling Stones

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ABSTRACT

Biodiversity in the Drakensberg is threatened by the expansion of farmlands exposing species to intense livestock grazing disturbance, which negatively impacts survival and sexual reproduction in plants, and subsequently the genetic diversity of populations. Assessing the vulnerability of species to grazing is required to ensure effective management of the biodiversity in the region, and this requires an integrated investigation of morphology and genetics.

This study aimed to assess the vulnerability of the near-endemic herb *Rhodohypoxis baurii* var. *platypetala* to grazing. Morphological difference was investigated by comparing traits between populations at 4 grazed and 4 ungrazed sites. Flower height was found to be 8mm (±2.94mm) taller in plants at ungrazed sites ($P = 0.009$) and had one more leaf on average ($P < 0.001$). Other traits, leaf table height, leaf length, number of flowers, leaf angle and above:below ground ratio did not show difference with grazing exposure. These results showed that *R. baurii* var. *platypetala* was not developing architecture that would facilitate grazing resistance. The small magnitude of the differences found along with the lack of observed grazing or evidence of grazing suggested that *R. baurii* var. *platypetala* plants are not affected by grazing disturbance.

The genetic investigation used AFLPs to measure genetic diversity within a population exposed to grazing and another not exposed to the disturbance. It was expected that populations of *R. baurii* var. *platypetala* exposed to grazing would have relatively lower genetic diversity and high differentiation. Expectations were not supported as genetic diversity was found to be moderate and similar in the population at the grazed site ($H_J = 0.345$) and the ungrazed site ($H_J = 0.361$). An AMOVA found low differentiation between the populations ($\Phi_{PT} = 0.096$) which is attributed to their recent establishment in the area. The moderate genetic diversity and low differentiation showed that it is unlikely that grazing is having a negative impact on the gene dynamics of *R. baurii* var. *platypetala*.

Comparison of the findings identified significant difference in morphology between the pair of populations, despite their low genetic differentiation and similarly moderate genetic diversity. With one population having 14.10mm taller flowers, 15.14mm taller leaf table height, and 13.35mm longer leaves (all $P < 0.001$), the morphological difference was extensive but as it was not reflected on a genetic level, it is likely attributed to phenotypic plasticity.

The integration of two forms of investigation allowed for comprehensive assessment of the vulnerability of *R. baurii* var. *platypetala* to livestock grazing. The results of both investigations aligned to show no negative impacts associated with grazing exposure, and an overall unlikelihood of grazing affecting the plants. The assessment framework is recommended for application in assessing other Drakensberg flora.

Keywords: grazing; Drakensberg; genetic diversity; grazing resistance; disturbance; vulnerability

GLOSSARY

$A_{260/230}$ – Secondary measure of nucleic acid sample purity. Based on average extinction coefficients of nucleic acids at 260nm and proteins at 230nm.

$A_{260/280}$ – Measure of nucleic acid sample purity based on protein contamination. Based on average extinction coefficients of nucleic acids at 260nm and proteins at 280nm.

AFLP – Amplified fragment length polymorphism.

AU/km^2 – Animal Unit per square kilometre where AU is the metabolic mass equivalent of a 455kg steer (Short *et al.* 2003)

CTAB – 'Cetyltrimethylammonium bromide' method of DNA extraction.

F_{IS} – Inbreeding coefficient. The proportion of the variance in the subpopulation contained in an individual.

F_{ST} – Wright's fixation index. The proportion of the total genetic variance contained in a subpopulation relative to the total genetic variance. Interpreted as genetic differentiation.

H_B – Genetic differentiation among populations.

H_E – Expected heterozygosity.

H_J – Nei's gene diversity. Analogous to H_E.

H_S – Heterozygosity of subpopulations.

H_W – Nei's gene diversity within populations. Analogous to H_S.

PCA – Principal component analysis.

PCoA – Principal co-ordinate analysis.

PCR – Polymerase chain reaction.

Φ_{PT} – Population genetic differentiation using binary data Analogous to F_{ST}.

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CHAPTER ONE:

GENERAL INTRODUCTION

Biodiversity conservation

Biodiversity preservation is key to the protection of ecosystems, as higher biodiversity has been shown to improve ecosystem functioning (Tilman *et al.*, 2014), make ecosystems more resilient to change (Isbell *et al.*, 2015), increase ecosystem productivity (Wagg *et al.*, 2017), and improve stability (Cadotte *et al.*, 2012). Under the threats of climate change and anthropogenically-driven land-use changes, ecosystem conservation, through biodiversity conservation (Hautier et al., 2015; Malcolm *et al.*, 2006), is key, and requires assessment of biodiversity levels and swift development of appropriate management plans for preservation (Essl *et al.*, 2015; Kohn *et al.*, 2006; Purvis and Hector, 2000).

Genetic diversity has been identified as one of one of the key types of biodiversity recommended for conservation (McNeely *et al.*, 1990), as genetic diversity facilitates evolution and improves population fitness (Reed and Frankham, 2003). The relationship between genetic diversity and fitness makes genetic monitoring an effective management tool that provides key insight into adaptability (Schwartz *et al.*, 2007). Species or populations with low adaptability are vulnerable to changes in their environment (Ford *et al.*, 2015), and this can be increased if the evolutionary history has driven other vulnerabilities. Both population size and isolation are linked to genetic vulnerability through decreasing levels of diversity and increased differentiation (Ellegren and Galtier, 2016; Frankham *et al.*, 2017; Hoffmann *et al.*, 2017; Willi *et al.*, 2006). Effective genetic monitoring requires assessment of the status of genetic diversity within species, as well as adaptability to disturbance.

Disturbance occurs naturally, operating both spatially and temporally on all levels of organisation (Pausas and Lavorel, 2003; Pickett *et al.*, 1989), and driving species and population dynamics through selection (Bello *et al.*, 2013; McPeek, 2017). While natural disturbance is part of ecosystem functioning, the type, rate, and intensity of disturbance is often greatly increased through anthropogenically-driven change (Johnson and Miyanishi, 2010; Turner, 2010). These disturbances can have strong, negative effects on genetic diversity (Banks *et al.*, 2013; Davies *et al.*, 2016) and so understanding how specific disturbances will effect particular species is a key component of genetic monitoring (Palsbøll *et al.*, 2007).

Grazing disturbance

The spread of grassy ecosystems across the globe 8-20 Ma ago presented novel challenges for non-grassy plants (Edwards *et al.*, 2010; Edwards and Smith, 2010). Paired with grassy ecosystems were frequent fire regimes, burning every two to three years, a frequency likely far greater than what those non-grassy plants had been exposed to before. New grazing fauna soon diversified and spread too, their mouth morphologies specifically designed to consume the dense basal leaf material grasses offer (Kleynhans *et al.*, 2011). These grazers reached great abundance in many tropical grasslands (Smith *et al.*, 2016). Nevertheless, a rich and diverse non-grass flora exists in all natural grasslands, often orders of magnitude more diverse than the grasses themselves (Murphy *et al.*, 2016). These plants have evolved traits to survive and persist in these frequently burned and heavily grazed environments, such as the thicker bark and the ability to resprout found in woody plants (Simon and Pennington, 2012). The responses of plants to disturbances such as grazing have shaped entire landscapes (Maron and Crone, 2006) and grazing disturbance has also been shown to alter species distribution and abundance (Anderson and Hoffman, 2011). While many plant species have developed finely balanced responses to grazing, others have been negatively impacted by the disturbance. Sexual reproduction has been negatively impacted by grazing in some species (Soons *et al.*, 2004; Thomann *et al.*, 2018), with some having reduced crossing potential (Dickson and Petit, 2006; Lortie and Aarssen, 1999) and others becoming dependent on clonal propagation to persist (Douhovnikoff *et al.*, 2005), changes that could hinder gene flow (Honnay and Bossuyt, 2005). Grazing may also drive fragmentation in species as it is often randomly distributed (McRae and Beier, 2007). The potential for grazing to drive such extensive, and often negative changes in plant species and communities, highlights the need for proper understanding of responses to grazing as the disturbance expands and changes.

One of the key shifts in grazing disturbance is the total alteration of the grazing fauna of our grasslands in recent centuries, with a diverse array of grazers being replaced by one or two livestock species (Craigie *et al.*, 2010). Land-use changes have also seen grazing disturbance expand into new areas (Green, 2005), exposing plant species to grazing which is either completely new, or is occurring at a greater rate and intensity than before (Cingolani *et al.*, 2005). Plant communities that do not have a long evolutionary history of grazing exposure are put at risk of irreversible diversity transitions due to the time required for adaptation (Cingolani *et al.*, 2005). Thus, grazing should be a key factor considered when biodiversity conservation is carried out in these areas. This is of particular importance in sub-Saharan Africa due to people's reliance on natural resources (Tibesigwa *et al.*, 2015; Twine, 2013), and thus grazing management requires balancing of the needs of communities along with preservation of biodiversity (Ceballos and Ehrlich, 2006). Preservation of biodiversity under expanding grazing disturbance requires identification of signs of adaptation to grazing to assess if a species is vulnerable or not.

Adaptation to grazing

Adaptations that allow plants to persist while exposed to grazing can develop through a variety of mechanisms that function at the individual or population level. On an individual level, surviving grazing requires the ability to either resprout after grazing has occurred or reduce the amount of tissue grazed. Plants that resprout allocate resources to below-ground storage organs that are protected from grazing, with this reservoir of resources allowing for rapid growth after defoliation (Thomas *et al.*, 2017). Reduction or avoidance of grazing is facilitated through changes in overall plant morphology, with plants changing their structure to avoid grazing or developing morphological or chemical deterrents (Briske, 1996). Extensive research has been carried out on grazing avoidance, and in an exhaustive summary by Díaz *et al.* (2007) the architecture favoured by grazing was summarised as short and prostrate. If persistence is not facilitated at an individual level, it may occur at the population level through changes to reproduction. Populations may develop seed banks to promote establishment, requiring development of seed dormancy

(O'Connor and Pickett, 1992; Sternberg *et al.*, 2003). The establishment of new individuals through seeds after grazing may also be easier as local competition has been removed (McIntyre *et al.*, 1999). Through strategies such as endozoochory, grazing can even be beneficial to seeds, providing improved germination conditions and facilitating dispersal (Dennis *et al.*, 2007). In order for a species to persist at the individual or population level through one of these mechanisms, trade-offs are usually required to facilitate the mechanism (Briske, 1996).

Individual persistence mechanisms such as resprouting or deterrents require redistribution of resources that could have been allocated to reproduction, or the loss of reproductive advantages through changes in morphology. Population level persistence mechanisms often trade-off individual survival to promote reproduction, by producing a greater number of seeds, or promoting grazing to improve establishment or facilitate endozoochory. The greater the commitment to a particular strategy, the greater the trade-off will need to be, but the greater the chance of success (Briske, 1996). The effects of the trade-offs made by a species or population to persist under grazing need to be considered in the context of genetic monitoring, as they may hinder sexual reproduction or gene-flow.

Plasticity

Phenotypic plasticity may be an alternative mechanism to facilitate persistence while exposed to grazing, allowing for adaptation mechanisms to be developed temporarily (Miner *et al.*, 2005; Pigliucci, 2005). Plasticity can develop within the lifespan of an individual (Young et al., 2003) or over generations (Agrawal *et al.*, 1999). Both allow plants to rapidly adapt to changes within the environment, and can impact local community dynamics, and in turn ecological processes (Schmitz *et al.*, 2003). Plasticity of key traits may enable plants to avoid or tolerate grazing, as a plant with plasticity of a trait such as height may be able to grow shorter to avoid defoliation, a common adaptation to grazing, but operating only at the phenotypic level. Developing grazing resistance through plasticity may be particularly useful as grazing tends to be localised and periodic, with grazers migrating to areas with more available biomass and water (Pringle and Landsberg, 2004). This would provide opportunities for plasticity to enable the temporary loss of grazing adaptation traits until grazers return, mitigating the individual or population level trade-offs.

Investigation of specific cases of plasticity and their ecological consequences is still considered to be under-investigated, despite their ecological importance (Miner et al., 2005; Pigliucci, 2005). This presents potential for meaningful investigation of evolutionarily important traits, particularly plastic responses to biotic factors such as grazing (Sultan, 2000). Understanding how grazing resistance mechanisms are facilitated within a species, through genetic variation or phenotypic plasticity, will inform how grazing is impacting the genetic diversity of a population.

AFLPs

Biodiversity conservation requires effective genetic monitoring, which has seen great expansion in the use of molecular markers and microsatellites in DNA fingerprinting as techniques are developed and improved (Hunter *et al.*, 2018). With various techniques available for investigating genetic diversity, selection is determined by the time required for application and prior information available on the genome of the species in question. When investigating genetic diversity for conservation, time is usually limited and prior information is often unavailable (Essl *et al.*, 2015), thus the amplified fragment length polymorphism (AFLP) technique has found favour in this context. First published by Vos *et al.* (1995), with an updated protocol by Vuylsteke *et al.* (2007), the technique detects polymorphisms based on PCR-amplification of fragments genomic DNA in a 5 step process:

1. **Digestion:** Genomic DNA from a single individual is digested by a pair of noncomplementary restriction enzymes (often *EcoR*I, a rare cutter, and *Mse*I, a frequent cutter) which cleave the DNA strands into restriction fragments.
2. **Ligation:** Adaptors matching the restriction enzymes are incubated with DNA ligase which binds the adaptors to their complementary fragments. The product of this step is unique DNA fragments with adaptors attached at both the 3´ and 5´ ends.
3. **Pre-amplification:** Due to the high number of unique DNA fragments produced by the pair of restriction enzymes, not all of the fragments are amplified to a detectable level. Primers that match the adaptor sequences are used, with selective nucleotides at the 3´end to ensure only a subset of the fragments undergo PCR-amplification.
4. **Selective amplification:** The final selective PCR-amplification with primers, one of which is radioactive or fluorescently labelled, to produce a detectable product.
5. **Gel electrophoresis:** The products of the AFLP process are viewed using gel electrophoresis, which separates the fragments by length.

AFLPs have grown popular due to their speed, reproducibility and the technique is particularly popular in ecology and conservation as it requires no prior knowledge of the genome being studied (Meudt and Clarke, 2007). The original purpose of the technique was for linkage-map development, but its role in DNA fingerprinting has developed further, with AFLPs finding extensive applications in genetic diversity estimations, parentage assignment, hybrid mapping, structure investigations of both natural and breeding populations, as well as phylogenetics (Vuylsteke *et al.*, 2007).

Other PCR-based techniques are available as alternatives to AFLPs, such as random amplified polymorphic DNAs (RAPDs), and inter simple sequence repeats (ISSRs), which have been found to produce similar results (Nybom, 2004). AFLPs remain preferable as they have greater reproducibility, provide more informative results, and have more robust applications (Meudt and Clarke, 2007). Blignaut *et al.* (2013) used known conservative regions of plant genomes to develop a transferable AFLP protocol, which reduces the time and cost of the technique as it is transferable across distantly related taxa and eliminates the need for screening of fluorescently labelled oligonucleotides. The versatility, reliability and speed of AFLPs have made them a key tool in genetic diversity assessment and management.

The Drakensberg Alpine Centre

As the risk of biodiversity loss increases under climate change, biodiversity hotspots require increased, effective conservation and monitoring (Malcolm *et al.*, 2006). The Drakensberg Alpine Centre (DAC) is one such area. Identified by Van Wyk and Smith (2001), the DAC is composed of the 40,000km^2 area spanning across both the Eastern Cape and KwaZulu-Natal Drakensberg, upward through the Lesotho Drakensberg to the eastern parts of the Free State province. An assessment by

Carbutt and Edwards (2003) found that native taxa numbered over 2800 (specific and infraspecific), with 16% angiosperm endemism.

The value of this area is not only ecological as numerous rural communities are dependent on the ecosystem services it provides (Ngwenya *et al.*, 2018). The area is also a key part of South Africa's tourism sector, with many communities involved in tourism strategies developed around it (Magi and Nzama, 2009). Ecosystem services and tourism are reliant on the biodiversity of the area, but this biodiversity is at risk as climate change threatens to contract already limited species ranges in the region (Bentley *et al.*, 2019). Climate change is also expected to alter plant-soil dynamics, threatening finely balanced relationships in the area (Carbutt and Edwards, 2015) and anthropogenically-driven increases to fire regimes have also placed the unique balance of biodiversity at risk (Gordijn *et al.*, 2018). Expansion of commercial and local farmlands has created conservation challenges, and exposed many plant species to increased grazing disturbance at a growing rate (Carbutt *et al.*, 2011; Jewitt *et al.*, 2015; Ramesh and Downs, 2015). Effective conservation of biodiversity in this area requires rapid investigation of the effect of grazing and other disturbances to ensure management can be implemented appropriately.

Grazing expansion

This study was based on grazing disturbance and while there is some literature on expansion of land used for commercial and subsistence farming of livestock in the Drakensberg area (e.g. O'Connor, 2005; Ramesh and Downs, 2015; Turpie *et al.*, 2007), little work has been done on quantifying the extent of this change, particularly on an evolutionary scale. The study by O'Connor (2005) estimated that expansion of livestock numbers in the Drakensberg and its surrounding areas has occurred at an alarming rate, finding that grasslands in the southern Drakensberg are exposed to livestock and wildlife at quantities six- to 20-times greater respectively, than during pre-settlement estimates of 0.0002 AU/km^2. While this study was comprehensive, it dealt with changes in grazing only of the last ~150 years and not all sources for estimates of livestock numbers were listed, with those that were dating back to the 1990's. Though small mammals also contribute to grazing, their abundance and thus the subsequent grazing intensity, are normally not great enough to drive large grazing responses (Granger *et al.*, 2017). With the homogenisation of large grazers as livestock (Craigie *et al.*, 2010), and the resulting intensity of grazing by livestock species, small mammals are often negatively impacted by the expansion of farms and rangelands (Herder *et al.*, 2016; Hoffmann and Zeller, 2005; Schmidt *et al.*, 2005). The loss of small mammals in these areas is a separate conservation issue that requires attention, but it shows that the contribution of small mammals to grazing in these areas can be considered negligible (Granger *et al.*, 2017). Livestock grazing remains the key driver of grazing adaptation and requires appropriately scaled assessments of change.

Although proper assessment of grazing change on an evolutionary scale would require a focused investigation, estimation of the extent of change was recently made possible due to work by Hempson *et al.* (2015) that estimated herbivore biomass across Africa ~1000 years ago. The results of the study placed the total biomass of herbivores of all forms at ±5091.7 kg/km^2 in the region. To compare this figure to modern herbivory, livestock biomass was calculated in the same method as Hempson *et al.* (2015) with current data on livestock populations (cattle, small ruminants and equines) in KwaZulu-Natal from GLiPHAs (2009). The resulting estimate places livestock biomass for the province at 15,075.1 kg/km^2. This figure does not include biomass of wild herbivores, as data is less accessible and often unreliable (Taylor *et al.*, 2016). Livestock biomass alone is nearly three times greater than the historic herbivore biomass of the region, and wild herbivory would only add to this figure, with private farms breeding larger herds to support hunting, auctioning and meat production (Taylor *et al.*, 2016). While more specialised study would be required to quantify grazing change in detail, with such a marked increase in the grazer biomass over the last millennium, it is clear that grazing is a disturbance that needs to be closely monitored.

Rhodohypoxis baurii

Rhodohypoxis baurii is one the many species of flora that contribute to the rich biodiversity of the DAC. This species of small, geophytic herbs of the Drakensberg, originally described by Baker (1878) and expanded on by Hilliard and Burtt (1978) who identified three varieties within the species. The plants are generally small and are associated with damp soils in rocky areas among short grasses at high altitudes (1800m – 3200m). Populations are not exclusive to rocky ridges though, spreading out onto flat grassland areas surrounding the ridges and outcrops, which could facilitate exposure to animals. The plants feature contractile roots and subterranean pseudostems that vary in length with soil depth (Figure 1.1). Vegetative propagation is prominent within the species, facilitated through stolons growing away from the parent plant and sprouting leaves before the stolon axis decays to form separate plants (Figure 1.2). The distribution of the species is centred in the KwaZulu-Natal Drakensberg and Lesotho plateau, but stretches from the northern Eastern Cape, across KwaZulu-Natal, and up into southern Mpumalanga. This region has been exposed to moderate historical grazing, with non-ruminant grazers contributing the greatest proportion of grazer biomass (Hempson *et al.*, 2015).

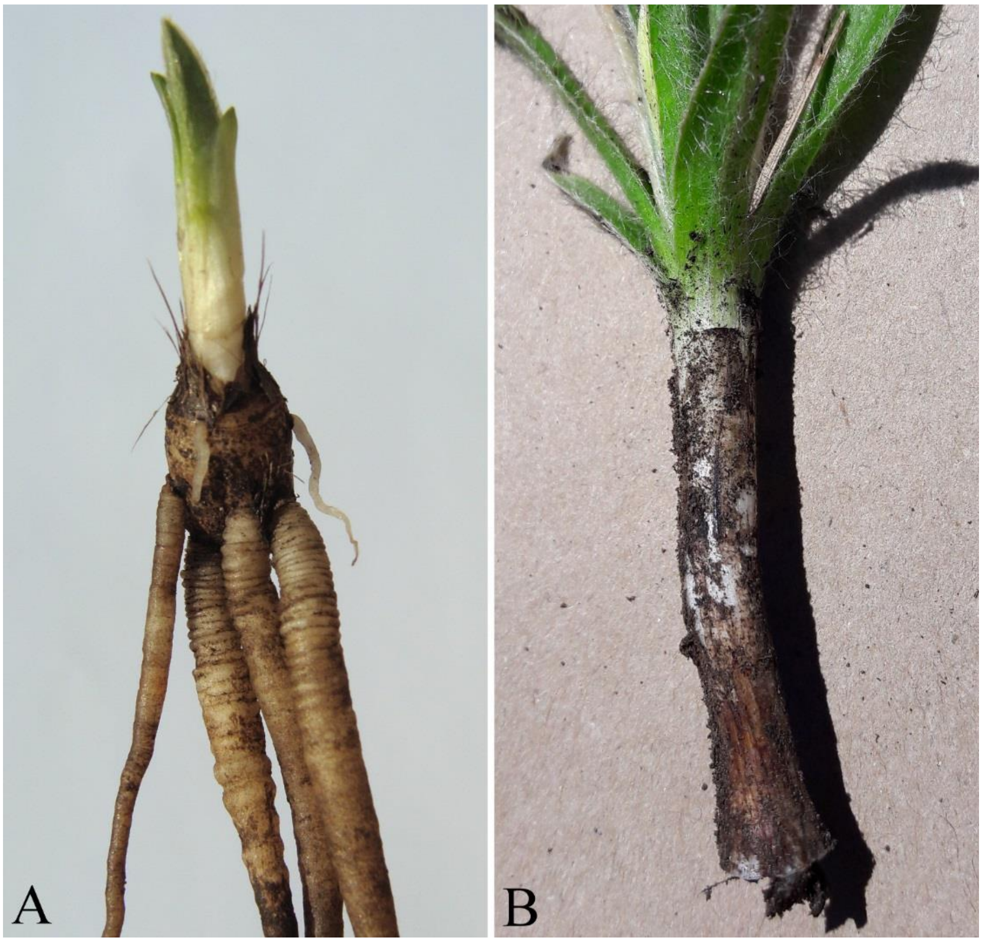

Figure 1.1. The robust subterranean axis and contractile roots of *Rhodohypoxis baurii* (A) and an extended subterranean pseudostem separated from the axis and roots (B).

Figure 1.2. Vegetative propagation in *Rhodohypoxis baurii* is facilitated through stolons that extend from the parent plant to establish new plants. Distance varies with stolon length and have been observed to range 1-8cm.

Three varieties within the species have been identified: *Rhodohypoxis baurii* var. *baurii*, *Rhodohypoxis baurii* var. *confecta*, and *Rhodohypoxis baurii* var. *platypetala*. The varieties featuring slightly varied morphology but with descriptions separating them mostly by variations in flower colour, ranging from white to deep pinks and red (Figure 1.3) but distinction remains difficult due to extensive hybridisation (Hilliard and Burtt, 1978). *R. baurii* var. *platypetala* is distinguished from other varieties by predominantly white, occasionally pale pink, flowers and a tendency to grow in drier, stony soils (Hilliard and Burtt, 1978). Populations are usually small, spanning less than $35m^2$ in varying densities, and are often isolated by several kilometres. The known distribution of *R. baurii* var. *platypetala* (Figure 1.4) extends laterally from the Eastern Cape Drakensberg, across the KwaZulu-Natal Drakensberg and further out into the province, with records near Nkandla. The longitudinal range of the variety ends at the southern Free State Drakensberg. *R. baurii* var. *platypetala* does not appear to extend into the Lesotho Drakensberg, with only a few records within the borders of Lesotho (BODATSA, 2016).

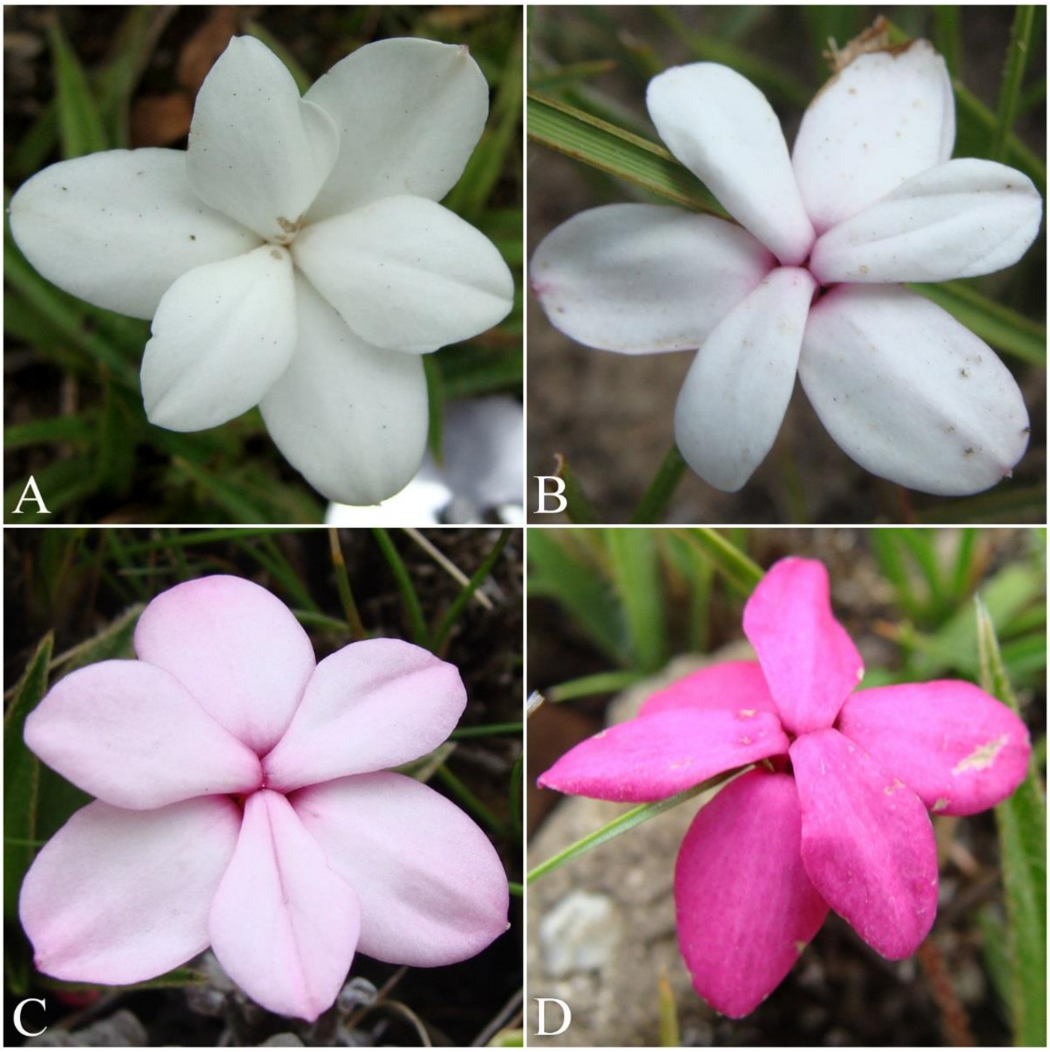

Figure 1.3. The variety of flower colours found in *Rhodohypoxis baurii*, completely white (A), white and pink combined (B), pale pink (C) and dark pink (D).

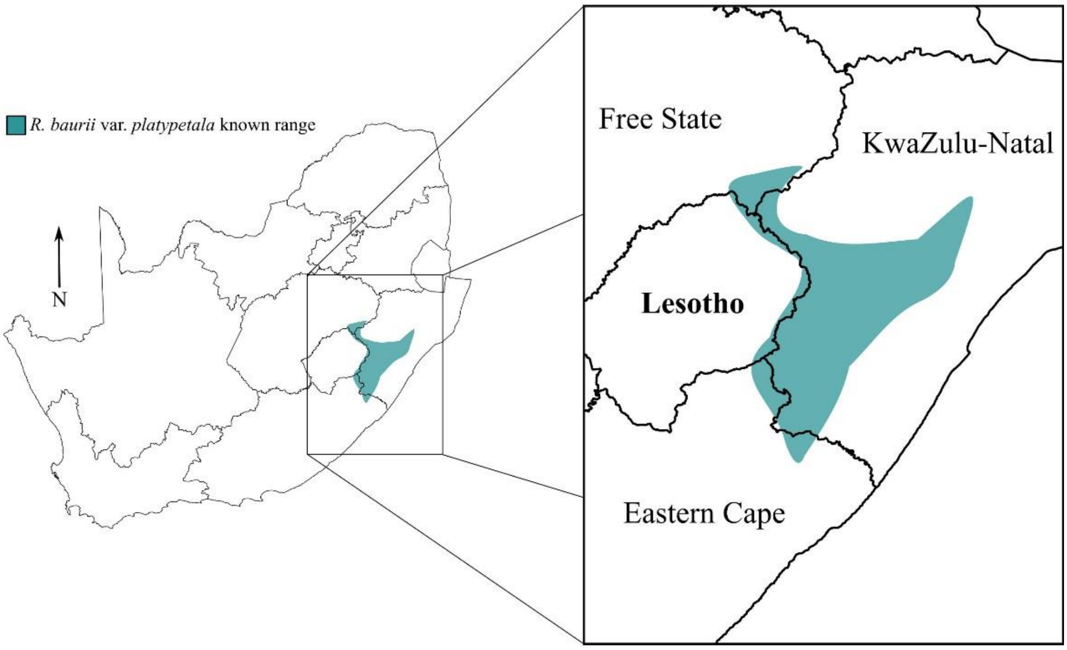

Figure 1.4. Distribution of *Rhodohypoxis baurii* var. *platypetala*. This range was estimated by assembling the records of Hilliard and Burtt (1978), the South African National Biodiversity Institute database (SANBI, 2016) and personal records of the variety.

Preliminary observation of populations of *R. baurii* var. *platypetala* located on commercial farms, thus exposed to livestock grazing, identified differences in morphology of these populations compared to those in areas unexposed to grazing (personal communication, Dr K.L. Glennon, November 2016). These morphological differences aligned with architecture known to confer grazing resistance (Díaz *et al.*, 2007), with plants at grazed sites appearing smaller and flatter, which suggested populations exposed to grazing may be undergoing grazing adaptation. As populations of *R. baurii* var. *platypetala* grow among grasses, often sprouting within dense grass tussocks, these populations may have been experiencing accidental grazing. As grazing has been shown to negatively impact plant species through direct impacts on sexual reproduction (O'Connor and Pickett, 1992; Thomann *et al.*, 2018), and through the trade-offs required for grazing adaptation (Briske, 1996), populations of *R. baurii* var. *platypetala* exposed to grazing were potentially at risk and required investigation.

Rationale

The spread of both commercial and subsistence farming in areas of the Drakensberg and its surroundings will undoubtedly expose more species to grazing disturbance (Carbutt *et al.*, 2011; Jewitt *et al.*, 2015). The rich biodiversity of the area is already at risk due to climate change (Bentley *et al.*, 2019; Carbutt and Edwards, 2015), and added grazing disturbance threatens to aggravate biodiversity loss as it negatively impacts sexual reproduction (Dickson and Petit, 2006; Douhovnikoff *et al.*, 2005; Honnay and Bossuyt, 2005; Thomann *et al.*, 2018) and drives adaptations that effect survival of both individuals and populations (Briske, 1996). The assessment of genetic diversity as a form of biodiversity monitoring allows for rapid investigation of the vulnerability of a species to change and disturbance (Hunter *et al.*, 2018). Investigation of plant morphology can identify development of adaptations to grazing as such strategies favour particular architectural changes (Díaz *et al.*, 2007). Preliminary observations of populations of *Rhodohypoxis baurii* var. *platypetala* exposed to grazing noted morphological differences that aligned with architecture favoured by grazing adaptation. As adaptation to grazing may occur too slowly to mitigate loss of genetic diversity (Cingolani *et al.*, 2005), or local adaptation may lead to isolation of populations (McRae and Beier, 2007), the effect of livestock grazing on *R. baurii* var. *platypetala* needed to be assessed. To enable this assessment to be carried out rapidly and without prior knowledge of the genome of

R. baurii var. *platypetala*, AFLPs were selected to measure genetic diversity. Combining investigation of both morphological grazing adaptation and genetic diversity would allow for effective assessment of adaptability or vulnerability to grazing in *R. baurii* var. *platypetala*. If vulnerability to the harmful effects of grazing was identified, populations of *R. baurii* var. *platypetala* would require protection from further exposure to grazing to conserve genetic diversity, contributing to biodiversity conservation of the region.

Study aims and objectives

This study aims to investigate if populations of *Rhodohypoxis baurii* var. *platypetala* exposed to livestock grazing are developing morphological grazing adaptations and determine if grazing is negatively impacting genetic diversity in these populations. The study also aims to recommend appropriate management if populations are found to be adapting to grazing and are at risk of loss of genetic diversity. The objectives of the study were as follows:

Objective 1: Assess whether morphological differences between populations of *R. baurii* var. *platypetala* exposed to livestock grazing and those unexposed to grazing are significant and facilitate grazing resistance.

Objective 2: Investigate genetic diversity within and between populations of *R. baurii* var. *platypetala* to determine if livestock grazing was driving loss of genetic diversity.

Objective 3: Assess the vulnerability *R. baurii* var. *platypetala* to livestock grazing and recommend appropriate management strategies.

Dissertation outline

The first chapter of this dissertation is a general introduction that reviews concepts relevant to the study. The second chapter is the investigation of morphological differences in populations of *R. baurii* var. *platypetala* and their link to grazing resistance (Objective 1). In chapter three the investigation into genetic diversity within and between populations is described (Objective 2). The final chapter synthesises the findings of the previous chapters to assess vulnerability of *R. baurii* var. *platypetala* to grazing to inform management strategies (Objective 3). This chapter also contains recommendations for future studies associated with grazing vulnerability and genetic monitoring.

This dissertation has been prepared and written in the format of scientific papers. This format may result in some repetition of information, but this has been minimised as far as possible.

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CHAPTER 2:

MORPHOLOGICAL DIFFERENTIATION

Abstract

Adaptation to grazing often requires specific trade-offs which may have negative implications for reproduction and genetic diversity. The potential threat of grazing to biodiversity is of growing concern as grazing disturbance expands due to increasing demand for cattle farming. This study aimed to investigate if populations of the near-endemic Drakensberg herb *Rhodohypoxis baurii* var. *platypetala* showed morphological differentiation due to grazing resistance developing in plants at grazed sites. Key morphological traits associated with grazing resistance were measured and compared between plants from 4 populations exposed to grazing and 4 from ungrazed sites (n = 224). Flower height showed a small but significant difference, being 8mm shorter in plants exposed to grazing ($P < 0.05$) and plants exposed to grazing also averaged 1 leaf less ($P < 0.001$). A lack of grazing observations, along with a low estimated likelihood of grazing and the few small morphological differences suggest that *R. baurii* var. *platypetala* plants are not experiencing grazing disturbance. While the study shows grazing may not be a cause for this variety, the impact grazing may have is highlighted for risk assessment in biodiversity conservation.

Keywords: Grazing; morphology; grazing resistance; *Rhodohypoxis baurii* var. *platypetala*; disturbance

Introduction

Grazing is a key disturbance factor that acts upon plant communities (Huntly, 1991), affects entire landscapes (Maron and Crone, 2006), alters the abundance and distribution of plant species (Anderson and Hoffman, 2011), and at the species-level can drive changes in plant architecture and morphology (Díaz *et al.*, 2007) as well as reproduction (Thomann *et al.*, 2018). The impact of grazing on plants at the species level may be beneficial or detrimental depending on the key persistence strategies employed by the species. Many plants are adapted to use grazing advantageously through endo- and ectozoochorous seed dispersal (Howe and Smallwood, 1982). Some Serengeti plant species have been shown to be adapted to endozoochorous dispersal by large herbivores (Anderson *et al.*, 2014). Grazing also removes local above-ground competition, facilitating establishment of new individuals adapted to be opportunistic (Pausas *et al.*, 2004). These strategies require a trade-off to be made favouring persistence of the population over survival of the individual, as if, for example, individuals were to deter grazing, seed dispersal and establishment would be hampered.

Alternatively, grazing may be detrimental to reproduction as reproductive organs, seeds, or both, are removed. The removal of flowers can reduce crossing potential and may have lasting effects with some plants showing reduced fecundity in response to long-term exposure to grazing (Knight, 2004). Intense grazing reduces the potential seed set of populations, with grazing by sheep reducing potential seed set in *Osteospermum sinuatum* (Asteraceae) by up to 90% (Milton, 1992). Grazing can also impact seed production, even when the flowers aren’t removed, with grazed plants showing lower seed set, as found in *Rumex obtusifolius* (Polygonaceae)

(Bentley *et al.*, 1980). Seed size was reduced by intense grazing in *Lespedeza davurica* (Fabaceae) but this reduction in size was coupled with enhanced germination characteristics (Chen *et al.*, 2017). Heavy grazing also reduced the size of seed banks in populations of Mediterranean grasses (Sternberg *et al.*, 2003). Grazing has also been shown to indirectly effect reproduction by altering pollinator richness abundance, with over-grazing leading to negative pollinator dynamics (Lázaro *et al.*, 2016; Tadey, 2015). The negative impacts of grazing will often be a driver for these plants to develop grazing resistance strategies in an attempt to reducing grazing: favouring individual survival over population level persistence.

Grazing resistance strategies allow plants to persist and reproduce while exposed to regular grazing (Briske, 1996). Resistance can be further divided into two key strategies, avoidance and tolerance, each with various mechanisms which facilitate resistance (Briske and Richards, 1995). Avoidance strategies involve mechanisms that reduce the direct physical impact of grazing thereby lowering the severity of damage as much as possible (Briske, 1996). Mechanical deterrents, biochemical compounds, and defensive symbiosis with toxin-producing fungi are mechanisms that actively deter grazers by reducing the forage value of the leaves. Alterations to growth form and architecture, as well as associations with species undesirable to grazing are passive mechanisms that attempt to avoid grazing by making plant biomass spatially inaccessible to grazers. Finally, inducible defences, asynchronous growth, and developmental resistance are temporal mechanisms that alter the expression of resistance based on grazing exposure (Briske, 1996).

Tolerance strategies enable plants to maintain, or even increase their fitness while exposed to herbivory (Strauss and Agrawal, 1999). Morphological tolerance mechanisms, such as alterations to the meristem source or number, act to resist grazing by ensuring primary growth by apical meristems is not affected. Seed availability is considered a tolerance mechanism as the development of a seed bank will promote establishment after grazing occurs (Noble and Slatyer, 1980). Finally, compensatory growth is a physiological tolerance mechanism facilitated by compensatory processes such as altered photosynthesis and resource allocation that compound to increase growth after grazing (McNaughton, 1983). A plant that displays an avoidance or tolerance strategy, or a combination of the two, will have a competitive advantage over other non-resistant grazed plants within the same community, and thus greater survivability (Briske, 1996). The strategy a plant develops will be suited to the frequency and intensity of grazing present, with infrequent grazing leading to overcompensation rather than adaptation (Turner *et al.*, 1993). The effect of grazing has also been found to be indirectly dependent on abiotic environmental factors, such as available precipitation, which drives biomass availability (Bat-Oyun *et al.*, 2016). Brief relief from grazing, due to disease-driven decreases in grazer population size, can provide opportunities for plants to pass grazing thresholds and become established, the effects of which can alter landscape dynamics (Holdo *et al.*, 2009). The amount of resistance a species has can be examined through an investigation of which avoidance and tolerance mechanisms are used by the plants.

Grazing resistance mechanisms are facilitated by combinations of key traits, and an extensive review by Díaz *et al.* (2007) identified the traits of plants that flourish in heavily grazed environments, which align with strategies of avoidance (e.g. low palatability), resistance (rosette architecture), and tolerance (annual life history strategy) (Table 2-1). Low palatability may result from biochemical avoidance mechanisms. When looking at canopy height and habit, plants favoured by grazing are short and prostrate, potentially indicating the development of avoidance as smaller, flatter plants reduce the amount of biomass accessible to grazers.

Table 2.1: Plant traits that are selected for by grazing (adapted from Díaz *et al.*, 2007)

Trait	**Grazing-favoured type**
Life history strategy	Annual
Canopy height	Short
Habit	Prostrate
Architecture	Stoloniferous, rosette
Growth form	Graminoid, forb, woody
Palatability	Low
Origin	Exotic

When adapting to grazing, trade-offs are made between individual survival and population-level persistence, and the development of grazing resistance mechanisms may lead to this trade-off in certain flowering plants. As plants become shorter and more prostrate, flower height is lowered which negatively impacts wind-based seed dispersal by reducing seed release height and making plants shorter than surrounding vegetation (Soons *et al.*, 2004). The potential impact of decreased height due to grazing was found to be particularly prominent for herbs surrounded by grasses in Central-European grasslands, with height being the trait that dispersal is most dependent upon (Diacon-Bolli *et al.*, 2013). Reduction in flower height also has implications for pollination, with pollinators favouring taller flowers, as was found in *Caladenia behrii* (Orchidaceae) (Dickson and Petit, 2006). Taller flowers in *Verbascum thapsus* (Scrophulariaceae) were also found to receive more pollen than shorter neighbouring flowers (Lortie and Aarssen, 1999). Reduced seed dispersal directly impacts on the establishment of new individuals within an existing population. The reduced effectiveness of sexual reproduction in grazed plants can be compensated for by clonal growth. However, if disturbance drives an increase in clonality it may also lead to a decrease in genetic variation, as found in populations of *Salix exigua* (Salicaceae), where populations with well-established clones showed lower genetic variation (Douhovnikoff *et al.*, 2005). These indirect effects of grazing that result from morphological changes are often only observed with long-term studies, such as in the 11-year study of *Primula farinosa* (Primulaceae) by (Thomann *et al.*, 2018). The authors found that grazing reduced inflorescence height, but it was only after the full study was completed that significant impact of pollinator mediated selection within the ungrazed plants was observed, as over time selection pressure became stronger as only taller plants persisted in grazing exclusions. Trading-off flower height may increase individual survival, but it has negative implications for population-level persistence, placing these populations at risk of directional selection or loss of genetic diversity.

Another change plants may exhibit in the presence of grazing is the alteration of biomass allocation between above-ground and below-ground structures. Findings have been mixed when investigating differences in biomass allocation based on disturbance despite the fact that below-ground storage of carbohydrates for resprouting is considered a key trait of disturbance-adapted plants (Iwasa and Kubo, 1997; de Moraes *et al.*, 2016). The relationship between grazing and differences in biomass allocation suggests that changes in allocation may be more intricately dominated by both disturbance and abiotic factors (Ma *et al.*, 2010; Zeng *et al.*, 2015; Klimešová *et al.*, 2017). Yet plants showing high below-ground biomass allocation can persist through and sustain cycles of herbivory, with the extent of allocation creating feedback effects within herbivore populations (Thomas *et al.*, 2017). This finding suggests that biomass allocation should be considered when investigating the impact of grazing. Changing biomass allocation from above-ground to below-ground structures would allow individual-level persistence through regular grazing but could impact population-level persistence if it is at the detriment of above-ground reproductive structures.

The effects of grazing are complex and operate through the highest to lowest levels of organisation within ecosystems, and as more plant species become exposed to grazing through the expansion of rangelands and farms, these effects need to be understood in order to preserve species diversity and richness (e.g. Scott-Shaw and Morris, 2015). Humans drive land-use changes through the demand for more farmlands which ultimately threatens biodiversity (Green, 2005) and is of particular concern in sub-Saharan Africa where more people are directly dependent on natural resources (Ceballos and Ehrlich, 2006). Mack and Thompson (1982) provided evidence that the impact of heavy grazing depends strongly on the adaptive strategies of the plants. They assessed the effect of increased livestock in grazer adapted, and non-grazer adapted grasslands in the mid-west of America. In Southern Africa, a similar phenomenon is occurring in the Drakensberg Midlands, where the expansion of farmlands and domestic livestock is changing the landscape (Ramesh and Downs, 2015). Such changes introduce grazing pressure to more Drakensberg plant species that may not be adapted to this increased level of disturbance.

One such species is *Rhodohypoxis baurii* (Hypoxidaceae), a small flowering herb that is endemic to alpine grasslands across the Drakensberg. In one variety, *Rhodohypoxis baurii* var. *platypetala*, observations showed morphological differentiation between populations in grazed and ungrazed areas, with plants at sites exposed to grazing appearing shorter and flatter (personal communication, Dr K.L. Glennon, November 2016). As the plants grow among tufts of grass that range from highly palatable such as *Acroceras macrum*, average palatability in *Harpochloa falx* and *Monocybium cereshforme*, to unpalatable grasses such as *Hyparrhenia cymbaria*, those exposed to grazing may be accidentally grazed along with surrounding grasses. Despite being associated with rocky terrain, many populations are also found on large, flat pans of grass that is easily traversed by livestock, and this had been observed with the preliminary morphological observations, with grazers among some populations. Thus, the differentiation in height observed, and its alignment with known grazing resistance mechanisms, indicated morphology might be influenced by grazer presence.

Seed dispersal in *R. baurii* var. *platypetala* is of particular interest as seeds are stored below the ovaries, at the base of the perigone tube within a seed capsule (Figure 2.1) and seed dispersal is passive. If seed dispersal is passive, this could put the seeds at a greater likelihood of being grazed as the flowers are the tallest structure of the plants. This removal of seeds from the population could greatly impact sexual reproduction within populations that are exposed to grazing – either increasing it if they are adapted for endozoochorous dispersal, or substantially reducing it.

Figure 2.1: The seed capsule of *Rhodohypoxis baurii* var. *platypetala* where seeds are held at the base of the flower. Flowers are abscised to expose the seeds, but no active release of seeds is known to occur.

I aimed to investigate whether there were significant morphological differences between populations of *R. baurii* var. *platypetala* where livestock grazing was present and absent, and if so, whether observed differences were linked with known grazing resistance adaptations.

Hypotheses

1. *R. baurii* var. *platypetala* from sites exposed to grazing would have shorter, flatter architecture in comparison to those at sites with no grazers.
2. Plants exposed to grazing would have proportionally larger below-ground biomass allocation than those not exposed to grazing.

3. Endozoochory would not be present in populations of *R. baurii* var. *platypetala*.

Methods

Study area

Eight sites were identified for sampling across the span of *R. baurii* var. *platypetala* distribution range (Figure 2.2; Table 2.2). Four sites were exposed to grazing and four sites were not. The plants at Hebron farm, Hebron Road, and Sunset farm were all exposed to grazing by cattle (*Bos taurus taurus*) while plants at the Golden Gate Highlands National Park site are exposed to grazing by several species of antelope, with zebra (*Equus quagga*) and black wildebeest (*Connochaetes gnou*) observed near the population during sampling. All grazed sites were open and flat enough for grazers to easily traverse among the studied populations, and grazers had been observed among the populations during preliminary scouting for the sites before data collection. The ungrazed sites were also flat and open, particularly those at the Giants Castle Game Reserve and Highmoor Nature Reserve, and all sampled populations were away from hiking trails and other travel routes. All sites featured consistently stony soils, with grasses being patchy but dominating surface cover in the area. The sites were selected for their similarity to improve comparability in order to reduce the influence of site-specific factors on the results.

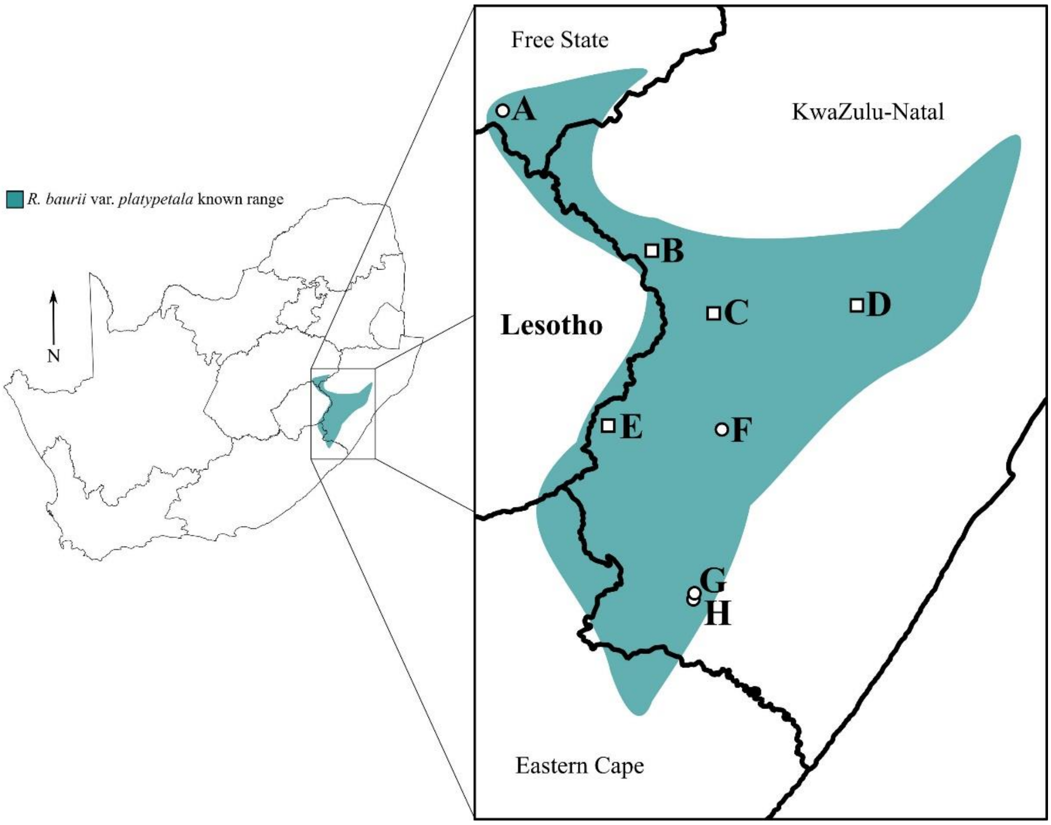

Figure 2.2: Sampling sites exposed to grazing (circles) and not exposed to grazing (squares). A: Golden Gate Highlands National Park; B: Giants Castle Game Reserve; C: Highmoor Nature Reserve; D: Karkloof Nature Reserve; E: Mkhomazi Wilderness Area; F: Sunset Farm; G: Hebron Road, and H: Hebron Farm. (for distance matrix see Chapter 3, Table 3.1)

Morphology

At each site sampling was conducted using 4 quadrats, each 25m^2 in size. Where possible, the quadrats were placed next to each other to form a larger quadrat (Figure 2.3). Within each quadrat, 7 flowering plants were selected spreading haphazardly across the quadrat as much as possible. The selection of flowering plants may have created some sampling bias, but this was unavoidable as flowering plants were needed to provide the appropriate data for the investigation.

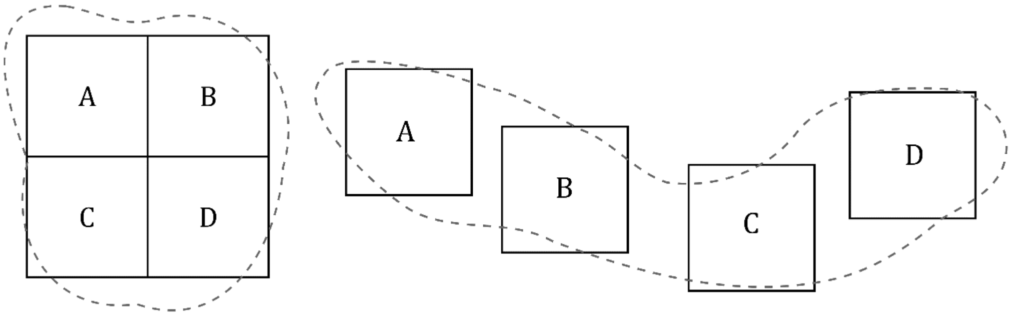

Figure 2.3: Sampling design using 12m^2 quadrats, arranged according to the shape of the population being sampled.

For all 28 plants sampled at each site, morphological traits were measured with callipers (Figure 2.4). Flower height was measured as the height from the ground to the top of each flower and is the tallest feature of the plants. Stem height was defined as height from the ground to the bottom of the seed capsule, observed externally by a change in colour along the stem below the flower (Figure 2.5). Leaf table height was the height below which 80% of the total leaf biomass fell, following O’Reagain and Mentis (1989). The number of leaves on each plant was counted, leaf length was measured for the 3 longest leaves on each plant, and those 3 longest leaves were also categorised by their angle by observation, recorded as 0°, 40°, 60°

or 90°. Damage as a result of grazing was also recorded for all sampled plants, with the extent of damage being recorded as an estimated percentage of total damaged above-ground tissue, and where possible the type of damage.

Figure 2.4: Sampling of morphological traits for individual plants. A: Flower height; measured to the top of the flower. B: Stem height; measured to the base of the seed capsule. C: Leaf table height; defined as the height of 80% of the leaf matter (O'Reagain and Mentis, 1989).

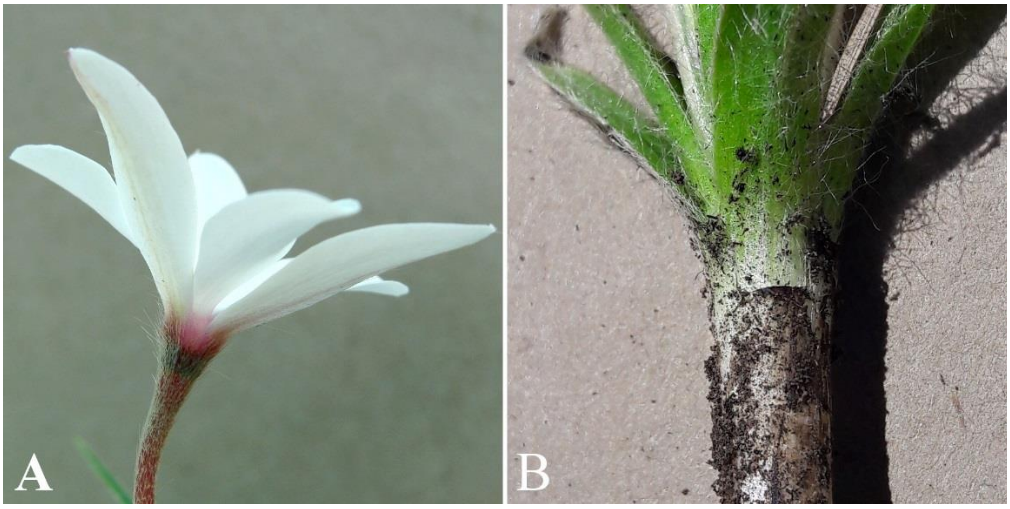

Figure 2.5: Change in colour A: along stem at the base of the seed capsule, B: of green tissue growing above-ground tissue to colourless below-ground tissue.

Biomass allocation

Of the 28 plants sampled at each site, 5 individuals were dug up and stored in paper envelopes, with root systems intact. These plants were cleaned to remove any soil in the roots, dried at 40°C for 24 hours and stored for 5 months. The plants were weighed whole, then all above-ground biomass was removed by cutting at the point of colour change from above-ground green tissue to below-ground white tissue (Figure 2.5), and the isolated below-ground biomass was weighed on a fine scale (0.001g).

Table 2.2: Details of sampling sites

	Code	**Map code**	**GPS co-ordinates**	**Grazing status**	**Grazers**	**Altitude**	**Mean annual precipitation**	**Mean annual temperature**
Karkloof Nature Reserve	KF	D	29°17'10.1"S 30°17'31.4"E	Ungrazed	None	1757m	800–900mm	16–18°C
Mkhomazi Wilderness Area	DG	E	29°44'39.0"S 29°12'30.2"E	Ungrazed	None	1874m	1000 – 2000mm	12–13°C
Highmoor Nature Reserve	HM	C	29°19'24.9"S 29°39'15.8"E	Ungrazed	None	1978m	1000 – 2000mm	13–14°C
Giant's Castle Game Reserve	MK	B	29°03'56.8"S 29°22'26.3"E	Ungrazed	None	2041m	700–800mm	14–16°C
Hebron Road	HR	G	30°23'41.6"S 29°33'55.5"E	Grazed	*Bos taurus taurus*	1628m	800–900mm	13–14°C
Hebron Farm	HF	H	30°24'53.6"S 29°33'49.2"E	Grazed	*Bos taurus taurus*	1678m	800–900mm	13–14°C
Sunset Farm	SF	F	29°45'28.3"S 29°38'24.9"E	Grazed	*Bos taurus taurus*	1934m	700–800mm	14–16°C
Golden Gate Highlands National Park	GG	A	28°30'10.3"S 28°39'07.8"E	Grazed	*Equus quagga* and *Connochaetes gnou*	2059m	500–600mm	14–16°C

Endozoochory

To test for endozoochory within *R. baurii* var. *platypetala*, fresh dung samples would need to be collected from grazers within the sampling area. To ensure the dung has not been infested with beetles that could eat seeds and potentially alter results, the dung samples should be fresh. They are and then air dried before germination testing following Anderson *et al.* (2014). Unfortunately, no grazers were present within the vicinity at the time of sampling and thus no fresh dung was available, and as it was not possible to return to the grazed sites for dung collection. As the *R. baurii* var. *platypetala* populations are small and isolated, this would also require very specific timing and continuous sampling within a few days of grazers feeding near the populations and seeds passing through their digestive system, which was not feasible.

Seed dispersal

Seed dispersal in *R. baurii* var. *platypetala* is facilitated through circumscissile dehiscence of the flower to expose the seed capsule, from which seeds are dispersed through exposure to wind and passing fauna (Hilliard and Burtt, 1978). To investigate the effectiveness of seed dispersal through this censer mechanism, two different strategies were used to investigate seed distribution at three sites using methods adapted from Chabrerie and Alard (2005). Sticky traps (Seabright Laboratories) were mounted 10cm above the ground to catch airborne seeds. A pair of simplified funnel traps were also placed at each of the sites to collect seeds dispersed by mechanical disturbance and in rain water. The sticky traps and funnel traps were left to collect seeds for a month after initial sampling, then collected and inspected for *R. baurii* var. *platypetala* seeds.

Data analysis

Data analyses were carried out in R (R Core Team, 2015) with R Studio (RStudio Team, 2016).

Grass height

Grass height was compared between grazed and ungrazed sites using a Welch two sample t-test to show if there was a distinct difference in grass height. No other environmental variables were taken into account as there were insufficient degrees of freedom to account for rainfall or temperature on grass height. It was assumed that grazing would be the main driver.

Correlation

A correlation matrix of all measured traits was constructed using the *rcorr()* function in the *Hmisc* package (Harrell, 2014), then visualised using the *corrplot* package testing for significant correlations between all combinations of traits at $\alpha = 0.01$. This served to identify initial relationships within the data, potentially highlighting key trait interactions.

Morphology

In order to compare plant traits between grazed and ungrazed populations, I conducted a series of tests. First, I used a principle component analysis (PCA) to examine potential groupings within the morphological data, serving as a secondary identification of relationships following the correlation matrix. These data were first log transformed to remove skewness (Venables and Ripley, 2002) the transformed data were centred and scaled. Biplots were generated using *ggbiplot*. Next I used mixed effect models to test if the traits leaf table height, flower height, leaf length, number of flowers and leaf angle, could be predicted as a function of grazing following Winter (2013) using *lme4* (Bates *et al.*, 2015):

$$trait \sim \text{Grazing} + (1|\text{Site})$$

Mixed effect models provided the robustness required as the data were not normally distributed, and it provided the ability to control for the effect of site-level distinctions. Values for individual flowers were used as the input data, which were logged as required, and all models were run with treatment as a fixed effect and the sites as a random effect to account for environmental variation. Null models were compared against the treatment models using an ANOVA for significant difference. Lastly, I used a Kruskal-Wallis rank sum test to compare the number of leaves on plants between grazed and ungrazed populations.

Biomass allocation

To assess allocation of biomass in the plants, the ratio of above- to below-ground biomass was compared between populations from grazed and ungrazed sites using a Welch two sample t-test. This provided a simple measure to show if biomass was distributed differently in individuals at grazed sites or not.

Results

We found no evidence that *R. baurii* var. *platypetala* individuals in grazed sites were actually grazed more than those in ungrazed sites. Observations showed no signs of large mammalian browsing damage on the plants.

However, clearly the grazed sites were more heavily used by animals, and the grass height at these sites was 111.25mm on average, 40.75mm less than on ungrazed sites, though this difference was not found to be significant with a Welch two sample t-test (Figure 2.6).

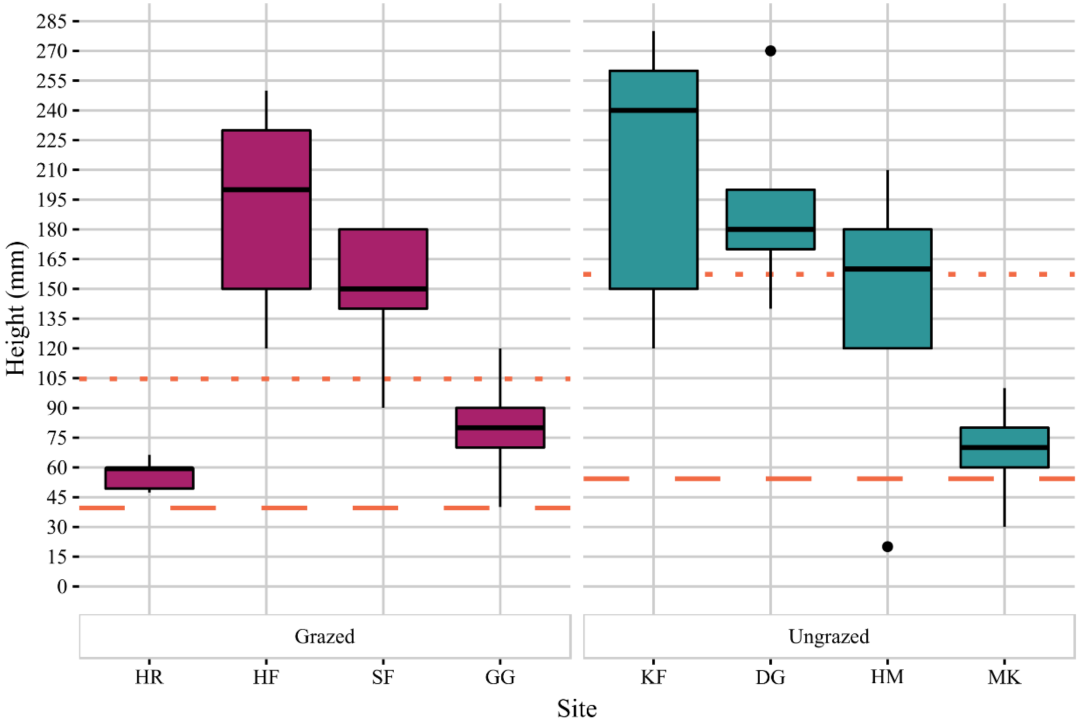

Figure 2.6: Average grass height at each of the sampling sites, grouped by grazing category. Bars represent mean values with Standard Error (SE). Average grass height is represented by the dotted line, with average flower height represented by the dashed line. Sites are ordered by increasing altitude from left to right within each category, and a trend of decreasing height is clear at higher altitudes, but this did not place flowers within grazing range. Despite the great range of heights, no significant difference was found in relation to grazing presence.

Altitude appeared to have an effect on grass height, with shorter grasses at higher altitudes, but even among the shortest grasses, flowers were below grass height.

Morphological traits

Correlation

Significant positive correlation was found between most pairs of morphological traits ($P < 0.01$; Figure 2.7). The strongest correlation was between flower height and stem height ($r = 0.99$, $R^2 = 0.98$) which was expected due to the shared axis of measurement and allowed for the exclusion of stem height in further analysis. Leaf table height and leaf length also correlated strongly ($r = 0.73$, $R^2 = 0.53$) given their similar axis of measurement.

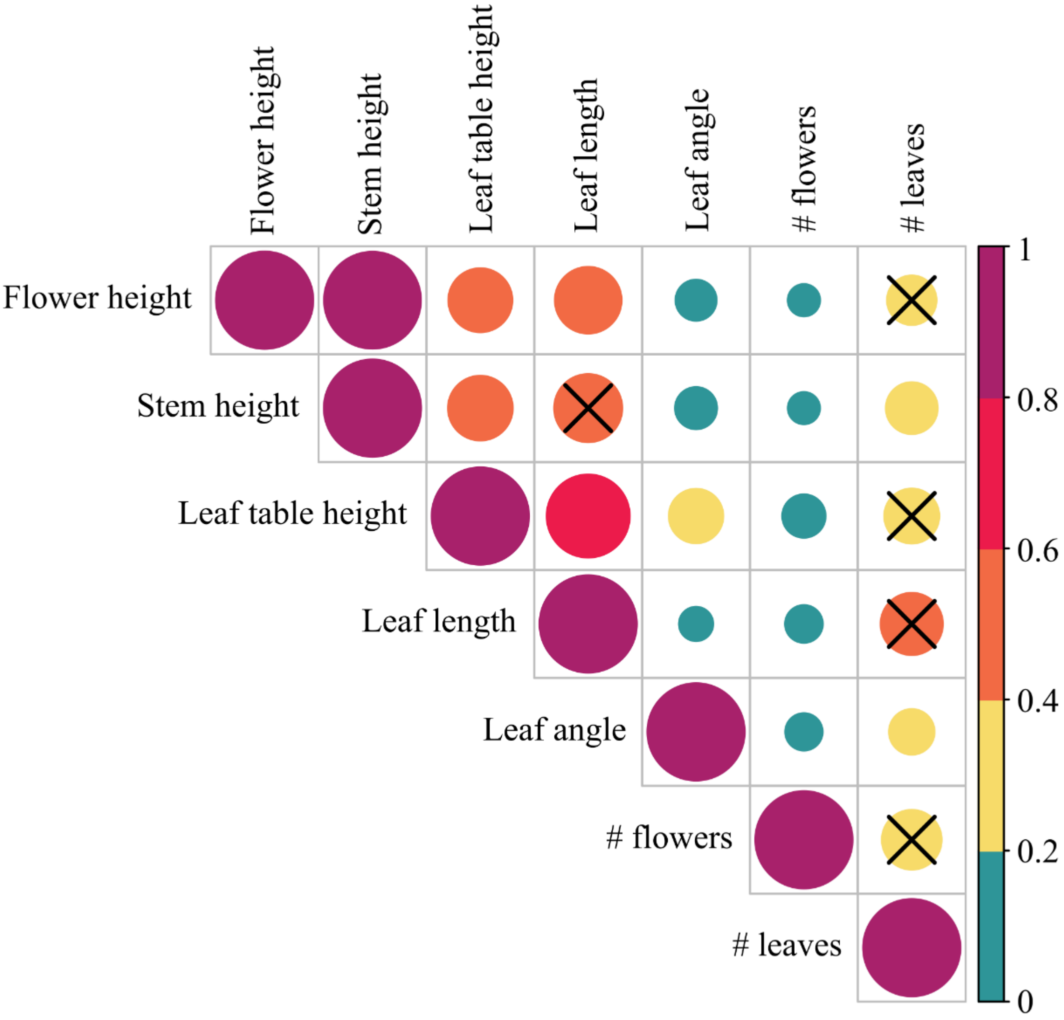

Figure 2.7: Correlation matrix of morphological traits with correlation coefficients, with circle size and colour scaling with the strength of the correlation (0 – 1). Most correlations were found to be significant (*P* < 0.01) with non-significant correlations marked with an X. No negative correlations were found.

Principal component analysis

The PCA (Figure 2.8) showed that PC1 accounted for the greatest proportion of variance (43.9%) with most of the traits loading along PC1 in the same direction, and only flower height and number of flowers showing a slight loading trend along PC2. Leaf table height and leaf length loaded very closely, almost overlapping. No clear separation was observed based on treatment category and there were numerous outliers from both the populations exposed to grazing and those unexposed to grazing. Cumulatively, PC1 through PC5 explained 95% of the variance (Figure 2.9).

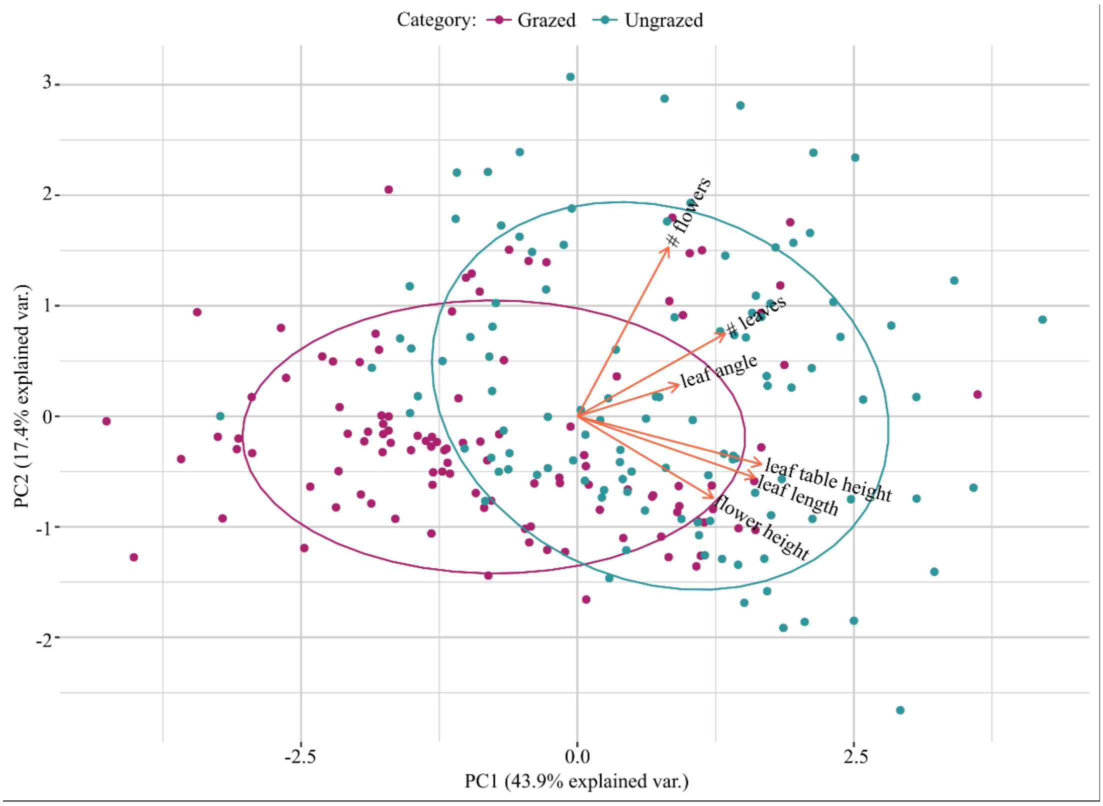

Figure 2.8: Principal component analysis of morphological traits loaded along PC1 and PC2.

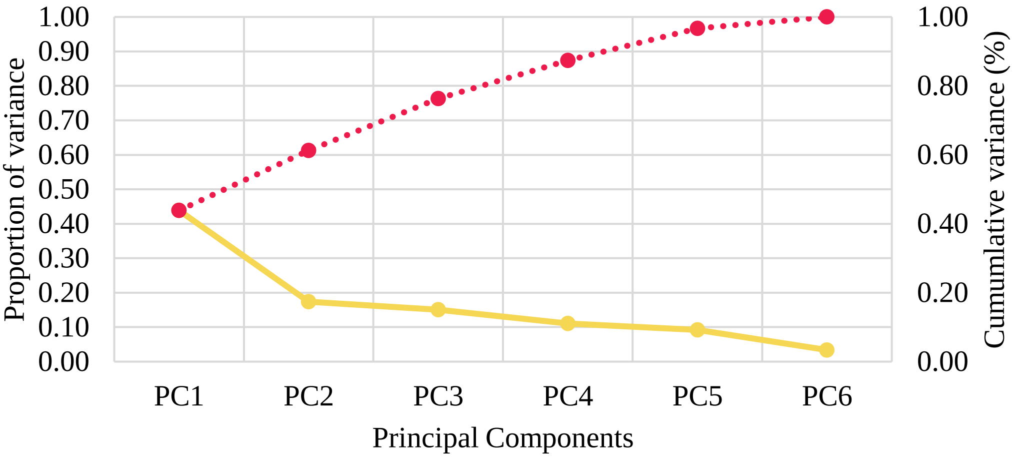

Figure 2.9: Proportion of variance explained by each principal component (solid line) and cumulative variance (dotted line). Explanation of 95% of variance is only cumulatively reached by PC5 by PC1 accounts for nearly half of the variance at 43.9%.

Grazing as a predictor of traits

The mixed effect models showed significant difference for only flower height ($\chi^2(1)$= 6.923, *P* = 0.009; Figure 2.10), with flowers in ungrazed populations being taller by 8.46mm (±2.94mm). For leaf table height and leaf length (Figure 2.10) as well as number of flowers (Figure 2.11) and leaf angle, no significant difference was found between grazed and ungrazed populations.

The Kruskal-Wallis rank sum test found significant difference in the number of leaves (χ^2 = 72.457, df = 1, *P* < 2.2e^{-16}) (Figure 2.11) with plants at ungrazed sites averaging 1 more leaf than those at grazed sites.

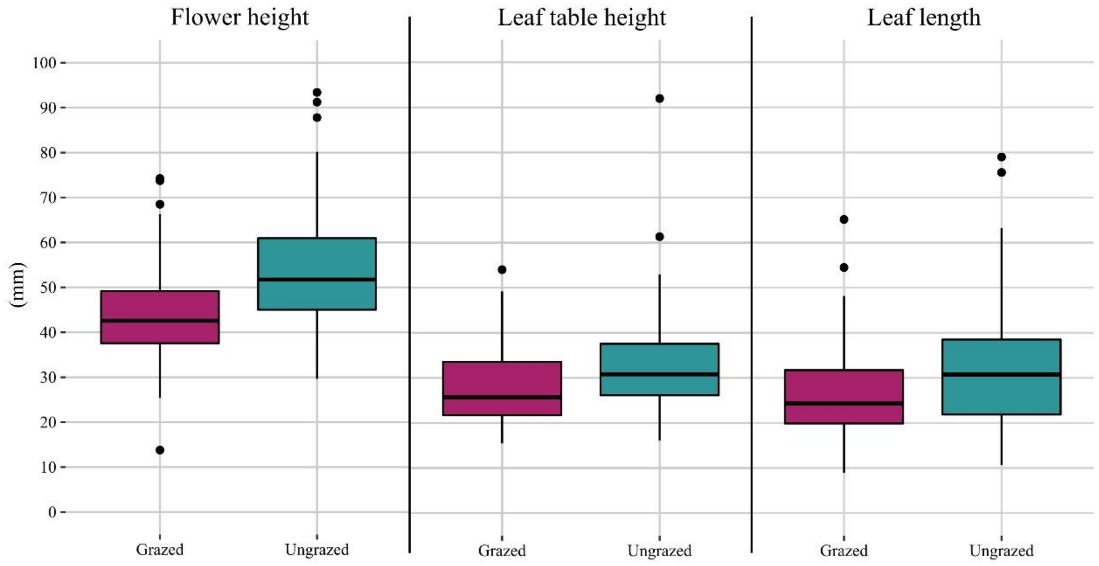

Figure 2.10: Flower height, leaf table height and leaf length compared between populations at grazed and ungrazed sites. Bars represent mean values with SE. Flowers at ungrazed sites were 8.46mm (±2.94mm) taller than those of populations exposed to grazing ($P < 0.05$). Leaf table height was only 1.67mm (±1.33mm) taller for plants in ungrazed sites and their leaf length was longer by 1.20mm (±1.19mm).

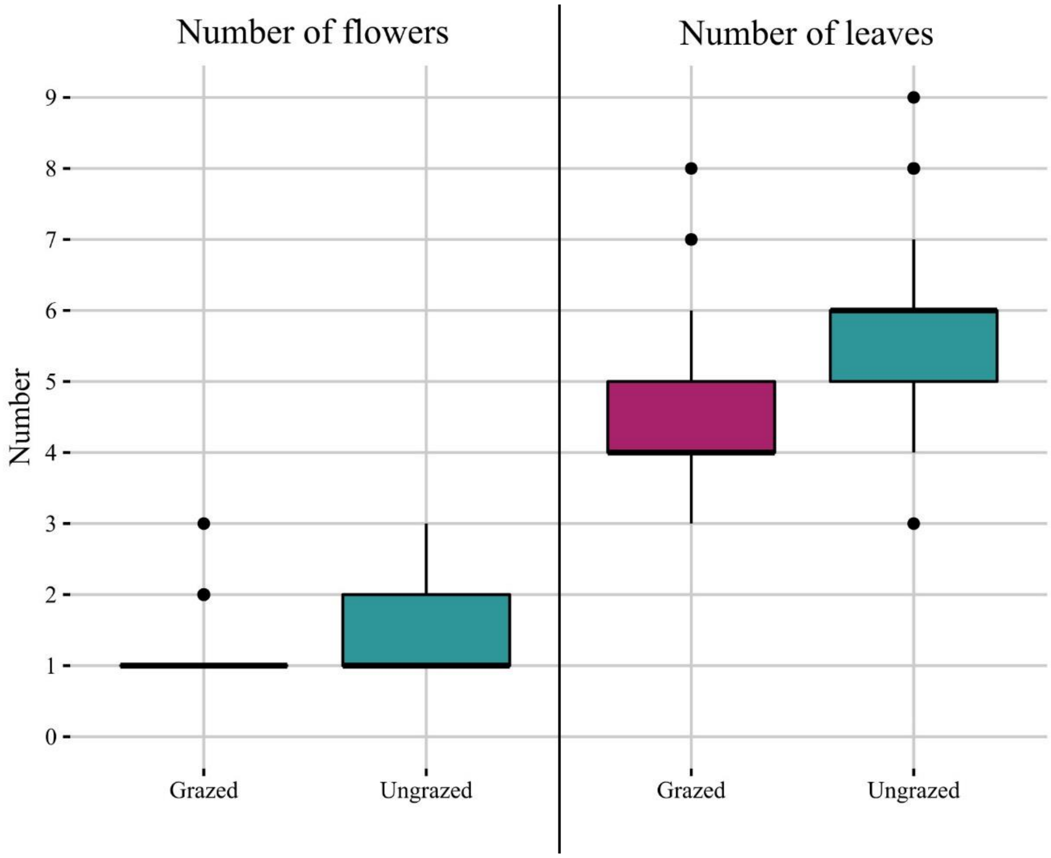

Figure 2.11: Number of flowers and leaves compared between plants at grazed and ungrazed sites. Bars represent mean values with SE. Plants not exposed to grazing had significantly more leaves ($P < 0.001$) than plants grazed populations.

Biomass allocation

The average above:below biomass ratio was 3.09 in populations not exposed to grazing, and 3.15 in populations from grazed sites, which was not significant in a Welch two sample t-test.

Seed dispersal

No seeds were found on the sticky traps or in the funnel traps at all sites suggesting the methods were not properly suited to scale of seed dispersal in *R. baurii* var.

platypetala, or that understanding of seed dispersal mechanisms in the variety require revision (see Chapter 4).

Discussion

Key morphological traits that are known to align with grazing resistance mechanisms were measured and compared between populations of *R. baurii* var. *platypetala* at sites exposed and unexposed to grazing. The relationship between the plants and their surrounding grasses was investigated through comparisons of height. The effect of grazing on seed dispersal was also considered, with preliminary investigations into passive seed dispersal carried out. Other grazing relationships such as endozoochory were considered but could not be effectively investigated.

Grass height

Average grass height varied a great deal between sites (Figure 2.6), from the very short 60mm at Hebron Road (HR) to 210mm at Mkhomazi Wilderness Area (DG). Despite this range of height, there was no significant difference in average grass height between grazed and ungrazed treatments. This is likely due to the grazed sites not having identical grazing patterns. Short sites such as Hebron Road were likely freshly grazed prior to measuring, while taller grazed sites such as Hebron Farm (HF) are known to be exposed to grazing but may not have been grazed as recently prior to measurement. The wide range of grass height within the ungrazed sites is likely attributed to other topographical drivers of grass height, such as litter presence and soil inorganic N (Bosco *et al.*, 2018; Sanaei *et al.*, 2018). While the grazed sites did not show as wide of a range of grass heights, topographical drivers will still be a factor in all patterns observed for these sites as well. Many studies that focus on the effect of grazing are designed to account for these factors through managed grazing exposure (Smyth *et al.*, 1997; Zeng *et al.*, 2015; Zhang *et al.*, 2015) but this was not feasible for this study, as it was not possible to control exposure to grazing in any form, much less at varied intensities, in populations of *R. baurii* var. *platypetala*. While it is possible to factor topographical drivers into statistical models, appropriate data is required which was beyond the range of this study. The use of "Site" as a fixed effect in the mixed effect models was intended to control for this as much as possible.

Morphology

The strong correlation between many of the measured traits of *R. baurii* var. *platypetala* (Figure 2.7) can be attributed to several of them having shared axes of measurement. This may make many of the traits appear redundant but, had grazing resistance been present, the combined response of each individual trait would have indicated development of this response. Grazing resistance often leads to flat and prostrate architecture (Díaz *et al.*, 2007), which would have been evident in the leaf table height and leaf length, as they would not be strongly correlated. While upright, longer leaves increase leaf table height, but were the plants to flatten, leaves could remain long while leaf table height decreased. As the flatter architecture was not present, the traits were strongly correlated and essentially shared an axis of measurement.

One of the traits found to be significantly different was flower height (Figure 2.11) with plants in ungrazed populations being 8.46mm (± 2.94mm) taller than plants in grazed populations. It is possible that this shorter height in populations exposed to grazing could be the early stages of grazing avoidance, but with no observations or evidence of grazing, this seems unlikely. There is a general ‘take half’ rule with cattle grazing that suggests cattle will bite their forage to a height that is half of the initial forage height (Laca *et al.*, 1992). Other findings have shown cattle will graze upon the upper levels of forage, above the half-point of forage height, only moving to biting lower down when the majority of the forage in the area has been grazed (Ungar *et al.*, 2001). Under these patterns of grazing, the small difference in height would have no impact on grazing avoidance, as flowers were generally far below bite thresholds. In *Primula farinosa* (Primulaceae), a comparable grassland herb of similar size and morphology to *R. baurii* var. *platypetala*, grazing exposure was found to reduce height at an increasing rate with each year of grazing exposure, ranging from an average of 0.5cm after 1 year to 15.7cm after 11 years (Thomann *et al.*, 2018). This dramatic change in height is not mirrored in *R. baurii* var. *platypetala*.

A shorter flower height might have an effect on reproduction by altering seed release height (Thomson *et al.*, 2011) but in the case of *R. baurii* var. *platypetala*, the lack of evidence of long-distance wind-based seed dispersal suggests there would likely be no impact on seed dispersal. In order to understand how the difference in flower height might impact seed dispersal in *R. baurii* var. *platypetala*, expanded investigation into seed morphology and dispersal syndrome is required, which can be combined with other trait data to estimate dispersal distance (Tamme *et al.*, 2014).

The number of leaves were significantly different between grazed and ungrazed sites, with plants in ungrazed sites averaging 1 leaf more than those exposed to grazing (Figure 2.11). This reduction in leaf number did not coincide with a significant difference in overall above-ground plant mass, nor a difference in the above:below ground biomass allocation ratio, suggesting that the reduced number of leaves does not effectively reduce the above-ground plant matter potentially exposed to grazing. The number of flowers was expected to be lower in populations exposed to grazing, as part of grazing avoidance to reduce available above-ground material and reduce grazing likelihood (Ehrlén and Ehrlen, 1997), but no difference was found. Together, these results suggest that *R. baurii* var. *platypetala* is not developing grazing avoidance through a reduction in above-ground material that could potentially be grazed. While site-level differences were controlled for in the construction of the mixed-effect models, the spread of study sites across such a large lead to the inclusion of a rainfall gradient, varied altitudes and other differences in local conditions. While this is not ideal, it was unavoidable due to study site availability. Identification of more sites will hopefully lead to better comparability in the future, along with the use of common garden experiments where these factors are controlled (see Chapter 4).

Grazing management

The results suggest that populations of *R. baurii* var. *platypetala* exposed to grazing are not developing grazing resistance mechanisms, and with the lack of observed grazing or evidence of grazing during this study, it appears unlikely that the plants are grazed. This could be further supported by studies such as cafeteria experiments (e.g. Alonso-Díaz *et al*., 2008; Lloyd *et al.*, 2010; Terrill *et al.*, 2004), where preference for grazing by cattle is directly tested for in the variety. If cattle are found to graze *R. baurii* var. *platypetala* when given the opportunity, it would suggest that grazing resistance or avoidance is necessary. Grazing could also be simulated through clipping experiments to investigate the impact of defoliation on the variety and better understand if observed differences in flower height can be attributed to natural variation or if they are the early stages of grazing resistance.

While it is unlikely that grazing is negatively impacting *R. baurii* var. *platypetala*, the expansion of farmlands is a growing concern in the Drakensberg area (O'Connor, 2005; Ramesh and Downs, 2015), thus the particular patterns of cattle grazing need to be considered for future management of the region. The 'take half' rule and this tendency for cattle to avoid lower-height grazing (Ungar *et al.*, 2001) can be used to ensure effective grazing management for *R. baurii* var. *platypetala* and other comparable species, by simply ensuring grazing rotation is carried out so that grasses surrounding populations are able to recover, and avoiding the harmful grazing patterns that result from intense depletion (Ungar and Ravid, 1999). This would likely eliminate the risk of *R. baurii* var. *platypetala* plants being grazed with the grasses. Although grazing may not appear to be affecting currently, the ability of grazing to drive extensive changes in plant morphology and reproduction (Díaz *et al.*, 2007; Dickson and Petit, 2006; Thomann *et al.*, 2018), along with the unmanaged exposure of populations of plants to grazing, are cause to develop management strategies for grazing that can be implemented in the future as needed.

Investigation of endemic species and development of management strategies for their conservation prior to the urgent need for the strategies can save valuable time in the future as land use changes occur (Batáry *et al.*, 2015; Fynn *et al.*, 2016) and species are exposed to new disturbance factors. With grazing in particular, effective management requires long-term study (Thomann *et al.*, 2018), and the more of this research that is carried out while the population is not at risk, the better, so conservation efforts can be implemented immediately should that status change. It would likely be in the best interest of *R. baurii* var. *platypetala* to ensure that populations unexposed to grazing remain protected from the disturbance until the variety and *R. baurii* as a species are better researched, to ensure disturbance does not lead to adverse effects.

Conclusion

There were few significant differences in morphology between populations of *R. baurii* var. *platypetala* in grazed and ungrazed areas, and differences that are present do not appear to be the result of grazing resistance. This is supported by the absence of observed grazing or grazing damage at any of the sites, suggesting that grazing is not a key disturbance factor acting upon populations of *R. baurii* var. *platypetala*. Thus, it appears that grazing need not be a factor of immediate concern when considering current management of *R. baurii* var. *platypetala*. There does appear to be a trend of smaller plants in grazed areas when looking at the results collectively, and while this size difference may not currently be significant, it is possible that it may become so if it is the beginning of a trend. As little is known about this species and its varieties in the context of disturbance and change, many questions are presented to further expand understanding. Direct grazing experimentation and improved understanding of seed dispersal are of priority to ensuring the response of *R. baurii* var. *platypetala* to the disturbance is understood. With the growing risk of grazing exposure in the Drakensberg, studies of this kind are of increasing importance as understanding disturbance dynamics is key for biodiversity conservation.

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CHAPTER 3

POPULATION GENETICS

Abstract

Assessing genetic diversity is key in biodiversity conservation and is of growing importance as climate and land-use change drive the need to assess vulnerability on a genetic level. Disturbances such as grazing can drive reduced genetic diversity within populations, as well as increased differentiation between those populations and others that are undisturbed. This study aimed to assess genetic diversity and differentiation within and between populations of the near-endemic herb *Rhodohypoxis baurii* var. *platypetala* of the Drakensberg, comparing populations exposed to livestock grazing to those unexposed to the disturbance. Genetic diversity was found to be moderate under both conditions (H_J grazed = 0.345; H_J ungrazed = 0.361) and differentiation was low ($\Phi_{PT} = 0.096$). While expanded assessment is required, moderate diversity along with low differentiation suggest that grazing is not having a negative effect on gene dynamics of *R. baurii* var. *platypetala*, and that the variety is not particularly vulnerable. The low differentiation is attributed to the evolutionary history of the variety and parent species, with recent establishment in the region. Investigation of genetic diversity is a key part of assessing vulnerability to disturbance and change, the need for which is highlighted as the region is exposed to expanding disturbance and climate change.

Keywords: genetic diversity; populations; *Rhodohypoxis baurii* var. *platypetala*; AFLPs; differentiation

Introduction

The available genetic diversity of a species determines its fitness through its ability to respond to environmental change (Hake and Ross-Ibarra, 2015; Reed and Frankham, 2003). Genetic diversity is also recognised by the IUCN as one of the 3 levels of biodiversity that should be conserved (Maunder and Byers, 2005). Gene dynamics are often in flux under natural conditions, driven by the environment and selection pressures, and are easily shifted by disturbances to the intricate dynamics of biotic and abiotic factors (McPeek, 2017). Anthropogenic actions can add to the amount of disturbance a species is exposed to, with large-scale actions altering entire landscapes (Turner, 2010). Thus, understanding how a species' genetic diversity is affected by disturbance, as well as how important genetic diversity is for their ability to respond to those changes, is increasingly important (Banks *et al.*, 2013; Manel and Holderegger, 2013). For endangered and rare species, knowledge of the genetic diversity is particularly important as it helps to implement effective conservation (Forcada and Hoffman, 2014; Frankham, 2005; Schwartz *et al.*, 2007; Vander Wal E. *et al.*, 2013). Species present in small and isolated populations are also of key concern when genetic diversity is considered, as they often have limited adaptive capacity and lose genetic diversity over time (Frankham *et al.*, 2017; Willi *et al.*, 2006). This was recently reaffirmed during a meta-analysis by Hoffmann *et al.* (2017) but rare exceptions can develop though mechanisms such as associative overdominance (Schou *et al.*, 2017). As anthropogenic disturbance can lead to fragmentation and isolation of populations, species can be put at risk of this loss of adaptability and genetic diversity (Delaney *et al.*, 2010). It is thus important to be aware of potential harmful effects of both anthropogenically-driven and natural environmental change when considering genetic diversity.

Not all disturbances are negative, with some leading to balancing selection to stabilise small populations and mitigate the loss of genetic diversity by selecting for heterozygous individuals, thus maintaining a higher level of genetic diversity within the population (Delph and Kelly, 2014; Funk *et al.*, 2016). This balancing selection is less common though, with disturbance and rapid environmental changes usually driving directional selection (Banks *et al.*, 2013; Pinsky and Palumbi, 2014; Voss-Fels *et al.*, 2015). Directional selection led to halved time-to-flowering within *Raphanus raphanistrum* (Brassicaceae) as a result of changes to harvesting techniques (Ashworth *et al.*, 2016). Directional selection even develops in long-lived species, such as the cycad *Zamia fairchildiana* (Zamiaceae), where anthropogenic habitat degradation drove genetic differentiation between subpopulations (Lopez-Gallego and O'Neil, 2014). It is important to effectively understand how change and disturbance have the ability to alter gene dynamics, particularly how they can drive differentiation.

Differentiation can result in genetic isolation, and there are several isolation hypotheses to explain how this occurs (Muñoz-Pajares *et al.*, 2017; Sexton *et al.*, 2014). Isolation-by-Distance (IBD) occurs when the increase in geographic distance between populations is correlated to a decrease in gene flow due to limitations in dispersal and drift (Slatkin, 1993). The IBD hypothesis explained patterns of genetic differentiation in populations of the alpine carnation *Silene ciliate* (Caryophyllaceae), with a latitudinal gradient developing across the mountains. The Isolation-by-Resistance (IBR) hypothesis is based on gene flow being hindered by geographic barriers (McRae, 2006) and explained fine-scale genetic differentiation in *Erysimum mediohispanicum* (Brassicaceae) (Muñoz-Pajares *et al.*, 2017). The Isolation-by-Environment (IBE) hypothesis is based on adaptation to local selection pressures, and can refer to abiotic (IBEa) or biotic (IBEb) factors (Sexton *et al.*, 2014). It was found that *Phlox drummondii* (Polemoniaceae) conformed to the IBEb hypothesis, with genetic differentiation in populations of the wildflower being linked to variations in pollinator communities (Hopkins *et al.*, 2012). Finally, the Isolation-by-Phylogeography (IBP) hypothesis is applied to explain patterns of genetic differentiation that are the result of historic processes that have persistent effects. IBP explained distinct genetic clustering in *Bromelia balansae* (Bromeliaceae) in South America (Leal *et al.*, 2018). These hypotheses often best explain observed patterns of genetic differentiation when used in combination, with some explaining broad-scale patterns and others the fine-scale trends.

The risks associated with isolation need to be considered when investigating genetic diversity, particularly as anthropogenic actions continue to drive fragmentation. Differentiation and isolation make management difficult as more populations require protection to maintain species-level genetic diversity (Frankham *et al.*, 2017), and many species are being placed at risk of this through climate change and anthropogenic fragmentation which prohibits gene flow (Ellstrand, 2014; Sexton *et al.*, 2014). Assessing genetic diversity levels and the extent of differentiation is thus critical, and presents the need for fast, effective and inexpensive techniques to do so.

Amplified fragment length polymorphism (AFLP) is a DNA fingerprinting technique that has become popular due its speed, robust applications, and ability to be used without prior knowledge of the species' genome (Mueller and Wolfenbarger, 1999; Vuylsteke *et al.*, 2007). AFLPs are used to investigate genetic diversity by constructing genetic maps and comparisons of marker variation, with AFLP profiles generated from selectively amplified restriction fragments from digested genomic DNA (Blears *et al.*, 1998; Vos *et al.*, 1995). This method is particularly popular in ecological studies of plants and recently, a low-cost, highly transferable protocol was outlined for application in plants by Blignaut *et al.* (2013). This protocol used three fluorescently labelled primers which target genomic regions that are highly conserved interspecifically, eliminating the need for primer screening and making the protocol very transferrable and cost-efficient. AFLPs have been used to quantify levels of genetic diversity within populations of *Glycine max* (Fabaceae) (Zargar *et al.*, 2017) and *Armoracia rusticana* (Brassicaceae) (Wedelsbäck Bladh *et al.*, 2014), and to compare inter- and intra-population genetic diversity in *Pinus pinaster* (Pinaceae) (Mariette *et al.*, 2001). AFLPs were also used to compare how anthropogenic and natural fragmentation affected genetic diversity in the orchid *Bulbophyllum occultum* (Orchidaceae) and provided insight into how different populations arose (Jaros et al., 2016). The diverse applications of AFLPs and their relative affordability have allowed them to become a useful tool when investigating genetic diversity and the technique is considered to have further potential as technology advances (Meudt and Clarke, 2007).

As techniques such as AFLPs make investigation of genetic diversity easier, more species can be studied to swiftly assess their potential adaptability and thus vulnerability to environmental change. A study by Gaudeul *et al.* (2000) of the endangered species *Eryngium alpinum* (Apiaceae), a perennial herb of the European

Alps, used AFLPs to detect high genetic differentiation between the isolated populations and recommended conservation of a "maximum number" of populations to maintain genetic diversity within the species. *Campanula thyrsoides* (Campanulaceae), another alpine plant species, was also found to have high genetic differentiation across its distribution range, with a reliance on outcrossing to maintain genetic diversity within populations (Ægisdóttir *et al.*, 2009). These investigations through AFLPs not only provide direct insight into the status of genetic diversity and differentiation in species, they also have potential applications as portions of vulnerability assessments.

Vulnerability to change and disturbance can be determined by various factors, and frameworks argue for multi-dimensional approaches for effective assessment. For example, Foden *et al*. (2013), advocated for assessment of sensitivity, exposure and adaptive capacity. Sensitivity was defined as the ability of a species to persist *in situ* under climate change, exposure was the projected extent of change the species' environment was expected to undergo, and adaptive capacity was the ability of the species to avoid the effects of change through "dispersal and/or micro-evolutionary change". In assessing adaptive capacity, potential evolvability was assessed through measures of genetic diversity, with low levels of diversity indicating poor evolvability and thus contributing to a status of low adaptive capacity (Foden *et al.*, 2013). As one of the key components of effective vulnerability assessments, the need for genetic diversity assessments is growing, as climate and land-use change put more species and populations at risk.

The expansion of rangelands for cattle grazing within parts of the Drakensberg mountain range (Ramesh and Downs, 2015) has exposed many species to grazing disturbance that would not naturally occur at such high levels. *Rhodohypoxis baurii* (Baker, 1878; Hilliard and Burtt, 1978) is a near-endemic species of small geophytic herbaceous perennials distributed in the KwaZulu-Natal Drakensberg and Lesotho plateau. Populations are generally small and isolated, found in rocky areas among grasses at high altitudes (1990m – 3200m). As the species grows in grasslands dominated by palatable grasses in some regions, some plants may be subject to grazing due to their proximity to grasses. In one variety, *Rhodohypoxis baurii* var. *platypetala*, preliminary observation showed morphological variation between populations exposed and unexposed to grazing, which suggested potential differentiation in response to grazing disturbance. The isolated nature and small size of *R. baurii* var. *platypetala* populations presented potential for pre-existing risk regarding their genetic diversity. With the addition of a new disturbance in the form of high intensity, anthropogenically-driven grazing (Cingolani *et al.*, 2005), the potentially already at-risk genetic diversity of the variety may be further compromised.

Grazing could lead to directional selection within populations of *R. baurii* var. *platypetala* and, as the selection is not acting upon all populations, this shift could lead to development of or an increase in differentiation within the variety. This differentiation could become very pronounced under the IBEb hypothesis if the disturbance was present for an extended period of time, relative to the generational turnover of the species. Grazing has also been shown to affect sexual reproduction in plants if they adapt by developing architecture that facilitates grazing resistance (Diacon-Bolli *et al.*, 2013; Soons *et al.*, 2004; Thomann *et al.*, 2018) which could negatively affect gene flow by reducing crossing (Dickson and Petit, 2006; Lortie and Aarssen, 1999). Clonal propagation can also increase as a result of grazing (Douhovnikoff *et al.*, 2005) and reliance on this for persistence is concerning as under certain conditions a species can permanently lose the ability to sexually reproduce (Eckert, 2002), and monoclonal populations can develop (Honnay and Bossuyt, 2005). As grazing sites are randomly distributed, they could contribute to fragmentation, particularly if sexual reproduction is hampered within a population that is a bridge for gene flow along part of the distribution (McRae and Beier, 2007).

I aimed to investigate the status of genetic diversity of *R. baurii* var. *platypetala* at both the variety- and population-level, as well as establish whether or not grazing pressure was negatively impacting genetic diversity and, if so, how severely. In order to do this swiftly and cost-effectively, AFLPs were selected for the investigation, as they would allow me to assess genetic diversity at both intra- and inter-population levels, and as they required no prior information of the genome of *R. baurii* var. *platypetala,* as none was available. Understanding the status of genetic diversity within *R. baurii* var. *platypetala* along with the effect livestock grazing has on the variety would add to effective management and biodiversity conservation. The investigation would also be an indicator of the risk grazing poses to comparable species in the Drakensberg, potentially aiding management planning in this region.

Hypotheses

1. Populations of *R. baurii* var. *platypetala* exposed to livestock grazing would have lower measures of genetic diversity when compared to those at sites with no grazing.

2. There will be high genetic differentiation between populations from grazed and ungrazed sites.

METHODS

Eight populations of *R. baurii* var. *platypetala* were sampled across the distribution range of the variety (Table 3-1; and see Chapter 2, Figure 2.2), with four of the populations exposed to grazing and 4 not. At each of the sites, a 25m^2 quadrat was set-up to sample the population and within the quadrat, 28 plants were selected at random for tissue collection, and their X and Y co-ordinates within the quadrat were recorded (Figure 3-1). This co-ordinate system allowed for extrapolation of spatial data for the individuals to properly asses population structure. Leaf tissue collected from the individuals was stored in filter paper with silica gel to dry and preserve the tissue until processing in the laboratory.

Table 3.1: Distance matrix of sampling sites arranged from North to South. All distances are in km and were calculated using *geosphere* (Karney, 2013).

	GG*	**MK**	**KF**	**HM**	**DG**	**SF***	**HR***	**HF***
GG*	*NA*							
MK	94.1	*NA*						
KF	182	92.6	*NA*					
HM	133.5	39.5	62.1	*NA*				
DG	147.8	76.9	116.7	63.6	*NA*			
SF*	169.1	80.9	82	48.2	41.8	*NA*		
HR*	227.7	148.5	141.6	119.1	79.9	71	*NA*	
HF*	229.6	150.7	143.6	121.3	81.9	73.2	2.2	*NA*

**site is exposed to grazing*

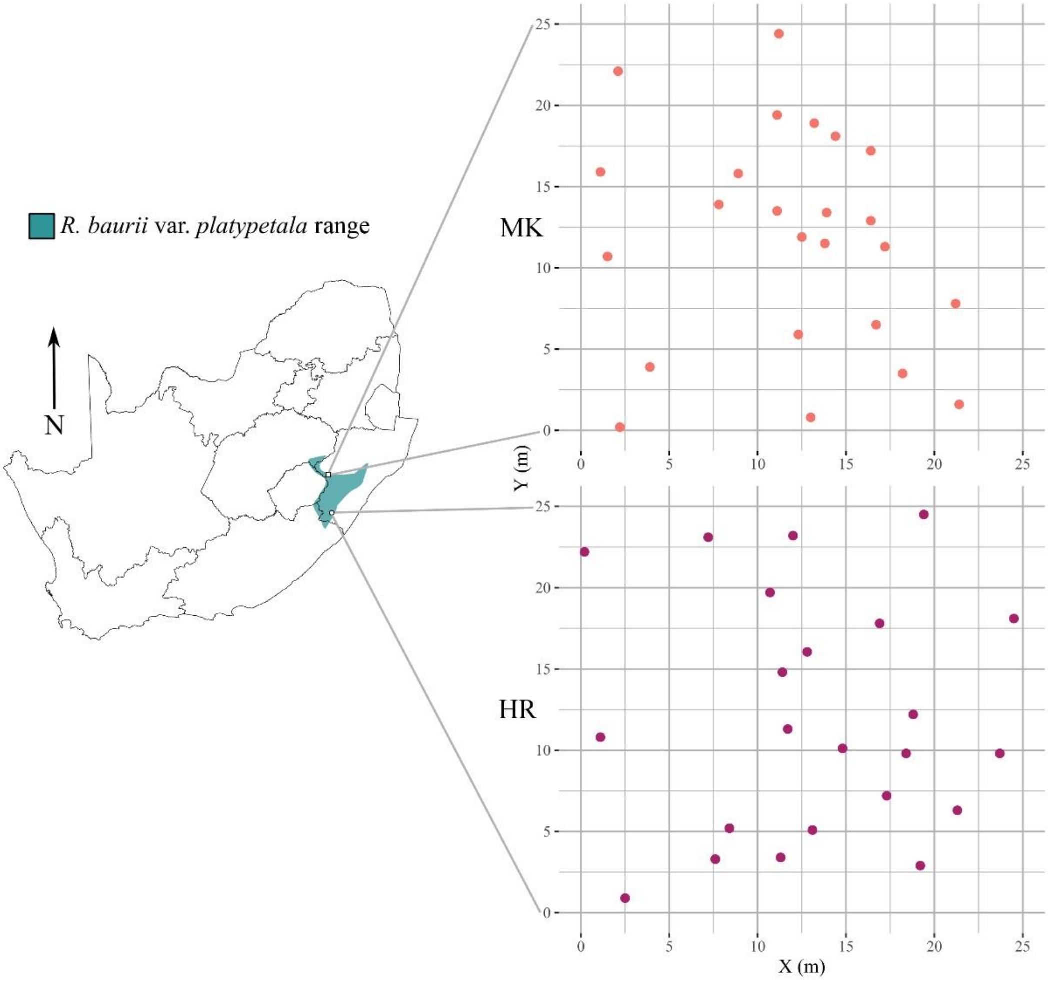

Figure 3.1: Spatial maps of sampling within the Hebron Road (HR) and Giant's Castle Game Reserve (MK) sites using 25m^2 quadrats. The quadrat was used to create an XY co-ordinate system to record spatial relationships between sampled individuals. Sampling had an average distance of 12.5m in the HR population, with the furthest individuals 29.0m apart and the closest sampling pair 2.1m apart. In the MK population the average distance was 11.5m, with a maximum of 28.2m and minimum of 1.4m.

DNA was extracted from the leaf tissue using the cetyltrimethylammonium bromide method (CTAB) following the Soltis Lab protocol (Cullings, 1992; Doyle and Doyle, 1987). The quality and concentration of DNA samples were checked with a UV-Vis spectrophotometer (ND-1000, NanoDrop, ThermoFisher Scientific, Wilmington, USA). Where possible, extraction was repeated to improve sample quality (ideal sample quality: $A_{260/280}$ ~ 1.8 and $A_{260/230}$ ~ 2.0), but due to limited availability of tissue this was not possible for all poor-quality samples.

Due to issues in the lab, delays were incurred that hampered the extent of sampling for this study. This resulted in the need for repetition of work which exacerbated time constraints. Given these difficulties, the AFLP step could not be carried out on all 224 DNA samples, and so a representative population was selected from the grazed sites and the ungrazed sites for comparison. Hebron Road (HR) was selected as the representative for those exposed to grazing and the Giant’s Castle Game Reserve population (MK) was selected from the populations unexposed to grazing, as these populations had the highest number of high-quality DNA samples available. This would allow for simple but informative investigation of the genetic diversity of R. *baurii* var. *platypetala* as well as the effect grazing may have, while allowing the study to be completed on time. However, it does limit statistical inferences that could be made due to a lack of replicates.

DNA samples were diluted to 100ng/µL for use in AFLPs following the protocol developed by Blignaut *et al.* (2013) with reagents prepared following Vuylsteke *et al.* (2007). For DNA samples below the suggested 100ng/µL concentration, the amount of dH_2O in the initial *EcoR*I digestion step was reduced, and the amount of DNA solution proportionally increased to ensure 200ng of DNA was available while maintaining the 20µL reaction volume. No alterations were made to Blignaut *et al.*'s (2013) protocol (Appendix I) other than using *Mse*I (Inqaba Biotechnical Industries, Pretoria, South Africa) rather than its isoschizomer, *Tru*I. Electrophoresis was carried out for 40 minutes at 65V after the pre-selective and selective PCRs, using 2µL of the respective products, and SybrSafe gel stain (ThermoFisher Scientific, Wilmington, USA) and Quick-Load Purple 50bp DNA Ladder (New England BioLabs, Ipswich, MA, USA supplied by Inqaba Biotechnical Industries, Pretoria, South Africa). While the two populations were selected for the availability of high-quality DNA samples, there were some poor quality samples that, despite repetition of the process, resulted in failed AFLPs. There where were 26 successful AFLP results from HR population and 24 from MK.

The AFLP amplification products were sent to the Central Analytical Facility at Stellenbosch University (Stellenbosch, South Africa) for fragment analysis with LIZ500 size standard. DNA fragments are loaded into a capillary array and migrate through, arranging by size in the process. The smaller fragments move more easily and so migrate under the detection window first, where the fluorescent labels are active by a laser beam. The fluorescence is detected and translated into peaks on an eletropherogram by a charge-coupled (CCD) camera, with larger peaks representing greater fluorescence intensity caused by a greater number of fragments. Peak calling was carried out for all three of the markers using Genemarker Version 3.0.0 (SoftGenetics, LLC, CA, USA) with the software pre-set for AFLP data, then confirmed manually. A binary data matrix was created, coding for presence (1) or absence (0) of peaks within all individuals, and the matrix was checked for monomorphic and spurious peaks to be removed before analysis.

Data analysis

AFLP-SURV (v1.0; Vekemans, 2002) was used to calculate descriptive statistics for the genetic diversity of the populations, using the software pre-sets that follow Lynch & Milligan (1994) to calculate both Nei's (Nei, 1987) and Reynolds' genetic distance (Reynolds *et al.*, 1983). Calculations were run with assumption of Hardy-Weinberg equilibrium ($F_{IS} = 0$) and 100 permutations were carried out to test F_{ST} to 5% confidence level. For each population, expected heterozygosity expressed as H_J (analogous to H_E), and within-population expected heterozygosity as H_W (analogous to H_S). It should be noted that the interpretation of F_{ST} values is not based on quantitative value, but rather contextual significance, as the robustness of genetic markers has increased since the measure was developed, altering interpretation of the quantitative scale of F_{ST} (Hedrick, 2011). Therefore F_{ST} values are used as identifiers of differentiation, but context and additional statistical tests, such as the Analysis of Molecular Variance (AMOVA; Excoffier *et al.*, 1992), are required for effective interpretation as they can standardise F_{ST} values.

Geographic and binary genetic distances were calculated for both populations using GenAlEx (v6.5; Peakall and Smouse, 2012, 2006), and in turn used to conduct Principal Co-ordinate analysis (PCoA). An AMOVA was also carried out in the software to calculate the proportion of genetic variance as Φ_{PT} (analogous to F_{ST}). Finally, GenAlEx was used to carry out Mantel tests (Mantel, 1967; Sokal, 1979) on each population to investigate how geographic distance related to genetic distance, using the population-level co-ordinate data.

Results

The 3 markers resulted in a total of 151 fragments identified, with a mean of 77.8 fragments per individual. For the HR population, 97.5% of loci were polymorphic, and 95.4% for the MK population ($\alpha = 0.05$).

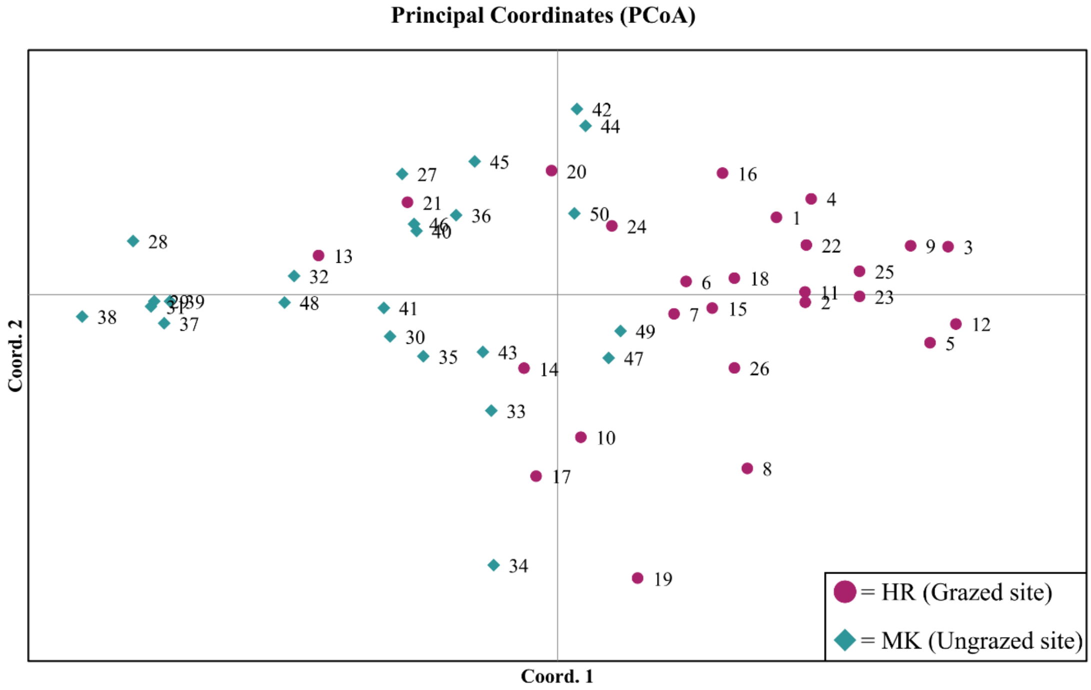

Figure 3.2: Principal Co-ordinate Analysis (PCoA) of the HR and MK populations, showing individuals ordinated by binary genetic dissimilarity matrix, with more similar plants closer together. Co-ordinate axis 1 accounted for 11.69% of variation and co-ordinate axis 2 accounted for 9.57%. The distribution was split by population along axis 1 with most of the MK individuals on the left and HR on the right. Within the populations, individuals did not cluster neatly, being spread mostly along axis 2.

PCoA of genetic similarity (Figure 3-2) showed general separating by population along Axis 1 which explained 11.69% of the variation. While populations did not cluster neatly, the majority of within-population variation was shown along Axis 2 which explained a further 9.57% of the variation. No identical individuals were identified. Some clustering of individuals within the populations was observed, particularly individuals 29, 31 and 39 in the MK population.

Within-population genetic diversity

Nei's gene diversity (H_J) in the HR population was 0.345 (S.E.= 0.011) and the MK population had a similar measure of 0.361 (S.E.= 0.011). Nei's gene diversity within populations was similarly moderate (H_W = 0.358; S.E = 0.003).

Mantel tests (Figure 3.3) run with 99 permutations for both populations found that for HR the genetic and geographic distances were positively correlated, and this was marginally significant (Rxy = 0.195), thus plants further away from each other within the population were less genetically similar. This trend was relatively weak though, indicating that there were factors other than geographic distance affecting plant relatedness: the R^2 value for HR was only 0.038. For the MK population there was no evidence of a relationship between geographic and genetic distance, the slight negative slope was not significant (Rxy = -0.112).

Between-population genetic diversity

Genetic differentiation between the pair found to be relatively low (H_B = 0.017; S.E. = 0.000). Gene diversity among populations was also low with F_{ST} only 0.045 (S.E. = 0.008). The observed genetic diversity was no higher than what was measured when individuals were randomly assigned to populations (100 permutations), with observed F_{ST} having the same value (0.045).

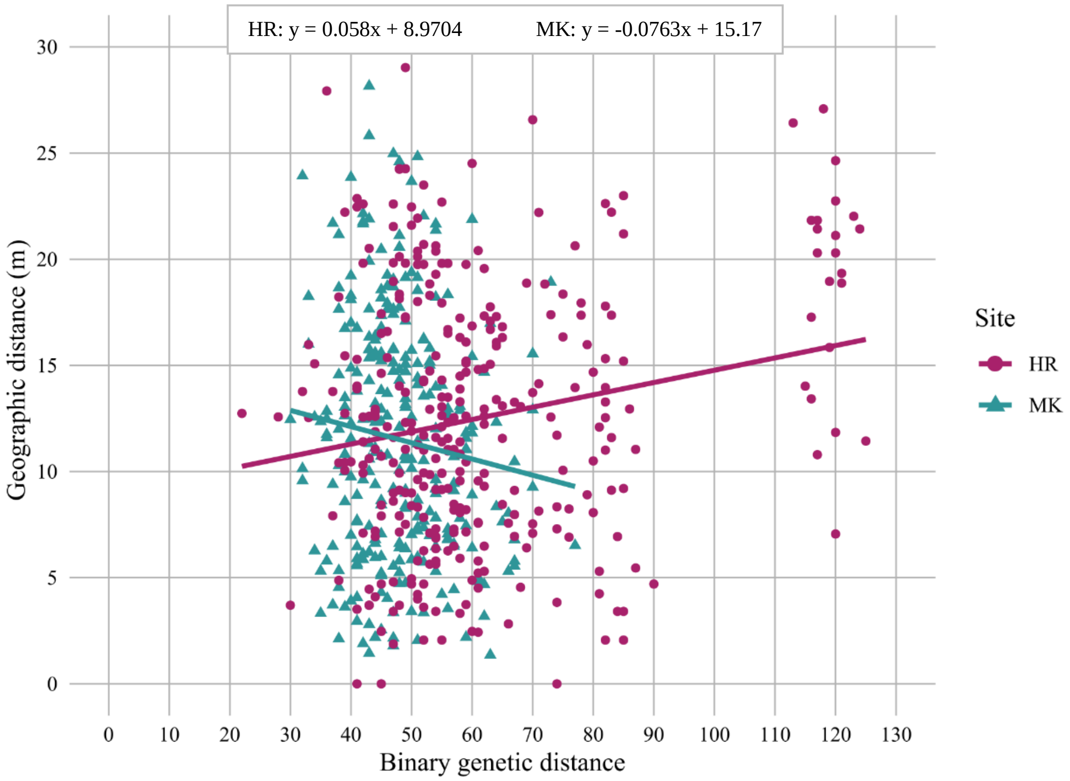

Figure 3.3: Geographic distance of individual plants plotted against binary genetic distance. Plants at HR were found to have a positive correlation (R*xy* = 0.195) thus an increase in genetic differentiation with increasing distance between individuals. Plants at MK had no significant correlation.

Table 3.2: Analysis of molecular variance (AMOVA) in *R. baurii* var. *platypetala*

Source of variation	**DF**	**SS**	**VC**	**PV (%)**	***P***	
Among populations	1	26.040	26.040	9.6	< 0.001	$\Phi_{PT} = 0.096$
Within populations	48	342.000	7.125	90.4	< 0.001	

DF: Degrees of Freedom; SS: Sum of squares; VC: Variance Components; PV: Percentage of variation.

Discussion

This study aimed to assess the genetic diversity of *R. baurii* var. *platypetala* both within and between populations, and identify if grazing was negatively impacting genetic diversity in populations exposed to the disturbance. Genetic diversity was measured within one population exposed to grazing and one unexposed population, then compared to quantify genetic differentiation between the pair.

The PCoA (Figure 3.2) shows how similar individuals are to each other and showed that most individuals of the same population loaded on the same side of the first axis, with some overlap, suggesting that the populations are somewhat genetically distinct from each other. Within the populations, individuals were not highly clustered, but were mostly spread along the second axis, suggesting that there is genetic variation within the populations. One small cluster is present, with a group of three individuals loading very closely and a fourth slightly further away. While R. *baurii* var. *platypetala* can vegetatively propagate, this would result in genetically identical individuals, which would overlap completely when loaded on the PCoA axes, which was not the case in this cluster. It is possible that this cluster is the result of a patch of highly inbred individuals (Berg and Hamrick, 1995), but this would only be confirmed by more extensive sampling of the population followed by spatial correlation.

Within-population genetic diversity

In a review by Nybom (2004), within-population genetic diversity measures were found to range from an average of 0.55 for short-lived perennials to 0.65 for outcrossing species. This range would suggest that the genetic diversity in the HR and MK populations is low, with H_J values of 0.354 and 0.361 respectively, along with the measure of Nei's gene diversity within populations ($H_W = 0.358$). However, comparisons to other species suggest that these values indicate moderate levels of genetic diversity. The alpine herb *Eryngium alpinum* (Apiaceae) has an altitudinal range of 1500m-2000m which is similar to *R. baurii* var. *platypetala* at 1600m – 2900m and was found to have within-population diversity of 0.198 (H_W), which the authors defined as intermediate through comparisons to other AFLP studies. Another alpine herb, *Campanula thyrsoides* (Campanulaceae), has an altitudinal range of 1000-2900m, which overlaps with but exceeds that of *R. baurii* var. *platypetala*, and was found to have within-population diversity of 0.76 (H_E) which the authors considered high and attributed to outcrossing (Ægisdóttir *et al.*, 2009). The results found in *R. baurii* var. *platypetala* can be deemed moderate in comparison to the findings of these alpine species. This is supported by measures of diversity in the orchid *Bulbophyllum occultum* (Orchidaceae) which were found to be very low (HE = 0.026 – 0.124) and this was attributed to self-fertilization (Jaros *et al.*, 2016). While it is expected that small populations lose genetic diversity over time due to their size (Willi *et al.*, 2006), assessing the risk of this for *R. baurii* var. *platypetala* would require long-term monitoring of genetic diversity within the populations, or sampling of more populations of different sizes.

The Mantel tests (Figure 3.3) found that genetic similarity decreased with an increase in geographic distance (R*xy* = 0.195) in the HR population, and while this trend was relatively weak (r^2 = 0.038) it shows that even at the population level, the IBD hypothesis can be observed. This could be attributed to pollinators only visiting plants closer to each other and thus not transporting genetic material across whole populations, or it could be the result of vegetative propagation being less prevalent or limited to small ranges within the population. No significant trend was found in the MK population.

Between-population diversity

The populations *R. baurii* var. *platypetala* examined in this study created potential for high differentiation between populations under the IBD hypothesis, but the results did not support this pattern. Nei's genetic differentiation between the two populations was 0.017 (H_B) and the analogous F_{ST} was calculated to be 0.045, which can be interpreted as very low differentiation. Phillips *et al.* 2012) concluded that species with effective dispersal had average F_{ST} values of 0.146, far greater than the results found in our analyses. This is supported by comparison to the previously discussed alpine species, with *Eryngium alpinum* having high differentiation (F_{ST} = 0.40; Gaudeul *et al.*, 2000) which was attributed to post-glacial founder events. Post-glacial migration patterns were also a driver of high differentiation in *Campanula thyrsoides* (G'_{ST} = 0.53), but the isolated habitat availability and its consequent restriction of gene flow, along with poor seed dispersal, were also considered vital factors (Ægisdóttir *et al.*, 2009). The F_{ST} analogue Φ_{PT} calculated through AMOVA was 0.096 (Table 3.2) and supports very low differentiation in *R. baurii* var. *platypetala*, compared to the high differentiation in *Bulbophyllum occultum* (Φ_{PT} = 0.387; Jaros *et al.*, 2016). The AMOVA analysis of *R. baurii* var. *platypetala* attributed only 9.6% of variation to the between-population level, further substantiating interpretation of low levels of differentiation. Given that the populations are 148.5km apart from each other, the very low differentiation between these populations was surprising. Absence of differentiation indicates maintained gene flow between populations, which could be facilitated through travelling pollinators, but confirmation of this would require further study as no pollinators have been observed for the variety nor other varieties within the species.

The lack of nutritious fruit or seeds likely eliminates endozoochory as a mechanism for gene flow and as seeds have passive dispersal, pollinators would likely be the key to maintaining gene flow across the distance between populations of *R. baurii* var. *platypetala*. It is possible that hybrid populations are key in maintaining this process, as they would act as bridges between populations of *R. baurii* var. *platypetala* and reduce the distance pollinators would need to travel to carry genetic material between populations. If pollinators are key to the maintained gene flow within *R. baurii* var. *platypetala*, it is puzzling that pollinators are yet to be observed, and thus is possible that the rate of gene flow required within the variety to prevent differentiation is low, given the apparent rarity of pollinators. Understanding pollination and its role in gene flow within both *R. baurii* var. *platypetala* and the parent species *R. baurii*, as well as the role of hybrid populations, appears to be key to understanding the maintenance of genetic similarity between such distant populations.

The more likely explanation for this pattern is that the populations are very newly diverged, only occupying these regions recently. With the majority of plant diversification occurring in the Miocene (Moldowan *et al.*, 1994), the relative recentness of the uplift of the Drakensberg in the Pliocene is likely the determinant of the low differentiation. This, along with the extensive hybridisation of all varieties within the parent species *Rhodohypoxis baurii*, suggests that these lineages may not be completely isolated.

Effects of grazing

Grazing was expected to negatively impact genetic diversity or gene flow within *R. baurii* var. *platypetala*, but the results did not support this expectation. The PCoA (Figure 3.2) showed individuals from the population exposed to grazing at HR were well distributed and not highly clustered, indicating moderate variation, which was not different from the one grazed site analysed. To properly test this, samples from more than just one ungrazed and one grazed site would need to be processed. Within-population genetic diversity was expected to be lower due to grazing exposure, but the HR population had an H_J value very similar to that of the MK population which is not exposed to grazing. Another concern was that grazing may drive isolation of populations, thus high differentiation was expected, but the results did not support this, with low differentiation measures between the pair. Overall, while grazing and similar disturbances have negatively affected many species, the preliminary results indicate that, these effects do not act on *R. baurii* var. *platypetala*. This is probably due to the low likelihood of the variety being grazed (see Chapter 2).

Future study

The robustness of the investigation could be expanded on both a broad and fine scale, to facilitate more in-depth understanding of the variety and populations within it. At the broader variety level, multi-population assessments can identify genetic trends, clines, and monitor differentiation between populations (Hunter *et al.*, 2018). Fine-scale analyses can be used to identify family group structures within populations, measure gene dispersal distances, and provide further understanding of how vegetative propagation factors into population persistence (Vekemans and Hardy, 2004).

The use of AFLPs when more advanced techniques are available was the result of practicality and cost, with AFLPs remaining inexpensive and require very little optimisation with robust protocols available (Blignaut *et al.*, 2013; Meudt and Clarke, 2007). The results of this study show that while not the most advanced technique available, AFLPs are still effective tools to answer key questions about the genetic diversity of a species and its populations in an ecological context. In countries such as South Africa where funding may be limited and conservation of biodiversity is of key concern (Jewitt *et al.*, 2015), the use of AFLPs to provide an overview of genetic diversity is recommended.

Expansion of the genetic investigation of *R. baurii* var. *platypetala* is recommended along with study focusing on reproduction, particularly pollination and dispersal, as this knowledge would allow for more effective assessment of the variety’s vulnerabilities to potential change and disturbance (Haig *et al.*, 2016). Many comparable species in the Drakensberg are exposed to the same changes and disturbances; this study highlights the need for effective assessment of other species of concern. Management and conservation of biodiversity is often difficult and expensive (Naidoo *et al.*, 2006), thus effective assessment of the appropriate strategies for particular species is key to ensuring resources are distributed effectively.

Conclusion

While assessment of several more populations is required for reliability, the results suggest that R. *baurii* var. *platypetala* is not currently vulnerable to change, having moderate genetic diversity within, and low differentiation between the populations investigated. These results also suggest that grazing is not a disturbance that is cause for concern when considering genetic diversity in R. *baurii* var. *platypetala*. Low genetic differentiation between populations is likely due to relatively new divergence and occupation of the region, presenting the need for further study to explore gene dynamics within the parent species.

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Appendix I

AFLP protocol (from Blignaut *et al.* 2013)

Digestion:

1. DNA samples quantified using micro-volume UV-Vis spectrophotometer. Good quality DNA ($A_{260/280}$ ~ 1.8 and $A_{260/230}$ ~ 2.0) diluted to a final concentration of 100ng/µL.
2. For each sample ca. 200ng of genomic DNA is digested with 5 units of *Eco*RI for 2 hours at 37°C in manufacturer's buffer in a 20 µL reaction volume.
3. After *Eco*RI digestion, 5 units of *Mse*I are added and the buffer concentration is again adjusted with manufacturer's buffer to a total volume of 30 µL.
4. The reaction is incubated at 37°C for 2 hours.
5. After incubation the enzymes are inactivated by incubating at 65°C for 30 minutes.

Ligation:

6. A 10 µL ligation reaction mix is made up consisting of 1 unit of T4 DNA Ligase, T4 DNA Ligase buffer, 50µM *Mse*I adaptor and 5µM *Eco*RI adaptor. This is added directly to the digestion reaction.
7. The digestion-ligation reaction is incubated overnight at 4°C.
8. Following ligation, the digestion-ligation mix is diluted 1:5 with sterile distilled water and used as a template for the pre-selective PCR.

Pre-selective amplification:

1. Each 15µL pre-selective PCR reaction contains 2.5µL of the diluted digestion-ligation reaction mix, 1µM *Mse*I+0 primer, 1µM *Eco*RI+0, readymix buffer (DreamTaq ReadyMix; Inqaba Biotechnical Industries, Pretoria, South Africa).
2. Pre-selective PCR amplification is done with an initial denaturing step of 94°C for 5 minutes, followed by 23 cycles consisting if denaturation t 94°C for 30 seconds, annealing at 56°C for 30 seconds, elongation at 72°C for 30 seconds, and a final elongation step at 60°C for 30 minutes.
3. Successful amplification is confirmed by running 5µL of the PCR product on a 1% aragose gel and observing a smear between 100 and 500 bp.

Selective amplification:

4. Following successful amplification, pre-selective PCR products are diluted with sterile distilled PCR-grade water (1:19 dilution) of which 5µL is used as a template for selective PCR amplification.
5. Each 20µL selective PCR reaction contains 0.25µM of fluorescently-labelled *Eco*RI+NNN and 1µM unlabelled *Mse*I+CTT and readymix.
6. PCR reactions are done without step-down PCR step following pre-selective PCR conditions but with 30 repeat cycles.

Analysis:

7. After amplification, 5µL of each fluorescently-labelled PCR product is mixed for each DNA sample.

CHAPTER 4:

SYNTHESIS AND CONCLUSION

Overview

Biodiversity conservation is of increasing importance as climate change and land-use change drive biodiversity loss (Hautier *et al.*, 2015; Malcolm *et al.*, 2006). Natural changes in disturbance regimes can dramatically affect patterns of genetic diversity, and anthropogenically driven changes have the potential to do the same (Banks *et al.*, 2013). Effective conservation requires assessment of a species' vulnerability to these changes to ensure management plans can be developed accordingly (Essl *et al.*, 2015; Kohn *et al.*, 2006; Purvis and Hector, 2000).

Many species in the Drakensberg are exposed to grazing as both commercial and subsistence farms expand to meet increasing demand (O'Connor, 2005; Turpie *et al.*, 2007). Grazing has the potential to drive the loss of genetic diversity as it can hinder sexual reproduction in populations of plants, which can drive reduced gene flow (Pausas and Lavorel, 2003; Thomann *et al.*, 2018). Even plants that adapt to grazing are at risk as the development of grazing resistance mechanisms requires trade-offs that affect the survival of individuals or populations, which could negatively impact genetic diversity (Briske, 1996; Díaz *et al.*, 2007).

This study aimed to assess the effects of grazing on the morphology (Chapter 2) and genetic diversity (Chapter 3) of populations of *Rhodohypoxis baurii* var. *platypetala*. This synthesis aims to draw conclusions from the results of the previous chapters to develop an overall assessment of the effect of grazing on *R. baurii* var. *platypetala* and its vulnerability to the disturbance, making recommendations for management of this variety. Opportunities for expansion of the study and its applications will also be highlighted.

General findings

Average grazing intensity is approximately three times the historical level in the Drakensberg region. As grazing is a patchy phenomenon the impacts of this for herbaceous biodiversity are likely to be experienced at two scales - firstly, more of the landscape will be exposed to very high levels of grazing. Secondly, there might be reduced possibility of gene-flow between ungrazed populations if the species is found to be negatively impacted by grazing. This investigation assessed vulnerability both by quantifying the morphological changes that occur to these species in heavily grazed sites, and by exploring the impacts that this might have on gene-flow.

The investigation of morphological differences in populations of *R. baurii* var. *platypetala* exposed to grazing in comparison to those unexposed to grazing found that morphology did not differ significantly in most of the traits measured. Flower height was significantly shorter in populations exposed to grazing, and this trait is one of the key indicators of adaptation to grazing, but contextualisation of this difference makes this scenario unlikely. The average difference in flower height was only 8mm, and the plants were already generally below the bite threshold relative to their surrounding grasses under the 'take-half' rule of grazing (Laca *et al.*, 1992). Additionally, no evidence of grazing was found on *R. baurii* var. *platypetala* individuals and no observations of grazing were made during the study. These factors suggest that it is very unlikely that *R. baurii* var. *platypetala* undergoes grazing, but with the difference in a key resistance trait, however small, extended study would be required for greater certainty.

Due to the reduction in scale, the investigation of genetic diversity in *R. baurii* var. *platypetala* functions only as a preliminary assessment, but the results provide key insight into the state of genetic diversity in *R. baurii* var. *platypetala*. Measures of genetic diversity were similar in the two populations and genetic differentiation between the pair was low, and as one population was exposed to grazing and the other not, these results suggest that it is unlikely grazing is negatively affecting *R. baurii* var. *platypetala*. The negative impacts of grazing were expected to drive reduced genetic diversity in populations exposed to the disturbance but with similar diversity values between the grazed and ungrazed sites, this is unsupported. Differentiation was also expected to be moderate given the isolated population structure of *R. baurii* var. *platypetala*, and grazing was expected to exaggerate this, but all measures of differentiation were low. This low differentiation can also be attributed to the evolutionary history of the species, with only recent establishment in these areas, but differentiation may increase given enough time, particularly if dispersal is poor. Measurement and comparison of genetic diversity in more populations will be needed to better support these results.

As grazing is a relatively new disturbance for *R. baurii* var. *platypetala*, it is possible that the effects of grazing require more time to manifest observably. Population dynamics have been found to lag behind changes in disturbance, with this delay often leading to extinction debt (Dullinger *et al.*, 2012). In their review of delayed responses of biodiversity to change, Essl *et al*. (2015) listed change in disturbance regime as one of the key mechanisms that contribute to this lag and highlighted the need for consideration of delay in management of biodiversity. Newly introduced disturbances also require long-term monitoring as they can result in directional selection that is only observable as it compounds over time, by which point the magnitude of the selection is exaggerated to alarming rates (Long *et al.*, 2011; Milesi *et al.*, 2016; Paixao and Barton, 2016). Many species are also slowly edged closer to their extinction threshold by gradual environmental challenges operating on long-term scales coupled with rapid introduction of new selection pressures. One such pressure, introduced grazing, can push the species past their extinction threshold (Bertram *et al.*, 2017). Long-term genetic monitoring is often encouraged due to potential response lag and the compounding effects of selection, and it might detect the impact of grazing on *R. baurii* var. *platypetala* in the future, but this is not expected due to the unlikelihood that the variety undergoes grazing.

An integrated approach

This study was built of two parts, a morphological and genetic assessment, each part reflecting similar studies on other species. While numerous studies assess morphological traits and their implications for the study species in an ecological context, and many studies measure genetic diversity within species, yet few studies feature a combination of the two, morphological/ecological and genetic investigations are almost always kept separate.

For example, Thomann *et al.* (2018) conducted an extensive, long-term study on the effects of grazing on inflorescence height of the endangered herb *Primula farinosa* (Primulaceae) and its effects on pollinator dynamics. *P. farinosa* is quite comparable to *R. baurii* var. *platypetala*, with similar size and morphology, semi-alpine distribution and scattered population structure. The species' relationship with grazing is different though, growing in meadows and wetlands with shorter grasses, and a dependence on continuous grazing for persistence. Thomann *et al.* (2018) found that exclusion of grazers led to an increase in plant height and strengthened pollinator-mediated selection for tall inflorescences. Their investigation of the changes in morphology of *P. farinosa* are comparable to findings presented here and their results show how the presence or absence of grazing can impact pollinator dynamics, driving directional selection, but they do not discuss the implications for this on genetic diversity. As pollination and flower height are critical in reproduction, and thus gene dynamics, along with the endangered status of the species and importance of genetic diversity in conservation, the absence of any discussion of how the findings might link to genetic diversity is conspicuous. Genetic information is available for *P. farinosa*, Reisch *et al.* (2005) assessed genetic diversity in 10 populations of the species, but their study makes no mention of grazing and its role in determining genetic diversity. A study by Lienert and Fischer (2003) investigated how *P. farinosa* was affected by habitat fragmentation, assessing fitness by evaluating population characteristics and morphological reproductive traits, and though they briefly mention grazing exposure, it is not incorporated into their study or mentioned in their discussion. The study that established *P. farinosa*'s dependency on grazing for persistence, Lindborg and Ehrlén (2002), was structured as an extinction-risk assessment, but makes no mention of genetic diversity and the role it plays in the species' vulnerability to extinction. Even a recent study by Gajewski *et al.* (2018) aimed at conservation of *P. farinosa* that assesses genetic structure of populations makes no mention of grazing at all. Despite extensive study and the threatened status of *P. farinosa,* a separation of ecological and genetic study is apparent, and crucial relationships are overlooked as a result.

While this trend of ecology and genetics being insufficiently integrated is highlighted in the case of *P. farinosa*, numerous other examples can be found. Many studies measure genetic diversity within species or populations and simply report these measures with no ecological context, even when studying endangered or rare species where such context should be vital, for example:

- Gaudeul *et al.* (2000) studied the genetic diversity of the endangered herb *Eryngium alpinum* (Apiaceae) and while they advocate for conservation of genetic diversity for adaptability, ecological context for this is not given to explain what the species might need to adapt to.
- Zargar *et al.* (2017) assessed genetic diversity in *Glycine max* (Fabaceae) and, as in the study by Gaudeul *et al.* (2000), state the importance of genetic diversity for adaptability with no ecological context.
- Rodrigues *et al.* (2013) found low levels of genetic diversity populations of the endangered herb *Mentha cervine* (Lamiaceae) and produced an extensive conservation plan aimed at preserving genetic diversity, but do not link this need for conservation of genetic diversity to an ecological need for adaptability.
- Wedelsbäck Bladh *et al.* (2014) found high levels of genetic differentiation between populations of *Armoracia rusticana* (Brassicaceae) but do not expand on the ecological implications of this differentiation.

While genetic studies often remain isolated from ecological context, ecological studies often exclude the genetic implications of their findings, even when dealing with reproductive dynamics that have direct impacts on genetic diversity and gene flow. Some examples based in grazing ecology include:

- Chen *et al.* (2017) found that overgrazing led to reduced seed size in the shrub *Lespedeza davurica* (Fabaceae), but do not discuss how this hinderance to sexual reproduction may affect genetic diversity.
- Johansen *et al.* (2016) found that clonal growth acts as a buffer to environmental change, grazing in particular, in the large herb *Knautia arvensis* (Caprifoliaceae) but did not discuss the implications of clonality for genetic diversity.
- A study by Lázaro *et al.* (2016a) found that grazing led to pollen limitation in the herb *Asphodelus ramosus* (Asphodelaceae) but the effect of this on genetic diversity is not postulated.
- Smyth *et al.* (1997) found that grazing reduced seed production by 87% in the invasive weed *Echium plantagineum* (Boraginaceae) but there is no discussion of how this could impact genetic diversity, and in turn adaptability, of this invasive species.
- A study of Oxalis corymbose (Oxalidaceae), a morphologically similar genus to *R. baurii* var. *platypetala,* assessed morphological response to grazing. This study by Suzuki *et al.* (2009) found that reproductive organs were smaller grazed sites and stems were also shorter. The potential consequences for gene-flow of this marked reduction in reproductive organ size paired lowered flower height on shorter stems are not discussed.

This is not to criticise the quality of these studies or suggest they were poorly executed; they are simply some examples of this persistent trend of isolated context. This separation of genetics and ecology could be problematic when assessing a species' vulnerability to change, as both aspects should be considered to ensure proper understanding of potential responses (McPeek, 2017). Measures of genetic diversity are used to predict adaptability, with high diversity making a species more adaptable, but these assessments are rarely expanded to scenarios where such adaptability is needed. Measuring genetic diversity does not provide evidence that the diversity can facilitate appropriate responses to disturbance and change (Booy *et al.*, 2000; McPeek, 2017). This trend also develops in ecological studies, where changes in morphology and reproduction are rarely given genetic context, despite their critical role in gene dynamics.

Phenomena such as phenotypic plasticity, which may facilitate adaptation that does not operate on a genetic level (Miner *et al.*, 2005; Nicotra *et al.*, 2010; Sultan, 2000), make this a more complex issue (Booy *et al.*, 2000). In the context of grazing disturbance, a species that has a level of phenotypic plasticity may develop morphology that aligns with grazing resistance, but this would not be reflected in a study that only measured genetic diversity. If the species was found to have high levels of genetic diversity, management plans would place it at low risk status, and its exposure to grazing may go unchecked. However, as grazing resistance adaptations have the potential to negatively affect sexual reproduction, and in turn genetic diversity, this lack of grazing management could have undesirable consequences. Inversely, an investigation of only morphology would find grazing resistance architecture in grazed populations and may conclude that grazed and ungrazed populations were distinct. This conclusion could lead to more extensive management of grazed and ungrazed populations to preserve diversity, which would be unnecessary and wasteful as the distinction is based in a plastic response. It is possible that plasticity is present in *R. baurii* var. *platypetala* and could be an explanation for the combination of almost equivalent genetic diversity in populations, but observable range of morphological variation within the variety. This would require proper investigation to be confirmed.

Despite almost similar genetic diversity within the Hebron Road (HR) and Giant's Castle Game Reserve (MK) populations, morphology was quite different. Comparison of morphological traits did not find significant differentiation associated with grazing exposure in Chapter 2, but significant variations in morphology are present independent of grazing exposure. When morphological traits were compared between the pair using Welch two sample t-tests with Tukey HSD post-hoc, significant difference was found for several traits, despite their similar genetic diversity and low differentiation. Flower height, leaf table height, and leaf length were all significantly different between the pair ($P < 0.001$; n = 56; Figure 4.1) with flowers and the leaf table being taller and leaves longer at the MK site. Number of leaves and leaf angle were also significantly different ($P < 0.001$; n = 56), with plants the HR population having 2 more leaves that were 39.6° flatter than those at MK. The number of flowers and above:below ground biomass ratio were not significantly different between the pair. With such extensive differentiation that was not attributed to grazing presence in Chapter 2, and not reflected on a genetic level in Chapter 3, it is possible that the variety has highly plastic morphology, as this would account for non-selective, non-genetic variation. Plasticity was considered as a factor at the beginning of this study and an experiment was intended to be incorporated into Chapter 2, the details of which are explained below as they present an opportunity for further study. Plasticity has been shown to facilitate adaptation and is therefore a key factor when assessing vulnerability to disturbance and change (Miner *et al.*, 2005; Nicotra *et al.*, 2010). As it was only through the comparisons that required a combination of morphological and genetic investigations that plasticity was identified, the need for integrated ecological and genetic assessments is emphasised.

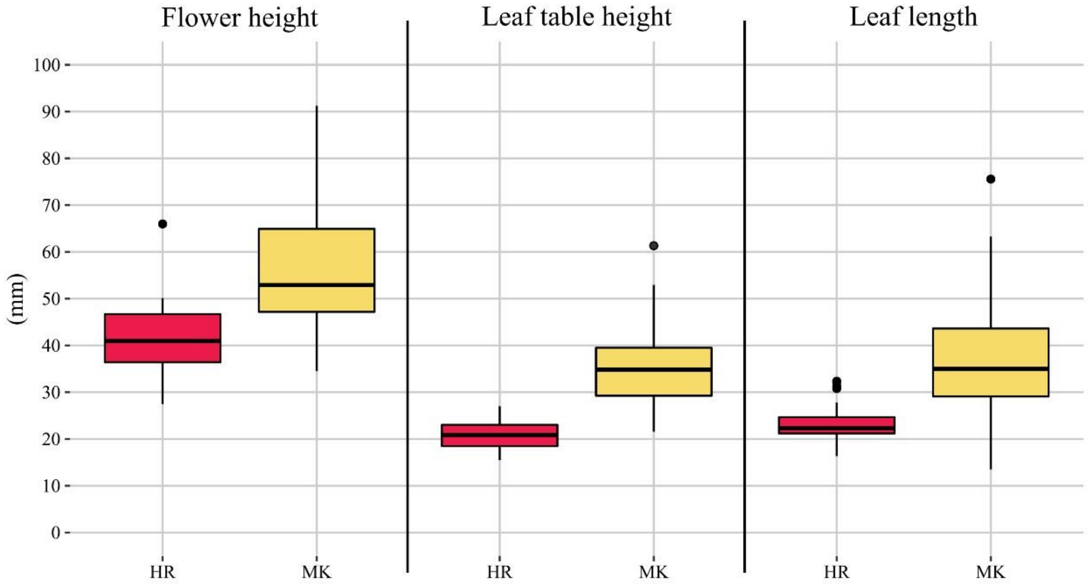

Figure 4.1: Flower height, leaf table height and leaf length compared the Hebron Road (HR) and Giant's Castle Game Reserve (MK) populations. These key morphological traits were found to be significantly different ($P < 0.001$) with plants at the MK site having flowers 14.10mm taller, leaf table height 15.14mm taller and leaves 13.35mm longer than those at the HR site. With no link found between morphology and grazing in Chapter 2, and very similar genetic diversity values and low genetic differentiation found for the pair in Chapter 3, it is likely that phenotypic plasticity has facilitated such extensive variation.

These combined findings of both morphological and genetic assessments suggest that it is unlikely that grazing is a disturbance that should be of immediate concern in *R. baurii* var. *platypetala*. The combination of approaches in this study allowed for comprehensive assessment of grazing and as the results of each aspect aligned, they added reliability to the findings and overall conclusion. The morphological assessment suggested that grazing does not impact *R. baurii* var. *platypetala*, as did the preliminary results of the genetic diversity assessment, but each had unique insights. The morphological assessment found that flower height was shorter with grazing exposure, and while monitoring is required to determine if this is the beginning of grazing resistance, this subtle difference would not have been identified by genetic analysis. The preliminary genetic diversity investigation found very low differentiation between populations at great distances, providing further insight into the evolutionary history of the variety that morphological assessments would not. Only through the combined assessment of both morphology and genetic diversity was the prevalence of phenotypic plasticity identified, a key factor for adaptability.

Our combined approach to assessing the vulnerability was framed in the context of expanding grazing disturbance threatening biodiversity, with land-use changes associated with agriculture found to be the second-largest threat to biodiversity behind over-exploitation (Maxwell *et al.*, 2016). The framework also has applications outside disturbance, with the strategies involved forming key parts of climate change vulnerability assessments. In a framework proposed by Foden *et al.* (2013), vulnerability to climate was determined by consideration of three factors: sensitivity, exposure and adaptive capacity. Sensitivity of a species was defined as its ability to persist within its natural habitat and was determined by factors such as specialised habitat requirements, tolerance thresholds, rarity, and specialised interspecific relationships. Exposure was defined as the degree to which the natural habitat would change which is usually determined through modelling and other predictive techniques. Adaptive capacity was defined as the species' ability to avoid or mitigate the negative effects of climate change, which is determined by its dispersal ability, and genetically-facilitated evolvability. Only through combined assessment of these three factors can a species' vulnerability to climate change be determined effectively. While evaluation on numerous dimensions requires extensive study, integrated frameworks would allow this to be carried out efficiently.

This study aimed to comprehensively investigate the vulnerability of *R. baurii* var. *platypetala* to grazing disturbance, but also provides fundamental knowledge for climate change vulnerability under Foden *et al.*'s framework (2013). The results contribute to knowledge of both sensitivity and adaptive capacity, leaving only exposure unassessed. The morphological assessment provided insight to both sensitivity and adaptive capacity, the genetic diversity informs the evolvability aspect of adaptive capacity, and finding plasticity adds another key factor for sensitivity assessment. While not a complete assessment, the results suggest that *R. baurii* var. *platypetala* has low sensitivity and moderate adaptive capacity, with limited dispersal standing out as a key factor that may contribute to vulnerability in the future. As more species require vulnerability assessments under expanding disturbance and global climate change, integrated studies speed up the process by providing information that would be lacking in purely ecological or genetic assessments.

An efficient framework

This study was able to assess both morphology and genetics in *R. baurii* var. *platypetala* due to its use of appropriately selected traits and molecular methods. The use of traits facilitates more rapid work in quantitative and predictive ecology by simplifying the function of a species relative to the investigation and allowing multiple species to be grouped based on their contextual functionality (Kimball *et al.*, 2016; Pausas *et al.*, 2004). Functional traits have been organised into many large-scale datasets for a variety of uses, such as LEDA (Life History Traits of the Northwest European Flora; Knevel *et al.*, 2003) and SID (Seed Information Database; Kew, 2008) and even global-scale sets such as GLOPNET (Global Plant Trait Network; Wright *et al.*, 2004) and TRY database (Kattge *et al.*, 2011). The use of functional traits allows for simplified approaches to investigating ecosystem processes such as disturbance, that is transferrable across various taxa, through reference to these databases and syntheses of trait-based studies. Díaz *et al.* (2007) developed an extensive synthesis of plant functional trait responses to grazing, simplifying the complex process of grazing adaptation to key traits, and these traits allowed the morphological assessment of this study to be conducted efficiently.

The use of functional traits in this study presents potential transferability to similar flora in the Drakensberg when conducting vulnerability assessments. Height was highlighted as the key trait of focus, with its proven responses to grazing (Briske, 1996; James *et al.*, 2001; Thomann *et al.*, 2018), and its link to sexual reproduction through pollination (Lázaro *et al.*, 2016b; Muñoz-Pajares *et al.*, 2017; Strauss, 1997) and dispersal (Bullock and Clarke, 2000; Soons *et al.*, 2004; Tackenberg, 2003; Tamme *et al.*, 2014; Thomson *et al.*, 2011), and thus subsequent implications for gene dynamics. When considering grazing height also allows for assessment of grazing likelihood when compared to grass height under known bite dynamics of grazers (Laca *et al.*, 1992; Ungar *et al.*, 2001; Ungar and Ravid, 1999). Additional architectural traits such as ‘flatness’ should also be considered but do not appear to have as extensive implications as height. After height, dispersal mechanism is key, particularly if reproduction is disrupted by grazing and generation time is long. Disruption of sexual reproduction and poor dispersal lead to the need for genetic investigation. Other traits, such as biomass allocation and number of leaves are given lower priority. Flower number has been shown to have balancing feedback loops in response to grazing, with grazed species often having fewer flowers (Bridle and Kirkpatrick, 2001; Briske, 1996; Díaz *et al.*, 2007; Lázaro *et al.*, 2016; Yoshihara *et al.*, 2008), but this reduction leading to increased pollinator visitation rates (Tadey, 2015). While morphological plasticity may facilitate adaptation to grazing without genetic response, the adaptations will still lead to trade-offs in reproduction that will affect gene dynamics. The prioritisation of height and subsequent traits will allow for simplified assessment of grazing risk in other flora following the presented decision sequence (Figure 4.2). Grass palatability will be a key factor, as palatable grasses would increase grazing likelihood, but unpalatable grasses should not be ignored. Increased dominance of palatable grasses would be linked to increased vulnerability and management strategies would need to account for this though their rotations and exclusions. Answering the questions in this framework would inform management strategies by highlighting if ecological or genetic monitoring is required, or a combination of the two. If genetic vulnerability is low, then management would not need to be as extensive as gene flow would likely mitigate the impacts of grazing

(Andrew *et al.*, 2013). Targeted strategies could be developed to ensure action is taken at the appropriate time of year to ensure seed dispersal is not hampered by grazing for the species, which might in turn moderate genetic vulnerability. Genetic assessments could also lead to re-classification if differentiation has become extensive, which could alter the protection status of the species. Simplification to this framework and the focus on height as a key trait provide a simple solution to developing targeted strategies without extensive research periods.

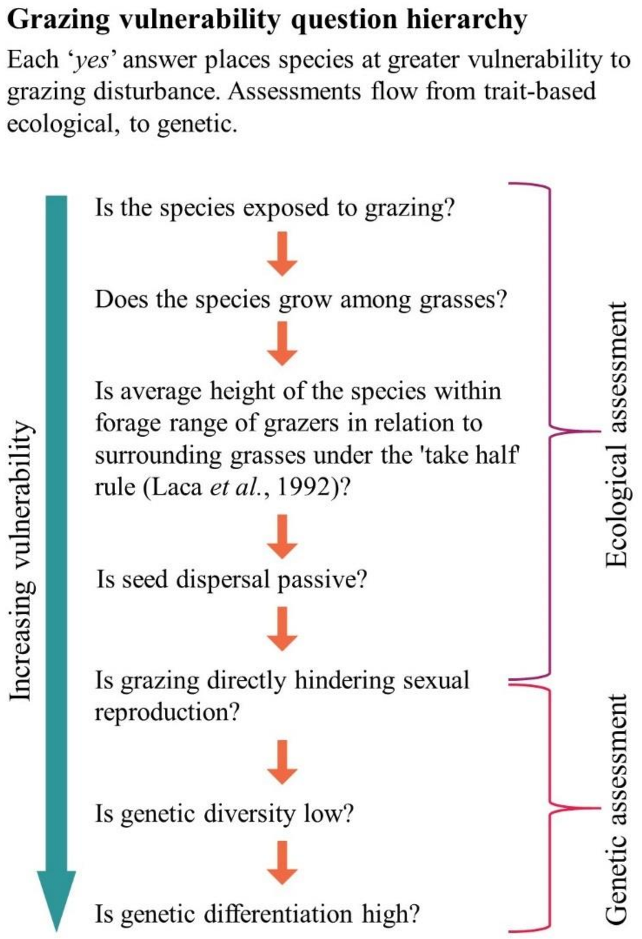

Figure 4.2: Grazing vulnerability question hierarchy

The identification of appropriate traits in combination with AFLPs (their efficiency and convenience are discussed in Chapter 1 and 3) is proposed as a template for effective integrated study of response and vulnerability in other species exposed to disturbance and change. There are 182 angiosperm species that are Red or Orange Data listed in the Drakensberg alpine Centre, 55% of which are endemic to the region (Carbutt and Edwards, 2006), and as grazing disturbance is expanding in this area, there is opportunity for this framework to be applied for efficient management strategy development.

Future study

Rhodohypoxis baurii var. *platypetala*

As discussed above, phenotypic plasticity may facilitate adaptation that does not operate through genetics, and this was a factor considered when developing this study. During field sampling, 10 of the plants at each site that had morphological traits measured for Chapter 2, were collected, potted in 15cm pots, and kept in a greenhouse. These plants were to form a common-garden experiment (e.g. Groot *et al.*, 2018) to monitor changes in morphology after removal of grazing disturbance, with plants from ungrazed sites acting as a control with identical water, light and soil conditions to ensure comparability. After a full year, the morphological trait measurements should be repeated to compare with the previous in-field measurements to test for significant changes. This monitoring under undisturbed conditions would provide insight to whether the morphological differences were genetically or phenotypically driven and if plants previously exposed to grazing had plasticity to return to the assumed ‘typical’ morphology of those from ungrazed sites. Most of individuals collected did not flower, and those that did were not mature in time for inclusion in this study, while *R. baurii* var. *platypetala* predominantly flowers in November, the few individuals that did flower in the greenhouse only developed in mid-January. While every effort was made to reduce the impact of collection on the individuals, with potting taking place as quickly as possible, the lack of flowering is likely attributed to the resource costs associated with re-establishment in the pots. While not part of this study, these individuals and the common-garden setup do provide potential to study plasticity in the variety in the future with continuous measurement. This investigation of plasticity would allow for proper assessment of the extent of morphological variation within *R. baurii* var. *platypetala* and as preliminary results above show that variation is quite prevalent, this should be a priority. In addition to quantifying variation within the variety, the results could highlight key factors that drive development of particular combinations of morphological traits as comparisons are expanded beyond grazing presence to abiotic factors.

Rhodohypoxis baurii

Apart from the original description by Baker (1878) and expansion 40 years ago by Hilliard and Burtt (1978), very little work has been published on *Rhodohypoxis baurii* and its varieties, presenting numerous opportunities for expanded study, most of which would answer questions related to reproduction. Work that does exist is based around its cultivation as an ornamental flower, being particularly popular in Japan (e.g. Matsuo *et al.*, 2011; Mori and Sakanishi, 1990) and Poland (Salachna *et al.*, 2015), but the plant has surprisingly low ornamental value in South Africa

(Duncan, 2003) which affords it protection from poaching. Currently, very little research on the species stems from South Africa aside from popular articles.

Understanding seed dispersal in *R. baurii* and its varieties also presents opportunity for expanded study. Passive seed dispersal through circumscissile dehiscence and subsequent censer dispersion though wind or agitation was described by Hilliard and Burtt (1978), but observations made during the study suggest this may need revision. In the majority of plants, seeds had difficulty falling from the seed capsule through wind exposure and agitation, even when manually aggravated. Instead, most seeds remained in the capsule until the stem underwent atrophy and collapsed from the base plant and would all germinate within range of where the capsule lay on the soil, often tightly packed together (Figure 4.3). While wind and rain would likely add variability to this, the seed dispersal experiments in Chapter 2 suggest they may not play a large role in seed dispersal. While proper study is required, these observations suggest that flower height could be a key determinant in ensuring seeds are established away from parent plants. This would have further implications if findings of lowered height in Chapter 2 are in fact the beginning of a trend of height reduction due to grazing exposure, as there could be consequences for dispersal and local competition in the future. Even if height does not become reduced, difficulty of dispersal would increase chances of like individuals mating with each other within populations, and over time could completely alter patterns of genetic diversity. With limited dispersal being a key factor of poor adaptive capacity in Foden *et al.*'s (2013) climate change vulnerability assessment framework, the variety and species could become threatened by climate change if these dispersal limitations are found to be extensive and persistent within populations. This places proper investigation of dispersal and reproduction at high priority when future study of the species is considered.

Figure 4.3: Observations of seed dispersal in *R. baurii* var. *platypetala*. Stems with seed capsules atrophy and collapse (A) and seeds germinate in proximity to the area where the capsule lands (B and C).

A pollinator of the species has not yet been identified, which leaves questions about pollen distribution unanswered. The distinct, closed-mouth structure of the flowers (Figure 4.4) suggests a pollinator with either a long proboscis to penetrate to the flower, or the strength to push through the bent perigone segments to access pollen. In expanding on this, it is recommended that nocturnal observations be included, as the lack of diurnal pollinator observations while carrying out this study suggests pollination by moth as flowers remain open at night. Non-pollinator mediated selection of floral characteristics in *R. baurii* is currently being expanded on (Gardiner and Glennon, unpublished data) and highlights the need to expand understanding of both how abiotic factors drive the diversity within the species, and how this affects gene dynamics.

Figure 4.4: The unusual flower structure of *R. baurii*, with perigone segments bent over reproductive structures, closing the mouth of the flower with pollen only partially visible.

Grazing expansion

As discussed in Chapter 1, quantification of grazing change in this region remains challenging. In order to effectively manage and preserve the biodiversity of the Drakensberg, high-quality data on the expansion of grazing land and quantity of grazers is required and should incorporate both domestic and wild livestock. With evolutionary-scale historic estimates now available, improved current data would allow for effective investigation of the role grazing has played in the development of modern flora. The role of smaller animals and their contribution to grazing pressure could also be expanded on, as there is potential that the corms would be attractive for foragers. This would likely be most prominent in undisturbed areas as disturbance, in particular livestock grazing, reduces small mammal abundance and diversity (Herder *et al.*, 2016; Hoffmann and Zeller, 2005; Schmidt *et al.*, 2005). Although there was no evidence of foraging by smaller animals during this study, targeted research strategies could potentially reveal unseen herbivory dynamics.

Conclusion

This study aimed to investigate the effect of grazing on populations of *R. baurii* var. *platypetala* to assess the vulnerability of the variety to this expanding disturbance. It was found that *R. baurii* var. *platypetala* does not currently appear to be negatively affected by grazing, and thus does not require prioritisation in management plans. The incorporation of both ecological and genetic assessments is intended to ensure this evaluation was comprehensive. The framework for this combined approach to assessment developed in this study, which is based on transferrable techniques, has applications for all plant species exposed to disturbance, particularly in the Drakensberg where expansion is prominent. As the need and urgency for conservation of biodiversity increases, so the associated challenges grow, requiring constant adjustment and improvement of research and assessment techniques to ensure efforts are efficient and effective.

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